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Biosynthesis of Jasmonic Acid by Several Plant Species[1]

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ABSTRACT

Six plant species metabolized ^{18}O-labeled 12-oxo-*cis,cis*-10,15-phytodienoic acid (12-oxo-PDA) to short chain cyclic fatty acids. The plant species were corn (*Zea mays* L.), eggplant (*Solanum melongena* L.), flax (*Linum usitatissimum* L.), oat (*Avena sativa* L.), sunflower (*Helianthus annuus* L.), and wheat (*Triticum aestivum* L.). Among the products was jasmonic acid, a natural plant constituent with growth-regulating properties. The pathway is the same as the one recently reported by us for jasmonic acid synthesis in *Vicia faba* L. pericarp. First, the ring double bond of 12-oxo-PDA is saturated; then β-oxidation enzymes remove six carbons from the carboxyl side chain of the ring. Substrate specificity studies indicated that neither the stereochemistry of the side chain at carbon 13 of 12-oxo-PDA nor the presence of the double bond at carbon 15 was crucial for either enzyme step. The presence of enzymes which convert 12-oxo-PDA to jasmonic acid in several plant species indicates that this may be a general metabolic pathway in plants.

Two previously independent areas of research have recently converged and suggest a link between polyunsaturated fatty acid metabolism and plant growth regulation. One research area concerns 3-oxo-2-(2′-pentenyl)-cyclopentaneacetic acid (jasmonic acid) and its methyl ester, which are constituents of many higher plants. Early interest in these compounds centered on their fragrant properties, and methyl jasmonate has now become an important ingredient in the perfume industry. More recently, jasmonic acid and methyl jasmonate have been the subject of investigations by plant physiologists who have shown that these compounds possess plant growth-regulating properties (4, 12, 14, 15, 22). Both compounds inhibit growth and promote senescence.

The other research area concerns the lipoxygenase-catalyzed oxygenation of polyunsaturated fatty acids which yields fatty acid hydroperoxides. The enzymic capability of plants to produce atty acid hydroperoxides has been a puzzling phenomenon to plant biochemists, because the highly reactive hydroperoxide products could potentially damage cellular components. In 1978, Zimmerman and Feng (24) demonstrated that the hydroperoxide of linolenic acid could be converted to an 18-carbon cyclic fatty acid, 12-oxo-PDA,[2] by the enzyme hydroperoxide cyclase. More recently, we have shown that, in *Vicia faba* L. pericarp, 12-oxo-PDA is further metabolized to the 12-carbon acid, jasmonic acid (19). The complete reaction sequence is illustrated in Figure 1.

In this report, we demonstrate that a variety of plant species possess the enzymes necessary to convert 12-oxo-PDA to jasmonic acid. The results suggest that regulation of plant growth may be one role for lipoxygenase in plants and that this function could be widespread within the plant kingdom.

MATERIALS AND METHODS

Chemicals. Linolenic acid was purchased from Nu Chek Prep, Inc.,[3] Elysian MN, and macerase was obtained from Calbiochem, La Jolla, CA. Soybean lipoxygenase and driselase were obtained from Sigma Chemical Co., St. Louis, MO. Isotopic $^{18}O_2$ gas (>99%) was purchased from Stohler Isotope Chemicals, Waltham, MA. Reversed phase $LKC_{18}F$ TLC plates were a product of Whatman Inc., Clifton, NJ, and normal phase TLC plates (Anasil HF) were from Foxboro/Analabs, North Haven, CT. Reversed phase TLC plates were developed in acetonitrile/water/acetic acid (95:5:1, v/v/v) and the normal phase TLC plates were developed three times in hexane/ethyl ether/acetic acid (50:50:1, v/v/v).

Plant Materials. Seeds of the following plants were germinated in moist paper towels wrapped in waxed paper for 6 d at 27°C in a 16-h light, 8-h dark cycle: corn, *Zea mays* L. cv NK PX443 (Northrup King); flax, *Linum usitatissimum* L. cv Linott; oat, *Avena sativa* L. cv Moore; sunflower, *Helianthus annuus* L. cv Sundak; and wheat, *Triticum aestivum* L. cv Thatcher. Eggplant fruit, *Solanum melongena* L., was obtained at a local produce market.

