Planta (1997) 202: 155–162

Planta

The jasmonate pathway is involved differentially in the regulation of different defence responses in tobacco cells

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Received: 4 July 1996 / Accepted: 23 November 1996

Abstract. Jasmonic acid, a product of the lipoxygenase (LOX) pathway, has been proposed to be a signal transducer of defence reactions in plants. We have reported previously that methyl jasmonate (MJ) induced accumulation of proteinase inhibitors in tobacco cell suspensions (Rickauer et al., 1992, Plant Physiol Biochem 30: 579–584). The role of this compound in the induction of this and of other defence reactions is further studied in this paper. Treatment of tobacco cell suspensions with an elicitor from *Phytophthora parasitica* var. *nicotianae* induced a rapid and transient increase in jasmonic acid levels, which was abolished when cells were preincubated with eicosatetraynoic acid (ETYA), an inhibitor of LOX. Pretreatment with ETYA also inhibited the induction of proteinase inhibitors by fungal elicitor, but not by MJ. Linolenic acid, a precursor of jasmonate biosynthesis, induced this defence response, whereas linoleic acid had no effect. Expression of defence-related genes encoding proteinase inhibitor II, hydroxyproline-rich or glycine-rich glycoproteins, glucanase and chitinase, was induced in a basically similar manner by fungal elicitor or MJ. However, ETYA did not inhibit, or only partially inhibited, the elicitation of these defence genes. Expression of the sesquiterpene cyclase (5-*epi*-aristolochene synthase) gene was not induced by MJ, but only by fungal elicitor, and ETYA pretreatment had no effect on this induction. The obtained results indicate that synthesis of jasmonate via the LOX pathway seems to be only part of a complex regulatory mechanism for the onset of many, but not all, defence reactions.

Key words: Defence reaction – Elicitor – Jasmonate – Lipoxygenase – *Nicotiana* – Signal transduction

Introduction

Jasmonic acid (JA) and its methyl ester are natural compounds occurring throughout the plant kingdom. Their effects on development-related physiological processes such as senescence and germination, tuber formation or ethylene biosynthesis when applied exogenously at low concentrations draw these substances close to classical phytohormones (reviewed by Sembdner and Parthier 1993).

An additional function has been attributed to JA since the discovery that methyl jasmonate (MJ) and its precursors induced proteinase inhibitor (PI) synthesis in tomato plants (Farmer and Ryan 1990, 1992). Originally described as wound-inducible defence compounds against herbivorous insects, PIs have since then been reported to be induced by pathogenic microorganisms and treatment with elicitors, and to inhibit fungal proteases (reviewed by Ryan 1990). It has been postulated that jasmonates might constitute lipid-derived messengers in the signal transduction chain preceding the activation of defence gene expression. This hypothesis has been strengthened by Gundlach et al. (1992), who reported that JA levels strongly increased in elicitor-treated cell suspensions of *Rauvolfia canescens* and *Eschscholtzia californica*. Methyl jasmonate treatment induced alkaloid accumulation in these cultures and phenylalanine ammonia-lyase activity in soybean, indicating induction of phenylpropanoid synthesis.

Jasmonic acid is synthesized from α-linolenic acid *via* the lipoxygenase (LOX) pathway, comprising oxidation of α-linolenic acid, cyclisation of the resulting hydroperoxide to 12-oxo-phytodienoic acid, and further conversion by reduction and β-oxidation (Vick and Zimmerman 1983). Lipoxygenase (EC 1.13.11.12) has been reported to be induced in various plants after

Abbreviations: EAS = sesquiterpene cyclase (5-*epi*-aristolochene synthase); ETYA = eicosatetraynoic acid; GRP = glycine-rich protein; HRGP = hydroxyproline-rich glycoprotein; JA = jasmonic acid; LOX = lipoxygenase; MJ = methyl jasmonate; PI = proteinase inhibitor; *Ppn* = *Phytophthora parasitica* var. *nicotianae*; PR = pathogenesis-related

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pathogen attack (e.g. Ruzicska et al. 1983; Croft et al. 1993; Fournier et al. 1993; Melan et al. 1993). Inhibition of the LOX pathway has been demonstrated to inhibit phytoalexin production in rice inoculated with *Pyricularia oryzae* (Li et al. 1991). The onset of defence reactions might thus be triggered by a rise in jasmonate levels, which are regulated by variations in LOX activity.

We have previously reported that LOX activity is induced in elicitor-treated tobacco cells prior to PI production (Fournier et al. 1986; Rickauer et al. 1990) and that the latter defence response is also induced by incubation with MJ (Rickauer et al. 1992). In this study, we demonstrate that jasmonate levels are strongly and transiently enhanced in tobacco cells treated with an elicitor preparation obtained from the cell walls of *Phytophthora parasitica* var. *nicotianae* (*Ppn*), a pathogen of tobacco. The role of LOX and jasmonate in PI induction is investigated by the use of LOX substrates and eicosatetraynoic acid (ETYA), an in-vitro inhibitor of LOX activity. Evidence is presented that an array of different defence-related transcripts is induced by MJ in tobacco, but seems to be regulated only partially by the LOX-catalysed jasmonate pathway. Phytoalexin synthesis however, assessed by the expression of the sesquiterpene cyclase (EAS) gene, seems to be unrelated to jasmonate production in tobacco.

Materials and methods

Tobacco cell culture. Cell suspensions were obtained from pith of *Nicotiana tabacum* L. cv. Wisconsin 38 and grown in a modified liquid Murashige-Skoog medium as described by Jouanneau (1973), under constant light (10 $W \cdot m^{-2}$) at 24 °C in Erlenmeyer flasks, on an orbital shaker at 100 rpm. The cells reached the stationary growth phase after 8 d and were subcultured every 14 d by diluting 34 ml of culture in 250 ml of fresh medium.

