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Effect of Abscisic Acid on the Linoleic Acid Metabolism in Developing Maize Embryos[1]

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ABSTRACT

Partially purified protein extracts from maize (*Zea mays* L.) embryos, whether treated or not with abscisic acid (ABA), were incubated with linoleic acid (LA) and 1-[^{14}C]LA. The resulting LA metabolites were monitored by high performance liquid chromatography with a radioactivity detector and identified by gas chromatography-mass spectrometry. α- and γ-ketol metabolites arising from 9-lipoxygenase activity were the more abundant compounds detected in the incubates, although the corresponding metabolites produced by 13-lipoxygenase were also present in the samples. In addition, a group of stereoisomers originating from two isomeric trihydroxy acids (9,12,13-trihydroxy-10-octadecenoic and 9,10,13-trihydroxy-11-octadecenoic acids) are described. Important variations in the relative proportions of the LA metabolites were observed depending on the embryo developmental stage and on ABA treatment. Two new ABA-induced compounds have been detected. These compounds are present in embryos at all developmental stages, being more abundant in old (60 days) embryos. Furthermore, ABA induction of these compounds is maximum at very young developmental stages, decreasing as maturation progresses. A tentative structure for these compounds (10-oxo-9,13-dihydroxy-11-octadecenoic acid and 12-oxo-9,13-dihydroxy-10-octadecenoic acid) is also provided. This study revealed an early stage in maize embryogenesis characterized by a higher relative sensitivity to ABA. The physiological importance of ABA on LA metabolism is discussed.

LOXs[2] are a group of enzymes that catalyze the oxygenation of fatty acids with a *cis,cis*-1,4-pentadiene system. A number of plant LOXs have been identified (2) and studied in terms of developmental regulation (9), genetic characterization (23), tissue specific distribution (30), or subcellular localization (25). Also, the DNA coding sequence for some plant LOXs genes has been obtained (3, 6, 22). However, no comparable data are available for maize where research has been focused mainly on LOX activity in seeds (10) and germinating seedlings (18, 26).

Although LOX activity is widespread throughout the plant kingdom, the physiological role of the enzyme is not well understood. It has been proposed that the enzyme may play a role during seed maturation. LOX catalysis may be responsible for the biosynthesis of certain growth regulatory substances in a variety of plant tissues (5). A LOX-derived metabolite, jasmonic acid, has been characterized in plants (27). Jasmonic acid, together with its methyl ester analog, are regulatory substances that inhibit growth and promote senescence (5). Interestingly, jasmonic acid methyl ester induces alteration of gene expression in different plant systems (15, 16), and some jasmonate-induced proteins seem to be electrophoretically and immunologically identical with ABA-induced proteins (16). The plant hormone ABA mediates a number of important physiological responses in plants. ABA is the major growth inhibitor present during the maturation period in seeds of many species (19). Developmental studies in maize embryos have shown that the endogenous levels of ABA increase during development (13) and induce the accumulation of specific mRNAs and proteins prior to the desiccation of the seed (17, 21, 29). However, the mode of action of the hormone remains unclear.

We have studied the effect of ABA on the metabolism of LA via the LOX pathway. Partially purified extracts were obtained from isolated embryos at different developmental stages treated or not with ABA and incubated with LA and 1-[^{14}C]LA. The resulting metabolites were monitored by HPLC with an on-line radioactivity detector, isolated, and identified by GC-MS.

MATERIALS AND METHODS

Plant Material

Isolated embryos of *Zea mays* L. inbred line W-64 of different developmental stages (from 15 d after pollination to dry embryos) were used as controls. ABA incubations were performed by culturing isolated embryos for 7 d in basal medium with 10 μM ABA as described previously (17).

Enzyme Extracts

Protein extracts from control and ABA-incubated embryos were prepared by grinding the fresh material in a mortar with liquid nitrogen. Samples of powder (100–200 mg) were immediately sonicated in 1 mL 0.05 M phosphate buffer, pH 7 (2% sodium metabisulfite). The samples were centrifuged and the supernatants were used for the incubation with linoleic acid.

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[2] Abbreviations: LOX, lipoxygenase; LA, linoleic acid; ME, methyl ester; MO, methoxime; TMS, trimethylsilyl.

