Regulation of tobacco lipoxygenase by methyl jasmonate and fatty acids

Régulation de la lipoxygénase de tabac par le méthyle jasmonate et les acides gras

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Abstract — Lipoxygenase (LOX) activity and gene expression have been described previously to be induced in tobacco by fungal infection and elicitor treatment. We now report that LOX activity is induced in tobacco cell suspensions by treatment with methyl jasmonate (MeJa). This compound had no effect on the in vitro activity of tobacco LOX. Induction of LOX activity is a dose-dependent response with a maximum around 890 µM MeJa. Linolenic acid, the precursor for jasmonate synthesis, also induces LOX activity. When applied together with fungal elicitor, linolenic acid drastically increases and prolongs the induction of LOX activity. LOX activity and gene expression in elicited tobacco cells are partially inhibited by pretreatment with eicosatetraenoic acid (ETYA), a potent inhibitor of tobacco LOX in vitro. The induction by methyl jasmonate, in contrast, was not inhibited by ETYA pretreatment. These data suggest that induction of LOX gene expression and activity upon elicitation are regulated at least partially by LOX products. © Académie des Sciences / Elsevier, Paris

fatty acids / lipoxygenase / methyl jasmonate / Nicotiana tabacum / transduction pathway

Résumé — Des travaux ont montré que l’activité lipoxygénase (LOX) et l’expression des gènes correspondants peuvent être induites par une infection fongique ainsi que par l’application d’éliciteurs. Nous montrons ici que l’activité LOX est stimulée dans les cellules de tabac traitées par le méthyle jasmonate. Ce composé n’a pas d’effet in vitro sur l’activité de la lipoxygénase de tabac. L’induction de l’activité LOX in vivo est une réponse dose-dépendante et présente un maximum pour une concentration en méthyle jasmonate autour de 890 µM. L’acide linoléique, le précurseur de la synthèse d’acide jasmonique est également inducteur de l’activité LOX. Lorsque l’acide linoléique est appliqué simultanément avec l’éliciteur, la stimulation de l’activité lipoxygénase est fortement augmentée et prolongée. L’activité LOX et l’expression des gènes correspondants dans les cellules élicitées sont partiellement inhibées par un pré-traitement avec l’acide eicosatétraénoïque (ETYA), un puissant inhibiteur de l’activité LOX in vitro. La stimulation par le méthyle jasmonate n’est pas inhibée par un pré-traitement avec l’ETYA. Les données présentées ici suggèrent que l’induction de l’expression des gènes