Elicitor preparation. Fungal elicitor was isolated by autoclaving a cell wall preparation of *Phytophthora parasitica* var. *nicotianae* (*Ppn*) as described previously (Pélissier et al. 1986). The ethanol-soluble fraction was used after dialysis with Spectrapor M6 (cut-off MW 1000; Polylabo, Strasbourg, France) against deionized water and lyophilisation.

Cell treatments. Seven-day-old tobacco cell cultures (in late logarithmic growth phase) were used for incubations and prepared under sterile conditions as follows: cells were allowed to settle by gravity, the medium was gently removed by suction, and the cells were resuspended in twice their volume of fresh medium. This procedure was carried out three times. For incubations, aliquots of this cell suspension were transferred into Erlenmeyer flasks, the compounds studied were added and the flasks were agitated under culture conditions for the times indicated in the text. Elicitor was dissolved in distilled water and lipophilic compounds in ethanol; control cells were incubated with the corresponding volume of either water or ethanol, respectively. Methyl jasmonate (a mixture of four stereoisomers) was purchased from Serva (Heidelberg, Germany), and fatty acids and ETYA from Sigma (St. Quentin Fallavier, France).

Viability staining. Aliquots of tobacco cells that had been incubated with water, elicitor or MJ for 40 h, were diluted tenfold with fresh medium and Evans' blue was added at a final concentration of 1 $mg \cdot ml^{-1}$. The cells were counted immediately afterwards under a microscope with a Fuchs-Rosenthal hemacytometer (PolyLabo, Strasbourg, France) and viability was determined as percent of stained, non-viable cells. At least 170 cells were examined for each measurement.

Proteinase inhibitor measurement. Generally, 25 ml of a cell suspension prepared as described above were incubated in 100-ml Erlenmeyer flasks for 40 h, harvested by filtration on a sintered glass filter, weighed and frozen immediately at −20 °C. The extraction of PIs and the proteinase inhibition assay were carried out according to Walker-Simmons and Ryan (1977) and as previously described (Rickauer et al. 1989). The crude extract was cleared by heat treatment at 65 °C for 10 min and centrifugation, before being used in the assay. Inhibition of trypsin was measured using azocoll (Sigma) as substrate and PI activity was expressed as μg trypsin inhibited · mg $protein^{-1}$. Protein content was measured after precipitation of the extracts with cold trichloroacetic acid (TCA) by the method of Lowry et al. (1951).

Jasmonate measurement. Aliquots of 60 ml of cell suspension (corresponding to ca. 0.3 g DW) were incubated in 250-ml Erlenmeyers flasks in the absence or presence of fungal elicitor, harvested after different time intervals by filtration, weighed and shock-frozen in liquid nitrogen. After addition of 100 ng of 9,10-dihydrojasmonic acid as an internal standard, the cells were homogenized and extracted twice with diethyl ether. The whole procedure of extraction, derivatisation and determination has been described in detail by Mueller et al. (1993) and Mueller and Brodschelm (1994). Briefly, extracts were purified on an aminopropyl solid-phase column by elution with ether/acetic acid (98:2, v/v), derivatized with pentafluorobenzyl bromide and finally analyzed by gas chromatography-mass spectroscopy (GC-MS).

Extraction of RNA and Northern blot hybridization. Total RNA was extracted from shock-frozen tobacco cells according to the method described by Haffner et al. (1978); concentrations were measured photometrically at 260 nm.

Generally, 20 μg of total RNA was subjected to electrophoresis in a denaturing gel of 1.2% agarose in 2.2 M formaldehyde and 20 mM 3-(N-morpholino)propanesulfonic acid, 8 mM sodium acetate, 1 mM EDTA, pH 7.0. Migration was performed in the presence of ethidium bromide in order to control whether equal amounts of RNA had been loaded. After destaining of the gel in distilled water and 10 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na-citrate, pH 7.0), RNA was passively transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) with 10 × SSC, and fixed by baking at 80 °C for 2 h under vacuum. The membranes were prehybridized for 4 h at 42 °C in 2 × SSC, 1 × Denhardt's solution, 50% formamide, 0.1% SDS and 100 $\mu g \cdot ml^{-1}$ denatured calf thymus DNA, then hybridized overnight at 42 °C in the same solution containing the radioactive probe.

The probes used in this study were pDC5A1 for hydroxyproline-rich glycoproteins (HRGPs; Chen and Varner 1985), the *Hind*III-*Eco*RI insert of pT2-47 for PI II (Graham et al. 1986), the *Pst*I insert of pNt517 for glucanase (Godiard et al. 1990), the *Pst*I insert of pCHN50 for chitinase (Shinshi et al. 1987), and the *Eco*RI-*Xho*I insert of pBS-TEAS for EAS (Facchini and Chappell 1992). Radioactive labelling was performed with [α-^{32}P]dCTP by random priming as described by Feinberg and Vogelstein (1983). After hybridization, the membranes were washed twice for 30 min at 60 °C (for PI II) or at 65 °C in 2 × SSC, 0.1% SDS. They were blotted dry, protected by Saran-wrap (Polylabo), and exposed to hyperfilm MP (Amersham, Les Ulis, France) at −80 °C.

Incubations of tobacco cells, RNA extractions and Northern blot hybridizations were carried out three times independently, and figures show representative results.

Results

Fungal elicitor induces a LOX-dependent increase in jasmonate levels in tobacco cells. We have previously demonstrated that treatment with MJ induced PI production in tobacco cells to the same extent as incubation with cell-wall-derived fungal elicitor (Rickauer et al. 1992). It has been postulated that defence responses such as PI production might be triggered by elicitor-induced jasmonate formation involving LOX, the first enzyme in the JA biosynthetic pathway.

