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Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression

(chalcone synthase/vegetative storage protein/proline-rich cell wall protein)

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ABSTRACT **Jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), are plant lipid derivatives that resemble mammalian eicosanoids in structure and biosynthesis. These compounds are proposed to play a role in plant wound and pathogen responses. Here we report the quantitative determination of JA/MeJA *in planta* by a procedure based on the use of [^{13}C,$^{2}H_3$]MeJA as an internal standard. Wounded soybean (*Glycine max* [L] Merr. cv. Williams) stems rapidly accumulated MeJA and JA. Addition of MeJA to soybean suspension cultures also increased mRNA levels for three wound-responsive genes (chalcone synthase, vegetative storage protein, and proline-rich cell wall protein) suggesting a role for MeJA/JA in the mediation of several changes in gene expression associated with the plants' response to wounding.**

Jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), are naturally occurring growth regulators found in higher plants (1, 2). JA was first isolated from culture filtrates of the fungus *Lasiodiplodia theobromae* Griff. & Maubl., and MeJA was described as a component of the essential oils of *Jasminum grandiforum* L. and *Rosmanus offincinalis* L. (3–5). Exogenous JA/MeJA caused leaf abscission and senescence and inhibited both growth and germination (6–13). These studies led some investigators to call JA/MeJA a senescence hormone (9, 14–16). However, whereas high concentrations of JA/MeJA (≈50 μM) induce senescence in cell cultures (17), lower concentrations (1–10 μM) alter protein and mRNA populations without inducing senescence (17, 18). Furthermore, qualitative estimates of JA/MeJA distribution in plant tissues revealed high concentrations in apical growing regions (especially developing flowers and fruit) (2). The observation that genes encoding vegetative storage proteins (*vsp*) are highly expressed in apical growing zones and are stimulated by exogenous JA/MeJA (18) suggests a role for this growth regulator in the biogenesis of vegetative and reproductive plant tissues.

The structures of JA and MeJA are similar to mammalian eicosanoids, which are potent modulators of smooth muscle contraction and inflammatory responses (Fig. 1 and refs. 19 and 20). Both eicosanoids and JA are derived from lipids in lipoxygenase-dependent pathways (1). Interestingly, inhibitors of lipoxygenase will attenuate mammalian inflammatory responses and some plant responses to wounding or pathogen attack (21, 22). It has been suggested that JA/MeJA (18, 23) and abscisic acid (ABA; ref. 24) are mediators of wound responses in plants. ABA has been reported to modulate the expression of a tomato proteinase inhibitor gene (24), but in the soybean seedling system this plant hormone had no effect on the expression of two wound-inducible genes (25). ABA levels increased in excised unwounded elongating tissue but did not cause changes in either extensin or *sbPRP1* gene expression (25).

As a first step in determining the role of JA/MeJA in wound responses in plants, we synthesized an internal standard to quantify the levels of JA/MeJA in control and wounded soybean stems. In this report, we demonstrate that wounding causes an increase in the levels of JA/MeJA. Previously, it has been demonstrated that two wound-responsive genes, proteinase inhibitors and *vsp*, were modulated by JA/MeJA (18, 23). To further test the hypothesis that JA/MeJA is a general mediator of wound responses, we determined whether MeJA could modulate the expression of two additional wound-responsive genes, chalcone synthase (*chs*, which encodes an enzyme in the phenylpropanoid biosynthetic pathway) and proline-rich cell wall protein (*PRP*, a cell wall structural protein). Here, we present evidence showing that MeJA can modulate *chs* and *PRP* gene expression.

MATERIALS AND METHODS

Plant Material. Nongrowing soybean (*Glycine max* [L] Merr. cv. Williams) hypocotyl tissue from 3-day-old dark-grown seedlings [6–30 g (fresh weight)] was either harvested and frozen immediately in liquid N_2 or wounded with a razor blade by cutting into 1- to 3-mm sections. Unwounded tissues used as controls were obtained from intact plants. After wrapping with a commercial plastic wrap and storage in darkness at 28°C and 100% relative humidity for various times, tissue was frozen in liquid N_2. Tissue was stored at −80°C until extracted.

Soybean suspension cultures were supplied by Suzanne Rogers (Department of Horticultural Sciences, Texas A&M University) and grown photomixotrophically as described (18).