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Version abrégée

Les lipoxygénases (LOX: linoléate: oxygène oxydoréductase, EC 1.13.11.12) sont des enzymes qui catalysent la formation d’hydroperoxydes via l’addition d’oxygène moléculaire sur les acides gras polyinsaturés (AGPs) contenant le motif cis, cis-1,4-pentadiène. Les AGPs tels que l’acide linoléique (C18:2) et l’acide linolénique (C18:3) représentent les principaux substrats de ces enzymes chez les plantes supérieures. Chez ces dernières, des lipoxygénases semblent jouer de nombreux rôles physiologiques, notamment dans la sénescence, la réponse à la blessure et aux attaques parasitaires, et dans la biosynthèse de régulateurs de croissance comme l’acide abscissique et l’acide jasmonique. Lors des interactions entre plantes et microorganismes pathogènes, des LOX pourraient participer à une cascade de transduction reliant la perception du microorganisme pathogène à l’expression des gènes de défense. Nous avons précédemment montré l’induction d’une lipoxygénase chez le tabac en réponse à l’infection par un champignon phytopathogène, Phytophthora parasitica var. nicotianae ainsi qu’après application d’éliciteurs issus du champignon. Il a été proposé que cette enzyme pourrait réguler la réponse de défense de la plante par la synthèse de produits secondaires dérivés du 13-hydroperoxyde, l’acide jasmonique et le méthyle jasmonate (Meja). Des études ont montré que l’acide linolénique, substrat de la LOX, et le Meja stimulent l’expression de gènes codant pour des inhibiteurs de protéase chez des plantules de tomate, ainsi que dans des cultures cellulaires de tabac, mais induisent également l’expression de protéines de réserve chez le soja, dont une lipoxygénase. Il était intéressant d’approfondir l’étude de la régulation de la lipoxygénase de tabac apparentée à la défense, notamment par ses propres substrats et le méthyle jasmonate. Nous avons d’abord recherché l’effet du Meja sur l’activité LOX in vitro. Le Meja n’a pas d’action inductrice ou inhibitrice sur l’activité LOX in vitro, alors que in vivo l’activité LOX est stimulée dans les cellules de tabac traitées avec cette même molécule. Cette stimulation est une réponse dose dépendante et présente un maximum pour une concentration autour de 890 μM en Meja. Une telle concentration a déjà été décrite comme optimale pour l’induction d’inhibiteurs de protéase dans des cellules de tabac. L’acide linolénique, le précurseur de la synthèse d’acide jasmonique est également inducteur de l’activité LOX, alors que l’acide linoléique ne l’est pas. Cependant il reste à déterminer si l’acide linolénique induit directement l’expression des gènes LOX, ou si il doit être converti en Meja pour stimuler cette expression. Quand il est appliqué simultanément avec les éliciteurs fungiques, l’acide linolénique augmente fortement la stimulation de l’activité lipoxygénase et la prolonge. L’activité LOX et l’expression des gènes LOX dans les cellules de tabac élicitées sont partiellement inhibées par un pré-traitement avec l’acide éicosatétraénioïque (ETYA), un puissant inhibiteur de l’activité LOX in vitro. En revanche, la stimulation par le méthyle jasmonate n’est pas inhibée par un pré-traitement avec l’ETYA, ce qui montre que les produits de la LOX, notamment le Meja, sont en partie nécessaires pour induire la production de cette enzyme.

Les résultats présentés dans ce manuscrit suggèrent que la production de la LOX peut être stimulée par ses substrats ainsi que par des produits formés après son action. Une telle régulation pourrait être mise en jeu pour amplifier la réponse initiale au cours des interactions plantes-microorganismes pathogènes.

1. Introduction

Lipoxygenases (LOX; linoleate: oxygen oxidoreductase, EC 1.13.11.12) are dioxygenases that catalyse the oxidation of polyunsaturated fatty acids (PUFAs) which present a cis, cis-1,4-pentadiene moiety. The major substrates for lipoxygenases in higher plants are linoleic acid (18:2) and linolenic acid (18:3). Linolenic acid is the most abundant fatty acid in most plant tissues, whereas linoleic acid is more abundant in plant seeds. LOXs appear to be ubiquitous among eukaryotic organisms. In animals, LOXs catalyse the oxygenation of arachidonic acid (released from phospholipids), and LOX-derived metabolites participate as signals in several biochemical and physiological processes [1]. In plants, LOX has been proposed to play a role in senescence, pathogen and wound responses, biosynthesis of the plant growth regulators abscisic acid and jasmonic acid, and mobilization of lipid reserves during seed germination [2, 3].

The involvement of LOX in plant–microbe interactions was suggested when arachidonic acid and eicosapentaenoic acid were isolated from the mycelium of Phytophthora infestans, a phytopathogenic fungus, and were shown to elicit phytoalexin accumulation and hypersensitive cell death in potato tissues, probably via metabolism by LOX [4]. The primary products of LOX, lipid hydroperoxides and their secondary derivatives such as free radicals, can exert deleterious effects on membranes, leading to cell death, so that a role for LOX in the hypersensitive response of a plant towards pathogens has been proposed [5]. Methyl jasmonate, a secondary product derived from 13-hydroperoxide has been described to increase expression of tomato and tobacco proteinase inhibitors [6, 7] which are part of the plant’s defence system.
Lipoxygenase has been previously shown to be induced in tobacco after infection with *Phytophthora parasitica* var. *nicotianae* [8] and treatment with fungal elicitors [9], and to be involved in the elicitor-induced production of jasmonic acid [10]. It has been proposed that this enzyme might regulate the onset of defence responses via the synthesis of jasmonic acid and methyl jasmonate. These compounds have been demonstrated to trigger the expression of defence-related genes in plants or plant cell cultures [7, 11], but also to induce expression of vegetative storage proteins in soybean [12], among them a lipoxygenase [13]. In barley, the expression of the *Lox 1 : Hv1* gene has been shown to be regulated during development and to be induced by MelA and wounding, but not by pathogen attack [14]. It was therefore interesting to study whether the defence-related lipoxygenase in tobacco was also affected by its substrates and by methyl jasmonate. The characterization of LOX activity and LOX expression in response to methyl jasmonate and fatty acids is described in this work.

