Plant Physiol. (1993) 102: 503–511

Effect of Jasmonic Acid on the Interaction of Barley (*Hordeum vulgare* L.) with the Powdery Mildew *Erysiphe graminis* f.sp. *hordei*

Patrick Schweizer*[1], René Gees, and Egon Mösinger

Sandoz Agro Ltd., Agrobiological Research Station, CH-4108 Witterswil, Switzerland

Jasmonic acid or its methyl ester induce de novo synthesis of a number of proteins of mostly unknown function in barley (*Hordeum vulgare* L.). In a topical spray application, 30 μg of jasmonic acid per plant effectively protected barley against subsequent infection by *Erysiphe graminis* f.sp. *hordei*. Examination of jasmonic acid-induced barley proteins revealed the presence of several acid-soluble (pH 2.8) proteins. Two prominent groups of 25 kD and 10 to 12 kD apparent molecular mass were present in the intercellular washing fluid. The set of extracellular, induced proteins showed no similarity to barley pathogenesis-related proteins. An in vivo test against *E. graminis* revealed no antifungal activity of the extracellular jasmonic acid-induced proteins. Experiments with the transcription inhibitor cordycepin showed no correlation between accumulation of jasmonic acid-induced proteins and protection. The application of jasmonic acid and *E. graminis* simultaneously resulted in independent extracellular accumulation of both jasmonic acid-induced proteins and of pathogenesis-related proteins. The data suggest that jasmonic acid directly inhibits appressoria differentiation of the fungus, and that it is not involved in the signal transduction mechanism leading to induction of pathogenesis-related proteins.

Jasmonates, i.e. JA or its methyl ester, occur in many plant species (Meyer et al., 1984) and have several effects on plants (see Parthier, 1990, for review). They accelerate leaf senescence (Ueda and Kato, 1980; Weidhase et al., 1987; Chou and Kao, 1992), inhibit pollen (Yamane et al., 1982) and seed (Wilen et al., 1991) germination, and induce accumulation of vegetative storage proteins (Anderson et al., 1989; Mason and Mullet, 1990) and of mRNAs encoding late embryogenesis-abundant proteins (Reinbothe et al., 1992a, 1992b). Furthermore, jasmonates induce accumulation of a number of proteins (JIPs) of unknown function in many plant species (Anderson, 1988; Herrmann et al., 1989; Reinbothe et al., 1992b) including barley (*Hordeum vulgare* L.) (Weidhase et al., 1987; Mueller-Uri et al., 1988; Maslenkova et al., 1992). The barley JIPs were named JIP 110, JIP 66, JIP 37, JIP 30, JIP 23, and JIP 10/12 according to the apparent molecular mass (in kD) in denaturing polyacrylamide gels. With one exception (Andresen et al., 1992), no function has been attributed to the barley JIPs.

Recently, JA has been proposed as an intracellular signal transducer in wounded or pathogen-attacked plants. This hypothesis is based on the observation that exogenously applied jasmonate induces wound-responsive (Creelman et al., 1992; Farmer et al., 1992; Farmer and Ryan, 1992; Hildmann et al., 1992) and pathogen-responsive (Dittrich et al., 1992; Gundlach et al., 1992) genes in several plants and secondary metabolites in suspension-cultured cells of 36 monocot and dicot species (Gundlach et al., 1992). Moreover, wounding (Creelman et al., 1992) and elicitor treatment (Gundlach et al., 1992) have been reported to cause a rapid increase of the endogenous JA pool.

Here we describe the protective effect of JA on barley against Egh, the powdery mildew fungus of barley. This protection is discussed with respect to JIP induction and a possible role of JA as signal transducer in the barley/Egh interaction. The results suggest a direct antifungal effect of JA. Furthermore, JA seems not to be involved in the signal transduction chain leading to PR protein induction during Egh infection.

MATERIALS AND METHODS

Plant and Fungal Material

Barley (*Hordeum vulgare* L.) seedlings, cv Golden Promise, without any known powdery mildew resistance gene, were grown in a growth chamber (25°C, 55% RH, 16 h of light, 20 plants/pot). Six-day-old plants were sprayed with 2 mL/pot of solutions of JA in chloroform:methanol (2:1). Under these conditions, at a JA concentration of 1 mg/mL, approximately 30 μg of JA was deposited on each plant. (±)JA was synthesized at Sandoz Agro Ltd.

The plants were challenge inoculated immediately after evaporation of the solvent, or 1 to 3 d later, with a mixed population of Egh. The pathogen was maintained on cv Golden Promise by weekly inoculation of fresh plants. Inoculation was done by blowing conidia from 15 to 20 heavily mildew-infected barley leaves into an inoculation tower over the test plants, which had been placed on a rotating platform. The vertical distance between blown-in inoculum and test plants was 1.05 m. After 10 min, the inoculated plants were transferred to a growth chamber (17°C, 55% RH, 16 h of

[1] Present address: Université de Fribourg, Institut de Biologie Végétale, 3, rue Albert Gockel, CH-1700 Fribourg, Switzerland.

* Corresponding author; fax 41–37–826–439.

Abbreviations: Egh, *Erysiphe graminis* f.sp. DC. *hordei* Em Marchal; IWF, intercellular washing fluid; JA, jasmonic acid; JIP, jasmonate-induced protein; 1-D, one-dimensional; PR, pathogenesis-related; 2-D, two-dimensional.

light). Disease severity on the apical half of primary leaves was monitored 7 d after inoculation by visually estimating the proportion of leaf area covered by mildew pustules.

For the induction of PR proteins and for microscopy, 6-d-old plants were inoculated by brushing two pots of Egh-infected plants directly over each pot of test plants.

Light Microscopy

Inoculated leaves were bathed in staining solution (0.3% [w/v] Coomassie G-250, 7.5% [w/v] TCA, 50% [v/v] methanol) for 10 min at room temperature. After dipping leaves in water to remove excess stain, the Coomassie-stained fungal structures were observed by light microscopy.

Isolation of JIPs and PR Proteins

All steps of the procedures were carried out at 0 to 4°C. IWF containing acid-soluble JIPs or PR proteins was isolated from primary leaves at pH 2.8 as described for extracellular peroxidase (Schweizer et al., 1989), except that the ammonium sulfate-precipitated proteins were resuspended (in 50 mM Tris-Cl, pH 7.5, 20% [v/v] glycerol) and desalted over a spin column of Sephadex G-25, equilibrated with the same buffer.