We therefore determined firstly if elicitor treatment of tobacco cells resulted in enhanced levels of JA under our experimental conditions. For this purpose, 7-d-old tobacco cell suspensions were incubated in the absence or presence of *Ppn* elicitor, harvested after different time intervals, and the JA content was determined by capillary GC-MS. In control cells, no significant changes were observed throughout the 24-h incubation period, whereas the addition of elicitor resulted in a rapid and transient increase in JA levels, identified as (3R/7S)-iso JA. A typical experiment is shown in Fig. 1A, with a maximum at 2 h, and a stimulation factor of 13. In five independent time-course studies we always observed a substantial and transient increase, but absolute values and timing of the peaks showed slight variations, which precluded calculation of mean values. Thus the maximum of jasmonate peaked between 1.5 and 3 h, and maximum values ranged from 30 to 80 ng JA per g DW; control values were between 5 and 9 ng JA per g DW in all assays during the whole incubation period. In all cases the increase was dectectable 1 h after elicitor addition to the cell cultures.

In order to determine if this increase involved synthesis of precursors by LOX, tobacco cells were preincubated with 20 μM ETYA before addition of elicitor, and JA production was measured thereafter. Eicosatetraynoic acid, a structural analogue of the LOX substrate arachidonic acid, efficiently inhibits tobacco LOX activity in vitro (data not shown). Pretreatment of the cells with ETYA resulted in a complete inhibition of the elicitor-induced rise in JA (Fig. 1B). Thus, LOX activity seems to be a prerequisite for the synthesis of this compound.

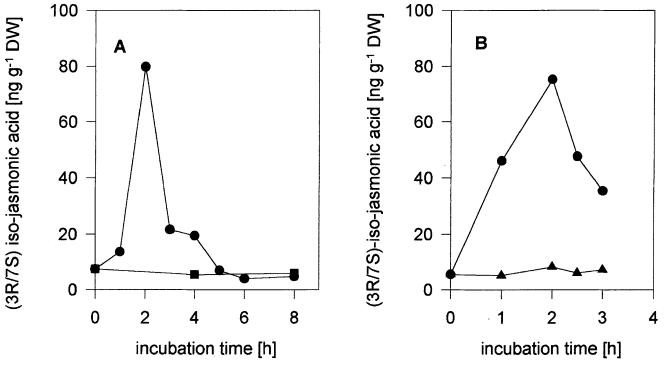

Fig. 1A,B. Production of JA in elicitor-treated tobacco cells. Seven-day-old tobacco cells were incubated in the absence or presence of *Ppn* elicitor at a concentration of 30 $\mu g \cdot ml^{-1}$ for varying time intervals, and JA content was determined by GC-MS. **A** Time course of JA production in untreated control cells (■) or in elicitor-treated cells (●). **B** Production of JA in elicitor-treated cells (●) or in cells that had been preincubated for 1 h with 20 μM ETYA before addition of elicitor (▲)

Proteinase inhibitor production is regulated via the LOX pathway. It has been reported previously that MJ induces accumulation of PI activity in tobacco cell cultures (Rickauer et al. 1992). However, the quantities of MJ required for this effect were significantly higher than those used by Farmer and Ryan (1992) for treatment of whole plants. A dose-response study of PI induction in a tobacco cell suspension by MJ showed that this response was maximal for a concentration of 890 μM; lower and higher concentrations had less effect (Fig. 2A). This optimal dose of MJ did not seem to have toxic effects, since viability staining of the tobacco cells with Evans' blue after treatment with fungal elicitor and 890 μM MJ showed the same values as for control cells (Fig. 2B). At higher concentrations, MJ showed some toxicity, as demonstrated by an increase in the amount of apparently dead cells after incubation with 1800 μM MJ. Similar results were obtained by viability staining with fluorescein diacetate (data not shown). Since the concentration of 890 μM MJ did not appear to be toxic, it was used for further studies on the defence responses in tobacco.

With the purpose of determining if the observed transient elicitation of jasmonate was required for PI production, the tobacco cell suspensions were pretreated with 20 μM ETYA before the addition of elicitor or MJ, and PI activity was measured after 40 h of incubation. Pretreatment with this inhibitor reduced the elicitation of PI activity to ca. 60% (Fig. 3A), indicating that LOX-

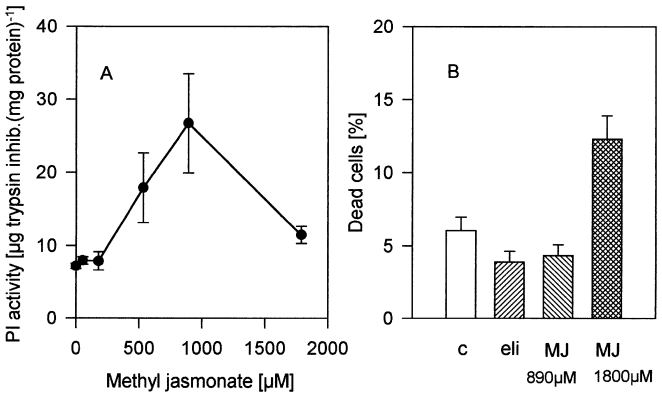

Fig. 2A,B. Dose response of PI activity in tobacco cells, and cell viability after treatment with MJ. **A** Seven-day-old suspension cultures were incubated in the presence of various amounts of MJ for 40 h, and PI activity was determined in crude extracts against trypsin. Data are the means of three independent experiments ± SD. **B** Suspension cultures were incubated in the absence (*c*) or presence of fungal elicitor (*eli*) at 30 $\mu g \cdot ml^{-1}$, or MJ at 890 μM (*MJ 890*) or 1800 μM (*MJ 1800*) for 40 h. Viability was determined immediately afterwards by staining with Evans blue and is expressed as percent of non-viable, stained cells. Values present the means of three independent experiments ± SD