2. Materials and methods

2.1. Tobacco cell culture

Cell suspension cultures of *Nicotiana tabacum* L. cv. Wisconsin 38 were grown as already described [15]. The cells were subcultured every 14 d and reached the stationary growth phase after 8 d.

2.2. Treatment of tobacco cells with methyl jasmonate and fatty acids

Seven-day-old tobacco cell cultures were used for incubations and prepared under sterile conditions as already described [15]. Elicitors were added as filter-sterilized solutions in water, at a final concentration of 30 μg dry weight mL⁻¹. Methyl jasmonate and fatty acids were added as aqueous solutions in 10% ethanol. Glycopeptide elicitors were prepared from the cell walls of *P. parasitica* Tuck var. *nicotianae* [16]. Linoleic acid (C18:2), linolenic acid (C18:3), arachidonic acid (C20:4) and eicosatetraenoic acid (ETYA) were purchased from Sigma and methyl jasmonate from Serva. The cells were incubated under culture conditions for the times indicated in the legends.

2.3. Lipoxygenase assay

Lipoxygenase was extracted from tobacco cells as described by Fournier et al. [17]. The enzyme assay was performed in the thermostatted cell of an oxygraph (Hansatech, DW1) at 30 °C, using a Clark oxygen electrode which was calibrated according to the method of Walker [18]. The enzyme extract (300 μL) was incubated in 0.25 M Na phosphate buffer pH 6.5 in a final volume of 790 μL; the reaction was started by adding 30 μL of linoleic acid to a final concentration of 2 mM. The initial velocity of oxygen uptake was converted into nanomoles per milligram of protein. A nanokatal was defined as the consumption of 1 nmol O₂ s⁻¹ at 30 °C. Appropriate controls in which the protein extract was heat-denatured or replaced by buffer were run simultaneously.

For the study of lipoxygenase inhibitors and methyl jasmonate on lipoxygenase activity in vitro, the crude protein extract was preincubated for 5 min at 30 °C, with increasing concentrations of methyl jasmonate or ETYA, before starting the enzymatic reaction by addition of lino-

2.4. RNA extraction and analysis

RNA was isolated from tobacco cells as described by Haffner et al. [19]. Total RNA (10 μg) was submitted to denaturing electrophoresis in a 1.2% (w/v) agarose formaldehyde gel. RNA was transferred to nitrocellulose membranes and fixed by baking for 2 h at 80 °C under vacuum. The membranes were prehybridized for 2 h at 42 °C in 50% formamide, 0.1% SDS, 1 × Denhardt's solution, 2 × SSC and 50 ng mL⁻¹ denatured calf thymus DNA, and hybridized overnight under the same conditions after addition of the radiolabelled tobacco LOX-cDNA probe [20, 21]. The 1.4-kb cDNA fragment (TL-35) was labelled with α³²P-dCTP by random priming as described by Feinberg and Vogelstein [22]. Membranes were then washed twice with 2 × SSC, 0.1% SDS at 55 °C before exposure to X-ray films at -80 °C.

2.5. Protein measurement

The protein content of the extracts was determined according to Lowry et al. [23] after precipitation with trichloroacetic acid. Soybean lipoxygenase type I (Sigma) was used as the standard protein.