For tests of antifungal activity, ammonium sulfate-precipitated proteins in the IWF were resuspended in 5 mM sodium phosphate, pH 6.0, and desalted over Sephadex G-25, equilibrated with the same buffer.

Homogenates were prepared from 1 g fresh weight of primary leaves in 5 mL of IWF buffer (Schweizer et al., 1989) or in 10 mL of 50 mM Tris-Cl, pH 7.5, containing 1 mM $MgCl_2$, 0.1 mM PMSF, 0.1% (v/v) 2-mercaptoethanol, and 0.5 g of polyvinylpolypyrrolidone. The filtered homogenate was centrifuged for 10 min at 25,000*g*. Proteins in the supernatant were precipitated with 0.5 g/mL of ammonium sulfate, and the pellets were desalted as described for the IWF.

PAGE of Proteins

Barley proteins were separated on 1-D 15% polyacrylamide-SDS gels (Laemmli, 1970) and stained with Coomassie blue. 2-D PAGE (IEF) was done using a Mini-Protean II 2-D Cell (Bio-Rad, Richmond, CA) according to the supplier's protocol. Conditions for separation in the second dimension were identical to those for 1-D gels. Proteins were silver stained according to the PhastSystem protocol (Pharmacia LKB, Uppsala, Sweden). Immunoblotting of proteins was done according to Towbin and Gordon (1984).

In Vivo Test for Antifungal Activity of Proteins in IWF

Barley leaf segments of 1 cm were placed on 0.5% agar in water in 24-well plates. Buffered protein solutions were sprayed onto each leaf segment, using an air-pressured microspraying device (DeVilbiss, Dietzenbach, Germany). Four replica leaf segments were treated on each plate with 1, 2, or 4 μg of BSA or IWF-localized proteins from barley. Eight control leaf segments on each plate were sprayed with buffer alone (5 mM sodium phosphate, pH 6.0, no wetting agent). After the surfaces of the leaf segments were dry, the segments were inoculated with Egh as described in "Plant and Fungal Material." Seven days after inoculation, the disease was monitored as described in "Plant and Fungal Material."

Cordycepin Treatment

Immediately after spray treatment with JA, shoots were cut at the stem base and each was transferred to 1 mL of distilled water containing 50 μg/mL of ampicillin and 0 to 0.8 mM cordycepin in glass tubes (6 cm high). For light microscopy, shoots were immediately inoculated with Egh and incubated as described in "Plant and Fungal Material." Development of the fungus on primary leaves was observed 24 h after inoculation. For the determination of JIP induction, primary leaves from noninoculated shoots were homogenized in IWF buffer 24 h after JA treatment.

RESULTS

Local Protection of Barley by JA

In a topical spray application, JA effectively protected barley primary leaves against Egh at a concentration of 1 mg/mL, equivalent to approximately 30 μg/plant (Fig. 1A). The solvent alone did not cause protection (data not shown). JA at 1 mg/mL did not cause phytotoxicity except for weak chlorosis; at 10 mg/mL, it caused necrosis of leaf tips. The spray application was most effective when the plants were challenge inoculated with Egh immediately after treatment (Fig. 1B). The decline in protection with increasing time between JA treatment and challenge inoculation clearly demonstrates that protection was not the result of steadily progressing, JA-induced leaf senescence (Weidhase et al., 1987).

Light microscopic analysis showed no inhibition of Egh

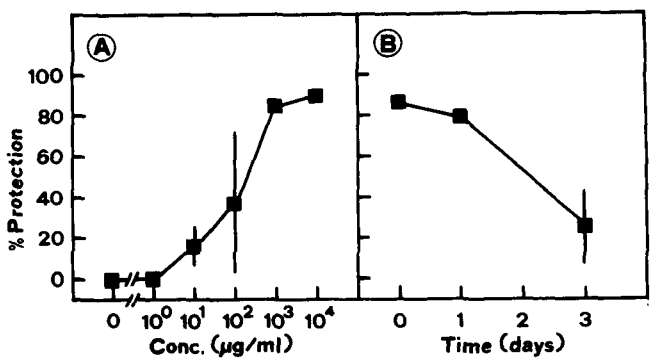

Figure 1. Protection of barley primary leaves by JA against Egh. A, JA was applied as a topical spray application in chloroform:methanol (2:1, v/v) at concentrations from 1 to 10,000 μg/mL. After the solvent had evaporated, plants were challenge inoculated with Egh. Seven days after inoculation, disease on primary leaves was monitored visually by estimating the percentage of leaf area covered with mildew pustules. B, JA was sprayed at a concentration of 1,000 μg/mL and the plants were challenge inoculated immediately after the solvent had evaporated, or 1 to 3 d later. A and B, The mean values of two independent experiments with approximately 25 plants per treatment are shown. Vertical bars indicate the range (difference between maximum and minimum value) and are omitted when they are smaller than the symbol size.