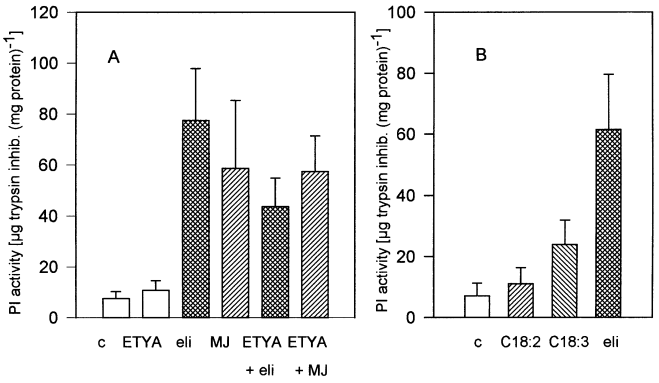

Fig. 3A,B. Proteinase inhibitor activity in tobacco cells after treatment with elicitor, MJ, ETYA and fatty acids. Cell suspensions were incubated in the absence or presence of elicitor, MJ, fatty acids or ETYA for 40 h and PI activity was determined in crude extracts against trypsin. **A** *c*, control cells incubated without addition of compounds; *ETYA*, incubated with 20 µM ETYA; *eli*, incubated with 30 µg · ml^{-1} *Ppn* elicitor; *MJ*, incubated with 890 µM MJ; *ETYA + eli*, *ETYA + MJ*: incubated 1 h with 20 µM ETYA before addition of elicitor or MJ, respectively. Data are the means of three independent experiments ± SD. **B** *c*, control cells; *eli*, incubated with 30 µg · ml^{-1} *Ppn* elicitor; *C18:2*, incubated with 150 µM linoleic acid; *C18:3*, incubated with 150 µM linolenic acid. Data are the means of three independent experiments ± SD

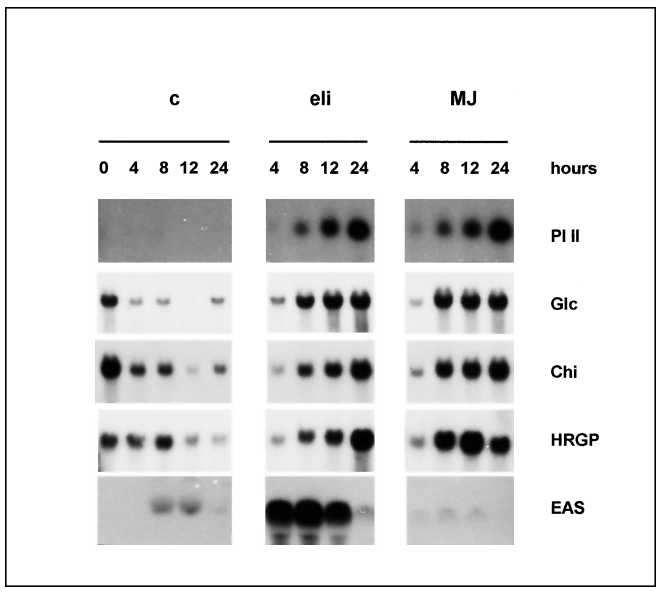

Fig. 4. Northern blot analysis of defence gene expression in tobacco cells treated with elicitor or MJ. Suspension cultures were incubated in the absence (*c*) or presence of 30 µg · ml^{-1} elicitor (*eli*) or 890 µM methyl jasmonate (*MJ*) for the times indicated and RNA was extracted. Twenty micrograms of total RNA was separated on a denaturing formaldehyde agarose gel, transferred to a nitrocellulose membrane and hybridized with a radiolabelled probe for PI II, glucanase (*Glc*), chitinase (*Chi*), HRGP and EAS, respectively. Ethidium bromide staining of the gels was performed in order to assure that equal amounts of RNA had been loaded. The figure shows one representative experiment out of three independent experiments

catalysed jasmonate production is necessary for maximal elicitation. In contrast, when pretreatment with ETYA was followed by incubation with MJ instead of fungal elicitor, induction of PI activity was roughly the same as with MJ alone.

The precursor for JA biosynthesis via LOX is α-linolenic acid. When tobacco cells were incubated with linoleic or linolenic acid, only the latter induced PI activity, with a mean stimulation factor of about four-fold above control values, whereas linoleic acid was without significant effect (Fig. 3B). This result further supports a putative role of the JA pathway in PI induction in tobacco cells.

Different defence responses are induced by MJ. The induction of PI activity and gene expression in tomato, tobacco and alfalfa plants by intermediate and final products of the JA pathway has been described by Farmer and Ryan (1992) and Farmer et al. (1992). It was of interest to study if this pathway was also regulating other defence reactions in tobacco. Hence we analysed the effect of elicitor or MJ on defence gene expression in cell suspensions by Northern blot hybridization, with molecular probes corresponding to PI II, β-1,3-glucanase, chitinase, HRGP and EAS.