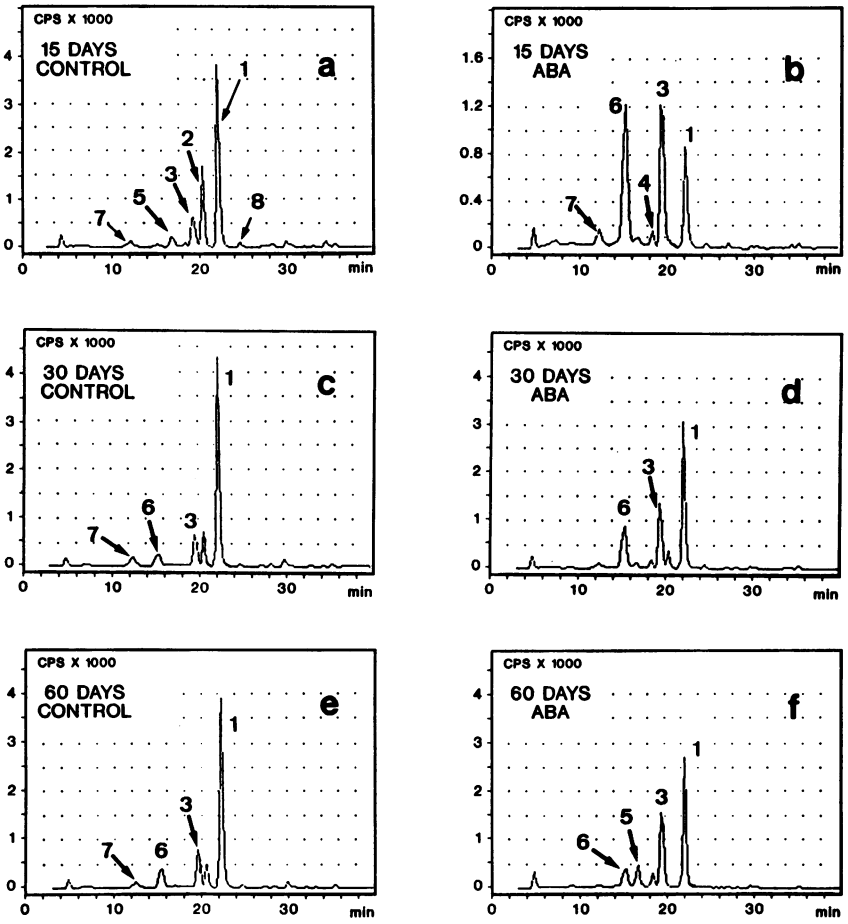

Figure 1. Radiochromatographic profiles of metabolites of 1-[^{14}C]LA incubated with an extract of maize embryos showing variations in controls (panels a, c, and e) at different times after pollination (15, 30, and 60 d) and in maize embryos treated with ABA (panels b, d, and f).

LA Oxidation Products

LA (100 μg) and 0.5 μCi of 1-[^{14}C]LA (59 mCi/mmol) were added to 4 mL of 0.05 M phosphate buffer pH 6 with 500 to 800 μL of the enzyme extract. To avoid the presence of LA autooxidation products, both LA and 1-[^{14}C]LA were previously purified and monitored by reverse phase HPLC. Incubation was carried out at room temperature during 30 min. Hydroperoxides were then reduced by addition of 500 μl $SnCl_2$ (1 mg/mL in EtOH). After 30 min reaction, lipids were extracted at pH 3 with ether. The ethereal extracts were desiccated with anhydrous sodium sulfate and evaporated to dryness with He. The material was redissolved in acetonitrile and stored at −80°C until analysis.

Liquid Chromatography

The HPLC separations were performed on a reverse phase 10 μm (30 × 0.4 cm) Spherisorb ODS-2 column. The mobile phase was H_2O (pH 3.5 with AcOH) with a gradient of acetonitrile from 30 to 95% in 30 min.

Radioactivity detection was carried out by means of an online LS detector (Ramona, RAYTEST, Isomess, Straubenhardt, Germany) or by radioactivity counting of sequential 30 s fractions in a Beckman LS counter detector.

Gas Chromatography-Mass Spectrometry

Radioactive fractions were individually subjected to standard methylation, methoximation procedures as described (1). Silylation was carried out with *N,O*-bistrimethylsilyltrifluoroacetamide in acetonitrile 1:1 for 1 h at 60°C. (20). Aliquots of these fractions were previously hydrogenated by bubbling hydrogen gas in an MeOH solution in the presence of 1 mg PtO_2 and submitted to the same derivatization steps (14).