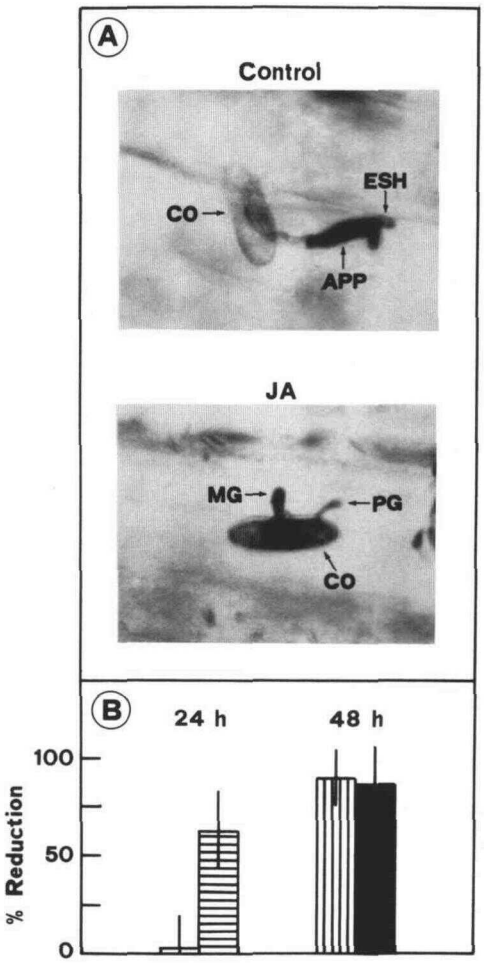

Figure 2. Effect of JA on early development of Egh. A, Coomassie-stained fungus on the surface of primary leaves was observed 24 h after inoculation of control plants or 1 mg/mL JA-treated plants. Plants were inoculated immediately after the solvent had evaporated. CO, Conidium; PG, primary germ tube; APP, appressorium; ESH, elongating secondary hyphae; MG, malformed germ tube. B, Germination (□) and formation of differentiated appressoria (■) on JA-treated leaves 24 h after inoculation; formation of elongating secondary hyphae (▥) and penetration efficiency (■) on JA-treated leaves 48 h after inoculation; percent reduction relative to development of nontreated leaves. Mean values from three independent experiments ± range are shown. A total of 409 to 637 conidia were observed per treatment 24 h after inoculation; a total of 621 to 674 conidia were observed per treatment 48 h after inoculation.

germination, but strong inhibition of appressoria differentiation on JA-treated barley leaves (Fig. 2B). Instead of mature appressoria, short, malformed germ tubes were formed in the presence of JA (Fig. 2A). Besides inhibition of appressoria differentiation, JA also reduced penetration efficiency (Fig. 2B). The solvent alone did not influence early development of Egh (data not shown). These results indicate either direct antifungal activity and instability on barley leaves of JA or rapid induction of transient plant defense reactions. A combination of direct and indirect effects of JA cannot be excluded either.

Induction of Extracellular Proteins by JA

Plant defense mechanisms against fungal development on the leaf surface or against fungal penetration must be postulated to be extracellular. Therefore, we analyzed the IWF from JA-treated primary leaves for accumulation of new proteins. Acidic IWF from plant leaves contains soluble, and probably also loosely cell-wall bound, apoplastic proteins. For two reasons, such proteins might be relevant for defense against Egh, although the fungus grows on the leaf surface. First, the primary germ tube of Egh comes into contact with the apoplastic space by penetrating the leaf cuticle (Kunoh et al., 1987). The primary germ tube seems to perceive a stimulus for appressoria formation, and damage of the primary germ tube by apoplastic antifungal components might inhibit appressoria differentiation (Carver and Ingerson, 1987). Second, the appressorial penetration peg breaches the epidermal cell wall and is, therefore, in close contact with apoplastic plant components (e.g. Takahashi et al., 1985).

Topical spray application of JA at 1 mg/mL caused massive accumulation of acid-soluble proteins in the IWF (Fig. 3, lane 2). The proteins had apparent molecular masses of 25 kD and of 10 to 12 kD. Reextraction of leaves used for isolation of IWF revealed three additional, acid-soluble proteins of 37, 30, and 16 kD (lane 4). This and the absence of two constitutive IWF-localized proteins of 22 to 23 kD in the reextracted proteins (compare lanes 1 and 3) show that the 25-kD and the 10- to 12-kD proteins did not merely leak from damaged

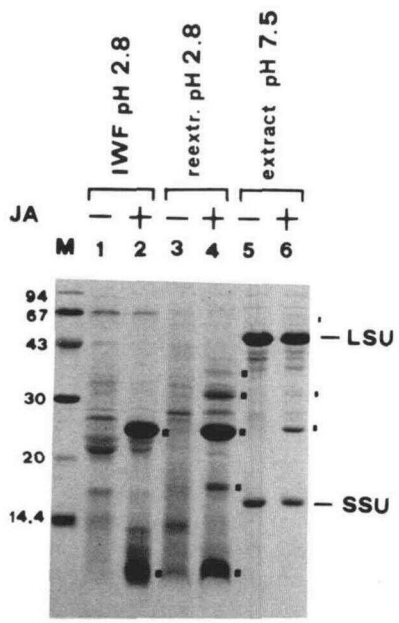

Figure 3. Induction and localization of barley JIPs. Acid-soluble proteins in the IWF of primary leaves (IWF pH 2.8) or proteins in total leaf homogenates (extract pH 7.5) were separated by SDS-PAGE and stained with Coomassie blue. After isolation of IWF, the same leaves were reextracted by homogenization (reextr. pH 2.8). Proteins were isolated from control leaves (–) or 3 d after spray treatment with JA (1 mg/mL, +). JIPs are marked by dots to the right of their respective lanes. LSU, Large subunit of Rubisco; SSU, small subunit of Rubisco; M, molecular mass markers.

