The Plant Cell, Vol. 6, 1077–1085, August 1994 © 1994 American Society of Plant Physiologists

Plant Defense Genes Are Synergistically Induced by Ethylene and Methyl Jasmonate

Yi Xu, Pi-Fang Linda Chang, Dong Liu, Meena L. Narasimhan, Kashchandra G. Raghothama, Paul M. Hasegawa, and Ray A. Bressan[1]

Center for Plant Environmental Stress Physiology, Purdue University, 1165 Horticulture Building, West Lafayette, Indiana 47907-1165

Combinations of ethylene and methyl jasmonate (E/MeJA) synergistically induced members of both groups 1 and 5 of the pathogenesis-related (PR) superfamily of defense genes. E/MeJA caused a synergistic induction of PR-1b and osmotin (PR-5) mRNA accumulation in tobacco seedlings. E/MeJA also synergistically activated the osmotin promoter fused to a β-glucuronidase marker gene in a tissue-specific manner. The E/MeJA responsiveness of the osmotin promoter was localized on a −248 to +45 fragment that exhibited responsiveness to several other inducers. E/MeJA induction also resulted in osmotin protein accumulation to levels similar to those induced by osmotic stress. Of the several known inducers of the osmotin gene, including salicylic acid (SA), fungal infection is the only other condition known to cause substantial osmotin protein accumulation in Wisconsin 38, a tobacco cultivar that does not respond hypersensitively to tobacco mosaic virus. Based on the ability of the protein kinase C inhibitor 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine to block ethylene induction of PR-1b mRNA accumulation and its inability to block osmotin mRNA induction by ethylene, these two PR gene groups appeared to have at least partially separate signal transduction pathways. Stimulation of osmotin mRNA accumulation by okadaic acid indicated that another protein kinase system is involved in regulation of the osmotin gene. SA, which is known to induce pathogen resistance in tobacco, could not induce the osmotin gene as much as E/MeJA and neither could it induce PR-1b as much as SA and MeJA combined.

INTRODUCTION

Of the many apparent plant defense responses to invasion by various pathogens, one of the most studied is the synthesis of a group of host-encoded proteins referred to as pathogenesis-related (PR) proteins. PR proteins were first discovered as polypeptides that accumulate in genotypes of tobacco that respond hypersensitively to infection with tobacco mosaic virus (TMV) (Gianinazzi et al., 1970; Van Loon et al., 1970). Since then, many PR proteins have been described as occurring in a wide variety of plant species (Cutt and Klessig, 1992). The PR proteins and genes that encode them have now been categorized into five major groups. A great deal of information concerning expression and function of these genes has been reported, and a number of reviews concerning PR proteins have appeared (Bol et al., 1990; Cutt and Klessig, 1992). Increasing evidence has mounted showing that many members of the PR superfamily have antifungal activity in in vitro assays (Bol et al., 1990). Three of the PR protein groups, PR-1, PR-3 (chitinases), and PR-5 (osmotins), have now been reported to contain gene members that can convey increased resistance to phytopathogenic fungi when overexpressed in transgenic plants (Broglie et al., 1991; Alexander et al., 1993; Liu et al., 1994). Besides the production of transgenic plants that overexpress PR proteins, another important approach toward understanding the antiphytopathogenic properties of these genes involves the elucidation of the signal transduction pathway(s) that controls their expression and thereby allows an effective means of testing their coordinated expression on resistance.

In recent years, we have examined in some detail the regulatory properties of a PR protein referred to as osmotin (Kononowicz et al., 1993). Osmotin, which has been classified as a basic homolog of family five PR proteins, was discovered as a protein that accumulates to high levels in cells of tobacco (cv Wisconsin 38 [W38]) after NaCl adaptation (Singh et al., 1987a, 1987b). Osmotin gene expression in tobacco is already known to be regulated by a large number of hormonal and environmental signals, which include NaCl, abscisic acid (ABA), ethylene, wounding, desiccation, UV light, cold, TMV infection, and fungal infection (Singh et al., 1989a, 1989b; Brederode et al., 1991; Stintzi et al., 1991; Cutt and Klessig, 1992; LaRosa et al., 1992; Nelson et al., 1992; Liu et al., 1994). Although many inducers of the osmotin gene have been described, most have been shown not to cause accumulation of osmotin protein in the tobacco cultivar W38 (LaRosa et al., 1992).

[1] To whom correspondence should be addressed.

There has been a well accepted assumption in recent years that factors that control the expression of a particular gene help define the function of that gene's product. Thus, there has been considerable effort to identify genes that function to produce a particular phenotype (e.g., disease or osmotic stress tolerance) by determining which genes will respond to environmental or hormonal signals that can induce the phenotype of interest (for instance, see Skriver and Mundy, 1990; Bray, 1993; Thomas and Bohnert, 1993). However, many genes, like osmotin, are induced by several apparently unrelated signals, such as osmotic stress and pathogen invasion, bringing into question the relationship between inductive signals and gene function. In this study, we present evidence that there are hyperinduced states of plant defense genes that result from particular signal combinations. We suggest that signal combinations may synergistically hyperinduce other plant defense genes and that such synergistic signals may be more specifically related to gene function than any single inductive signal.

We specifically demonstrated that osmotin and PR-1b, members of the PR gene superfamily, which have not been reported to be regulated by methyl jasmonate (MeJA), responded to this signal through its cooperation with other hormonal factors in regulating the expression of PR genes. We have described a dramatic and tissue-specific synergistic effect of ethylene and MeJA on activating the expression of the osmotin promoter. The *cis* element pattern of hyperresponsiveness of the osmotin promoter to combinations of ethylene and MeJA (E/MeJA) indicated that the responsive sequences are present on the same DNA fragment (−248 to +45) where responsiveness to other signals has been mapped (Raghothama et al., 1993).