Flax cotyledons, oat leaves, and wheat leaves were partially digested with enzymes before the application of substrate. Each tissue was rinsed for 10 s in ethanol and brushed with 320 grit carborundum on the underside. The tissues were rinsed with distilled H_2O, the oat and wheat leaves cut into 1.5-cm segments, and 10 each of cotyledons or leaves were floated for 30 min on a solution of 2% macerase and 1% driselase containing 0.5 M sorbitol and 10 mM $CaCl_2$ at pH 5.5. For corn, the epidermis was removed from 10 coleoptiles and the coleoptiles were cut into 1.5-cm segments. For sunflower, three cotyledons were sliced longitudinally into six flat, thin sections. A cork borer was used to extract a 1.1-cm diameter cylinder of eggplant fruit, from which seven discs of about 1-mm thickness were cut.

Preparation of Substrates. 12-Oxo-PDA was enzymically synthesized from linolenic acid and an extract of flaxseed according to Zimmerman and Feng (24). 12-[^{18}O]Oxo-PDA was prepared as described previously (19). Figure 2 shows the structure of 2, an epimer of 12-oxo-PDA at carbon 13. It was formed by heating 12-oxo-PDA at 150°C for 30 min and then purifying it by normal phase TLC. The preparation of 3 (OPC-8:0), a derivative of 12-

[1] Conducted in cooperation with the North Dakota Agricultural Experiment Station, Paper No. 1307.

[2] Abbreviations: 12-oxo-PDA, 12-oxo-*cis,cis*-10,15-phytodienoic acid; OPC-8:0, 3-oxo-2-(2′-pentenyl)cyclopentaneoctanoic acid; OPC-6:0, 3-oxo-2-(2′-pentenyl)cyclopentanehexanoic acid; OPC-4:0, 3-oxo-2-(2′-pentenyl)cyclopentanebutanoic acid.

[3] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

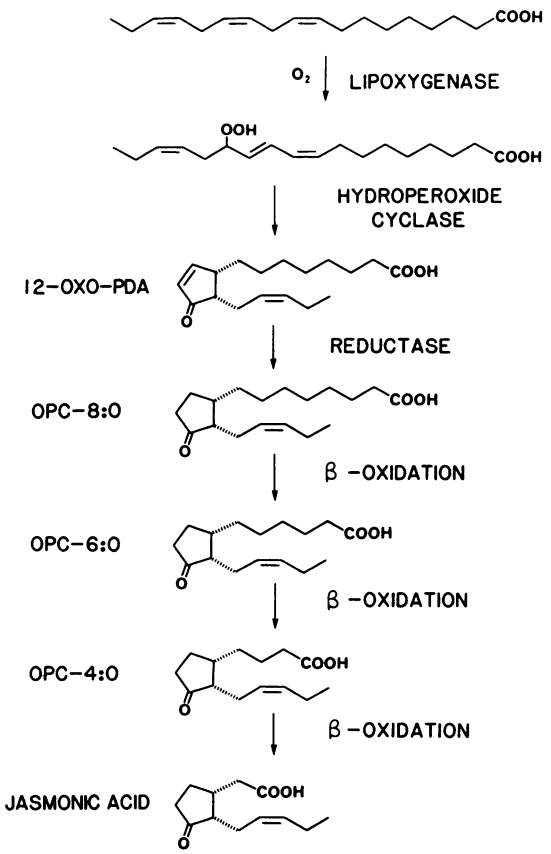

FIG. 1. The biosynthesis of jasmonic acid from linolenic acid. The projections shown represent the relative stereoconfiguration of the side chains rather than the absolute configuration, which is not known.

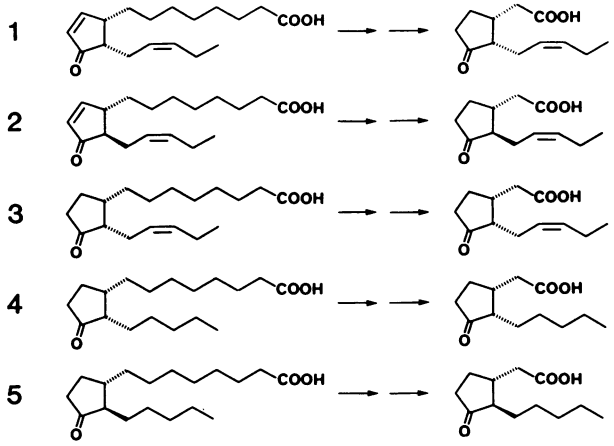

FIG. 2. Some effective substrates for the cyclopentenone reductase and β-oxidation enzymes and their products. The projections shown represent the relative stereoconfiguration of the side chains rather than the absolute configuration, which is not known.

oxo-PDA in which the ring double bond is saturated, has been reported earlier (19). Two saturated epimers of 3-oxo-2-pentanylcyclopentaneoctanoic acid, **4** and **5**, were prepared from 12-oxo-PDA and its 13-epimer by bubbling H_2 through a methanolic solution of each epimer (5 mg in 2 ml) for 5 min with about 4 mg of platinum oxide as catalyst. The catalyst was removed by centrifugation, and **4** and **5** were purified by normal phase TLC.