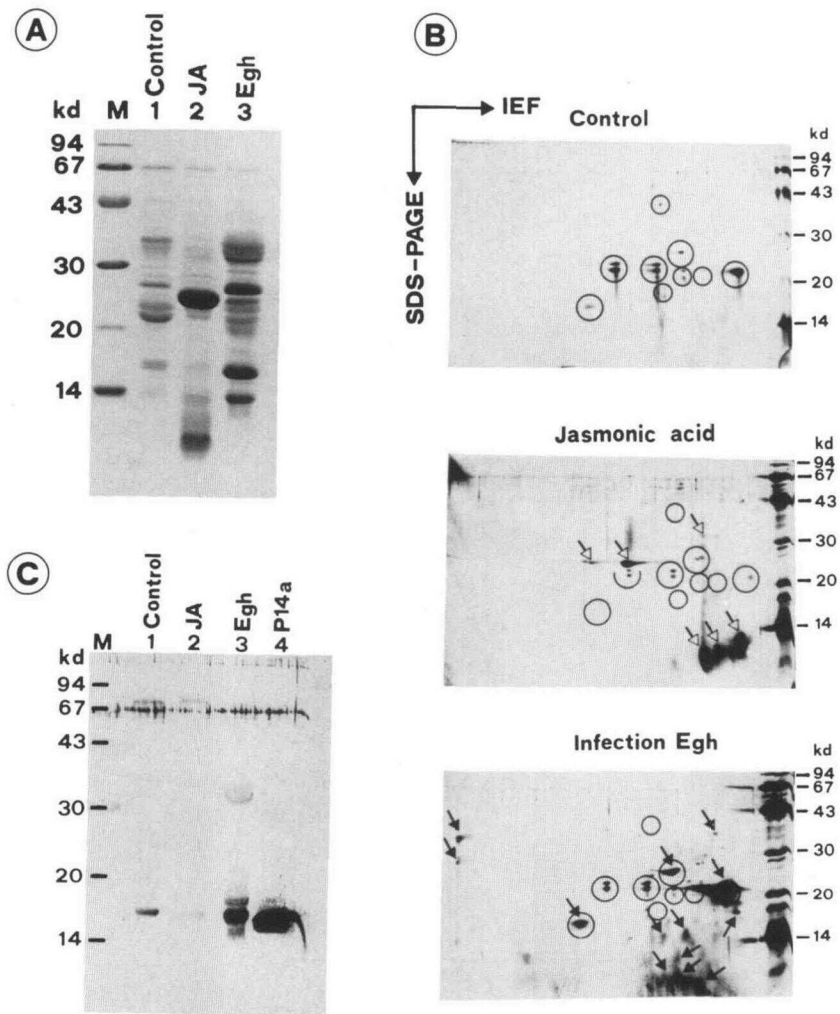

Figure 4. Comparison of JIPs to PR proteins. A, IWF-localized proteins were separated by 1-D SDS-PAGE and stained with Coomassie blue. IWF from control leaves (lane 1), from leaves 3 d after JA (1 mg/mL) treatment (lane 2), and from leaves 3 d after Egh inoculation (lane 3) were compared. Proteins equivalent to 0.5 g leaf fresh weight were loaded per lane. M, Molecular mass marker. B, The same protein samples as in A were separated on 2-D IEF SDS-PAGE and silver stained. The pH range for IEF was from pH 9 (left) to pH 5 (right). Proteins equivalent to 0.5 g leaf fresh weight (except jasmonic acid, 0.25 g leaf fresh weight) were loaded per gel. Positions of proteins in control IWF are circled in all three gels to facilitate orientation; JIPs and PR proteins are marked by open and closed arrows, respectively. C, Immunoblot of the IWF-localized proteins shown in A. Proteins of the PR1 group were detected by antiserum against tomato P14 (Fischer W et al., 1989). As a positive control, 1 μg of tomato P14 was loaded in lane 4. M, Molecular mass markers.

cells during IWF isolation. For unknown reasons, the 16-kD protein was not always present in JA-treated leaves. The presence of the 25-kD and the 10- to 12-kD proteins in both the IWF and homogenate from reextracted leaves suggests extracellular and intracellular localization, possibly reflecting differential targeting of JA-induced protein families. Comparison with a total leaf extract of pH 7.5 revealed strong enrichment of the 25-kD and the 10- to 12-kD proteins in the acid-soluble fractions (compare lanes 2 and 4 to lane 6), whereas an additional protein of 66 kD was present only in the neutral leaf extract. The solvent alone did not induce accumulation of any proteins (data not shown).

The apparent molecular masses of the set of JA-induced proteins described here are very similar to those of the barley JIPs (Mueller-Uri et al., 1988). Therefore, we will refer to the proteins as JIPs 10/12, 23, 30, 37, and 66, according to Mueller-Uri et al. (1988). We believe that the 25-kD protein described here is identical to barley JIP 23, because both protein bands are the most abundant JIPs in barley leaves (Andresen et al., 1992). The group of low molecular mass proteins is referred to as JIP 10/12 because these proteins were poorly separated in the gel system used.

Comparison of the IWF-localized JIPs with IWF-localized PR proteins, which accumulated in barley leaves during Egh infection, revealed no similarity in the protein patterns (Fig. 4, A and B). The proteins accumulating in infected leaves were clearly plant encoded because they also accumulated in leaves treated with a synthetic chemical compound (data not shown). Our finding that JIPs 23 and 10/12 are composed of several proteins is in agreement with an earlier report (Mueller-Uri et al., 1988).

Immunoblots of IWF-localized JIPs and PR proteins were probed with an antiserum raised against a PR protein of group 1 (P14 of tomato, Fischer W et al., 1989). The antiserum detected a barley protein of 16 kD (Fig. 4C). There was a background level of barley PR1 in control leaves and a strong accumulation in Egh-infected leaves but no increased levels in JA-treated leaves. These data led us to the conclusions that JA does not induce IWF-localized PR proteins in barley and that the extracellular JIPs do not represent a subset of PR proteins.

The kinetics of accumulation of extracellular JIPs showed detectable amounts of JIPs 23 and 10/12 at 9 h after JA treatment (Fig. 5). At 9 h after inoculation, Egh appressoria were not yet fully differentiated under the growth conditions described here (data not shown). Thus, the extracellular

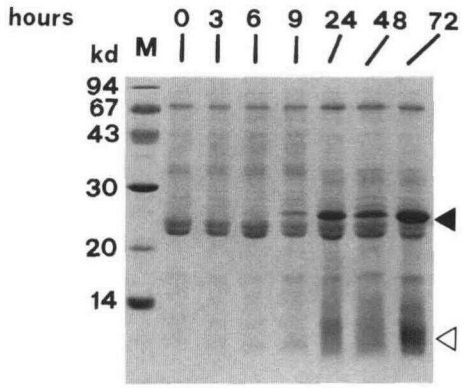

Figure 5. Kinetics of accumulation of extracellular JIPs. IWF was isolated 0, 3, 6, 9, 24, 48, and 72 h after JA (1 mg/mL) treatment, separated on SDS-PAGE, and stained with Coomassie blue. Proteins equivalent to 0.5 g leaf fresh weight were loaded per lane. The closed and open arrowheads mark the positions of JIP 23 and JIP 10/12, respectively. M, Molecular mass markers.

accumulation of JIPs might have been responsible for reduced appressoria differentiation and reduced penetration efficiency of Egh, even when plants were challenge inoculated immediately after JA treatment (Fig. 2B). In contrast, no correlation between massive accumulation of JIPs and protection was evident when plants were challenge inoculated 3 d after JA treatment (Fig. 1B).

Test for Antifungal Activity of Extracellular JIPs

To obtain functional evidence for or against involvement of extracellular JIPs in protection by JA, we tested the IWF from JA-treated plants for antifungal activity against Egh in an in vivo test on barley leaf segments. BSA and IWF from control plants were tested for comparison. In this test, IWFs from control and JA-treated leaves had antifungal activity that was reduced by heat treatment (Fig. 6A). We assume that the heat-sensitive part of the antifungal activity was protein based and therefore think that the test described here allows functional analysis of putative antifungal proteins against an obligate biotrophic pathogen. At a protein concentration of 4 μg/leaf segment, an effect, probably nonspecific, of BSA was observed that was weaker than the effect of the IWFs. The heat-sensitive antifungal activity in the IWF from JA-treated leaves was smaller than the same activity in the IWF from control leaves.