The derivatized samples were analyzed by electron impact GC-MS in a Hewlett-Packard 5995 quadrupole instrument. The GC column was a 25 m Ultra-1 also from Hewlett-Packard, using helium as carrier gas.

The column temperature was programmed from 80 to 170°C at 15°C/min and then to 270°C at 3°C/min. Injector, GC-MS interface, ion source, and analyzer temperatures were 260, 280, 250, and 200°C, respectively. Mass spectra were obtained at 70 eV.

RESULTS

The HPLC profile resulting from incubating 1-[^{14}C]LA with extracts from maize embryos obtained at 15, 30, and 60 d, after pollination and treated (b, d, f) or not (a, c, e) with ABA is shown in Figure 1. As illustrated in this figure, LA metabolites in these incubates can be readily and selectively detected

by radioactive monitoring. Incubation of maize embryos with labeled LA affords up to eight radioactive signals, labeled 1 through 8 in Figure 1. None of these peaks were observed when protein was deactivated by heat (100°C, 1 min) before incubation. Only very small amounts of nonmetabolized LAs were detected under the incubation conditions used.

Depending on the embryo developmental stage, important variations in the relative proportions of the HPLC signals can be observed (see peaks 2, 3, 5, and 6), both in control and in ABA-treated embryos.

The more dramatic change is observed in the relative abundances of peak 6 when young embryos were treated with ABA. ABA induction of peak 6 is maximum at very young developmental stages, decreasing as maturation progresses and being null in dry embryos. Thus, these results show differences in the response to ABA which are related to the age of the embryos. Peak 6 also appears during normal embryogenesis when the endogenous levels of ABA are at a maximum (13), and it is also present in dry embryos.

Characterization of peak 6 and some of the major peaks on the HPLC profile was carried out by GC-MS analysis of the adequate derivatives. Derivatives were selected based on the functional moieties that could be expected to be present in the various LOX metabolites of LA. The principal pathways for the formation of the lineal chain LA metabolites by 9- and 13-LOX activity in plants are shown in Figure 2. The R1 group in these chemical structures is $(CH_2)_6COOH$. Correlation between chemical structures established by GC-MS analysis of suitable HPLC fractions and the radioactive peaks appearing in the radiochromatograms is presented in Tables I and II.

A detailed study of the fragmentation patterns of the resulting derivatives allows the following conclusions to be drawn regarding peak identification.

The expected reduction product of the 9-hydroperoxide of LA (9-OOH C18:2), the 9-hydroxy-10-*trans*-12-*cis*-octadecadienoic acid (9-OH C18:2) (Ia), could be observed only as a very minor peak in these extracts (peak number 8, Fig. 1).

The ME, TMS ether, and MO derivatives of peak 1 in Figure 1 gave two major GC peaks with different mass spectral patterns and different relative abundances. Analysis of the hydrogenated HPLC fraction and the use of other derivatization sequences (ME, TMS, and TMS derivatives) show that the mass spectra of these peaks (Tables I and II) are consistent with the structure of the MO *syn*- and anti-isomers of the methyl 9-trimethylsilyloxy-10-methoxime-12-octadecenoate. Thus, the major component of peak 1 is the 9-hydroxy-10-oxo-12-octadecenoic acid (see Va in Fig. 2). This compound has been reported as one of the major metabolites of LA arising through LOX activity in several plant sources (28).

Compound Va is generated by the addition of water to the allene oxide IIa formed by the action of a hydroperoxide dehydrase upon the 9-OOH C18:1 (16), as illustrated in Figure 2. The allene oxide intermediate has not been detected in these samples due to its low half-life ($t_{1/2}$ = 33 s at 0°C) in aqueous solutions (12). Nucleophilic attack of water could also take place at C13 in IIa affording the γ-ketol IVa. This γ-ketol was detected as the major component of HPLC peak 3. The 4-hydroxy-2-alkenone moiety in compound IVa is highly reactive towards diazomethane, thus showing an unexpected behavior in the derivatization processes. Differential derivatives were found depending on the order of the derivatization sequence (see Fig. 3). If esterification with diazomethane is carried out first (Fig. 3A), the derivatization reagent itself can be incorporated into the analyte structure through heterocyclic ring formation, as shown in the figure. This is prevented by MO formation prior to esterification (Fig. 3B).

