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Labeling of major plant lipids and jasmonic acid using [1-^{14}C] lauric acid

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Abstract

A medium chain length fatty acid, [1-^{14}C] lauric acid (12:0) was administered to the detached leaves of *Artemisia* and was incorporated into major lipids, including phospholipids and galactolipids. [1-^{14}C]12:0 was elongated and desaturated into linolenic acid (18:3). In detached leaves of both *Artemisia* and *Arabidopsis thaliana* ecotype Columbia, radioactivity from [^{14}C]18:3 was incorporated into jasmonic acid (JA) and methyl jasmonate (MJ). Higher amounts of [^{14}C]JA were measured in *Artemisia* than *Arabidopsis* leaves. In *Artemisia*, [^{14}C]JA was actively metabolized into [^{14}C]MJ. Extracts prepared from the leaves of *Artemisia*, exhibited higher in vitro JA methyltransferase activity than those from *Arabidopsis*.

Keywords: *Arabidopsis thaliana: Cruciferae*; *Artemisia tridentata: Compositae*; Jasmonic acid; JA methyltransferase; Lauric acid

1. Introduction

Galactolipids in plastid membranes and phospholipids in the plasma membrane of plant leaves contain large amounts of linolenic acid (18:3) **3** (see Fig. 1). The latter apparently can be released by the action of lipolytic acyl hydrolases (LAH) or phospholipase A_2 (PLA_2). 18:3 (**3**) that is released from phospholipids or galactolipids by hydrolytic enzymes could potentially be metabolized into jasmonic acid (JA) **7**. JA **7** is a powerful inducer of plant defense genes. 12-Oxo-phytodienoic acid (OPDA) **5**, the precursor to JA **7** is known to be involved in mechano-transduction (Stelmach et al., 1998; Stelmach et al., 1999), and in resistance to insect and fungal attack (Stintzi et al., 2001). In *Arabidopsis*, the galactolipid monogalactosyldiacylglycerol (MGDG) has OPDA **5** esterified at the *sn*-1 position (Stelmach et al., 2001). Whether this esterified OPDA **5** is derived from the free 18:3 **3** pool, or synthesized from 18:3 esterified to the sn-1 position of MGDG, is still not clear. Furthermore, the role of OPDA **5** esterified to a chloroplast lipid has yet to be demonstrated. Nonetheless, the pathway of JA **7** biosynthesis, starting with 18:3 **3** has been described in previous studies (Vick and Zimmerman, 1983; Schaller, 2001). In addition, Vick and Zimmerman (1984) identified all three intermediates between OPDA **5** and JA **7** after saturating the pathway with exogenous [^{18}O]OPDA **5**. However, labeling of the intermediates and end products downstream of 3-oxo-2-(pent-2′-enyl)-cyclopentane-1-octanoic acid **6** (OPC-8:0) have been limited because the label from the commercially available [1-^{14}C]18:3 is lost during β-oxidation of OPC-8:0 to JA **7**. In the present study, we used

Abbreviations: AdoMet, *S*-adenosyl-L-methionine; DGDG, digalactosyldiacylglycerol; JA, jasmonic acid; JAMT, jasmonic acid methyltransferase; MGDG, monogalactosyldiacylglycerol; MJ, methyl jasmonate; 12:0, lauric acid; 18:2, linoleic acid; 18:3, linolenic acid.

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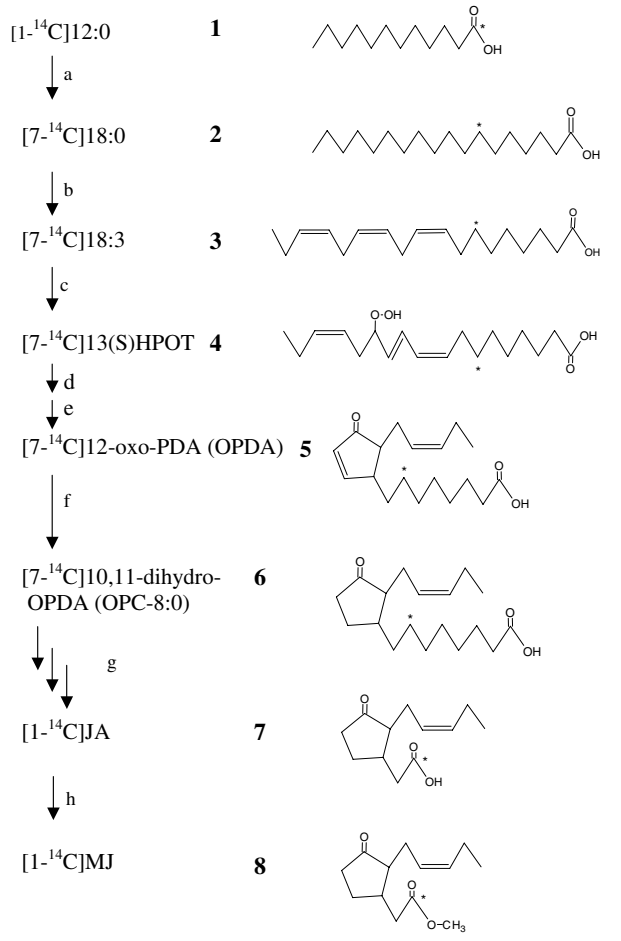