RESULTS

Synergistic Effect of Ethylene and MeJA on the Osmotin Promoter

Ethylene caused osmotin mRNA accumulation in young root and leaf tissues (LaRosa et al., 1992). After 24 hr of ethylene treatment, 5-day-old seedlings containing an osmotin promoter–β-glucuronidase (GUS) fusion gene (GUS1B) (Nelson et al., 1992) exhibited a fivefold increase in GUS activity. As seen in Figure 1A, 5 ppm ethylene is above the concentration required to produce maximum activation of the osmotin promoter. The osmotin promoter was not responsive to treatment with MeJA alone (Figure 1A). However, when MeJA was applied in combination with a response saturating level of ethylene (5 ppm), the activity of the osmotin promoter was induced dramatically beyond that seen with either ethylene or MeJA in whole GUS1B seedlings (Figures 1B and 1C). When increasing concentrations of MeJA were applied with 5 ppm ethylene, an optimal response of the promoter was seen at 45 μM MeJA (Figure 1C). Synergistic induction of the osmotin promoter by E/MeJA was confirmed by RNA gel blot analysis that indicated synergistic accumulation of osmotin mRNA (Figure 2A).

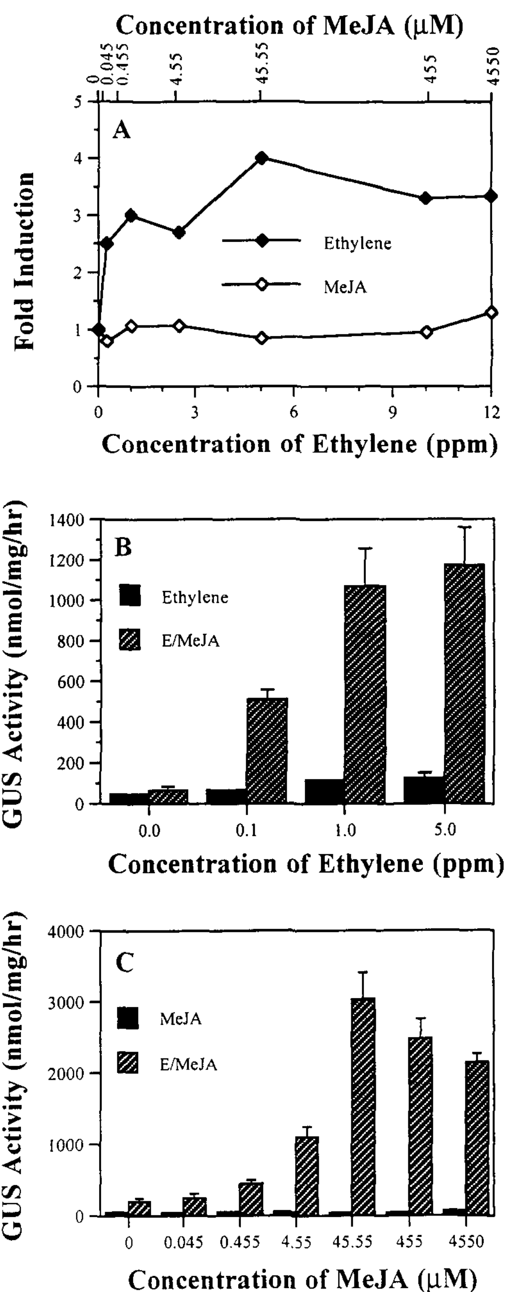


Figure 1. GUS Activities of GUS1B Seedlings Treated with MeJA, Ethylene, and Ethylene Plus MeJA (E/MeJA).

(A) Effect of ethylene and MeJA on osmotin promoter–GUS fusion gene activity. Seedlings were treated for 24 hr with the indicated concentrations of MeJA or ethylene. The mean GUS activity of untreated control tissues was 45.6 nmol of methylumbelliferone per mg of protein per hr.

(B) Synergistic effect of 45.5 μM MeJA and increasing concentrations of ethylene on osmotin promoter–GUS fusion gene activity. Seedlings were treated for 24 hr.

(C) Synergistic effect of 5.0 ppm ethylene and increasing concentrations of MeJA on osmotin promoter–GUS fusion gene activity. Seedlings were treated for 24 hr.

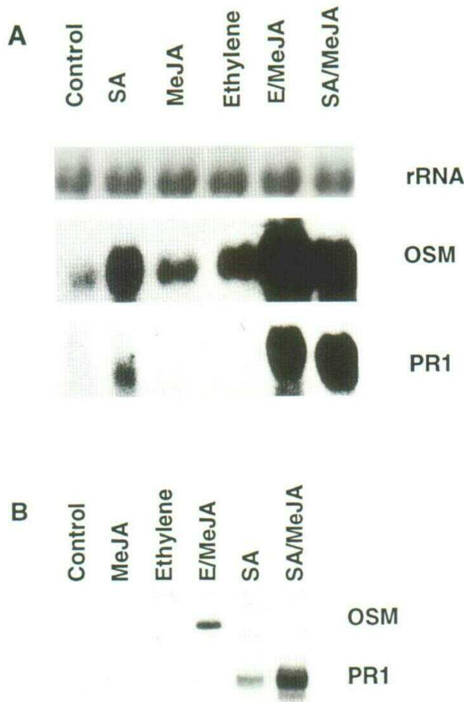


Figure 2. Effects of Inducers and Combinations of Inducers on Osmotin and PR-1 mRNA and Protein Accumulation.