From these data, together with the results from microscopic analysis of development of Egh (Fig. 6B), two conclusions can be drawn. First, barley contains constitutively expressed, heat-sensitive antifungal components against Egh in the extracellular space. The fungus efficiently formed appressoria and secondary elongating hyphae on control leaves, which indicates that the constitutive antifungal components were more active on the leaf surface, where they were in close contact with the fungus, than inside the leaf. Second, the reduced differentiation of appressoria and formation of secondary elongating hyphae on JA-treated leaves was not correlated to enhanced, heat-sensitive antifungal activity in the IWF from JA-treated leaves. This argues strongly against involvement of the massively accumulating, extracellular JIPs 23 and 10/12 in JA-mediated protection. The weaker antifungal activity, compared with IWF from control leaves, might have been due to dilution of constitutive antifungal proteins by JIPs 23 and 10/12. IWF from JA-treated leaves also did not inhibit in vitro mycelial growth of 10 phytopathogenic fungi or of *Erwinia carotovora* and *Xanthomonas campestris* (data not shown). IWF from JA-treated leaves prepared with water (pH approximately 5.5) was not more active than acidic IWF (pH 2.8, data not shown).

Protection by JA in the Presence of Cordycepin

Although extracellular JIPs were probably not antifungal, JA might have induced other defense mechanisms. To examine this question, we inhibited host transcription with cordycepin and analyzed fungal development in the presence or absence of JA (Fig. 7A). Cordycepin was found to be a useful inhibitor for the study of host response in a barley coleoptile/Egh system (W.R. Bushnell, personal communication). Treatment of cut barley shoots with cordycepin did not inhibit appressoria differentiation. On the other hand, JA also inhibited appressoria differentiation on cordycepin-treated shoots. The concentrations of cordycepin used for microscopic analysis clearly inhibited accumulation of JIP 23 as a marker for transcriptionally regulated gene products (Fig. 7B). Although the degree of inhibition at 0.4 mM cordycepin

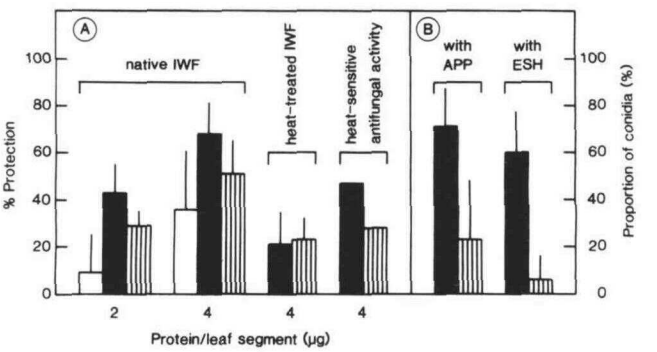

Figure 6. Test for antifungal activity of IWFs. A, BSA (□) or barley IWF (2 or 4 μg of protein/leaf segment) from control leaves (■) or JA-treated leaves (▥) was sprayed onto barley leaf segments. After the leaf surfaces were air dried, they were inoculated with Egh and disease was monitored as described in Figure 1. The heat-treated IWF was autoclaved for 20 min and centrifuged, and the supernatant was sprayed onto leaf segments. The heat-sensitive antifungal activity of the IWFs was calculated by subtracting the activity of the heat-treated IWFs from the activity of the native IWFs. Mean values from three independent experiments and one-half the range are shown. B, Development of Egh on whole plants to be related to antifungal activity of IWFs (A). Microscopic analysis of fungal development on control (■) and JA-treated (▥) primary leaves 48 h after inoculation. APP, Appressorium; ESH, elongating secondary hyphae. The results are expressed as percent of conidia having formed APPs and ESH. Mean values from five (■) and three (▥) independent experiments with a total of 902 and 621 counted conidia, respectively, are shown. Vertical bars represent one-half the range.

varied between approximately 50 and 100%, these results suggest that the inhibition of appressoria differentiation was not mediated by transcriptionally regulated plant defense mechanisms.

Independent Induction of JIPs and PR Proteins

As shown with 1-D and 2-D polyacrylamide gels (Fig. 4, A and B), JA and Egh induced completely different sets of acid-soluble proteins in the IWF. To see whether JA influenced PR protein induction by Egh and vice versa, we compared accumulation kinetics of JIPs and PR proteins after JA treatment, Egh infection, or both (Fig. 8). Applying JA and Egh simultaneously to barley leaves resulted in induction of both sets of proteins with the same kinetics as when the agents were applied alone. Therefore, JA did not influence PR protein induction by Egh or vice versa. There might be some negative interference between the treatments, resulting in less JIP 10/12 accumulation at d 3 and 4.

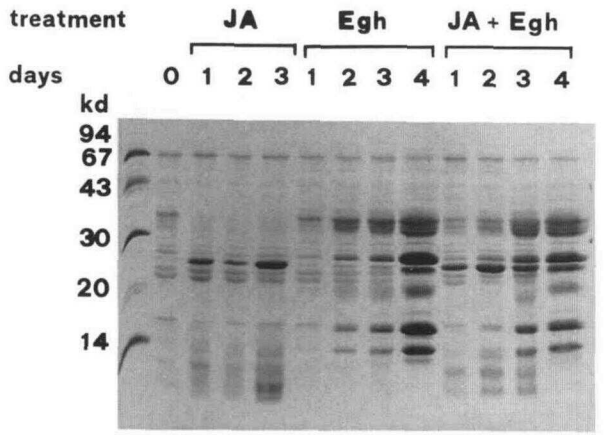

Figure 8. Kinetics of JIP and PR protein induction by JA and Egh, respectively. Six-day-old plants were spray treated with JA (1 mg/mL) and/or inoculated with Egh at day 0. IWF was isolated on the days indicated above the gel. Proteins (equivalent to 0.5 g leaf fresh weight) were separated by SDS-PAGE and stained with Coomassie blue.