Fig. 1. A proposed scheme of labeling JA and MJ using [1-^{14}C]12:0. a, elongation; b, desaturation; c, lipoxygenase; d, allene oxide synthase; e, allene oxide cyclase; f, reductase; g, β-oxidation (three steps); h, JA methyltransferase (AdoMet is a methyl donor). Names of molecules shown: **1**, lauric acid; **2**, stearic acid; **3**, linolenic acid; **4**, 13-hydroperoxy-octadecatrienoic acid; **5**, 12-oxo-phytodienoic acid; **6**, 10,11-dihydro-phytodienoic acid; **7**, jasmonic acid; **8**, methyl jasmonate.

[1-^{14}C] lauric acid **1** to label 18-carbon fatty acids, including 18:3 **3** and traced incorporation of the label into JA **7** and methyl jasmonate (MJ) **8**.

2. Results and discussion

2.1. Incorporation of [1-^{14}C] lauric acid *1* into lipids

[1-^{14}C] Lauric acid (12:0) **1** was administered through the petiole of excised leaves of *Artemisia tridentata* for 6 h in a ventilated hood under constant illumination. Radioactivity from [1-^{14}C]12:0 **1** was incorporated into phospholipids and galactolipids during incubation (Fig. 2). During 5 h of incubation in non-radioactive solution, the amount of radioactivity in phosphatidylcholine (PC) decreased while the radioactivity in MGDG increased. A previous study by Norman and St. John (1986) has shown similar results where a decline in the radioactivity of PC coincided with an increase in MGDG labeling, indicating that PC is a precursor for MGDG. In another study, an inverse relation between PC and MGDG was demonstrated in seedlings of *Capsicum annum* (pepper) treated with Cd (Jemal et al., 2000). PC accumulated in the leaves, while the amount of MGDG was lowered.

Analysis of the fatty acid methyl esters revealed that [1-^{14}C]12:0 **1** was elongated into 18 carbon fatty acids and desaturated into linoleic acid (18:2) and 18:3 **3** (Table 1). Surprisingly, radioactive oleic acid (18:1) was not detected. Since 18:1 is an intermediate between stearic acid (18:0) and 18:2, it is possible that separation of 18:1 from saturated fatty acids was not achieved. Large amounts of radioactivity, 72–93% was associated with saturated fatty acids. Saturated fatty acids were not separated to determine the composition of 12:0, 16:0 and 18:0. A time course study was carried out for 5, 15 and 24 h in non-radioactive solution after [1-^{14}C]12:0 **1** was administered to detached leaves. In this study, the amount of radioactivity in saturated fatty acids decreased progressively with prolonged incubation. The decrease in the radioactivity of saturated fatty acids was by 5.3×10^6 radioactivity counts between 0 and 5 h, while 18:2 + 18:3 **3** gained 0.3×10^6 counts. Between 5 and 15 h of incubation in non-radioactive solution, saturated fatty acids lost 1.8×10^6 radioactive counts, while 18:2 + 18:3 **3** gained 1.2×10^6 counts. Thus, a 1:1 ratio between radioactivity loss in saturated fatty acids and the gain in 18:2 + 18:3 **3** was apparent between 5 and 15 h. This indicates that saturated fatty acids were converted into 18:2 and 18:3 **3**. However, between 0 and 5 h of incubation, the loss of radioactivity from saturated fatty acids cannot be completely accounted for by the gain in 18:2 + 18:3 **3**. This anomaly may be explained by assuming that between 0 and 5 h of incubation, there could have been a rapid lipid turnover. [^{14}C] Unsaturated fatty acids might have been lost via β-oxidation in the peroxisomes. In addition, [^{14}C] might have entered the mitochondrial TCA cycle, where loss of ^{14}C might have occurred as [^{14}C]CO_2 through respiration. In this study, we did not measure released [^{14}C]CO_2. Another possibility is that unsaturated fatty acids could have been metabolized as they were formed. As shown in Table 1, radioactivity in 18:2 but not 18:3 **3** increased at 15 h of incubation in non-radioactive solution. The observation suggests that the desaturation of [^{14}C]18:2 into [^{14}C]18:3 **3** might have been limiting. Alternatively, [^{14}C]18:3 **3** might have been metabolized almost as rapidly as it was formed.