Reaction of Substrates with Plant Tissues. Approximately 400 μg of substrate was dispersed by sonication in 100 μl of 50 mM K-phosphate, pH 7.0, containing 1 mM DTT and 0.5 M sucrose. The plant tissues were placed in small Petri dishes lined with moist filter paper. The substrate solution was applied to the plant tissue and the dish was covered. After 2 h at 22°C, the tissues were extracted according to the method of Bligh and Dyer (2) by homogenizing them in a mortar and pestle with 6 ml of methanol/chloroform (2:1, v/v) solvent.

Following the addition of 2 ml of chloroform and 2 ml of water, the chloroform layer was concentrated by evaporation under N_2. The residue was chromatographed alongside jasmonic acid and 12-oxo-PDA standards on a reversed phase TLC plate. The area of the TLC plate which chromatographed just above 12-oxo-PDA and included jasmonic acid was eluted from the gel with diethyl ether. The area of the TLC plate which cochromatographed with 12-oxo-PDA was also eluted. This fraction contained both 12-oxo-PDA and its ring-saturated metabolite, OPC-8:0. OPC-8:0 was separated from the unreacted substrate by normal phase chromatography with authentic OPC-8:0 cochromatographed as a reference. The OPC-8:0 fraction was eluted with diethyl ether and recombined with the jasmonic acid fraction from the reversed phase chromatography step, and the sample was esterified with diazomethane. The recombination step permitted a single GC-MS analysis devoid of large amounts of unreacted substrate.

Metabolite Analysis by GC-MS. Samples were analyzed with a Hewlett-Packard 5992 GC-MS equipped with a capillary column and a cool, on-column injector. The 25 m × 0.32 mm i.d. CP Sil 5 CB fused silica column (Chrompack, Inc., Bridgewater, NJ) was operated isothermally at 70°C for 2 min, then heated at 10°C/min to 250°C.

Metabolites were identified by their retention times and characteristic fragment ions of their methyl esters (19). For methyl jasmonate (R_t = 9.9 min), the identifying ions and, in parentheses, their relative intensities were m/z 226 (13) $[M]^+$, 206 (11) $[M\text{-}H_2{}^{18}O]^+$, 195 (9) $[M\text{-}OCH_3]^+$, 158 (13) $[M\text{-}C_5H_9\text{+}H]^+$, 153 (24) $[M\text{-}CH_2COOCH_3]$ and 85 (100) $[C_5H_6{}^{18}O\text{+}H]^+$. For methyl-OPC-4:0 ($R_t$ = 12.4 min), the characteristic mass fragments were m/z 254 (2) [M] 234 (11) $[M\text{-}H_2{}^{18}O]^+$, 223 (2) $[M\text{-}OCH_3]$ 186 (7) $[M\text{-}C_5H_9\text{+}H]^+$, 153 (24) $[M\text{-}(CH_2)_3COOCH_3]^+$, and 85 (100) $[C_5H_6{}^{18}O\text{+}H]^+$. The characteristic ions for methyl-OPC-6:0 (R_t = 14.5 min) were m/z 282 (5) $[M]^+$, 262 (7) $[M\text{-}H_2{}^{18}O]^+$, 251 (6) $[M\text{-}OCH_3]^+$, 214 (33) $[M\text{-}C_5H_9\text{+}H]^+$, 153 (75) $[M\text{-}(CH_2)_5COOCH_3]^+$, and 85 (100) $[C_5H_6{}^{18}O\text{+}H]^+$. Finally, methyl-OPC-8:0 (R_t = 16.4 min) was identified by fragment ions at m/z 310 (0.5) $[M]^+$, 290 (2) $[M\text{-}H_2{}^{18}O]^+$, 279 (2) $[M\text{-}OCH_3]^+$, 242 (6) $[M\text{-}C_5H_9\text{+}H]^+$, 153 (27) $[M\text{-}(CH_2)_7COOCH_3]^+$, and 85 (100) $[C_5H_6{}^{18}O\text{+}H]^+$.