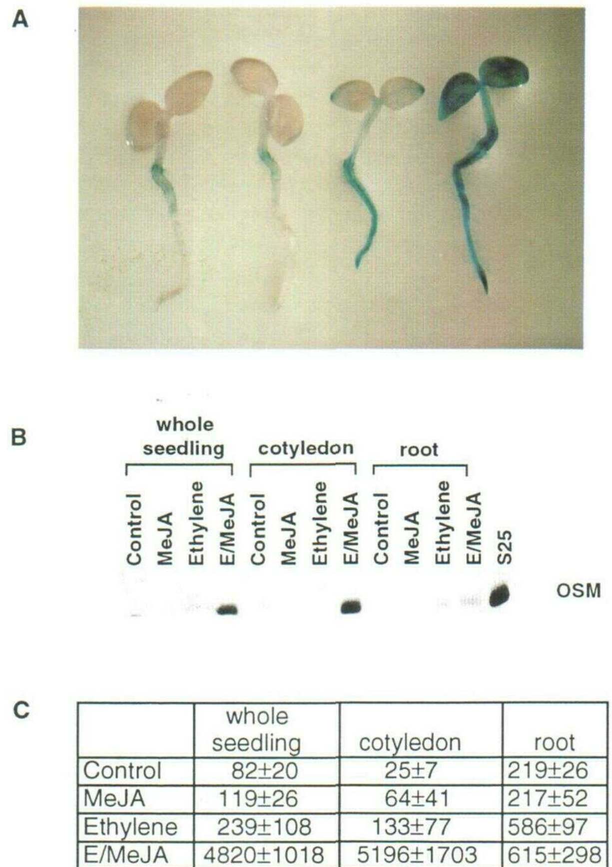
(A) Induction of osmotin (OSM) and PR-1 mRNA. Gel blots of osmotin and PR-1 RNA (15 μg of total RNA per lane) from the untreated control and seedlings treated with 1 mM SA, 45.5 μM MeJA, 5 ppm ethylene, E/MeJA (5 ppm and 45.5 μM), and SA/MeJA (1 mM and 45.5 μM) are shown; rRNA hybridization is shown to illustrate the relative amount of RNA loaded and transferred. RNA extractions were made 24 hr after treatments.

(B) Induction of osmotin and PR-1 proteins. Shown are osmotin and PR-1 immunoblots of an SDS–polyacrylamide gel of proteins (30 μg protein per lane) from seedlings treated for 3 days under the same conditions as given in **(A)**.

In contrast to ABA, the activation of the osmotin promoter by ethylene (Nelson et al., 1992) and by a combination of ethylene and MeJA was not affected by age of the seedlings (data not shown).

Ethylene and MeJA Induce Osmotin mRNA and Protein Accumulation

We have reported previously that although several factors can induce osmotin mRNA accumulation, most do not cause protein accumulation (LaRosa et al., 1992). The combination of E/MeJA induced both osmotin mRNA and protein to accumulate to levels nearly as high as those found in salt-adapted cells (Figures 2A, 2B, and 3B). This accumulation of protein occurred within 3 days of treatment, similar to the time required for protein to accumulate in response to fungal infection (Liu et al., 1994) and salt adaptation (LaRosa et al., 1992). In fact, the levels of protein accumulation induced in roots and shoots of tobacco seedlings correlated well with the induction of the promoter as evidenced by GUS staining (Figure 3). Compared to control levels, ethylene and especially MeJA caused a larger accumulation of osmotin mRNA than when they stimulated the osmotin promoter fused to GUS (Figures 1A and 2A). This suggests that these signals may have effects both on osmotin promoter induction and on stabilization of osmotin mRNA.



	whole seedling	cotyledon	root
Control	82±20	25±7	219±26
MeJA	119±26	64±41	217±52
Ethylene	239±108	133±77	586±97
E/MeJA	4820±1018	5196±1703	615±298

Figure 3. Tissue-Specific Pattern of Osmotin Promoter Activity in GUS1B Seedlings Treated with MeJA, Ethylene, and E/MeJA.

(A) Histochemical localization of GUS gene expression (from left to right): untreated seedlings and seedlings after 24 hr of treatment with 45.5 μM MeJA, 5 ppm ethylene, or E/MeJA (5 ppm/45.5 μM).

(B) Osmotin (OSM) protein induction in roots, cotyledons, and whole seedlings. Osmotin immunoblot of an SDS–polyacrylamide gel of proteins (30 μg of protein per lane) from whole seedlings, cotyledons, or roots 3 days after they were treated with the same concentrations of inducers as indicated in **(A)**. Osmotin accumulation in salt-adapted (S25) tobacco cells is shown for comparison.

(C) Quantitative assay of GUS activities. Given are GUS activities (nmoles of methylumbelliferone per milligram of protein per hour) in the whole seedlings, cotyledons, or roots treated as described in **(A)**.

Although we have found that MeJA has no effect on the level of GUS mRNA, we cannot rule out the possibility that it destabilizes GUS protein.