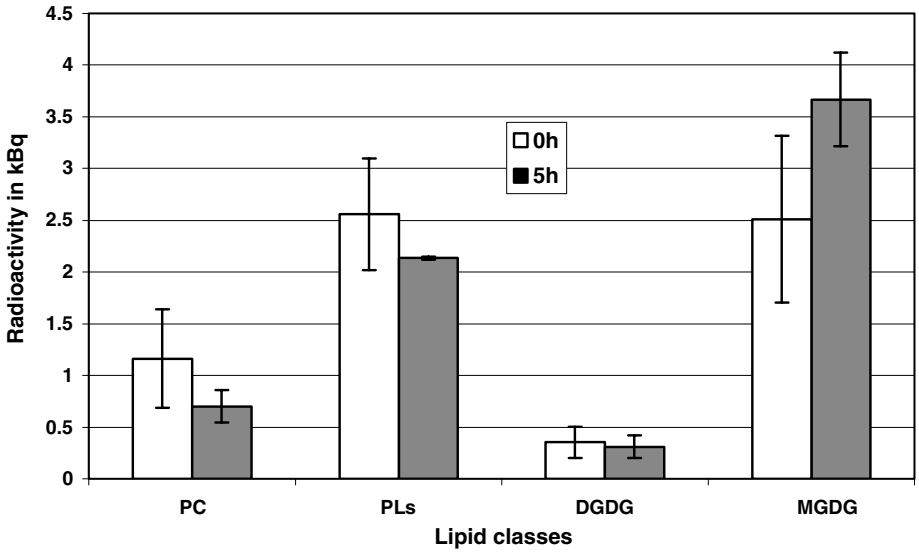

Fig. 2. Incorporation of radioactivity from [1-^{14}C]12:0 into major lipids in the leaves of *Artemisia*. Detached leaves were incubated with [1-^{14}C]12:0 with a 6 h pulse. Leaves were incubated for an additional 5 h in a non-radioactive solution (5 h). Lipids were extracted as described under a section for extraction of radioactive lipids. Data are the average of three replicates. PLs, phospholipids excluding PC.

Table 1
Relative levels of radioactive methyl esters of saturated fatty acids, 18:2 and 18:3 **3** extracted from the leaves of *A. tridentata* subsp. *vaseyana*

Incubation time after a 6 h [1-^{14}C]12:0 **1** pulse	Relative phosphoImager counts × 10^6		
	Saturated fatty acids	18:2	18:3 **3**
0 h	13.32 ± 0.55	0.74 ± 0.10	0.17 ± 0.00
5 h	8.00 ± 1.46	0.94 ± 0.19	0.22 ± 0.02
15 h	6.12 ± 1.97	2.08 ± 1.03	0.28 ± 0.11
24 h	5.75 ± 0.88	1.33 ± 0.19	0.35 ± 0.06

Detached leaves were incubated with [1-^{14}C]12:0 with a 6 h pulse. Some batches of leaves were incubated further in non-radioactive mineral solution for 5, 15 or 24 h. Data are the average of three replicates.

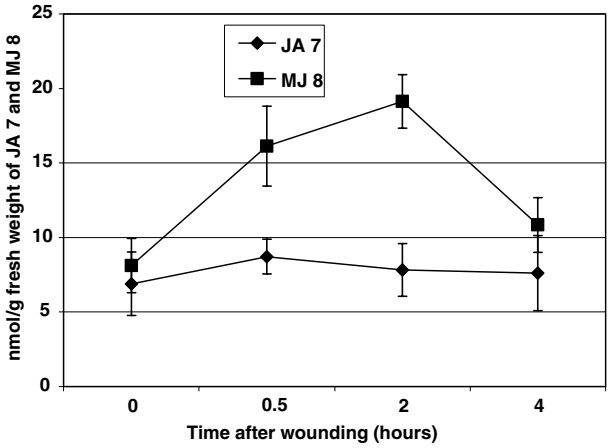

Fig. 3. Levels of JA and MJ extracted from the leaves of *A. tridentata* subsp. *vaseyana*. Time points in JA and MJ are averages of six replicates from three independent experiments.