In control cells, the levels of glucanase, chitinase and HRGP transcripts were relatively high at the beginning of the experiment, but decreased rapidly thereafter; PI II and EAS transcripts were absent or only faintly detected in controls (Fig. 4). Addition of elicitor or MJ enhanced the transcript levels revealed with PI II, glucanase, and chitinase probes. In all cases the maximum stimulation was around 24 h. Five transcript species of about 7.0, 5.0, 3.3, 2.5 and 1.4 kb were detected in control cells and elicitor-treated cells when Northern blots were probed with a carrot HRGP clone (data not shown). Only the most prominent species of about 1.4 kb showed a substantial increase which started after 12 h of treatment. This effect was similar for both elicitor and MJ. The term "HRGP transcript" used in this study refers to the probe rather than to the identity of transcripts. In fact, due to the complementary of codons for (hydroxy-) proline and glycine, a double-stranded HRGP probe would thus be able to hybridize with HRGP and glycine-rich proteins (GRP) transcripts. We suppose that the 1.4-kb transcript revealed with the HRGP probe in tobacco cells corresponds to a GRP gene which has been reported to be induced by fungal glucan in tobacco plants, and which shows a transcript of the same size (Brady et al. 1993). Expression of the EAS gene was induced very rapidly in the presence of elicitor; transcript levels were already at maximum after 4 h of incubation, declining after 12–24 h. Methyl jasmonate, however, did not induce any increase in EAS gene expression.

Taken together, it can be stated that expression of the four defence-related genes which were induced by elicitor and MJ (PI II, glucanase, chitinase and HRGP) showed similar kinetics by both compounds; the phytoalexin-related EAS gene, in contrast, was induced only by fungal elicitor.

Expression of defence genes is affected differentially by ETYA. In order to determine if there was a causal relationship between elicitor, MJ, and defence responses,

the effect of the LOX inhibitor ETYA on defence gene expression was analyzed by Northern blot hybridization. Tobacco cells were preincubated with ETYA and then further incubated for 8 and 24 h in the presence of elicitor or MJ. These incubation periods were retained since they correspond to the maxima of expression of the different genes under study.

Treatment of tobacco cells with ETYA alone had no effect on levels of PI II, HRGP and EAS transcripts, but induced slight increases of glucanase and chitinase transcripts (Fig. 5). This effect, however, was always weaker than induction by fungal elicitor or MJ.

Transcript induction by *Ppn* elicitor was reduced by ETYA only in the case of PI II and glucanase, but this inhibition was never very strong. In contrast, elicitor-induced chitinase gene expression was strongly enhanced by ETYA after 8 h of incubation, and EAS gene expression was slightly, but reproducibly, stimulated after 24 h of incubation. Induction of HRGP transcripts by elicitor did not change appreciably after ETYA pretreatment. Thus the elicitor-induced increase in defence gene expression was either not affected, or only partially affected by inhibition of JA synthesis with an in-vitro inhibitor of LOX in tobacco cells.

Transcript induction by MJ was also influenced by ETYA pretreatment, the effect depending on the gene under study. Whereas PI II induction remained unchanged, increases of glucanase, chitinase and HRGP transcript levels were reduced by ETYA pretreatment. However, in the case of glucanase transcripts this effect was strong only after 8 h of incubation and disappeared at 24 h, whereas it was equally apparent at 8 and 24 h in the case of chitinase and HRGP transcripts. Levels of HRGP transcripts were reduced even below the levels detected in control cells. The non-inducibility of EAS transcripts by MJ was not changed by ETYA treatment. It appears that for three out of the five defence responses studied, even the induction by externally added MJ is affected by an inhibitor of its biosynthesis.

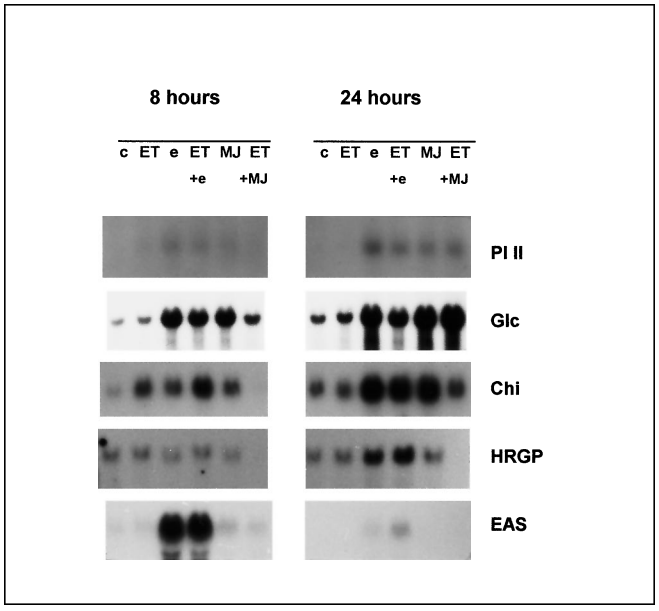

Fig. 5. Elicitation of defence-gene expression in tobacco cells after ETYA pretreatment. Suspension cultures were preincubated for 1 h in the absence or presence of 20 μM ETYA before addition of 30 μg · ml^{-1} *Ppn* elicitor or 890 μM MJ. Incubation continued for 8 and 24 h, and 20 μg of total RNA was analyzed by Northern blot hybridization with radiolabelled probes for PI II, glucanase (*Glc*), chitinase (*Chi*), HRGP and EAS, respectively. *c*, non-treated control cells; *ET*, ETYA pretreated, no compound added afterwards; *e*, elicitor-treated; *ET* + *e*, ETYA pretreated, then elicitor-treated; MJ, MJ-treated; ET + MJ, ETYA pretreated, then MJ-treated. Loaded amounts of RNA were checked by ethidium bromide staining of the gel. The figure shows one representative experiment out of three independent experiments

Discussion

During the last few years evidence has accumulated that the lipid-derived compounds JA and MJ are regulatory compounds for the wound response in plants (Farmer and Ryan 1990, 1992; Staswick et al. 1991; Creelman et al. 1992; Peña-Cortès et al. 1993), and might also regulate the defence response against microbial attack (Gundlach et al. 1992). Indeed, they have been shown to induce an array of defence reactions in various plants, and to be produced in response to wounding and elicitor treatment. Jasmonic acid might thus participate in a transduction cascade linking pathogen perception to defence gene expression. The present study attempts to unravel the importance of LOX and JA for the induction of defence responses in tobacco cells.