Response of Whole Seedlings to Ethylene and MeJA Is the Result of Synergistic Action on Roots and Nearly Codependent Action on Cotyledons

The GUS expression patterns of 5-day-old GUS1B seedlings treated for 5 days with water, MeJA, ethylene, and E/MeJA are shown in Figure 3A. In the control seedlings and seedlings treated with MeJA, there was a constitutive expression of GUS only in root tissues especially near the transition zone. The seedlings treated with ethylene alone showed increased GUS activity in roots and cotyledon tips. The treatment of E/MeJA significantly increased GUS activity in roots and caused dramatic expression in otherwise nonexpressing cotyledon tissue (Figure 3A). The quantitative measurement of GUS activity also showed that induction of the osmotin promoter in cotyledons was virtually codependent on the presence of both ethylene and MeJA, which elicited a 200-fold increase in promoter activity (Figure 3C). The variation between different tissues in degree of response of the osmotin promoter to individual and combinations of signals could be explained by different endogenous levels of the signal molecules in different tissues. Higher levels of endogenous MeJA in roots, cotyledon tips, and mature expanded leaves could result in our observation that the osmotin promoter was more responsive to ethylene and less responsive to E/MeJA in these tissues.

The induction of large amounts of osmotin protein occurred only in treatments with E/MeJA, and only the cotyledons responded by accumulating protein to levels similar to those seen in salt-adapted cells (Figure 3B).

Responsiveness to Ethylene and E/MeJA Is Associated with the Same Region of the Osmotin Promoter as Other Inducers

We previously reported that analysis of 5′ deletions of the osmotin promoter by transient expression in microprojectile-bombarded leaves illustrated that the promoter sequence from −248 to +45 bp is absolutely required for reporter gene activity (Raghothama et al., 1993). The responsiveness of the promoter to ABA, ethylene, salt, desiccation, and wounding also appears to be associated with the −248/+45-bp sequence of the promoter (Raghothama et al., 1993). This region contains three imperfect G-box motifs (Raghothama et al., 1993). The pattern of expression of GUS induction by E/MeJA treatment in stable transformants carrying osmotic promoter deletion fragments fused to GUS revealed that the same 5′ sequence from −248 to +45 bp is required for the synergistic induction of the promoter (Figures 4A and 4B). Although gel retardation experiments with the −248/+45-bp fragment showed that no changes in gel retardation were observed after E/MeJA treatment, the protein synthesis inhibitor cycloheximide blocked E/MeJA induction of the osmotin promoter.

Ethylene and MeJA Can Synergistically Induce a PR-1 Gene

Besides osmotin, the PR-1b gene also was induced synergistically by E/MeJA (Figure 2A). This synergism was at least as dramatic as that seen with osmotin. It appears likely that the other PR gene families will be synergistically activated by E/MeJA.

PR-5 (Osmotin) and PR-1 Are Induced through Different Signal Transduction Pathways

Recently, it was reported that the protein kinase C inhibitor 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7) could inhibit the induction of PR-1 transcript by ethylene (Raz and Fluhr, 1993). However, H7 was unable to inhibit the induction of osmotin transcript accumulation by ethylene in mature expanded leaves (Figure 5A). In contrast, we also found, as reported by Raz and Fluhr (1993), that H7 inhibited ethylene induction of the PR-1b message (Figure 5A). Apparently, another protein kinase system is involved in osmotin gene expression because the phosphatase inhibitor okadaic acid stimulated osmotin gene expression (Figure 5B) as it did PR-1 expression (Raz and Fluhr, 1993). These results strongly suggest that two of the PR gene family subgroups, PR-1 and PR-5 (osmotin), are induced through separate signal transduction pathways, both involving protein kinases. Because several primary signals induce both of these genes, it is quite possible that some secondary signal steps are shared by PR-1 and PR-5 genes.

Roles of Salicylic Acid, Ethylene, and MeJA in Coordinating Defense Gene Responses

A great deal of evidence has been reported to show that salicylic acid (SA) plays a key role in coordinating plant defense gene expression (see Enyedi et al., 1992). Indeed, exogenously applied SA significantly affects resistance to pathogens (see Enyedi et al., 1992). We have found the curious result that the osmotin gene, which was also induced by SA, was induced to a greater extent by E/MeJA (Figure 2A). Even more interesting is our observation that in W38 tobacco, which does not respond hypersensitively to TMV, SA was not able to induce a large accumulation of the osmotin protein (Figure 2B), whereas E/MeJA strongly induced the protein (Figures 2B and 3B). In addition, although SA substantially induced the accumulation of PR-1b protein in W38 tobacco, the combination of MeJA and SA induced PR-1 protein to accumulate severalfold more (Figure 2B). Although MeJA, in combination with ethylene or SA, hyperinduced osmotin and PR-1b mRNA accumulation