The mass spectral pattern of the ME, TMS ether derivative of HPLC peak 7 (Table I) is coincident with the spectra reported by Ustumes *et al.* (24) for two isomeric trihydroxy

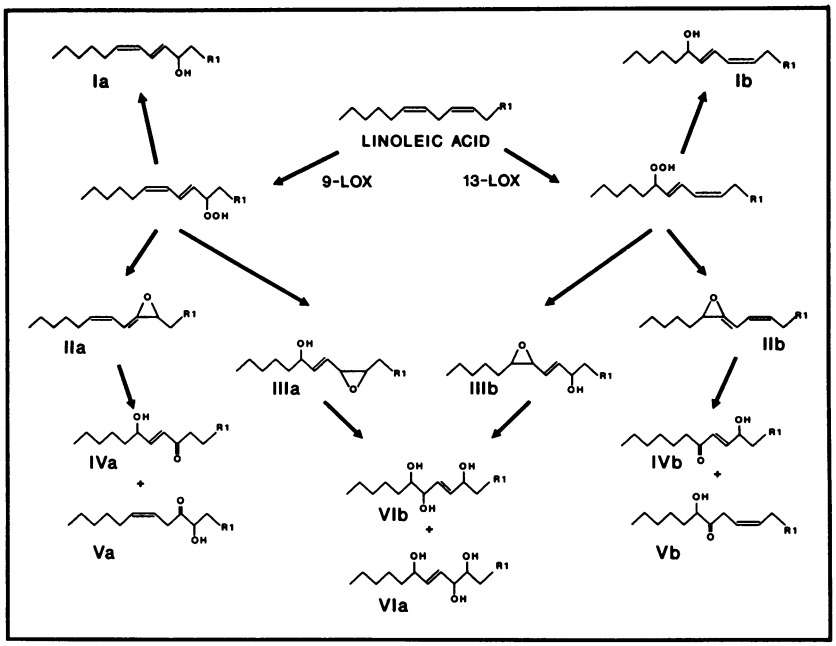

Figure 2. LOX metabolic pathway of LA. R1 is $(CH_2)_6COOH$. (From refs. 4, 12, and 24.)

Table I. *Metabolite Identification by GC-MS*

HPLC Peak	Compound Structure	GC Derivative	Mol Wt	MS Ions
1	Va	MEMOTMS	427	396, 326, 259, 240, 168
				412, 396, 271, 270, 259, 240, 155
		MO(TMS)[a]2	485	470, 454, 364, 317, 271, 240
				454, 364, 317, 271, 240, 229
		(TMS)2	456	441, 351, 317, 277 (2)
		(TMS)3	528	528, 513, 438, 317, 313, 237
	Vb	MEMOTMS	427	396, 326, 254, 173
				412, 357, 326, 230, 173
3	IVa	MEMO(TMS)2	541	510, 440, 369, 338, 336, 173 (2)
		MOMETMS	427	427, 396, 356, 306, 240
		(TMS)2	456	456, 385, 351, 295, 199
		ME(TMS)2	512	412, 339, 340, 255, 173 (2)
		MO(TMS)2	485	454, 414, 384, 364, 284, 240
	IVb	MEMO(TMS)2	541	510, 420, 283, 282, 259, 252 (2)
6	VIIa	MEMO(TMS)3	629	598, 528, 456, 238, 173 (2)
	VIIb	MEMO(TMS)3	629	598, 371, 370, 280, 259, 238 (2)
7	VIa	ME(TMS)3	560	460, 387, 259, 173 (3)
	VIb	ME(TMS)3	560	460, 301, 259, 173 (3)
8	Ia	METMS	382	382, 311, 225, 130

[a] Numbers in parentheses following MS ions are the number of isomers found in GC as separate, well resolved entities with similar mass spectral patterns.

octadecenoic acids identified in onion bulbs (VIa and VIb in Fig. 2). Compounds VIa and b are known to be formed through hydrolysis of IIIa and b due to nucleophilic attack at an epoxide ring carbon atom or at the double bond center. Actually, both of these compounds appear in the GC-MS chromatogram as a family of stereoisomers which can be partially resolved by monitoring the characteristic ion fragments.