2.2. Endogenous levels of non-radioactive JA 7 and MJ 8

In a time course study that was conducted after wounding, levels of JA **7** remained constant (Fig. 3). However, wounding resulted in an increase in levels of MJ **8** that reached a peak 0.5 h after wounding. The elevated levels of wound-induced MJ remained constant until 2 h, after which MJ **8** declined to basal levels. This observation mirrors a reported study (Seo et al., 2001), in which transgenic *Arabidopsis* plants that overexpressed JAMT had threefold elevated levels of MJ **8** but normal levels of JA **7**. Our data indicates an active flow of carbon into JA **6**, but this flow was exceeded by the conversion of JA **7** into MJ **8**.

2.3. Incorporation of radioactivity into jasmonates

[1-^{14}C] Lauric acid **1** was administered through the cut petiole and the incorporation of radioactivity into jasmonates **7** and **8** (JA + MJ) was measured. The major goal of the study was to label JA **7** without losing the label during β-oxidation of OPC-8:0 **6** into JA **7**. Secondly, using this novel approach, we wanted to understand the basis of the difference in jasmonates accumulation between *Arabidopsis* and *Artemisia*. In *Arabidopsis*, the uptake and recovery of radioactivity were 93% and 38%. In *Artemisia*, uptake and recovery were 83% and 30%. As shown in Fig. 4, radioactivity from [1-^{14}C]12:0 **1** was incorporated into JA **7** and MJ **8** in both plant species. Higher amounts of [^{14}C]JA **8** and [^{14}C]MJ **7** were measured in *Artemisia* compared to *Arabidopsis*. Interestingly, a higher amount of [^{14}C]MJ **8** than [^{14}C]JA **7** was measured in *Artemisia* but not in *Arabidopsis*. As expected, an in vitro enzyme

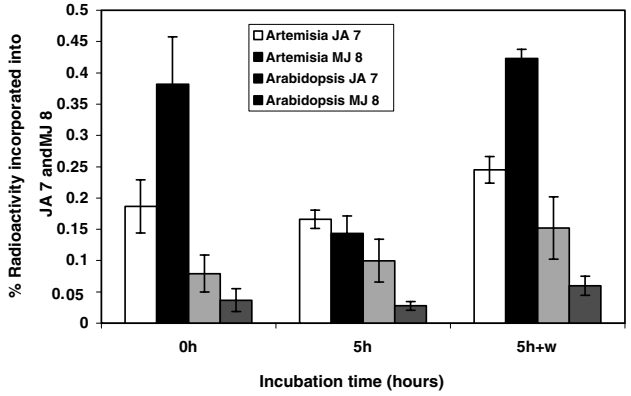

Fig. 4. Incorporation of radioactivity from [1-^{14}C]12:0 into JA and MJ. Detached leaves were fed [1-^{14}C]12:0 through the cut petiole with a 6 h pulse. After administering, leaves were incubated for an additional 5 h in non-radioactive solution (5 h). Some batches were wounded and incubated for an additional 1 h (5 h + W). Data are the average of six replicates from three independent experiments.

