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Effect of Volatile Methyl Jasmonate on the Oxylipin Pathway in Tobacco, Cucumber, and Arabidopsis[1]

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The effect of atmospheric methyl jasmonate on the oxylipin pathway was investigated in leaves of tobacco (*Nicotiana tabacum* L.), cucumber (*Cucumis sativa* L.), and *Arabidopsis thaliana* (L.). Differential sensitivities of test plants to methyl jasmonate were observed. Thus, different concentrations of methyl jasmonate were required for induction of changes in the oxylipin pathway. Arabidopsis was the least and cucumber the most sensitive to methyl jasmonate. Methyl jasmonate induced the accumulation of lipoxygenase protein and a corresponding increase in extractable lipoxygenase activity. Atmospheric methyl jasmonate additionally induced hydroperoxide lyase activity and the enhanced production of several volatile six-carbon products. It is interesting that lipid hydroperoxidase activity, which is a measure of hydroperoxide lyase plus allene oxide synthase plus possibly other lipid hydroperoxide-metabolizing activities, was not changed by methyl jasmonate treatment. Methyl jasmonate selectively altered the activity of certain enzymes of the oxylipin pathway (lipoxygenase and hydroperoxide lyase) and increased the potential of leaves for greatly enhanced six-carbon-volatile production.

MJ and JA are thought to be involved in numerous aspects of plant biochemistry. These include wound-induced defense (Farmer and Ryan, 1992; Pena-Cortes et al., 1993) and inhibition of plant growth and promotion of senescence (reviewed by Hamberg and Gardner, 1992), as well as the induction of tuberization in potato (Koda et al., 1991) and yam plants *Dioscorea* spp. (Koda and Kikuta, 1991). Mueller-Uri et al. (1988) demonstrated that jasmonins altered the expression of certain proteins they designated as jasmonate-induced proteins. Parthier (1991) and Reinbothe et al. (1994) reviewed the possible function of JA and MJ as signaling molecules.

Both JA and MJ are derived from linolenic acid via the oxylipin pathway (Vick and Zimmerman, 1984; Song and Brash, 1991), and there has been speculation that they may fulfill a biochemical and regulatory signaling role in plants similar to that of leukotrienes and prostaglandins in animals. Anderson et al. (1989) and Francheschi and Grimes (1991) showed that exogenously applied MJ induced the accumulation of vegetative storage proteins in soybeans, that the 94-kD vegetative storage protein was a member of the LOX gene family (Tranbarger et al., 1991), and that MJ induced an active form of LOX (Bell and Mullet, 1991; Grimes et al., 1992).

A product of the oxylipin pathway, MJ, may have a positive feedback effect on one of the enzymes involved in its own production, LOX. The work reported below is an attempt to clarify the importance of the effects of MJ on the enzymes and the products of the oxylipin pathway.

MATERIALS AND METHODS

Biological Material

Tobacco (*Nicotiana tabacum* L. cv KY14) and cucumber (*Cucumis sativus* L. cv Wisconsin SMR-58) plants were grown in a greenhouse at 23 to 33°C under a 14-h photoperiod. *Arabidopsis thaliana* ecotype Columbia plants were grown in a growth chamber at 20°C under a 12-h photoperiod.

Chemicals

Yeast alcohol dehydrogenase, NADH, soybean LOX 1, leupeptin, linoleic acid, linolenic acid, heptadecanoic acid, and Triton X-100R were from Sigma. Chloroform, methanol, and hexane were from Fisher. 13(*S*)-Hydroperoxide of linolenic acid was prepared with soybean LOX 1 following the procedure described by Gardner (1975), and the concentration of hydroperoxide was measured spectrophotometrically at 235 nm with 25,000 M^{-1} cm^{-1} as the extinction coefficient. Hexanal, *E*-2-hexenal, and *Z*-3-hexenyl acetate were from Aldrich, and *Z*-3-hexen-1-ol was from Bedoukian (Danbury, CT).

Plant Treatments and Enzyme Assays

Plants were individually enclosed in airtight 12.5-L bell jars. One microliter of neat MJ on a piece of a filter paper

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Abbreviations: HPL, hydroperoxide lyase; JA, jasmonic acid; LHP, lipid hydroperoxidase; LOX, lipoxygenase; MJ, methyl jasmonate.

was applied per jar for tobacco (6–7 weeks old), 50 μL of neat MJ for Arabidopsis (2–3 weeks old), and 1 μL of MJ diluted 1:100 in ethanol for cucumber (3–4 weeks old). Three plants were used for treatment with MJ. For the control, plants were kept in similar jars either untreated (tobacco, Arabidopsis) or treated with 1 μL of ethanol (cucumber). The plants were analyzed for up to 5 d after treatment.