Synthesis of [^{13}C,$^{2}H_3$]MeJA. To obtain JA, the methyl ester of MeJA (Bedoukian Research, Danbury, CT) was hydrolyzed with methanolic KOH, the resulting solution was acidified, and the JA was partitioned into ether. To convert the carboxylic acid to the deuterated form, 0.25 g (1 mmol) of JA in ether (0.5 ml) was washed with $^{2}H_2O$ (0.5 ml) four times. $^{13}C^{2}H_2N_2$ (2.5 mmol) was generated from *N*-methyl-*N*-[^{13}C]-methyl[$^{2}H_3$] diazald (99 atom % ^{13}C, 99.5 atom % ^{2}H; Aldrich) using a minidiazald apparatus according to the manufacturer's instructions (Aldrich). [^{13}C,$^{2}H_3$]MeJA was synthesized from $^{2}H_2O$-washed JA in the presence of deuterated solvents as recommended by the manufacturer (Aldrich).

Extraction, Purification, and Quantification Procedures. Plant samples, after addition of 0.875–3.5 μg [^{13}C,$^{2}H_3$]MeJA and distilled water (10–20 ml), were homogenized in acetone. The acetone was removed by rotary evaporation, the aqueous phase was acidified to pH 2.5, and JA/MeJA was

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Abbreviations: JA, jasmonic acid; MeJA, methyl jasmonate; ABA, abscisic acid.

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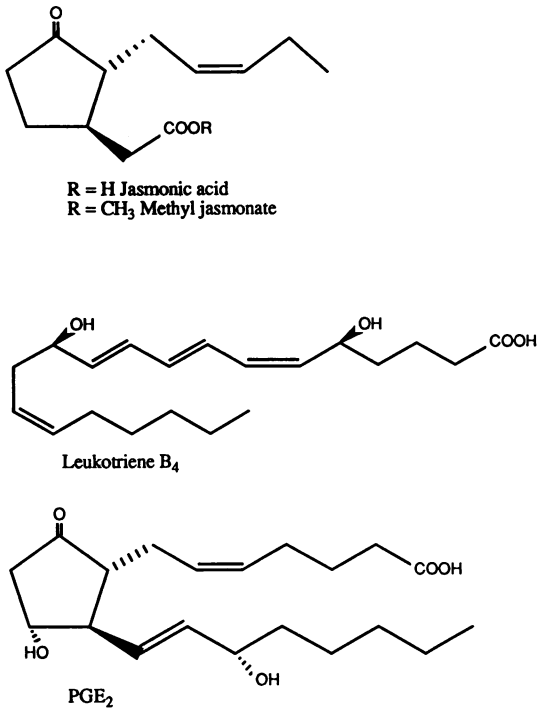

FIG. 1. Structure of JA, MeJA, and the mammalian eicosanoids leukotriene B_4 and prostaglandin E_2 (PGE_2).

partitioned into ether. The ether phase was partially dried with sodium sulfate and was removed by rotary evaporation. Increased losses occurred when the ether phase was dried exhaustively with rotary evaporation. The wet residue was partitioned into a small volume (about 400 μl) of 89% hexane/10% ethyl acetate/1% acetic acid (vol/vol). MeJA and JA were further purified by isocratic (89% hexane/10% ethyl acetate/1% acetic acid) silica gel [5 μm (particle size), 4.5 × 250 mm] HPLC at a flow rate of 2 ml/min. Fractions corresponding to MeJA (3.0 min) and JA (8.0 min) were collected, dried, dissolved in a small volume of ethyl acetate (usually 20–100 μl), and analyzed by GC/mass spectrometry-selected ion monitoring as described below. Before analysis, JA was methylated with diazomethane to give MeJA. The recovery of [^{13}C,$^{2}H_3$]MeJA ranged from 40 to 60%. In a mock experiment where a known amount of JA was added to the extract, the recovery was similar to MeJA. Because of this, JA content was estimated by assuming the percent recovery of JA to be identical to that of MeJA. A calibration curve using various amounts of unlabeled and [^{13}C,$^{2}H_3$]MeJA was used to estimate endogenous MeJA levels (26).