assay showed that methylation of JA **7** was about 13 pmol min^{-1} mg protein^{-1} in *Artemisia* and 3 pmol min^{-1} mg protein^{-1} in *Arabidopsis* (data not shown). The data support our observation that in the leaves of *Artemisia*, in vivo methyltransferase activity, which converts JA **7** into MJ **8**, is higher than that in *Arabidopsis*. Therefore, in *Artemisia*, accumulation of radioactive JA **7** and MJ **8** mirrors that observed with the non-radioactive endogenous pool (Fig. 3). A radioactive band, which co-migrated with OPDA **5**, was detected only in *Arabidopsis* (data not shown). Free OPDA **5** and OPDA **5** esterified to a chloroplast lipid have been reported in *Arabidopsis* (Stelmach et al., 2001). Previous studies including ours (quantification of oxylipins in the *ped* 1 mutant) have reported high levels of OPDA **5** in the unwounded leaves and cotyledons of *Arabidopsis*. Upon wounding, however, OPDA **5** level decreased dramatically, while levels of JA **7** increased significantly. The accumulation of radioactive OPDA **5** in *Arabidopsis* suggests that the conversion of OPDA **5** into OPC-8:0 **6** might be a tightly regulated process in this plant species. Schaller and Weiler (1997) suggested that OPDA reductase was a key point of regulation in the JA pathway. In *Artemisia*, radioactive OPDA **5** appears to have been rapidly metabolized as it was formed. In another study, MJ **8** that was released from the leaves of *Artemisia* was measured in headspace (data not shown). In labeling studies, incubation of leaf tissues for 5 h in non-radioactive solution resulted in the loss of [^{14}C]MJ **8** in *Artemisia* (Fig. 4). It seems reasonable to assume that with prolonged incubation, [^{14}C]MJ **8** could have been volatilized. We, however, did not measure radioactive MJ **8** in the headspace.

When compared to the amount of radioactivity measured after administering [1-^{14}C]12:0 **1** for 6 h, wounding did not increase the radioactivity in JA **7** and MJ **8** in both plant species. The failure of wounding to increase radioactive JA **7** and MJ **8** may in part be due to the "cold" pool diluting the "hot" pool of 18:3 **3**. Vick and Zimmerman (1984) reported similar results where no labeling was found in JA **7**, 2 h after feeding of [^{18}O]OPDA **5** in oat and wheat leaves. They speculated that the formation of JA **7** from the endogeous substrates was favored. In the present study, wounding of the leaves might have resulted in a substantial amount of [^{14}C]18:3 **3** being channeled into the hydroperoxide lyase (HPL) pathway. In another study, we have demonstrated that HPL products were synthesized de novo and released in large quantities upon wounding of leaf tissues (data not shown). In conclusion, our results demonstrated that [1-^{14}C]12:0 **1** was elongated and desaturated into [^{14}C]18:3 **3**. The latter was metabolized into [^{14}C]JA **7** and [^{14}C]MJ **8** in both plant species. The accumulation of [^{14}C]JA + MJ (**7** + **8**) was five-fold higher in *Artemisia* compared to *Arabidopsis*. In *Artemisia*, [^{14}C]JA **7** was actively metabolized into [^{14}C]MJ **8**. An in vitro enzyme assay confirmed a higher JAMT activity in *Artemisia* compared to *Arabidopsis*. In *Arabidopsis*, the conversion of OPDA **5** into JA **7** appears to be a tightly regulated process. Although inconclusive, our data suggest that enzymes of the JA **7** pathway were functioning near their maximum in unwounded leaves of *Artemisia*. We propose a scheme that shows the position of the label from [1-^{14}C]12:0 **1** during JA **7** biosynthesis (Fig. 1). This novel approach of labeling jasmonates should be useful in the study of mutants, which have a block in the JA **7** pathway. Since new tools are now available to accurately measure these compounds, this novel approach can be used to simultaneously measure the "cold" and "hot" pools of jasmonates.

3. Experimental

3.1. Plant growth and the incubation conditions

Artemisia tridentata subsp. *vaseyana* plants were germinated from seeds (a gift from Dr. Durant McArthur, Salt Cave Hollow, Utah) and plants were grown in the greenhouse. Seeds of *Arabidopsis thaliana* ecotype Columbia were sown in soil and cold-treated at 4 °C. Plants were grown in growth chambers under 14-h light/10-h dark cycles with cool white fluorescent light of 100 μmol m^{-2} s^{-1} at 23 °C.

Young expanding leaves of *Artemisia* and rosette leaves from 4 to 6-week-old *Arabidopsis* were used in all labeling studies. Radioactivity was administered through the petiole of the excised leaves via the transpiration stream. Excised leaves were incubated for 6 h at 26 °C under continuous light in 92.5 kBq of [1-^{14}C] lauric acid **1** dissolved in a mineral solution containing 0.025% Tween-20. The mineral solution was prepared as described by Somerville and Ogren (1982), and stored at 4 °C not for more than 14 days. After incubation,

leaves were washed in non-radioactive mineral solution to remove exogenous radioactivity. At this point a batch of leaves was weighed, frozen in liquid nitrogen and stored at −80 °C (i.e. 0 h). Other batches were further incubated in non-radioactive mineral solution for specified times. For the incorporation of [1-^{14}C] lauric acid **1** into major polar lipid classes, fatty acids, and jasmonates, radio-labeled leaves were further incubated in non-radioactive solution for either 5, 15 or 24 h.