DISCUSSION

JA and its methyl ester have been reported to induce a number of wound- or pathogen-responsive plant defense genes (Andresen et al., 1992; Creelman et al., 1992; Dittrich et al., 1992; Farmer et al., 1992; Gundlach et al., 1992). Furthermore, octadecanoid precursors of JA strongly induced proteinase inhibitors in tomato (Farmer and Ryan, 1992). Lipoxygenase, which is involved in the proposed biosynthetic pathway of JA, has been found to be induced in bean by an avirulent race of *Pseudomonas syringae* (Croft et al., 1990), in tobacco cells by elicitor treatment (Rickauer et al., 1990), and in soybean by methyl jasmonate (Stephenson et al., 1992). Therefore, there might exist an autocatalytic, JA-mediated signaling pathway in pathogen-attacked plants. Indeed, a role of JA in signal transduction after wounding or pathogen attack has recently been proposed (Farmer and Ryan, 1992). However, the potential of jasmonates to protect plants against pathogens or herbivores by activating plant defense responses remains to be examined.

It is well known that resistance in cereals against *E. graminis* can be induced by a primary inoculation with compatible, incompatible, or nonpathogenic powdery mildew (e.g. Schweizer et al., 1989, and refs. therein). The rationale behind our approach was to apply directly a possible signal transducer and to test its potential to induce resistance against

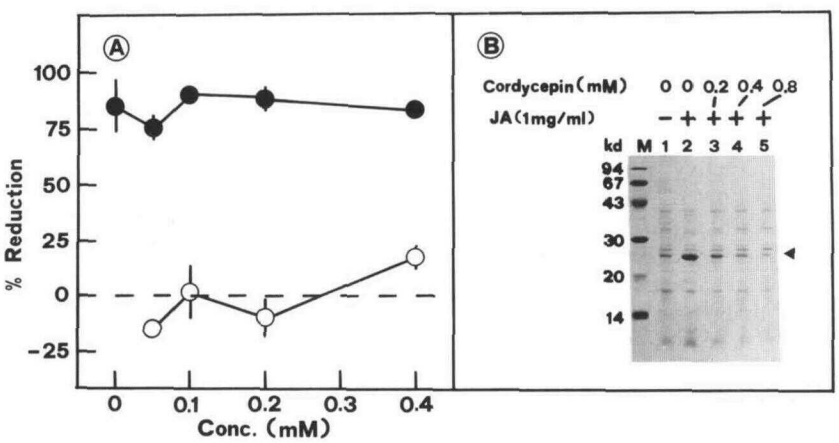

Figure 7. Reduction of appressoria formation by JA on cordycepin-treated barley leaves. A, JA (1 mg/mL) was sprayed onto 6-d-old barley plants that were subsequently cut, and the shoots were placed on solutions containing the indicated concentrations of cordycepin. Inoculation by Egh was performed immediately after transferring shoots to cordycepin solution. Egh appressoria formation was determined by light microscopy 24 h after inoculation. O, Control; ●, JA-treated. Mean values of two independent experiments are shown. Vertical bars represent the range and are omitted when they are smaller than the symbol size. An average of 246 conidia was observed per treatment. B, Cordycepin-dependent repression of JIP 23 induction in cut shoots. Total leaf homogenate at pH 2.8 was prepared 24 h after spray treatment with JA (1 mg/mL) and proteins were separated by SDS-PAGE and stained with Coomassie blue. Four micrograms of protein were applied per lane. M, Molecular mass markers.

Egh. Indeed, the observed protection by JA first suggested an important role of JA in signal transduction, leading to accumulation of barley defense gene products. However, several lines of evidence pointed to a direct antifungal effect of JA and argued against induced resistance. First, protection by JA rapidly declined with time, whereas local protection of barley by primary inoculation with the nonpathogenic *E. graminis* f.sp. *tritici* persisted for at least 3 d (data not shown). The rapid decline in protection was negatively correlated to massive accumulation of extracellular JIPs. The view that JA acted directly against Egh and was metabolized is supported by the finding that barley metabolizes exogenously applied dihydrojasmonic acid as well as JA (Meyer et al., 1989, 1991). Second, local protection by JA was not correlated to enhanced antifungal activity of IWF that contained extracellular JIPs as possible candidates for defense gene products. And third, inhibition by JA of appressoria differentiation was not abolished by cordycepin treatment of the plants. Cordycepin repressed JIP 23 accumulation as a marker for transcriptionally regulated gene products. It also repressed JA-induced chlorosis and the accumulation of violet pigments, probably anthocyanins (data not shown).

These data argue against protection by JA-induced, rapid, and transient defense reactions. However, we cannot exclude induction of translationally regulated defense reactions, metabolism of JA to a fungicidal compound by plant or fungal enzymes, or involvement of defense reactions only in reduced penetration efficiency. We were unable to test the last hypothesis due to experimental limitations. First, protection by JA was strictly confined to the treated leaf surface (data not shown), which did not allow separation of direct and indirect effects by studying systemic protection. Second, cordycepin partly inhibited fungal development beyond the appressorial state (data not shown). Therefore, we could not test by microscopy whether reduced penetration efficiency was due to transcriptionally regulated, JA-induced defense reactions.

The probable direct antifungal effect of JA indicates that this compound does not affect only plant cells. It seems not to be fungitoxic, because it did not inhibit in vitro mycelial growth of 10 different phytopathogenic fungi (data not shown). Therefore, JA might act as an antifungal compound by inhibiting fungal differentiation processes. It is tempting to speculate about a possible antifungal function of endogenous JA accumulating in infected tissues. However, the amounts per gram fresh weight of accumulated, endogenous JA after wounding (Creelman et al., 1992) or elicitor treatment (Gundlach et al., 1992) are 2 to 3 orders of magnitude lower than the amount applied here (approximately 300 μg/g fresh weight). Recently, an antifungal substance from *Oryza officinalis* has been identified as JA (Neto et al., 1991), thus confirming our observations.

The extracellular localization of a subset of barley JIPs indicates some function in cell wall modification or in pathogen or insect defense. The probable lack of antifungal activity of JIPs 23 and 10/12 leaves this question open. A cDNA for one member of barley JIP 23 has recently been cloned, but the sequence showed no similarity to known protein sequences, although another cDNA was found to correspond to a JA-inducible barley leaf thionin precursor (Andresen et al., 1992). Barley leaf thionins are known to be antifungal (Bohlmann et al., 1988). Moreover, they were detected in the outer epidermal cell wall of Egh-infected barley leaves (Ebrahim-Nesbat et al., 1989). The JA-induced thionin was localized in the vacuole (Andresen et al., 1992), but the authors did not exclude accumulation of other JA-induced, extracellular thionins. Therefore, it remains uncertain whether the lack of enhanced antifungal activity in IWF from JA-treated leaves, compared with IWF from control leaves, was due to the absence of JA-induced thionins or to a lack of antifungal activity of such thionins in our test system.