RNA Isolation and Analysis. Total nucleic acid was isolated from soybean suspension cultures treated with 20 μM MeJA as described (27). In another experiment, total nucleic acid was isolated from either wounded mature or elongating hypocotyl tissue. Unwounded tissue used as controls was from intact plants. A cDNA clone corresponding to soybean *chs1* (27) was obtained from a soybean cDNA library constructed using mRNA from the elongating region of water-deficient soybean seedlings. RNA levels were determined using antisense RNA probes for *vspB* (18) and random-primer-labeled probes for *chs1* and pDC16 as described (27–29). The carrot cDNA clone pDC16 hybridizes to mRNAs encoding the soybean proline-rich cell wall proteins, sbPRP1, and sbPRP2 (28, 29). In RNA blots, the upper and lower bands correspond to mRNA encoding sbPRP1 and sbPRP2, respectively (28, 29).

Mass Spectrometry. GC/mass spectrometry analysis of compounds was performed using a Hewlett–Packard model 5890 gas chromatograph coupled to a Hewlett–Packard model 5970B mass selective detector. GC conditions were isothermal for 1 min at 80°C followed by temperature programming to 180°C at 25°C/min using a 25-m DB-1 (0.25-mm i.d., 1-μm phase thickness) column at a column pressure of 30 kPa with He as carrier. The retention time of MeJA was ≈10 min. GC/mass spectrometry-selected ion monitoring was performed by monitoring m/z 151, 156, 160, 177, 181, 193, 224, and 228. Dwell time for all ions was 100 msec. Sensitivity limits (m/z 224) were 0.5–1 ng/μl.

RESULTS AND DISCUSSION

In previous studies, only qualitative estimates of JA/MeJA content in plant tissue were possible due to lack of a suitable JA or MeJA internal standard labeled with stable isotopes (2). An internal standard was synthesized for accurate quantitation by methylating JA with $^{13}C^{2}H_2N_2$ to generate [^{13}C,$^{2}H_3$]-MeJA with a new average molecular weight of 227.6 (Fig. 2). This compound was used to quantify MeJA levels in plant tissue by the method of isotope dilution (26). Another plant growth regulator found in the mature regions of soybean seedlings is ABA, a compound that ameliorates several effects of water deficit (30). The levels of JA and MeJA in mature unwounded regions of soybean seedling stems (Fig. 3) were similar to those found for ABA (25, 30). However, when this tissue was wounded by chopping it into 1- to 3-mm sections, JA and MeJA levels rapidly increased within 2 h. Levels of JA/MeJA increased further by 8 h after wounding and remained elevated for at least 24 h.

In plants, the expression of at least four classes of genes are modulated in wounded tissue (for reviews, see refs. 31–33). Chitinase and β-glucanase gene expression increases in response to the production of wound ethylene. These genes encode enzymes capable of hydrolyzing fungal cell walls (34–36). A second class of wound-inducible genes include *chs*, phenylalanine ammonia lyase, and chalcone isomerase that encode enzymes in the phenylpropanoid/flavonoid bio-

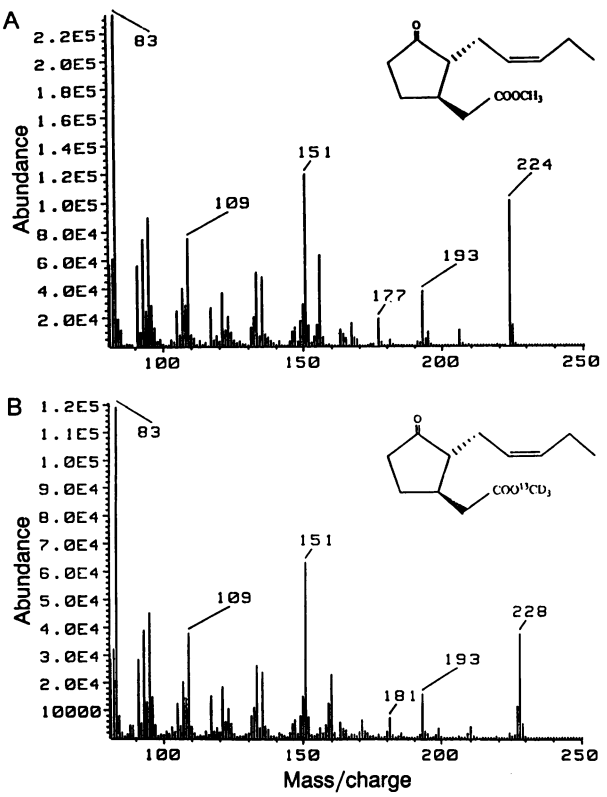

FIG. 2. Mass spectra of authentic MeJA (*A*) and [^{13}C,$^{2}H_3$]MeJA (*B*).