RESULTS

Metabolism of 12-[^{18}O]Oxo-PDA by Various Plant Tissues. Corn, eggplant, flax, and sunflower plants each converted 12-[^{18}O]oxo-PDA to three detectable products labeled with ^{18}O. OPC-8:0, OPC-4:0, and jasmonic acid were identified as metabolites of 12-oxo-PDA on the basis of their characteristic mass spectral fragmentation patterns. The mass spectra were indistinguishable from those published previously for *Vicia faba* L. (19). In oat and wheat plants, only OPC-8:0 and OPC-4:0 were detected with ^{18}O label. Jasmonic acid was detected in both, but it was not labeled with ^{18}O, and it presumably arose from endogenous substrates before the addition of 12-[^{18}O]oxo-PDA. It is likely that the enzymes which convert OPC-4:0 to jasmonic acid are present in oats and wheat just as they are in the other tissues, but that our experimental conditions were not favorable for this conversion.

We were not able to observe the presence of OPC-6:0 as a metabolite with any of the tissues examined here. This compound was detected as a metabolite in *Vicia faba* L. pericarp (19), but only when a large amount of concentrated sample was

injected into the GC-MS. OPC-6:0 is most likely an intermediate in each of the above plants also, even though it was not detected. None of the tissues tested showed conversion of 12-oxo-PDA to fatty acids with an odd number of carbons, indicating that the chain-shortening reactions are a result of β-oxidation and not α-oxidation.

Substrate Specificities of the Disubstituted Cyclopentenone Reductase and β-Oxidation Enzymes. The five compounds in Figure 2 were prepared and applied to corn coleoptile sections to determine which were effective substrates for conversion to short chain alkylcyclopentanone carboxylic acids. All were effective. As reported above, 12-oxo-PDA (**1**) was converted to OPC-8:0, OPC-4:0, and jasmonic acid, all having a *cis* configuration of the side chains with respect to the plane of the ring. The 13-epimer of 12-oxo-PDA (**2**) produced the same products, except that the two side chains were in the *trans* configuration. The two epimers are easily distinguished by GC because the *trans* forms elute 0.4 to 0.6 min earlier than the *cis* forms. Thus, the stereoconfiguration of the pentenyl side chain at carbon 13 is not important for activity of either the reductase enzyme or the β-oxidation enzymes.

As expected, 3-oxo-2-(2′-pentenyl)cyclopentaneoctanoic acid (**3**) yielded OPC-4:0 and jasmonic acid. This compound is an intermediate in the conversion of 12-oxo-PDA to jasmonic acid. The fully saturated epimers **4** and **5** also underwent β-oxidation to give the corresponding dihydro analogs of OPC-4:0 and jasmonic acid. Both analogs were recognized by the mass fragments of their methyl esters at *m/z* 83 (100) $[C_5H_6O+H^+]^+$ and 153 (20) $[M\text{-}CH_2COOCH_3]^+$ (relative intensities shown in parentheses). In addition, methyldihydro-OPC-4:0 was characterized by a mass fragment at *m/z* 184 (7) $[M\text{-}C_5H_9+H]^+$ and methyldihydrojasmonate by a mass fragment at *m/z* 156 (20) $[M\text{-}C_5H_9+H]^+$. These results show that the double bond of the pentenyl side chain of **3** is not required for recognition by the β-oxidation enzymes. We earlier showed that this double bond, originally at carbon 15 of linolenic acid, was required for the hydroperoxide cyclase reaction (20).

DISCUSSION

Most reports about the stereochemistry of methyl jasmonate and jasmonic acid have shown that the naturally occurring compounds are levorotatory. Demole *et al.* (5) proposed that (−)-methyl jasmonate has the *trans* configuration of the side chains with respect to the plane of the ring. Hill and Edwards (10) later concluded that the absolute stereochemistry of (−)-jasmonic acid (as its methyl ester) was (1*R*,2*R*) 3-oxo-2-(2′-pentenyl)cyclopentaneacetic acid (Fig. 3A). We note that several recent reports have correctly illustrated (−)-methyl jasmonate, but have incorrectly referred to the stereochemistry as 1*S*,2*R* rather than 1*R*,2*R* (6, 14, 15).