Although compounds VIa and b are normally detected in systems where hydroperoxides are formed (4, 11, 24), they had not been described before in maize.

The HPLC fraction corresponding to the ABA-induced HPLC peak 6 shows by GC-MS four major GC peaks with mass spectral patterns consistent with the stereoisomers of 10-oxo-9,13-dihydroxy-11-octadecenoic acid and 12-oxo-9,13-dihydroxy-10-octadecenoic acid (VIIa and VIIb, respectively, in Fig. 4). The relative total ion abundances of the two positional isomers in the GC-MS chromatogram indicate that compound VIIa could be the more abundant one (4:1). The mass spectra of the two positional isomers (VIIa and b) are shown in Fig. 5. They are characterized by major fragment ions at m/z 598 (M − CH_3O), 456 (M − $CH_3(CH_2)_4$-

Table II. *Metabolite Identification (Hydrogenated Compounds) by GC-MS*

HPLC Peak	Compound Structure	GC Derivative	Mol Wt	MS Ions
1	Va	MEMOTMS	429	398, 331, 300, 259, 212, 170
				414, 398, 273, 272, 259, 242, 170, 155
		MO(TMS)[a]2	487	456, 389, 357, 317, 273, 242, 170
				456, 366, 317, 273, 272, 242
3	IVa	MEMOTMS	429	398, 358, 308, 256, 243, 242, 187 (2)
		MO(TMS)2	487	472, 416, 366, 314, 301, 243, 242 (2)
6	VIIa	MEMO(TMS)2	517	486, 396, 361, 300, 259, 173
		MO(TMS)3	575	544, 454, 402, 361, 317, 300, 173
	VIIb	MEMO(TMS)2	517	486, 447, 396, 259, 173
		MO(TMS)3	575	544, 505, 454, 317, 173
7	VIa	ME(TMS)3	562	401, 359, 303, 259, 213, 173, 129 (2)
	VIb	ME(TMS)3	562	389, 299, 259, 173, 129 (2)

[a] Numbers in parentheses following MS ions are the number of isomers found in GC as separate, well resolved entities with similar mass spectral patterns.

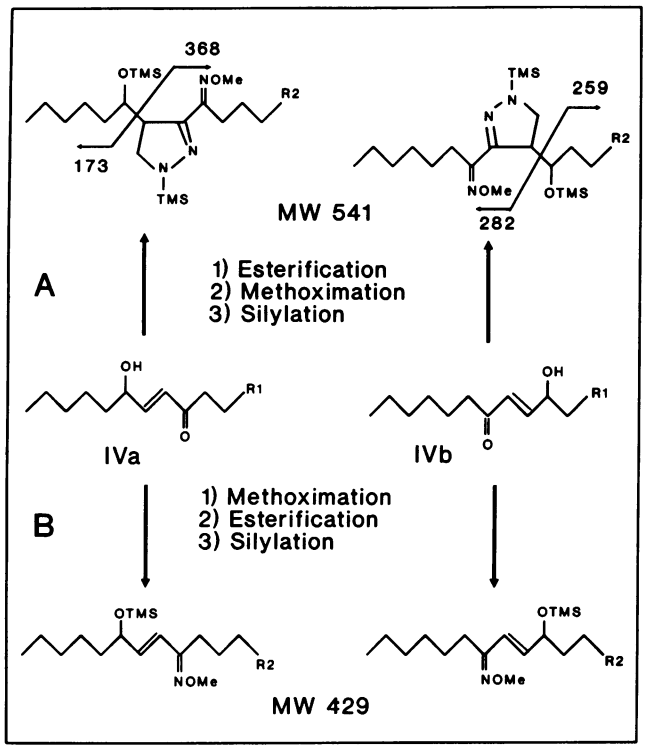

Figure 3. Derivatization scheme for metabolites IVa and IVb. Different derivatives are formed when (A) methylation is the first derivatization step or (B) methoximation is carried out before methylation. In B, addition of diazomethane to the double bond is prevented by methoximation. R1, as in Figure 2; R2 = $(CH_2)_6COOCH_3$.