3. Results

3.1. Dose–response of LOX activity during methyl jasmonate treatment

It has been demonstrated previously that LOX activity and LOX gene expression are induced by methyl jasmonate in tobacco cell suspensions [24]. A concentration of 890 μM methyl jasmonate was used in these experiments, which has been previously shown [10] to be optimal for proteinase inhibitor induction in tobacco cell suspensions. In order to establish if LOX induction occurred at the same concentrations, tobacco cells were incubated with increasing amounts of methyl jasmonate for 24 h, and LOX activity was determined polarographically in extracts with linolenic acid as substrate. A significant inducing effect of MelA was observed at 50 μM, but maximum response needed concentrations between 600 and 900 μM (figure 1). At still higher concentrations, however, LOX induction became less pronounced, probably owing to a toxic effect of MelA [10]. Control assays where LOX activity was measured in extracts obtained from elicitor-
treated cells, in the presence of increasing amounts of Mela, showed that Mela had no effect on LOX activity in vitro. Similarly, the low basal activity observed in extracts from untreated cells was not affected by Mela (data not shown).

3.2. Effect of fatty acids on LOX activity

Linoleic and linolenic acids are natural substrates of lipoygenase in plants, but only linolenic acid is converted into the precursor for jasmonate synthesis. Arachidonic acid occurs in significant amounts only in animals and fungi, but is a substrate for both animal and plant lipoxynases.

The effect of these polyunsaturated fatty acids on LOX induction was studied by incubating tobacco cells for 24 h in the presence of 150 μM of either compound. This concentration has been found to give maximal response during preliminary assays (data not shown). Linolenic acid and arachidonic acid showed stimulatory effects on LOX activity, with stimulation factors of three- and two-fold respectively (figure 2). The induction of LOX activity by linolenic acid was further investigated by a time course study. The stimulation factor compared to control cells did not change significantly during the incubation period (figure 3). When both elicitor and linolenic acid were added to the cell suspension, a significant difference to treatment with elicitor alone appeared after 24 h (figure 3). Whereas LOX activity was transient in elicitor-treated cell suspensions, its decrease after 24 h was prevented by the presence of linolenic acid; LOX activity at 30 h was about five-fold higher in cells treated with both compounds than in elicitor-treated cells.

3.3. An inhibitor of LOX activity prevents LOX induction

The induction of lipoygenase by elicitor, methyl jasmonate and by linolenic acid suggests that the initial response triggered after elicitor perception might be am-

Figure 1. Dose-response measurement of methyl jasmonate induced lipoxynase activity.
After incubation of tobacco cells for 24 h in the presence of methyl jasmonate at increasing concentrations, lipoxynase activity was measured in crude extracts by a polarographic method. Values are the means of three independent experiments ± SE.

Figure 2. Effect of fatty acids on LOX activity.
Tobacco cells (10 mL) were incubated for 24 h in the presence of 150 μM linoleic acid (C18:2), 150 μM linolenic acid (C18:3), 150 μM arachidonic acid (C20:4) in water containing 10% ethanol (control), or with 30 μg mL⁻¹ elicitors (Eli). Lipoxynase activity was measured in crude extracts by polarography. Values are the means of three independent experiments ± SE.
4. Discussion

Several studies have shown that two compounds of the lipoxygenase pathway, jasmonic acid and methyl jasmonate are regulatory compounds for the wound response in plants [6, 25], and might also regulate the defence response against microbial attack [10, 11, 26, 27]. Methyl jasmonate has been shown to trigger defence reactions in various plants, and to be produced in response to wounding and elicitor treatment [10]. Recently, we have reported that treatment of tobacco cell suspensions with elicitor from Phytophthora parasitica var. nicotianae induced a rapid and transient production of JA, which was abolished by ETYA, an inhibitor of LOX activity in vitro [10]. It has also been demonstrated that LOX activity and LOX gene expression were induced similarly in tobacco cells treated with methyl jasmonate [24] or with elicitors [8]. Lipoxy-