The first result of this work shows that tobacco cells respond to an elicitor of a fungal pathogen of tobacco by a rapid increase in JA levels. A time course study indicated that, as in the case of *Rauvolfia canescens* and *Agrostis tenuis* (Gundlach et al. 1992; Mueller et al. 1993), the production of JA is transient with a "spike profile", which reaches its maximum around 2–3 h of elicitor treatment before returning to control values after 5–6 h. This pattern is also very similar to the previously reported induction of ethylene by the same elicitor in this system (Rickauer et al. 1990).

A further study of JA production in the presence of ETYA indicated that LOX activity is required for its synthesis, since pretreatment of the cell suspension with this compound inhibited the elicitor-induced rise in JA content. Eicosatetraynoic acid is an irreversible inhibitor of animal and plant LOX (Kühn et al. 1984), and preliminary results showed that it is an efficient inhibitor of tobacco LOX in vitro (data not shown). The fact that elicitation of LOX in tobacco cells has been shown to extend for a longer period of time than the very transient production of JA (Rickauer et al. 1990) suggests that, in addition to JA, other products are generated by the enzyme. Indeed, several categories of fatty-acid-derived molecules are known to occur in plants (reviewed by Farmer 1994). The nature of these putative end-products in tobacco still remains to be elucidated. In *Pseudomonas syringae*-inoculated bean plants, the LOX pathway has been reported to form volatile compounds with antimicrobial activity (Croft et al. 1993).

The channeling of the primary LOX products towards JA or other end-products can be regulated at different levels of the pathway. First, depending on the substrate released prior to LOX action, different products are generated. A high phospholipase activity is induced in elicitor-treated tobacco cells, in a manner consistent with LOX elicitation (Roy et al. 1995). Preliminary data indicate that different enzymes are involved in phospholipid hydrolysis, making it likely that a variety of potential LOX substrates is released. Second, in most plants there occur different LOX isoforms, and in tobacco cells there might be one isoform active at early stages of elicitation which leads to formation of JA, whereas a second isoform whose action is different, appears at later stages. Indeed, the tobacco LOX that we have previously characterized at the maximum of induction by elicitors (24 h) forms mainly the 9-hydroperoxide from α-linolenic acid in-vitro and only a small proportion of the 13-hydroperoxide which is the direct precursor of JA (Fournier et al. 1993). Finally, JA synthesis could be regulated by changes in the levels of enzymes required for the later steps of the pathway. One or more of these mechanisms might account for the transient induction of JA by elicitor treatment, while high levels of LOX activity persist in the cells.

In order to address the role of JA/MJ in signal transduction, we first compared the effects of MJ and fungal elicitor on defence-gene expression. Of the four classes of genes that were retained, only the one related to phytoalexin synthesis responded exclusively to the elicitor, and was not induced by MJ. This result is in agreement with the work of Choi et al. (1994) who reported that the phytoalexin pathway is induced by elicitor, but not by MJ or wounding in potato tubers. The three remaining classes corresponding to enzyme inhibitors (PI), pathogenesis-related (PR) proteins, and cell-wall structural proteins are equally induced by MJ and elicitor. Elicitation by MJ showed a similar time course as induction by the fungal elicitor, which is consistent with a role in elicitor transduction. These results enlarge the spectrum of defence-related genes which are induced by MJ. After PIs (Farmer and Ryan 1990), LOX (Tranbarger et al. 1991), phenylalanine ammonia-lyase (Gundlach et al. 1992), proline-rich proteins and chalcone synthase (Creelman et al. 1992), berberine-bridge enzyme (Kutchan 1993), and PR-1 and PR-5 (Xu et al. 1994), we now report induction of glucanase, chitinase and HRGP (or GRP) transcripts in tobacco by this compound.

A dose-response study of PI induction by MJ showed that relatively high concentrations of this compound were needed for maximum response. Several reasons might account for this. Besides the fact that the actual concentration of the biologically active isomer (3R/7S)-iso-JA is only 5–10% of the commercial mixture, the physiological state of the cell suspension might be important. Indeed, in most studies with cell suspensions, the cultures were used 3 d after subculturing (Mueller et al. 1993; Blechert et al. 1995), whereas in the present work the cells were in late logarithmic phase, 7 d after subculturing. This phase has previously been found to be most responsive with regard to elicitor-induced ethylene and LOX production (Rickauer et al. 1990), but it cannot be excluded that it is less responsive to MJ. Finally, tobacco might simply be less sensitive to MJ than other plant species. Indeed, a recent publication of Ellard-Ivey and Douglas (1996) showed that wound-inducible gene expression in tobacco required 100-fold higher JA concentrations than in parsley.