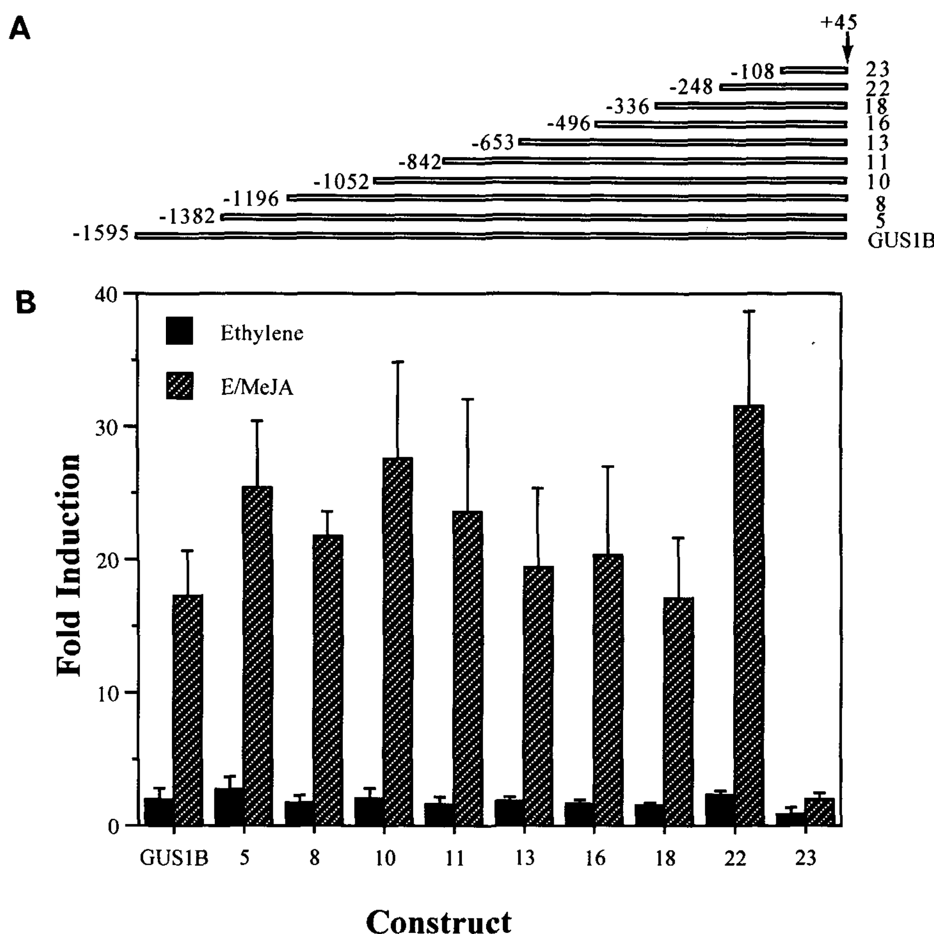


Figure 4. Responsiveness of Stable Transgenic Plants Carrying Osmotin Promoter Fragments Fused to GUS That Were Treated with Ethylene and MeJA.

(A) 5′ Deletion fragments of the osmotin promoter. The positions of the 3′ and 5′ nucleotide ends of each fragment are indicated relative to the transcription start.

(B) Relative induction of GUS fused to promoter fragments. The fold induction is given as the GUS activity after 24 hr of treatment with 5 ppm ethylene or 5 ppm ethylene and 45.5 μM MeJA divided by the GUS activity of control plants (untreated). All data for every osmotin promoter–GUS fusion construct were obtained from several hundred seedlings of three independent transformants carrying that construct. The mean GUS activities of the control (untreated) for GUS1B and constructs 5, 8, 10, 11, 13, 16, 18, 22, and 23 were 83, 83, 136, 169, 65, 77, 60, 23, 11, and 1 nmol of methylumbelliferone per mg of protein per hr, respectively. The GUS activities of seedlings treated only with 45.5 μM MeJA for GUS1B and constructs, 5, 8, 10, 11, 13, 16, 18, 22, and 23 were 67, 98, 79, 177, 49, 88, 63, 12, 25, and 1 nmol of methylumbelliferone per mg of protein per hr, respectively. These values illustrate that several independent transformants that respond to ethylene and E/MeJA do not respond to MeJA alone.

(Figure 2A), osmotin protein accumulated to very high levels only when MeJA was combined with ethylene, and PR-1b protein did so only when MeJA was combined with SA (Figure 2B). Apparently, SA and ethylene exert some translational or protein stability effects on PR-1b and osmotin, respectively. It will be extremely interesting to compare SA to combinations of signals in their abilities to induce the accumulation of other defense proteins in different tobacco genotypes. This appears not to have been studied with specific antibody probes to date. Because our results clearly indicated that different members of the PR defense gene superfamily are not regulated by a single signal pathway and that maximal accumulation of more than one defense protein requires multiple signals, the possible role of signal combinations in inducing acquired resistance should be considered and studied.

DISCUSSION

In this study, we have demonstrated the synergistic effect of ethylene with MeJA on activating PR gene expression.

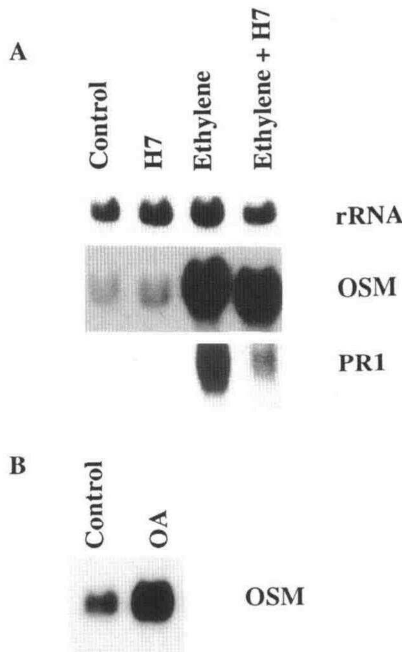


Figure 5. Comparison of the Regulation of a PR-1 Gene with That of the Osmotin Gene.

(A) H7 blocks ethylene-induced PR-1 but not osmotin (OSM) mRNA accumulation. Total RNA was extracted from leaves of 4-week-old GUS1B plants (with six expanded leaves) that were treated with 100 μM H7 with or without 12 ppm ethylene. Fifteen micrograms of total RNA was loaded onto each lane and transferred after electrophoresis to nitrocellulose. RNA was hybridized with ^{32}P-labeled probes of osmotin or PR-1 cDNAs. rRNA hybridization of the same blot is shown to illustrate the relative amount of RNA loaded and transferred.
(B) Induction of osmotin mRNA accumulation by okadaic acid. Shown is a gel blot of RNA (15 μg total RNA per lane) from untreated tobacco cells and tobacco cells treated with 0.5 μM okadaic acid (OA) after hybridization with ^{32}P-labeled osmotin cDNA.