3.2. Chemicals

[1-^{14}C] Lauric acid **1** of specific activity 1850 MBq/mmol was purchased from ICN, Irvine, CA. *S*-Adenosyl-L-[methyl-^{14}C]methionine (specific activity 2,220 MBq/mmol) was purchased from Amersham Pharmacia Biotech. [U-^{14}C] linolenic acid **3** of specific activity 5.92 MBq/mmol was synthesized as described previously (Vick and Zimmerman, 1983). All authentic, non-radioactive, lipid standards were obtained from Sigma Chemical Company, St. Louis, MO. Methyl jasmonate **8** was a gift from Bedoukian Research, Inc and 12-oxo-phytodienoic acid **5** obtained from Cayman Chemical Company. JA **7** was prepared from MJ **8** by saponification. Radioactivity counts were obtained using a 1500 Tri-Carb Liquid Scintillation Analyzer (Packard).

3.3. Extraction of radioactive lipids

Lipids were extracted with 2-propanol/$CHCl_3$ (1:2, v/v). 1 M oxalic acid was added to the extracts to lower the pH to 4 and extracts were washed with 0.2 M H_3PO_4/1 M KCl. The collected $CHCl_3$ layer was evaporated under a stream of N_2 gas.

For separation of lipid classes, the $CHCl_3$ was evaporated under a stream of N_2, and the residue was dissolved in MeOH/$CHCl_3$ (1:1, v/v). Samples were loaded on $(NH_4)_2SO_4$-impregnated, heat activated silica TLC plates (Baker S_i 250) and developed in acetone/benzene/H_2O (91:30:8, v/v/v) (Lightner et al., 1994). Bands were visualized with iodine staining, scraped and counted using liquid scintillation counting. Authentic lipids were used as chromatographic standards.

For analysis of fatty acid methyl esters, the $CHCl_3$ was evaporated under a stream of N_2. The residue was methylated with excess ethereal diazomethane and transmethylated with sodium methoxide. Samples were diluted with H_2O and fatty acid methyl esters were extracted with hexane. The collected hexane layer was washed with 0.9% KCl and hexane was evaporated under a stream of N_2. The residue was dissolved in a known small volume of MeOH/$CHCl_3$ (1:1, v/v) that contained 0.01% butylated hydroxytoluene (BHT). Samples were loaded onto heat activated silica TLC plates (Baker S_i 250) that had been agitated in 5% $AgNO_3$ in CH_3CN and dried. The loaded plates were developed in toluene/CH_3CN (97:3, v/v) and exposed to the phosphoImager for 20 h. Methylated [1-^{14}C]12:0 **1**, [^{14}C]18:2 and [U-^{14}C]18:3 **3** were used as chromatographic standards to identify saturated and unsaturated fatty acids.

3.4. Extraction of labeled JA 7 and MJ 8

JA **7** and MJ **8** were extracted as described previously (Miersch et al., 1986; Meyer et al., 1989). Leaf tissues were extracted with MeOH and re-extracted with 80% MeOH–H_2O (4:1). MeOH was evaporated under a stream of N_2 and the remaining aqueous phase was frozen overnight at −20 °C. Thawed samples were centrifuged; supernatants were decanted, acidified to pH 3 with 1 N HCl and extracted with EtOAc. The EtOAc soluble material was dried (anhydrous Na_2SO_4), filtered and the filtrate evaporated under a stream of N_2. The resulting residue was dissolved in a known small volume of MeOH/$CHCl_3$ (1:1, v/v) that contained 0.01% BHT. Samples were loaded onto $(NH_4)_2SO_4$-impregnated, heat activated TLC plates (Baker S_i 250) and developed in hexane/EtOAc/AcOH (60:40:1, v/v/v). After development, TLC plates were dried with a stream of warm air and sprayed with *p*-anisaldehyde reagent [0.5 mL of *p*-anisaldehyde in 100 mL of MeOH/H_2SO_4/AcOH (90:5:5, v/v/v)] (Stahl and Glatz, 1982; Sigma, product A-3957). Plates were heated for 5 min at 110 °C; JA **7** and MJ **8** were identified by co-chromatography with authentic standards. Authentic 12-oxo-phytodienoic acid **5** and 13-HPOT **4** also were used as chromatographic standards. Bands that co-chromatographed with authentic standards were scraped from plates and counted using liquid scintillation counting. For all labeling studies, counts were obtained from the incubation and washing solutions, dry pellet, aqueous phase and organic fraction. These counts were used to calculate the amount of radioactivity taken up by the tissue, recovered from the tissue and incorporated into either neutral or polar lipids, or into oxylipins.