The testing of IWF from barley leaves for antifungal activity in an in vivo test system with barley leaf segments revealed an interesting side aspect of this study. IWF from control leaves contained a constitutive, heat-labile antifungal activity against Egh. This activity seems to be different from a heat-stable, ethanol-soluble compound described by Hiramoto et al. (1992). The constitutive antifungal activity described here might be based on a constitutive low level of expression of barley PR proteins. This assumption is supported by immunoblot experiments, which revealed constitutive expression of barley PR1 and PR5 proteins (Fig. 4C and data not shown). IWF from Egh-infected leaves did not have a higher antifungal activity, based on protein content, than IWF from control leaves. On the other hand, the antifungal activity, based on leaf fresh weight, of IWF from Egh-infected leaves was clearly higher than the activity of control IWF (data not shown), which probably reflects massive accumulation of PR proteins in IWF from Egh-infected leaves. Finally, because Egh is an obligate biotrophic pathogen, we had to test the IWFs on living leaf tissue. Therefore, the data obtained did not allow us to discriminate between direct or indirect (i.e. resistance-inducing) antifungal activity of the IWFs. Nevertheless, we believe that at least IWF from control leaves acted directly as an antifungal agent, because otherwise a constitutive, induced resistant phenotype of the leaves from which it was isolated would have to be observed. This clearly was not the case with cv Golden Promise.

The phytopathological data described here raise a question about the central role of JA in signal transduction in the barley/Egh interaction. We addressed this question further by comparing extracellular proteins induced by JA and by Egh. The results clearly demonstrate that extracellular JIPs are not a subset of Egh-induced extracellular PR proteins, i.e. JA was not capable of inducing extracellular PR proteins in barley. Moreover, exogenous JA did not alter the kinetics of PR protein induction by Egh. The independent induction of both JIPs and PR proteins in leaves that had been treated with JA and simultaneously inoculated with Egh strongly suggest that JIPs and PR proteins were induced via two independent signal transduction pathways. Therefore, a JA-independent signal transduction mechanism seems to mediate induction of at least a subset of host-response genes in barley. In contrast, an additive stimulating effect of pathogen-derived signals and of methyl jasmonate on proteinase inhibitor activity in tobacco cells has been reported (Rickauer et al., 1992).

Leaf thionin has recently been identified as one major barley JIP (Andresen et al., 1992). This shows that jasmonates can also induce a barley gene belonging to a pathogen-

responsive gene family (Bohlmann et al., 1988). However, barley leaf thionins seem to accumulate in response to a wider array of stresses than the PR proteins described here (Fischer R et al., 1989). Together with recent evidence (Reinbothe et al., 1992), our results indicate that, in barley, jasmonates are involved in mediating forms of stress such as desiccation but are not primarily involved in mediating responses to pathogen attack. The situation might be different in dicots, where jasmonates were found to cause not only local, but also systemic, protection against a fungal pathogen (Cohen et al., 1993). Further studies, e.g. using JA-insensitive mutants (Staswick et al., 1992), might be able to test the hypothesis that jasmonates lead to changes in host-gene expression that partly overlap with changes induced by pathogen attack.

ACKNOWLEDGMENTS

We would like to thank T. Niderman and C. Weinmann for the immunoblot experiment, and Dr. F. Baker for critical reading of the manuscript.

Received November 10, 1992; accepted February 26, 1993.
Copyright Clearance Center: 0032-0889/93/102/0503/09.

LITERATURE CITED

Anderson JM (1988) Jasmonic acid-dependent increases in the level of specific polypeptides in soybean suspension cultures and seedlings. J Plant Growth Regul **7:** 203–211

Anderson JM, Spilatro SR, Klauer SF, Franceschi VR (1989) Jasmonic acid-dependent increase in the level of vegetative storage proteins in soybean. Plant Sci **62:** 45–52

Andresen I, Becker W, Schlüter K, Burges J, Parthier B, Apel K (1992) The identification of leaf thionin as one of the main jasmonate-induced proteins of barley (*Hordeum vulgare*). Plant Mol Biol **19:** 193–204

Bohlmann H, Clausen S, Behnke S, Giese H, Hiller C, Reimann-Philipp U, Schrader G, Barkholt V, Apel K (1988) Leaf-specific thionins of barley—a novel class of cell wall proteins toxic to plant-pathogenic fungi and possibly involved in the defence mechanism of plants. EMBO J **7:** 1559–1565

Carver TLW, Ingerson SM (1987) Responses of *Erysiphe graminis* germlings to contact with artificial and host surfaces. Physiol Mol Plant Pathol **30:** 359–372

Chou CM, Kao CH (1992) Methyl jasmonate, calcium, and leaf senescence in rice. Plant Physiol **99:** 1693–1694

Cohen Y, Gisi U, Niderman T (1993) Local and systemic protection against *Phytophthora infestans* induced in potato and tomato plants by jasmonic acid and jasmonic-methyl-ester. Phytopathology (in press)

Creelman RA, Tierney ML, Mullet JE (1992) Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proc Natl Acad Sci USA **89:** 4938–4941

Croft KPC, Voisey CR, Slusarenko AJ (1990) Mechanism of hypersensitive cell collapse: correlation of increased lipoxygenase activity with membrane damage in leaves of *Phaseolus vulgaris* (L.) inoculated with an avirulent race of *Pseudomonas syringae* pv. *phaseolicola*. Physiol Mol Plant Pathol **36:** 49–62

Dittrich H, Kutchan TM, Zenk MH (1992) The jasmonate precursor, 12-oxo-phytodienoic acid, induces phytoalexin synthesis in *Petroselinum crispum* cell cultures. FEBS Lett **309:** 33–36

Ebrahim-Nesbat F, Behnke S, Kleinhofs A, Apel K (1989) Cultivar-related differences in the distribution of cell-wall-bound thionins in compatible and incompatible interactions between barley and powdery mildew. Planta **179:** 203–210