CHOTMS), 238 and 173 ($CH_3(CH_2)_4CHOTMS$) (VIIa), and 598 (M − 31), 370 (M − $TMSOCHCH_2R2$), and 259 ($TMSOCHCH_2R2$) (VIIb). The position of the hydroxyl and ketone substituents was further verified by the mass spectra of the MO, TMS derivatives and by the corresponding hydrogenated HPLC aliquot. Hydrogenation affords two GC peaks showing major fragments (ME, MO, TMS derivative) at m/z 486 (M − CH_3O), 396 (486 − TMSOH), 361 (rearrangement, M − CH_2R2 + H), 300, 259 (M − $TMSOCHCH_2R2$), and 173 ($CH_3(CH_2)_4CHOTMS$ (VIIa), and 486, 447 (rearrangement, M − $CH_3(CH_2)_4$ + H), 396, 259, and 173 (VIIb) (see Table II).

The derivatization behavior of these compounds is similar to that observed for compounds IV and b, in agreement with the structure proposed. Thus, the molecular weight of the ME, MO, TMS derivatives (m/z 629) of VIIa and b is too high if compared with that of the MO, TMS derivatives (m/z 573, not shown in tables) or the corresponding hydrogenated compounds (m/z 517), showing the addition of diazomethane to the hydroxy-alkenone system. In addition, hydrogenation of VIIa and b affords, through hydrogenolysis, minor peaks with the same retention time and mass spectra that occur with the hydrogenated compounds Va and b, which confirms the presence of the α-ketol structure. To our knowledge, these types of structures arising from LOX metabolism of LA have not been reported before.

DISCUSSION

Linoleic acid was oxidized by maize embryo extracts resulting in the accumulation of eight main metabolites. The GC-MS determination of the metabolites indicates that all of those labeled Ia, IVa and b, Va and b, VIa and b, and VIIa and b in Figures 2 and 5 have been detected in the maize embryo incubates, with metabolites VI and VII being described for the first time in maize. Except for VI, the isomers arising from 9-LOX activity were the more abundant during embryo development, although the corresponding 13-lipoxygenase metabolites also have been detected in lower amounts. This is in agreement with the proportion of 9-isomers detected in dry maize seeds reported by other authors (10). Compounds VIa and VIb show minor differences in their relative abundance due to the possibility of formation of both VIa and VIb from IIIa or IIIb, depending on the site of water attack.

Two LOX isoenzymes, L1 and L2, from maize have been isolated and characterized (18). L2 catalyzes oxygenation at C9 with C18 fatty acids as substrates and is present in maize dry seeds. L1 isoenzyme leads to 13-hydroperoxides, is barely present in dry seeds, and gradually increases with the progress of seed germination. None of the cyclic metabolites of LA precursors of jasmonic acid have been detected among the LA metabolites of maize embryo homogenates, whether treated or not with ABA. However, both 13-LOX and hydroperoxide cyclase, the enzymes involved in jasmonic acid

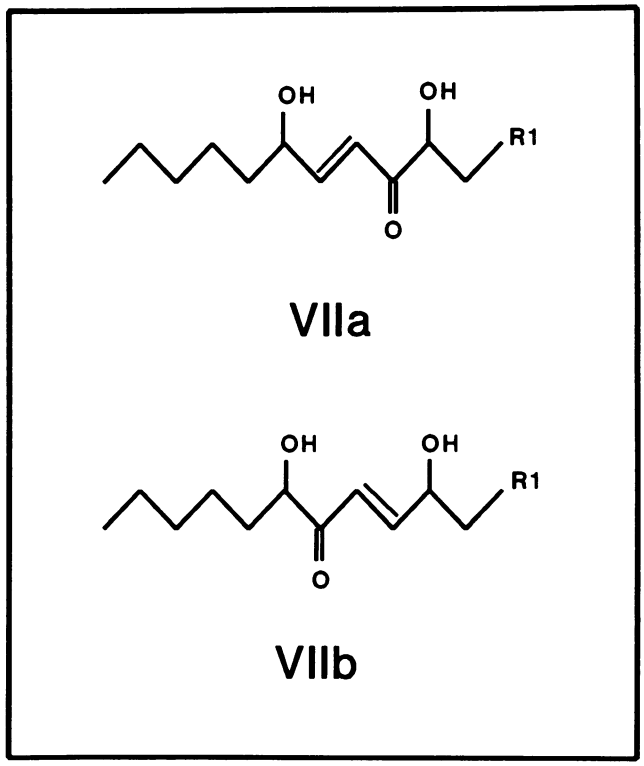

Figure 4. Structure proposed for metabolite VII. Double bond is depicted in the *trans*-configuration only for drawing purposes. The stereochemistry of the double bond and the hydroxy groups has not been elucidated in the present studies.