Although both the stimulation and inhibition of ethylene biosynthesis by MeJA have been reported (Saniewski et al., 1987; Bailly et al., 1992; Chou and Kao, 1992), the ability of MeJA and ethylene to coregulate the osmotin gene is not a result of any effect of MeJA on ethylene production. As shown in Figure 1A, there is a fivefold induction of GUS activity by ethylene in GUS1B seedlings, and this induction is not increased by higher concentrations of ethylene. The ability of MeJA to increase the induction of the osmotin promoter occurs at ethylene concentrations above this level of saturation.

Mason et al. (1992) have shown that MeJA and soluble sugars synergistically stimulate the accumulation of soybean vegetative storage protein (*vsp*) mRNAs, whereas little accumulation occurred when these compounds were supplied separately. To date, this is the only report indicating a synergistic effect of MeJA with other factors in regulating gene expression. For this synergistic effect, two hypotheses are mentioned by Mason et al. (1992). One is that the sugar is a signal molecule and acts with MeJA to induce the expression of the *vsp* gene. The other is that MeJA is the signal molecule for induction of *vsp* transcription, and the sugar is an energy source to enhance this process. How does MeJA interact with ethylene and coregulate osmotin gene expression? The binding of ethylene to its receptors on the plasma membrane might sensitize MeJA receptors on the membrane. Seedlings pretreated with ethylene for several hours were then treated with MeJA in the absence or presence of further ethylene treatment. Osmotin promoter activity was synergistically induced by MeJA with continuous ethylene treatment but not by MeJA with only an ethylene pretreatment (data not shown). This indicates that the continuous presence of ethylene is required for the synergistic effect of MeJA and ethylene on inducing the osmotin gene, i.e., the continuous binding of ethylene with its receptors might be required or that a short-lived participant in signal transduction is induced.

Although several signal molecules have been shown to be involved in the induction of genes that participate in the defense against plant pathogens and pests (Lamb et al., 1989; Ward et al., 1991; Enyedi et al., 1992), none of these genes has been shown to be synergistically hyperinduced by a combination of such signals. However, some environmentally induced genes have been found to respond synergistically to multiple signals (Bostock and Quatrano, 1992). Bostock and Quatrano (1992) also suggested that NaCl sensitized plant cells to ABA, resulting in a NaCl–ABA synergistic response of the Em gene.

The demonstration of such hyperactivation raises the possibility that the functional specificity of defense genes in general may be related to particular combinations of signal molecules that produce a more specific "signature" set of inducers that initiate a specific environmental warning. Such a mechanism would allow a very complex set of specific signals by using only a handful of primary signal messengers and their primary receptors. The evolution of such a cross interactive signal transduction system could also explain the common occurrence of "cross-talk" between signaling molecules because cross-activation to produce synergistic responses would need to evolve through common biochemical recognition domains. An important question raised by such a hypothesis is the functional relationship between ordinary gene induction by single signals and hyperinduction by synergistic combinations of signals. Single-signal inductions could represent evolutionary remnants of archeotype genes, or they could be part of a tiered system involving multiple degrees of responsiveness, each tier representing a more severe and more clear warning of a changing environment that evokes a stronger gene expression response. This view is in contrast to an interesting interpretation expressed by Enyedi et al. (1992) that the signaling transduction system of plants can simply fail to distinguish signals specifically, and sometimes functionally improper genes are induced, much like the animal immune system falsely recognizes allergens as potential threats. Such

signal recognition failures could be the result of repeated counter-evolutionary responses by hosts and pests to thwart each other's attack and defense strategies.

A significant issue arising from our observation of hyperinducibility of defense genes is whether hyperinducing signal combinations can induce resistance to pathogens. This, of course, would greatly depend on the ability of a given set of hyperinducing signals to turn on the proper combination of defense genes. The ability of SA to induce multiple defense genes is consistent with the observation that resistance can be induced in plants by SA treatment. Other signals, such as ethylene, can induce multiple defense genes, and ethylene, to a limited extent, can increase resistance. Although very high levels are required to affect resistance, these levels are much higher than those usually produced by pathogen exposure (see Enyedi et al., 1992). However, our results suggest that even saturated ethylene treatment alone is not able to cause maximum gene expression changes and thereby may not affect resistance. The fact that both osmotin (PR-5) and PR-1 gene expression is synergistically induced by E/MeJA suggests that other defense genes and thus resistance might be induced by these signals.

Recent experiments have shown that the ability to synthesize SA is required to induce resistance by exposure to a pathogen (Gaffney et al., 1993). It remains to be seen whether other signals or hyperinducing signal combinations can induce resistance in the absence of SA. The E/MeJA–mediated hyperinduction of osmotin does not appear to involve SA because this level of induction cannot be achieved by SA. This is consistent with the view expressed by Enyedi et al. (1992) that at least two separate systems of induction of resistance exist, one involving pathogens through SA and another involving wounding mediated by insect damage, which often facilitates pathogen invasion. However, it remains unclear as to whether all pathogens induce defense genes and resistance through SA, or as suggested here, other hyperinducing signal combinations also may be involved.

METHODS

Plant Material

Tobacco cell cultures that are adapted to grow in the presence of 428 mM NaCl (S25) were maintained as described by Singh et al. (1987b). Homozygous seeds of tobacco transformant GUS1B, which expressed a high level of β-glucuronidase (GUS) activity (Nelson et al., 1992), were germinated on two layers of Whatman No. 1 filter paper in 9-cm sterile Petri dishes with 5 mL of 0.1 × Murashige and Skoog (MS) salts. Five-day-old seedlings were used for all treatment experiments.