Lipoxygenase inhibitors have been successfully used to inhibit wound- or elicitor-induced responses in various plants (Staswick et al. 1991; Peña-Cortès et al. 1993; Choi and Bostock 1994; Ellard-Ivey and Douglas 1996). We used ETYA, a structural analogue of the LOX substrate arachidonic acid, in order to determine if LOX activity is necessary for defence induction in tobacco. When the jasmonate pathway was blocked by a low concentration of ETYA, the induction by elicitor of PI II and glucanase transcripts was only partially inhibited, and induction of chitinase and HRGP transcripts was either not affected or even stimulated. This indicated that, even though MJ is sufficient to induce these defence reactions when supplied exogenously, it is not the only compound involved in their induction when it is produced in planta in response to elicitor. Other compounds, related to the same or to different pathways might overcome the lack of JA. It is possible that products derived from other LOX isoforms whose synthesis is not inhibited by ETYA in vivo, are also active in defence induction. Finally, we cannot exclude the possibility that ETYA might be destroyed in vivo after some time, which would result in a later reappearance of LOX activity. The partial inhibition by ETYA of MJ-induced glucanase, chitinase and cell-wall protein gene expression might be due to the fact that MJ also induces LOX activity in tobacco cells (Véronési et al. 1996). In this case, ETYA would inhibit the MJ-induced LOX, thereby affecting some defence responses. In order to avoid the potential problems related to the use of in-vitro inhibitors, future work will aim at obtaining tobacco plants with genetically modified LOX gene expression.

Finally, it should be kept in mind that other signalling pathways, notably involving ethylene and salicylic acid, are induced in plants in response to microbial attack (Enyedi et al. 1992). Ethylene and salicyclic acid do not induce PI activity in tobacco cell cultures (Rickauer et al. 1990), but are known to regulate other defence markers such as PR proteins and HRGP. It has been reported that PR-1 and PR-5 gene expression is synergistically induced in tobacco seedlings by MJ and salicylic acid, or MJ and ethylene, respectively (Xu et al. 1994). Induction of these two defence-related genes seems to be regulated by partially different signal transduction pathways, involving two different protein kinases. More and more it appears that defence responses in plants are regulated by a complex network of signal transduction pathways interacting with each other, and when one pathway is blocked, the signal may be shunted to another one. Eventually, certain defence responses might be preferentially activated by one distinct pathway. Thus in potato (Choi et al. 1994) and tobacco (this work), the phytoalexin

pathway is induced by fungal elicitors, but not by MJ, and in maize PRms gene expression is also induced only by elicitor (Cordero et al. 1994). These results lead us to the hypothesis that in certain plants, notably in members of the *Solanaceae* family, jasmonate is part of a network which regulates the expression of genes known to be both wound- and elicitor-inducible, whereas infection- and elicitor-specific responses such as the accumulation of sesquiterpenoid phytoalexins are regulated by a different signal pathway, unrelated to this fatty-acid-derived compound.

The authors would like to thank Drs. J. Chappell (University of Lexington, Ky., USA), Y. Marco (INRA Toulouse, France), F. Meins (University of Basel, Switzerland), C.A. Ryan (University of Pullman, Wash., USA) and J. Varner (University of St. Louis, Mo., USA) for their gift of pBS-TEAS, pNt517, pCHN50, pT2-47 and pDC5A1, respectively. Thanks also to P. Sanchez for elicitor preparation and G. Putinier for typing the manuscript, and to Drs. M.H. Zenk and T. Kutchan (both University of München, Germany) for helpful discussions. Work at München was supported by SFB 369 of the Deutsche Forschungsgemeinschaft, Bonn.

References

Blechert S, Brodschelm W, Hölder S, Kammerer L, Kutchan TM, Mueller MJ, Xia Z, Zenk MH (1995) The octadecanoic pathway: signal molecules for the regulation of secondary pathways. Proc Natl Acad Sci USA 92: 4099–4105

Brady KP, Darvill AG, Albersheim P (1993) Activation of a tobacco glycine-rich protein gene by a fungal glucan preparation. Plant J 4: 517–524

Chen J, Varner JE (1985) An extracellular matrix protein in plants: Characterization of a genomic clone for carrot extensin. EMBO J 4: 2145–2151

Choi D, Bostock RM (1994) Involvement of de novo protein synthesis, protein kinase, extracellular Ca^{++} and lipoxygenase in arachidonic acid induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase genes and isoprenoid accumulation in potato (*Solanum tuberosum* L.). Plant Physiol 104: 1237–1244

Choi D, Bostok RM, Avdiushko S, Hildebrand DF (1994) Lipid-derived signals that discriminate wound- and pathogen-responsive isoprenoid pathways in plants: Methyl jasmonate and the fungal elicitor arachidonic acid induce different 3-hydroxy-3methylglutaryl-coenzyme A reductase genes and antimicrobial isoprenoids in *Solanum tuberosum* L. Proc Natl Acad Sci USA 91: 2329–2333

Cordero MJ, Raventos D, San Segundo B (1994) Expression of a maize proteinase inhibitor gene is induced in response to wounding and fungal infection: systemic wound response of a monocot gene. Plant J 6: 141–150

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, Jüttner F, Slusarenko AJ (1993) Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* var. *phaseolicola*. Plant Physiol 101: 13–24

Ellard-Ivey M, Douglas CJ (1996) Role of jasmonate in the elicitor- and wound-inducible expression of defence genes in parsley and transgenic tobacco. Plant Physiol 112: 183–192

Enyedi AJ, Yalpani N, Silverman P, Raskin I (1992) Signal molecules in systemic plant resistance to pathogens and pests. Cell 70: 879–886

Facchini PJ, Chappell J (1992) Gene family for an elicitor-induced sesquiterpene cyclase in tobacco. Proc Natl Acad Sci USA 89: 11088–11092

Farmer EE (1994) Fatty acid signalling in plants and their associated microorganisms. Plant Mol Biol 26: 1423–1437

Farmer EE, Ryan CA (1990) Interplant communication: Airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc Natl Acad Sci USA 87: 7713–7716

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

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

Feinberg AP, Vogelstein B (1983) A technique for labelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132: 6–13