Fully expanded leaf tissues (0.5 g) were homogenized in a chilled mortar and pestle in a total of 0.5 mL of Hepes buffer (50 mM, pH 7.5) containing 0.5% (v/v) Triton X-100R at 0°C. The homogenate was then centrifuged at 14,000*g* for 15 min, and the supernatant was used for measurement of enzymatic activities.

LOX activity was measured using a standard spectrophotometric assay involving the measurement of conjugated diene formation at 235 nm at pH 6.8 (Hildebrand et al., 1991). HPL activity was detected by NADH oxidation at 340 nm in the presence of yeast alcohol dehydrogenase and 13(*S*)-hydroperoxide of linolenic acid (Vick, 1991), and the decrease in A_{235} of conjugated diene substrate (LHP activity) was measured.

Electrophoresis and Western Blotting

SDS-PAGE was performed using 10% separating and 5% stacking gels as described by Laemmli (1970). Western blotting was performed as described by Wang and Hildebrand (1987) using rabbit polyclonal antibodies raised against soybean leaf peak 3 LOX (Grayburn et al., 1991). Fifty micrograms of the protein extracted from tobacco leaves were analyzed on a 10% SDS gel and probed with polyclonal antibodies raised against soybean leaf peak 3 LOX isozyme.

C_6-Aldehyde Measurement

Five discs cut with an 8-mm cork borer from tobacco or cucumber leaves (the same leaves that were analyzed for enzymatic activities) or five whole leaves of Arabidopsis were put into 1.8-mL screwcap vials and stored at −80°C until the assay. In preparation for C_6-aldehyde measurement, the vials were placed in a 30°C water bath for 20 min followed by 5 min at 80°C, and then 250 μL of gas from the headspace of the vials were withdrawn using a gas-tight 500-μL syringe and injected directly into a Varian (Sunnyvale, CA) 3700 gas chromatograph with a 30-m × 0.53-mm DB-Wax (PEG) fused silica column operated under the following conditions: injector, 220°C; oven temperature, 50°C for 5 min and then 3°C min^{-1} to 150°C; flame ionization detector, 240°C; helium carrier flow rate, 6 mL min^{-1}. All quantitative analyses were repeated at least four times. The identity of compounds was determined by co-chromatography with authentic standards.

Time Course of MJ Treatment

For the time course of the MJ-treatment studies, tobacco plants (six to seven fully developed leaves) were treated with 2 μL of neat MJ for 5 d in the bell jars. Control plants were kept in similar bell jars. Every day the bell jars were opened, an aliquot of leaf tissue was collected (0.5 g), and the plants were put under bell jars again with a new aliqout of MJ added. Leaf tissues were kept at −80°C until the analysis.

Aliquots of leaf tissues (0.5 g) were ground in a mortar with liquid nitrogen with 2 mL of the grinding buffer as described by Radetzky et al. (1993) with some modifications. The final composition of buffer was 15% Suc, 10 mM KCl, 1.5 mM EDTA, 0.1 mM $MgCl_2$, 10 μM leupeptin, 150 mM Tris-HCl, pH 7.5. This was the only buffer among several that are used routinely for LOX analysis where the full-size LOX band was seen intact on western blots. The extracts were centrifuged for 15 min at 12,000*g*, and the supernatants were used for the enzymatic activity analyses. For western blots, supernatants were mixed 1:1 (v/v) with loading buffer (Laemmli, 1970) and boiled for 5 min.

RESULTS

Effects of MJ on Oxylipin Pathway Enzymes

Tobacco plants were treated with MJ in airtight 12.5-L bell jars for 5 d. One microliter of neat MJ was applied per jar. Plants thus treated showed symptoms of senescence, visible as slight chlorosis that was lacking in controls. Treated cucumber plants exhibited tissue collapse within 10 h of treatment with 1 μL of neat MJ. An application of 1 μL of MJ diluted 1:100 in ethanol (less than 1 nL L^{-1} final concentration) caused no visible tissue collapse but caused a significant increase in LOX and HPL activities (Table I) and LOX/HPL product formation (Table II). Arabidopsis plants were little affected when treated at the same level of MJ that significantly affected tobacco. A 50-fold higher level of MJ was needed to give a similar induction of C_6-aldehyde formation in Arabidopsis as in tobacco. At this level of MJ treatment, Arabidopsis plants exhibited growth inhibition. For example, such plants had a drastically reduced growth of the flower stalk to approximately 5 to 10% of the length of those of control plants.