Farmer EE, Johnson RR, Ryan CA (1992) Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. Plant Physiol **98:** 995–1002

Farmer EE, Ryan CA (1992) Octadecanoic precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell **4:** 129–134

Fischer R, Behnke S, Apel K (1989) The effect of chemical stress on the polypeptide composition of the intercellular fluid of barley leaves. Planta **178:** 61–68

Fischer W, Christ U, Baumgartner M, Erismann KH, Mösinger E (1989) Pathogenesis-related proteins of tomato. II. Biochemical and immunological characterization. Physiol Mol Plant Pathol **35:** 67–83

Gundlach H, Müller MJ, Kutchan TM, Zenk MH (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc Natl Acad Sci USA **89:** 2389–2393

Herrmann G, Lehmann J, Peterson A, Sembdner G, Weidhase RA, Parthier B (1989) Species and tissue specificity of jasmonate-induced abundant proteins. J Plant Physiol **134:** 703–709

Hildmann T, Ebneth M, Pena-Cortés H, Sanchez-Serrano JJ, Willmitzer L, Prat S (1992) General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. Plant Cell **4:** 1157–1170

Hiramoto T, Tobimatsu R, Shiraishi T, Yamada T, Ichinose Y, Oku H (1992) Endogenous elicitor present in barley seeds. II. Mechanisms of the induction of the resistance in barley leaves to *Erysiphe graminis*. J Phytopathol **135:** 167–176

Kunoh H, Tsuzuki T, Ishizaki H (1978) Cytological studies of early stages of powdery mildew in barley and wheat. IV. Direct ingress from superficial primary germ tubes and appressoria of *Erysiphe graminis hordei* on barley leaves. Physiol Plant Pathol **13:** 327–333

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Maslenkova LT, Miteva TS, Popova LP (1992) Changes in the polypeptide patterns of barley seedlings exposed to jasmonic acid and salinity. Plant Physiol **98:** 700–707

Mason HS, Mullet JE (1990) Expression of two soybean vegetative storage protein genes during development and in response to water deficit, wounding, and jasmonic acid. Plant Cell **2:** 569–579

Meyer A, Gross D, Vorkefeld S, Kummer M, Schmidt J, Sembdner G, Schreiber K (1989) Metabolism of the plant growth regulator dihydrojasmonic acid in barley shoots. Phytochemistry **28:** 1007–1011

Meyer A, Miersch O, Büttner C, Dathe W, Sembdner G (1984) Occurrence of the plant growth regulator jasmonic acid in plants. J Plant Growth Regul **3:** 1–8

Meyer A, Schmidt J, Gross D, Jensen E, Rudolph A, Vorkefeld S, Sembdner G (1991) Amino acid conjugates as metabolites of the plant growth regulator dihydrojasmonic acid in barley. J Plant Growth Regul **10:** 17–25

Mueller-Uri F, Parthier B, Nover L (1988) Jasmonate-induced alteration of gene expression in barley leaf segments analyzed by in-vivo and in-vitro protein synthesis. Planta **176:** 241–247

Neto GC, Kono Y, Hyakutake H, Watanabe M, Suzuki Y, Sakurai A (1991) Isolation and identification of (−)-jasmonic acid from wild rice, *Oryza officinalis*, as an antifungal substance. Agric Biol Chem **55:** 3097–3098

Parthier B (1990) Jasmonates: hormonal regulators or stress factors in leaf senescence? J Plant Growth Regul **9:** 57–63

Reinbothe S, Machmudova A, Wasternack C, Reinbothe C, Parthier B (1992a) Jasmonate-induced proteins in cotton: immunological relationship to the respective barley proteins and homology of transcripts to late embryogenesis abundant (Lea) mRNAs. J Plant Growth Regul **11:** 7–14

Reinbothe S, Reinbothe C, Lehmann J, Parthier B (1992b) Differential accumulation of methyl jasmonate-induced mRNAs in response to abscisic acid and desiccation in barley (*Hordeum vulgare*). Physiol Plant **86:** 49–56

Rickauer M, Bottin A, Esquerré-Tugayé MT (1992) Regulation of proteinase inhibitor production in tobacco cells by fungal elicitors, hormonal factors and methyl jasmonate. Plant Physiol Biochem **30:** 579–584

Rickauer M, Fournier J, Pouénat ML, Berthalon E, Bottin A, Esquerré-Tugayé MT (1990) Early changes in ethylene synthesis

and lipoxygenase activity during defence induction in tobacco cells. Plant Physiol Biochem **28:** 647–653

Schweizer P, Hunziker W, Mösinger E (1989) cDNA cloning, in vitro transcription and partial sequence analysis of mRNAs from winter wheat (*Triticum aestivum* L.) with induced resistance to *Erysiphe graminis* f.sp. *tritici*. Plant Mol Biol **12:** 643–654

Staswick PE, Wenpei S, Howell SH (1992) Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. Proc Natl Acad Sci USA **89:** 6837–6840

Stephenson LC, Franceschi VR, Grimes HD (1992) Molecular regulation of soybean lipoxygenase responsive to atmospheric methyl jasmonate (abstract No. 720). Plant Physiol **99:** S-121

Takahashi K, Aist JR, Israel HW (1985) Distribution of hydrolytic enzymes at barley powdery mildew encounter sites: implications for resistance associated with papilla formation in a compatible system. Physiol Plant Pathol **27:** 167–184

Towbin H, Gordon J (1984) Immunoblotting and dot immunobinding—current status and outlook. J Immunol Methods **72:** 313–340

Ueda J, Kato J (1980) Isolation and identification of a senescence-promoting substance from wormwood (*Artemisia absinthium* L.). Plant Physiol **66:** 246–249

Weidhase RA, Kramell HM, Lehmann J, Liebisch HW, Lerbs W, Parthier B (1987) Methyl jasmonate-induced changes in the polypeptide pattern of senescing barley leaf segments. Plant Sci **51:** 177–186

Wilen RW, van Rooijen GJH, Pearce DW, Pharis RP, Hobrook LA, Moloney MM (1991) Effects of jasmonic acid on embryo-specific processes in *Brassica* and *Linum* oilseeds. Plant Physiol **95:** 399–405

Yamane H, Takagi H, Abe H, Yokota T, Takahashi N (1981) Identification of jasmonic acid in three species of higher plants and its biological activities. Plant Cell Physiol **22:** 689–697