Methyl Jasmonate, Ethylene, and Salicylic Acid Treatments

All treatment experiments were repeated at least three times with three replications each. For methyl jasmonate (MeJA) and salicylic acid (SA) treatments, dishes were flooded with 0.1 × MS solution containing desired MeJA (Bedoukian Research, Danbury, CT) or SA (Sigma) concentrations and decanted, and 1 mL of fresh solution was added. Dishes for control and ethylene treatments received 1 mL of 0.1 × MS solution. Ethylene treatments were made by sealing the dishes in 9-L desiccator chambers or 25-L plastic chambers. Ethylene concentrations were determined by gas chromatography.

Assay for GUS Enzyme Activity

After treatment, seedlings were extracted with the luciferase-GUS extraction buffer (50 mM K_2HPO_4, pH 7.8, 1 mM EDTA, 10 mM DTT, and 5% glycerol) using a polytron homogenizer. After centrifugation at 12,000*g* for 5 min in a microcentrifuge, the supernatant was collected and used for protein determination by the Bradford reagent assay and for measuring GUS enzyme activity. GUS activity was measured as amount of methylumbelliferone formed using 4-methylumbelliferyl β-D-glucuronide as substrate as described by Nelson et al. (1992).

Histochemical Assay for GUS Enzyme Activity

Histochemical staining for GUS activity was performed according to the procedure of Jefferson et al. (1987), with the modifications proposed by Koltunow et al. (1990). Seedlings were fixed for 5 min in fixing solution (0.1 M Na_2HPO_4, pH 7.0, 0.1% formaldehyde, 0.1% Triton X-100, and 0.1% β-mercaptoethanol) and then rinsed twice with 0.1 M Na_2HPO_4, pH 7.0, containing 0.1% β-mercaptoethanol, followed by a rinse with 0.05 M Na_2HPO_4, pH 7.0. For the GUS reaction assay, a buffered solution (0.05 M Na_2HPO_4, pH 7.0) of 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium salt (Biosynth AG, Staat, Switzerland) containing 0.1% β-mercaptoethanol and 0.1% Triton X-100 was used. After the GUS reaction, seedlings were fixed for 2 hr in a solution with 50% ethanol, 10% formaldehyde, and 5% acetic acid. Chlorophyll was removed by 95% ethanol, followed by 70% ethanol.

RNA Extraction and Gel Blot Hybridization

Tissues were frozen in liquid N_2 and ground to a fine powder with a mortar and pestle. Total RNA was extracted by the guanidine isothiocyanate method as described by Casas et al. (1992). Fifteen micrograms of total RNA was loaded to a formaldehyde–formamide denatured 1.2% agarose gel and then transferred to nitrocellulose membrane as described by Sambrook et al. (1989). Hybridization conditions were as described (Singh et al., 1989a). ^{32}P-labeled DNA probes were prepared from osmotin and pathogenesis-related PR-1b coding regions by random primer labeling. Filters were washed at room temperature in 2 ×, 1 ×, and 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) with 0.1% SDS sequentially and exposed to x-ray films.

Protein Gel Blot Detection of Osmotin and PR-1

Seedlings were ground in liquid N_2 and proteins were precipitated with ice-cold acetone. Osmotin protein was detected on gel blots of proteins that were separated by SDS-PAGE and reacted with polyclonal anti-osmotin antibody (Singh et al., 1987b) or monoclonal anti-PR-1 antibody.

ACKNOWLEDGMENTS

We wish to thank Jean Clithero for excellent technical assistance. We also thank Dan Klessig, Rutgers University, Piscataway, NJ, for the generous gift of the PR-1 antibody. This research was supported in part by National Science Foundation Grant No. DCB 9005216, a Rockefeller Foundation Fellowship to D.L., and a McKnight Foundation Fellowship to P.-F.L.C. This is Journal Paper Number 14,220 of the Purdue University Agricultural Experiment Station.

Received March 11, 1994; accepted June 2, 1994.

REFERENCES

Alexander, D., Goodman, R.M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Ahl-Goy, P., Luntz, T., Ward, E., and Ryals, J. (1993). Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. Proc. Natl. Acad. Sci. USA **90**, 7327–7331.

Bailly, C., Corbineau, F., and Come, D. (1992). The effect of abscisic acid and methyl jasmonate on 1-aminocyclopropane-1-carboxylic acid conversion to ethylene in hypocotyl segments of sunflower seedlings, and their control by calcium and calmodulin. J. Plant Growth Regul. **11**, 349–355.

Bol, J.F., Linthorst, H.J.M., and Cornelissen, B.J.C. (1990). Plant pathogenesis-related proteins induced by virus infection. Annu. Rev. Phytopathol. **28**, 113–138.

Bostock, R.M., and Quatrano, R.S. (1992). Regulation of Em gene expression in rice. Plant Physiol. **98**, 1356–1363.

Bray, E.A. (1993). Molecular responses to water deficit. Plant Physiol. **103**, 1035–1040.

Brederode, F.T., Linthorst, H.J.M., and Bol. J.F. (1991). Differential induction of acquired resistance and PR gene expression in tobacco by viral infection, ethephon treatment, UV light and wounding. Plant Mol. Biol. **17**, 1117–1125.

Broglie, K., Chet, I., Hollidat, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C.J., and Broglie, R. (1991). Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science **254**, 1194–1197.

Casas, A.M., Nelson, D.E., Raghothama, K.G., Paino, D.M., Singh, N.K., Bressan, R.A., and Hasegawa, P.M. (1992). Expression of osmotin-like genes in the halophyte *Atriplex nummularia* L. Plant Physiol. **99**, 329–337.