MJ in the gaseous phase increased LOX activity in tobacco leaf tissues 3.2-fold after 5 d of treatment. HPL activity was enhanced more than 2-fold, whereas LHP activity was not significantly affected (Table I). After 5 d of MJ treatment, plants were removed from the bell jars and left in normal greenhouse conditions. The increase in both LOX and HPL activity was persistent and did not significantly change for at least 7 d after termination of MJ exposure. The increases in LOX and HPL activities

Table I. *Enzymatic activities (nmol s^{-1} μg^{-1} protein) in tobacco and cucumber plants treated with MJ for 5 d*

MJ significantly increased LOX and HPL activities at the 1% level but had no significant effect on LHP.

Plant and Treatment	LOX	HPL	LHP
Tobacco			
Control	0.18 ± 0.06	0.19 ± 0.01	0.29 ± 0.04
MJ	0.58 ± 0.16	0.53 ± 0.06	0.26 ± 0.01
Cucumber			
Control	0.83 ± 0.13	0.73 ± 0.11	0.23 ± 0.02
MJ	2.05 ± 0.45	1.19 ± 0.26	0.25 ± 0.07

Table II. *Effects of MJ treatment on volatile fatty acid oxidation product content in tobacco, cucumber, and Arabidopsis leaves*

Units are gas chromatograph-flame ionization detector area units and represent means ± SE of three experiments.

Retention Time	Compound	Tobacco		Cucumber		Arabidopsis	
		Control	MJ	Control	MJ	Control	MJ
2.8	Hexanal	133 ± 18	523 ± 71	33 ± 5	83 ± 26	44 ± 10	261 ± 72
6.5	*E*-2-hexenal	2062 ± 410	3689 ± 612	289 ± 62	462 ± 83	128 ± 37	586 ± 124
10.2	*Z*-3-hexenyl acetate	nd[a]	50 ± 4	4 ± 1	5 ± 1	nd	nd
12.9	*Z*-3-hexen-1-ol	61 ± 12	99 ± 17	68 ± 13	70 ± 19	nd	nd

[a] nd, Not detected.

were readily apparent after 2 d of exposure to MJ, with maximum enhancement seen after 4 d of exposure to MJ (Fig. 1A). The absolute level of LOX and HPL activities varied from experiment to experiment, depending on leaf and plant age as well as environmental conditions, but the relative increase due to MJ treatment was highly reproducible.

MJ treatment of cucumber plants produced results similar to MJ treatment of tobacco in the change of oxylipin pathway enzymatic activities. LOX activity was increased 2.5-fold, HPL activity was increased by 62%, whereas LHP activity was not significantly altered (Table I).

An increase of LOX protein was apparent in tobacco leaf tissues upon treatment with MJ as shown by immunoblot analysis (Fig. 1B). Similar results were obtained for cucumber and Arabidopsis leaves (data not shown).

Effects of MJ Treatment on C_6-Aldehyde Production

MJ treatment of tobacco plants significantly affected the formation of C_6-aldehydes, which are the products of the oxylipin pathway via HPL activity as shown by co-chromatography with authentic compounds (Table II). The amount of hexanal was increased more than 3-fold and the amount of the most abundant aldehyde, *E*-2-hexenal, by 80%. *Z*-3-Hexenyl acetate, not detectable in control plants, accumulated in the leaf tissues treated with MJ. There was a 60% increase in the amount of the C_6-alcohol *Z*-3-hexen-1-ol.

Cucumber plants showed a similar trend in C_6-aldehyde production (Table II). MJ treatment increased the amount of hexanal 2.5-fold and the amount of *E*-2-hexenal by 60%. *Z*-3-Hexenyl acetate was detected at a very low level, and its concentration, as well as the concentration of *Z*-3-hexen-1-ol, was not affected by MJ.

In Arabidopsis plants, MJ treatment increased the amount of hexanal production more than 5-fold and that of *E*-2-hexenal 4.6-fold (Table II).

DISCUSSION

JA and MJ appear to be important signaling molecules in plants (Farmer and Ryan, 1990, 1992; Song and Brash, 1991). They have been shown to be able to alter expression of many plant proteins when applied exogenously (Mueller-Uri et al., 1988; Farmer and Ryan, 1990; Bell and Mullet, 1991; Francheschi and Grimes, 1991; Grimes et al., 1992). MJ also seems to be regulatory for some enzymes directly involved in the oxylipin pathway, since it induces the accumulation of LOX in soybean (Grimes et al., 1992), Arabidopsis (Melan et al., 1993), and *Phaseolus vulgaris* (K.P.C. Croft, unpublished data). We provide evidence here that MJ can increase levels of LOX and of HPL in tobacco, Arabidopsis, and cucumber while leaving some other enzymes associated with the oxylipin pathway unchanged in activity.