In each of the plant tissues we examined in this report, the results showed that a *cis* configuration of the side chains was maintained during the enzymic conversion of 12-oxo-PDA to jasmonic acid. Although we believe that the *cis* form is the natural stereoisomer of jasmonic acid, we have not yet established the absolute stereoconfiguration for the cyclopentane substituents. However, if it is assumed that naturally occurring jasmonic acid has *R* stereochemistry at carbon 1 of the ring as Hill and Edwards (10) concluded, then a *cis* configuration of the side chains would mean that carbon 2 must have *S* stereochemistry (Fig. 3B).

The *cis* form of jasmonic acid easily epimerizes under acidic conditions (6), and 12-oxo-PDA undergoes a similar transformation in the presence of acid, base, or high temperature, presumably through an enol intermediate (21). Therefore, the *trans* form of methyl jasmonate or jasmonic acid reported by others may have been the result of epimerization of the *cis* compound

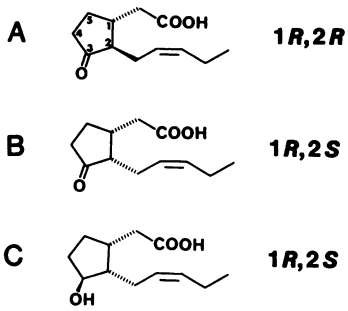

FIG. 3. Some possible stereoconfigurations of jasmonic acid A, (1*R*,2*R*) 3-oxo-2-(2′-pentenyl)cyclopentaneacetic acid; B, (1*R*,2*S*) 3-oxo-2-(2′-pentenyl)cyclopentaneacetic acid. C, Curcurbic acid, (1*R*,2*S*) 3*S*-hydroxy-2-(2′-pentenyl)cyclopentaneacetic acid.

at carbon 2 during the purification procedure (1, 3–5, 14). Each of these procedures employed either acidic extraction conditions or high temperature fractional distillation. Furthermore, Fukui *et al.* (6) demonstrated that cucurbic acid, which is probably derived from jasmonic acid, has the stereochemistry 1*R*,2*S* as shown in Figure 3C (incorrectly referred to as 1*S*,2*S* in their paper). The presence of the hydroxyl group at carbon 3 of cucurbic acid precluded epimerization through an enol intermediate, and cucurbic acid was isolated in its natural stereoconfiguration.

We recently reported that many plant species, including those described in this paper, were able to convert 13-hydroperoxylinolenic acid to 12-oxo-PDA by hydroperoxide cyclase (17). The additional discovery reported here that many plant species convert 12-oxo-PDA to jasmonic acid presents a new approach to the investigation of lipoxygenase function. Many recent reports have implicated jasmonic acid or methyl jasmonate in growth inhibition (1, 4, 6, 12, 14, 15, 22). The rapid increase in lipoxygenase and hydroperoxide cyclase activities observed shortly after germination (18) may indicate a role for this pathway in regulating certain cellular activities during plant development.

We believe that the function of lipoxygenase differs among plants, and may even vary at different locations within the same plant. Some plant lipoxygenases catalyze O_2 incorporation predominantly at carbon 9, others at carbon 13. Jasmonic acid is a product only when oxygenation occurs at carbon 13. Some species, such as corn, catalyze oxygenation predominantly at carbon 9. The proportion of 9-isomer formed by corn lipoxygenase varies from 63% in young leaves (18) to 88% in the germ (9), yet corn readily synthesizes jasmonic acid from exogenous 12-oxo-PDA, a metabolite of the 13-isomer. The 9-hydroperoxy isomer presumably follows a different course of metabolism. Both the 9- and the 13-isomer can be converted to α- and γ-ketols by hydroperoxide isomerase (8, 25), but no physiological function has yet been assigned to these compounds. Some plants have hydroperoxide lyase enzymes (7, 11, 13, 16) which cleave 9- or 13-hydroperoxides to oxoacids and aldehydes. The physiological roles of these compounds also are not established, although 12-oxo-*trans*-10-dodecenoic acid, a metabolite of the 13-hydroperoxide isomer, was identified as the active component of traumatin, a wound hormone (23). Because of the similarity in structure of many of these metabolites to the eicosanoids of mammalian metabolism, it would not be surprising to discover that the "octadecanoids" (lipoxygenase products) of plants also behave as potent metabolic regulators.

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