3.5. Assay of methyltransferase activity

All reactions were carried out at 4 °C using a previously described procedure (Wang et al., 1997). Harvested leaves were immediately frozen in liquid nitrogen, polyvinylpolypyrrolidone (PVPP) was added and the tissue pulverized in a pre-cooled mortar and pestle. The tissue was extracted with ice-cold 50 mM Bis-Tris–HCl, pH 6.9, 10 mM of 2-mercaptoethanol, 5 mM $Na_2S_2O_5$, 1% (w/v) PVP-40, 10% (v/v) glycerol. A protease inhibitor cocktail (inhibits serine-, cysteine-, aspartic- and metallo-proteases, and aminopeptidases) was purchased from Sigma Chemical Company, and the inhibitor was added to the homogenization buffer immediately before use. The slurry was centrifuged at 12,000*g* for 10 min and the resulting supernatant was

used as a source of crude enzyme. Protein content was measured using the Bradford method (Bradford, 1976). The assay medium in a final volume of 1 mL consists of an assay buffer (250 mM Tris–HCl, pH 7.5; 10 mM DTT), 1 mM JA final concentration, 0.925 kBq of *S*-adenosyl-L-[methyl-^{14}C] methionine and deionized H_2O. The reactions were started by adding the enzyme and stopped by adjusting the pH to 3 with 1 N HCl. Incubations were carried out at 22 °C for 5 min. Products were extracted using EtOAC and an aliquot was counted for radioactivity using liquid scintillation counting. Specific activity of [^{14}C]AdoMet was used to convert dpm into moles of [^{14}C]MJ formed.

Products were verified by loading an aliquot of the sample on a TLC plate, developing in *n*-hexane/EtOAC/AcOH (60:40:1, v/v/v) and exposing to a phosphoImager. Standards used were authentic [^{14}C]AdoMet, non-radioactive JA **7** and MJ **8**. Standards of JA **7** and MJ **8** were visualized by spraying the plate with *p*-anisaldehyde reagent and the plate heated at 110 °C for 5 min. The areas of a TLC plate corresponding to the authentic standards were scraped off, and the amount of radioactivity measured using liquid scintillation counting.

3.6. Extraction of non-radioactive endogenous JA 7 and MJ 8

Where indicated, leaves were wounded twice across the main vein with a hemostat. Leaves were harvested at indicated times, weighed, frozen in liquid nitrogen and pulverized. Leaves of *Artemisia* were extracted with acetone and the extract dried in vacuo at 40 °C. The residue was resuspended in MeOH–H_2O (4:1) containing 0.2% acetic acid, acidified to pH 3 with 1 N HCl and passed through pre-rinsed Sep-Pak C_{18} cartridges (Waters). The filtrate was made to MeOH–H_2O (1:4) with 0.2% AcOH, passed through pre-rinsed Sep-Pak C18 cartridges and washed with MeOH–H_2O (1:4) containing 0.2% AcOH. JA **7** and MJ **8** were eluted with MeOH–H_2O (4:6 and 6:4) containing 0.2% AcOH, respectively. The eluates were partitioned with peroxide free Et_2O. The organic phase was dried with anhydrous $MgSO_4$ and evaporated in vacuo at 35 °C. Only the JA **7** containing fraction was methylated with excess ethereal diazomethane and the Et_2O evaporated. Both JA **7** and MJ **8** residues were extracted with hexane and analyzed by gas chromatography (Hewlett Packard 5890 with flame ionization detector) on FFAP column of 14 m × 0.25 mm, 0.33 μm film thickness. Temperature program started from 140 °C for 1 min, then increased to 235 °C at a rate of 10 °C/min and held at this temperature for 20 min. Identity of the compounds was confirmed by GC-MS (HP5 column, 30 m × 0.25 mm, 0.25 μm film thickness) with a temperature program of 60 °C for 1 min, increased to 220 °C at 2 °C/min, followed by 10 °C/min to 270 °C, and held at this temperature for 10 min. Helium was the carrier gas at a flow rate of 1 mL/min.

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