In all three test plant species used in this series of experiments, increases in the activities of specific enzymes in-

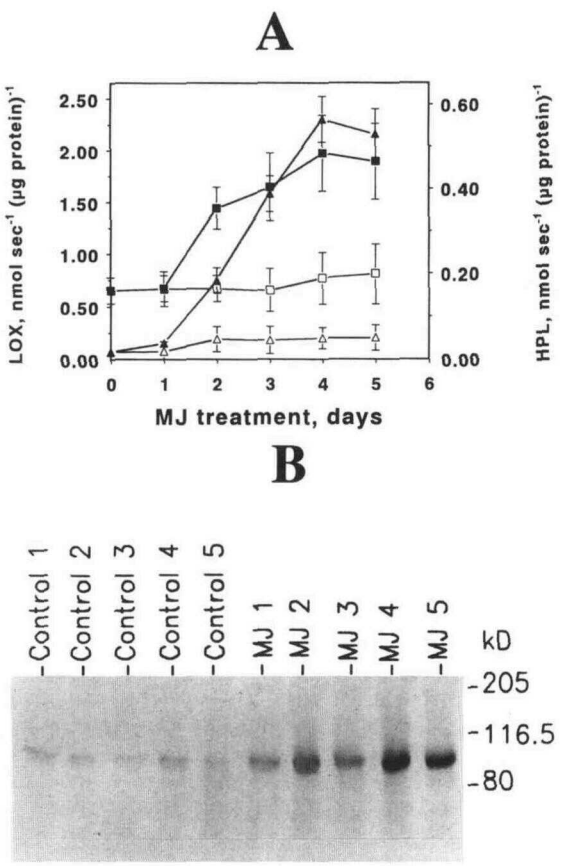

Figure 1. Time course of enzymatic activities of LOX and HPL (A) and of LOX protein as detected by western blot (B) in tobacco leaves after treatment with 2 µL of MJ in the bell jars. A, Triangles, LOX activity; squares, HPL activity; filled symbols, MJ treatment; open symbols, control. B, Fifty micrograms of the protein were loaded in each lane. The legend represents samples from control and MJ-treated leaves 1 to 5 d after application of MJ. Soybean LOX peak 3 antibodies were used to decorate the blot. Molecular mass standards are indicated on the right side of B.

volved in the LOX/lyase pathway were observed after exposure to MJ, as were changes in certain products of the pathway. In vitro assays for LOX activity and western blot analysis of protein extracts from MJ-treated tobacco and cucumber plants showed that MJ is a potent enhancer of LOX. Western analysis of MJ-treated Arabidopsis showed a similar increase in LOX protein. It is interesting that there was a wide variation in the degree of sensitivity to MJ between the plant species tested. Arabidopsis was the most insensitive, requiring 50-fold more MJ than tobacco to achieve a similar degree of LOX increase, whereas cucumber appeared to be approximately 100-fold more sensitive than tobacco. How this affects the importance and use of MJ as an inter- and intracellular signaling molecule in different plant species is yet to be investigated.

Other enzymes of the oxylipin pathway were also enhanced by MJ. Tobacco and cucumber exhibited an increase in HPL of 2- and 0.6-fold, respectively, following MJ treatment. Once the fatty acid hydroperoxide [e.g. 13(*S*)-hydroperoxide of linolenic acid] is formed because of LOX activity, at least two possible metabolic routes are available in the oxylipin pathway (Vick and Zimmerman, 1987; Hildebrand, 1989). First, HPL will cleave the 18-carbon chain into C_6- and C_{12} moieties and give rise to the C_6-aldehydes and C_6-alcohols and the C_{12} product 12-oxo-*E*-10-dodecenoic acid (traumatin). Alternatively, the fatty acid hydroperoxide may be metabolized by allene oxide synthase and a cyclase, causing the cyclization of the hydroperoxide, which, following reduction and several rounds of β-oxidations, will form JA (Song and Brash, 1991). The data presented here provide evidence that MJ directly or indirectly enhances certain enzymes of the oxylipin pathway and thereby affects the direction the pathway takes and ultimately the type of fatty acid peroxidation products formed. In both tobacco and cucumber, only HPL showed good induction of enzymatic activity, whereas LHP levels were not significantly different between control and MJ-treated plants. From this result one would expect to see a shift in the balance of oxylipin pathway products after MJ treatment to increased production of C_6 volatiles and traumatin from the HPL branch. When the ability of MJ-treated tissues to evolve C_6 volatiles was compared with that of control tissue, this was the case. All three plant species showed greater potential to form a variety of C_6 volatiles than controls. Hexanal, derived from linoleic acid, increased 2.5-fold in cucumber, 3-fold in tobacco, and 5-fold in Arabidopsis. Similarly, *E*-2-hexenal, derived from linolenic acid, increased above control plants by 80% in tobacco, 60% in cucumber, and 460% in Arabidopsis. If the levels of linoleic and linolenic acids available to LOX-catalyzed peroxidation are limiting in the pathway, the increase in HPL could shift the oxylipin pathway away from production of JA by allene oxide synthase.

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