Table I. Effect ETYA on LOX activity in vitro.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Compound added</th>
<th>LOX activity (^{b}) (nkat mg(^{-1}) protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated cells</td>
<td>none</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>Elicitor-treated cells(^{a})</td>
<td>ETYA 50 µM</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>5 µM</td>
<td>1.90 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>10 µM</td>
<td>1.25 ± 0.33</td>
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<tr>
<td></td>
<td>20 µM</td>
<td>0.27 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>50 µM</td>
<td>0.25 ± 0.05</td>
</tr>
</tbody>
</table>

\(^a\) In all cases elicitor was added to a final concentration of 30 µg dry weight mL\(^{-1}\) to the cell suspension; \(^b\) values are the means of three independent experiments ± SE.
Genase might thus participate in a transduction cascade, linking pathogen perception and defense, where Mejα concomitantly acts as an inducer of defense gene expression and amplifies the initial signal. The aim of the present work was to further elucidate the importance of methyl jasmonate and fatty acids for lipoygenase regulation in tobacco cells.

In this study it is shown that tobacco LOX was induced by Mejα over a wide concentration range with a maximal response around 890 μM. Thus, the dose dependence of LOX induction by Mejα highly resembles the response of proteinase inhibitors to Mejα [10], suggesting a link between these two responses. The apparently high optimal concentration of Mejα is only a theoretical value since the commercial solution contains only 5–10% of the biologically active isomer [10]. As proposed in a former work [10] it is possible that tobacco cell cultures are less sensitive to Mejα than other plant material; this difference might be species-specific or due to the physiological state of the cell culture. LOX activity extracted from elicitor-treated cells was not affected by jasmonate in vitro. Thus, induction of LOX activity by methyl jasmonate is related to stimulation of gene expression without a direct effect on the enzyme. Linolenic acid, the precursor for JA biosynthesis, was able to induce LOX activity in tobacco (this work), and to promote proteinase inhibitor accumulation [28]. Linoleic acid, a LOX substrate which is not a jasmonate precursor, presented no stimulatory effect on either LOX activity or proteinase inhibitor synthesis [28]. It is not known if linolenic acid induced LOX gene expression directly, or if it has to be converted into jasmonate or into a jasmonate precursor. In potato tubers it has been demonstrated that the elicitor activity of arachidonic acid depended on its conversion by LOX, but the active metabolite has not been identified [4]; LOX activity itself increased rapidly after treatment with the fatty acid [29]. The effect of linolenic acid has not been investigated in this system. The addition of linolenic acid to elicitors had a synergistic effect on LOX activity and prolonged the induction of LOX activity in tobacco cells. It has been reported that elicitor treatment induced phosphatidase activity in tobacco [30] and soybean [31], as well as increases in free linolenic acid in Eschscholzia californica cells [32]. These data and our results indicate that the availability of substrate might be a limiting step in the LOX pathway. The induction of tobacco LOX by fungal elicitor was partially inhibited by ETYA. This is consistent with a positive feedback regulation of the enzyme by one (or several) of its products, e.g., jasmonate. According to this model ETYA should not suppress LOX induction by Mejα treatment. This was indeed the case, but at 24 h of ETYA + Mejα treatment, LOX induction was even stronger than after treatment with Mejα alone. Such a stimulation suggests a more subtle regulation of the LOX pathway. It must be kept in mind that the fatty acid hydroperoxides formed by LOX can be transformed into a multitude of compounds, such as free radicals, hydroxides, cetones, aldehydes, etc. The occurrence of these compounds and their putative biological activities have yet to be characterized in tobacco. It is possible that one (or several) of them exert negative regulation on LOX, and the inhibition of its (their) formation might thus enhance LOX induction by methyl jasmonate.

So far, our data indicate a complex regulation of LOX by its substrates and reaction products. It will be of particular interest to study the other enzymes involved in the so-called octadecanoid pathway [6], notably allene oxide synthase and allene oxide cyclase which are involved in the further conversion of LOX products. The importance of LOX in the incompatible interaction between tobacco and Phytophthora parasitica var. nicotianae race 0 has been demonstrated recently by the generation of transgenic plants expressing antisense LOX sequences [33]. Further studies concerning defense gene expression in these plants after treatment with elicitors or lipid compounds will help to unravel the regulation of this enzyme and its importance in the elicitation process.

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References