Chou, C.M., and Kao, C.H. (1992). Stimulation of 1-aminocyclopropane-1-carboxylic acid-dependent ethylene production in detached rice leaves by methyl jasmonate. Plant Sci. **83**, 137–141.

Cutt, J.R., and Klessig, D.F. (1992). Pathogenesis-related proteins. In Genes Involved in Plant Defense, T. Boller and F. Meins, eds (New York: Springer-Verlag), pp. 209–243.

Enyedi, A.J., Yalpani, N., Paul, S., and Raskin, I. (1992). Signal molecules in systemic plant resistance to pathogens and pests. Cell **70**, 879–886.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261**, 754–756.

Gianinazzi, S., Martin, C., and Vallée, J.C. (1970). Hypersensibilité aux virus, température et protéines solubles chez le *Nicotiana Xanthi* n.c. Apparition de nouvelles macromolécules lors de la répression de la synthése virale. C.R. Acad. Sci. Paris **D270**, 2283–2386.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: β-Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6**, 3901–3907.

Koltunow, A.M., Truettner, J., Cox, K.H., Wallroth, M., and Goldberg, R.B. (1990). Different temporal and spatial gene expression patterns occur during anther development. Plant Cell **2**, 1201–1224.

Kononowicz, A.K., Raghothama, K.G., Casas, A.M., Reuveni, M., Watad, A.-E.A., Liu, D., Bressan, R.A., and Hasegawa, P.M. (1993). Osmotin: Regulation of gene expression and function. In Plant Response to Cellular Dehydration during Environmental Stress, T.J. Close and E.A. Bray, eds (Rockville, MD: American Society of Plant Physiologists), pp. 144–158.

Lamb, C.J., Lawton, M.A., Dron, M., and Dixon, R.A. (1989). Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell **56**, 215–224.

LaRosa, P.C., Chen, Z., Nelson, D.E., Singh, N.K., Hasegawa, P.M., and Bressan, R.A. (1992). Osmotin gene expression is posttranscriptionally regulated. Plant Physiol. **100**, 409–415.

Liu, D., Raghothama, K.G., Hasegawa, P.M., and Bressan, R.A. (1994). Resistance to the pathogen *Phytophthora infestans* in transgenic potato plants that over-express osmotin. Proc. Natl. Acad. Sci. USA **91**, 1888–1892.

Mason, H.S., DeWald, D.B., Creelman, R.A., and Mullet, J.E. (1992). Coregulation of soybean vegetative storage protein gene expression by methyl jasmonate and soluble sugars. Plant Physiol. **98**, 859–867.

Nelson, D.E., Raghothama, K.G., Singh, N.K., Hasegawa, P.M., and Bressan, R.A. (1992). Analysis of structure and transcriptional activation of an osmotin gene. Plant Mol. Biol. **19**, 577–588.

Raghothama, K.G., Liu, D., Nelson, D.E., Hasegawa, P.M., and Bressan, R.A. (1993). Analysis of an osmotically-regulated pathogenesis-related osmotin gene promoter. Plant Mol. Biol. **23**, 1117–1128.

Raz, V., and Fluhr, R. (1993). Ethylene signal is transduced via protein phosphorylation events in plants. Plant Cell **5**, 523–530.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Saniewski, M., Nowacki, J., and Czapski, J. (1987). The effect of methyl jasmonate on ethylene production and ethylene-forming enzyme activity in tomatoes. J. Plant Physiol. **129**, 175–180.

Singh, N.K., Bracker, C.A., Hasegawa, P.M.,Handa, A.K., Buckel, S., Hermodson, M.A., Pfankoch, E., Regnier, F.E., and Bressan, R.A. (1987a). Characterization of osmotin. Plant Physiol. **85**, 529–536.

Singh, N.K., LaRosa, P.C., Handa, A.K., Hasegawa, P.M., and Bressan, R.A. (1987b). Hormonal regulation of protein synthesis associated with salt tolerance in plant cells. Proc. Natl. Acad. Sci. USA. **84**, 739–743.

Singh, N.K., Nelson, D.E., Kuhn, D., Hasegawa, P.M., and Bressan, R.A. (1989a). Molecular cloning of osmotin and regulation of its expression by ABA and adaptation to low water potential. Plant Physiol. **90**, 1096–1101.

Singh, N.K., Nelson, D.E., LaRosa, P.C., Bracker, C.E., Handa, A.K., Hasegawa, P.M., and Bressan, R.A. (1989b). Osmotin: A protein associated with osmotic stress adaptation in plant cells. In Environmental Stress in Plants, NATO ASI Series, Vol G19, J.H. Cherry, ed (Heidelberg: Springer-Verlag), pp. 67–87.

Skriver, K., and Mundy, J. (1990). Gene expression in response to abscisic acid and osmotic stress. Plant Cell **2**, 503–512.

Stintzi, A., Heitz, T., Kauffmann, S., Legrand, M., and Fritig, B. (1991). Identification of a basic pathogenesis-related thaumatin-like protein of virus-infected tobacco as osmotin. Physiol. Mol. Plant Pathol. **38**, 137–146.

Thomas, J.C., and Bohnert, H.J. (1993). Salt stress perception and plant growth regulators in the halophyte *Mesembryanthemum crystallinum.* Plant Physiol. **103**, 1299–1304.

Van Loon, L.C., and Van Kammen, A. (1970). Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. 'Samsun' and 'Samsun NN.' II. Changes in protein constitution after infection with tobacco mosaic virus. Virology **40**, 199–211.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Métraux, J.-P., and Ryals, J.A. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3**, 1085–1094.