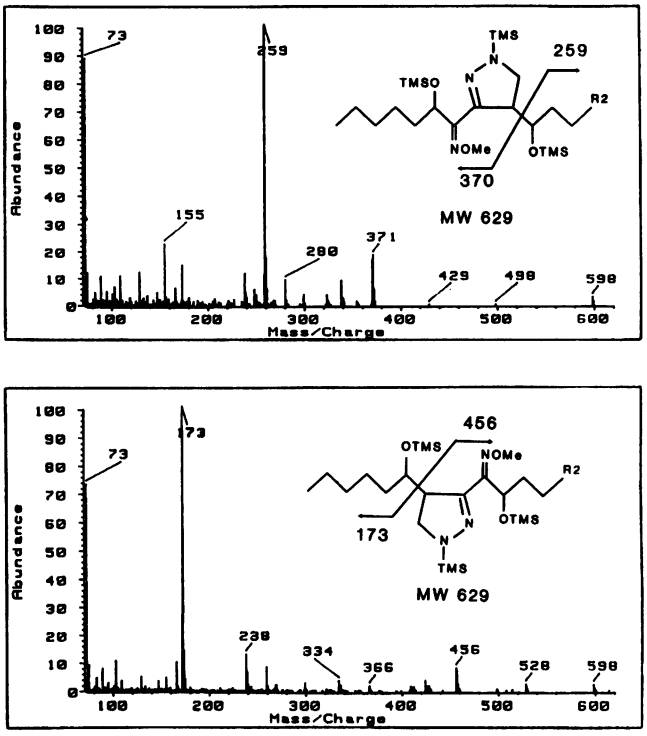

Figure 5. Mass spectral pattern of metabolites VIIa and VIIb as ME, MO, TMS derivatives.

synthesis, are low in seeds and increase during early seedling growth (26). Whether or not treatment with ABA affects 13-LOX metabolism in seedlings remains to be determined.

The metabolism of linoleic acid shows important variations during normal embryogenesis. ABA induces changes in the metabolism of LA in embryos at a very early developmental stage. Other authors have reported that ABA promotes accumulation of long chain fatty acids (7). The study of the major metabolites which show significant quantitative changes in ABA-treated young embryos revealed the presence of an unknown metabolite (VIIa and b) appearing as peak 6 in Figure 1 with the putative ketodiol structure, as indicated in Figure 4.

The biosynthetic pathway to compound type VII cannot be determined from our data, although it would seem that production of VII would correlate with the synthesis of the γ-ketols (IV) and with diminished amounts of α-ketols (V).

Although no specific metabolic function has yet been ascribed to the ketol products of hydroperoxide isomerase, it has been reported that the chemical characteristics of the alkenone system could account for the marked inhibition of sulfhydryl enzymes such as glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase (28). In this regard, compounds IV and VII could have an important regulatory role in enzyme activity during maturation.

The effect of ABA on the induction of peak 6 during embryo development indicates differences in the response depending on the age of the incubated embryos. These results reveal a stage in maize embryogenesis characterized by a higher relative sensitivity to ABA. It is interesting that during maize seed development, biochemical and molecular specific responses to ABA have been obtained only in young embryos. ABA seems to play an important role in the control of dormancy (19). Treatment of maize embryos with fluoridone, a pyridinone inhibitor of carotenoid biosynthesis and, consequently, of ABA, induces viviparism (lack of dormancy) only in embryos which are at a very young developmental stage (8). Furthermore the ABA-insensitive viviparous mutants of maize do not respond to exogenous ABA. However, synthesis of specific ABA-induced mRNAs can be effectively induced by ABA treatment in young (15 d after pollination) embryos (M. Pla and M. Pagès, unpublished results).

ABA treatment of young maize embryos induces dramatic changes in protein synthesis pattern, and more than 20 different proteins have been detected as precociously induced by ABA treatment in maize (21). Whether or not the enzymes involved in the formation of the ketodiol product VII are ABA-induced proteins remains to be determined. This research, together with the investigation of the metabolic pathway for ketol VII formation and its biological significance, may help understand the complex mechanism of hormone action.

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