Fournier J, Pélissier B, Esquerré-Tugayé MT (1986) Induction d'une activité lipoxygénase dans les cellules de tabac *Nicotiana tabacum* en culture, par des éliciteurs d'éthylène de *Phytophthora parasitica* var. *nicotianae*. CR Acad Sci Paris 303: 651–654

Fournier J, Pouénat ML, Rickauer M, Rabinovitch-Chable H, Rigaud M, Esquerré-Tugayé MT (1993) Purification and characterization of elicitor-induced lipoxygenase in tobacco cells. Plant J 3: 63–70

Godiard L, Ragueh F, Froissard D, Marco Y, Grosset J, Chartier Y, Meyer Y (1990) Analysis of the synthesis of several PR proteins in tobacco leaves infiltrated with water, compatible and incompatible isolates of *Pseudomonas solanacearum*. Mol Plant-Microbe Interact 3: 207–213

Graham JS, Hall G, Pearce G, Ryan CA (1986) Regulation of synthesis of proteinase inhibitors I and II mRNAs in leaves of wounded tomato plants. Planta 169: 399–405

Gundlach H, Mueller MJ, Kutchan TM, Zenk MH (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc Natl Acad Sci USA 89: 2289–2293

Haffner MH, Chin MB, Lane BG (1978) Wheat embryo ribonucleases. XII: Formal characterization of terminal and penultimate nucleoside residues at 5′ end of "capped" RNA from imbibing wheat embryos. Can J Biochem 56: 729–733

Jouanneau JP (1973) Contribution à l'étude du rôle moléculaire des cytokinines dans le contrôle de la mitose chez les cellules de tabac. Thèse Doct. es Sciences, Paris, CNRS AO 7045

Kühn H, Holzhütter HG, Schewe T, Hiebsch C, Rapoport SM (1984) The mechanism of inactivation of lipoxygenases by acetylenic fatty acids. Eur J Biochem 139: 577–583

Kutchan TM (1993) 12-oxo-phytodienoix acid induces accumulation of berberine bridge enzyme transcript in a manner nalogous to methyl jasmonate. J Plant Physiol 142: 502–505

Li WX, Kodama O, Akatsuka T (1991) Role of oxygenated fatty acids in rice phytoalexin production. Agric Biol Chem 55: 1041–1047

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275

Melan MA, Dong X, Endara ME, Davis KR, Ausubel FM, Peterman TK (1993) An *Arabidopsis thaliana* lipoxygenase gene can be induced by pathogens, abscisic acid, and methyl jasmonate. Plant Physiol 99S: 220

Mueller MJ, Brodschelm W, Spannagl E, Zenk MH (1993) Signalling in the elicitation process is mediated through the octadecanoid pathway leading to jasmonic acid. Proc Natl Acad Sci USA 90: 7490–7494

Mueller MJ, Brodschelm W (1994) Quantification of jasmonic acid by capillary gas chromatography-negative chemical ionization-mass spectrometry. Anal Biochem 218: 425–435

Pélissier B, Thibaud JB, Grignon C, Esquerré-Tugayé MT (1986) Cell surfaces in plant-microorganism interactions. VII. Elicitor preparation from fungal pathogens depolarize plant membranes. Plant Sci 46: 103–109

Peña-Cortés H, Albrecht T, Prat S, Weiler EW, Willmitzer L (1993) Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. Planta 191: 123–128

Rickauer M, Fournier J, Esquerré-Tugayé MT (1989) Induction of proteinase inhibitors in tobacco cell suspension culture by elicitors of *Phytophthora parasitica* var. *nicotianae*. Plant Physiol 90: 1065–1070

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

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

Roy S, Pouénat ML, Caumont C, Cariven C, Prévost MC, Esquerré-Tugayé MT (1995) Phospholipase activity and phospholipid patterns in tobacco cells treated with fungal elicitor. Plant Sci 107: 17–25

Ruzicska P, Gombos Z, Farkas GL (1983) Modification of the fatty acid composition of phospholipids during the hypersensitive reaction in tobacco. Virology 128: 60–64

Ryan CA (1990) Protease inhibitors in plants: Genes for improving defenses against insects and pathogens. Annu Rev Phytopathol 28: 425–449

Sembdner G, Parthier B (1993) The biochemistry and the physiological and molecular actions of jasmonates. Annu Rev Plant Physiol Plant Mol Biol 44: 569–589

Shinshi H, Mohnen D, Meins F Jr (1987) Regulation of a plant pathogenesis-related enzyme: Inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissue by auxin and cytokinin. Proc Natl Acad Sci USA 84: 89–93

Staswick PE, Huang JF, Rhee Y (1991) Nitrogen and methyl jasmonate induction of soybean vegetative storage protein genes. Plant Physiol 96: 130–136

Tranbarger TJ, Franceschi VR, Hildebrand DF, Grimes HF (1991) The soybean 94-kilodalton vegetative storage protein is a lipoxygenase that is localized in paraveinal mesophyll cell vacuoles. Plant Cell 3: 973–978

Véronési C, Rickauer M, Fournier J, Pouénat ML, Esquerré-Tugayé MT (1996) Lipoxygenase gene expression in the tobacco-*Phytophthora parasitica nicotianae* interaction. Plant Physiol, 112: 997–1004

Vick BA, Zimmerman DC (1983) The biosynthesis of jasmonic acid: A physiological role for plant lipoxygenase. Biochem Biophys Res Comm 111: 470–477

Walker-Simmons M, Ryan CA (1977) Wound induced accumulation of trypsin inhibitor activities in plant leaves. Plant Physiol 59: 437–439

Xu Y, Chang PL, Liu D, Narasimhan ML, Raghothama KG, Hasegawa PM, Bressan RA (1994) Plant defence genes are synergistically induced by ethylene and methyl jasmonate. Plant Cell 6: 1077–1085