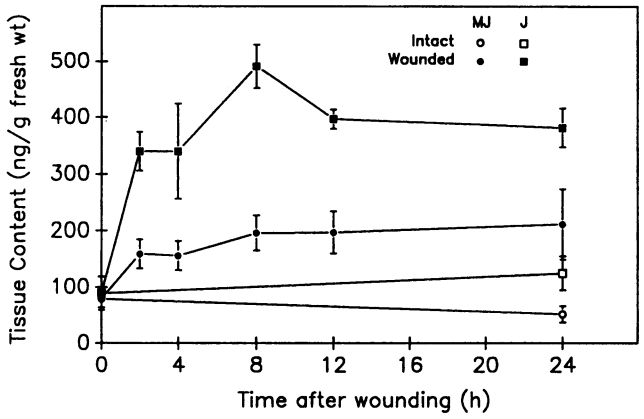

FIG. 3. Wounding modulates MeJA/JA levels. MeJA (○, ●) and JA (□, ■) accumulation in wounded (solid symbols) or unwounded (open symbols) nongrowing hypocotyl tissue. Data presented are the mean ± SD.

synthetic pathways (32, 33). Phytoalexins derived from this pathway are involved in plant disease resistance. Another class of wound-modulated genes code for structural proteins found in the cell wall, such as extensins, glycine-rich proteins, and proline-rich cell wall proteins (28, 29, 31). The fourth class of genes that respond to wounding encode proteinase inhibitors and the soybean vegetative storage proteins (18, 23, 37). Genes of this class are regulated by JA/MeJA (18, 23, 37).

Further information about the relationship between MeJA levels and changes in wound-responsive genes was gathered by determining the levels of soybean chs1, vspB, and sbPRP1 mRNA in soybean suspension cells treated with 20 μM MeJA (Fig. 4). A slight increase in chs1 mRNA level could be detected within 1 h; mRNA levels then reached a maximum between 4 and 12 h and subsequently declined. Accumulation of vspB and sbPRP1 mRNA occurred more slowly. Differences in the accumulation of these mRNAs in response to MeJA could result from differences in mRNA stability or the response of these genes to MeJA. No effect of 20 μM MeJA on β-tubulin mRNA level was observed (data not shown). The stimulation of four wound-inducible genes [proteinase inhibitor, *vspB*, *chs1*, and *sbPRP1* (Figs. 4 and 5 and refs. 18 and 23)] suggests that increased levels of JA/MeJA in wounded tissue modulate specific changes in gene expression associated with the plant's response to wounding.

A linkage between JA/MeJA and changes in the expression of *chs1* and *sbPRP1* is suggested by the dominant *I* mutant in soybeans (38). This mutation inhibits the accumulation of both anthocyanins and sbPRP1 protein in soybean seed coats (38). Since anthocyanin accumulation (39) and expression of *chs1* and *sbPRP1* can be modulated by JA/MeJA, the *I* gene

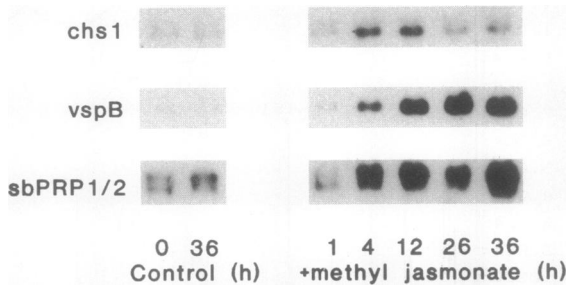

FIG. 4. Effect of 20 μM MeJA on chs1, vspB, and sbPRP1/2 mRNA abundance in soybean suspension cells. Total nucleic acid for Northern blot analysis was harvested from untreated soybean cells at 0 and 36 h or from soybean suspension cells treated with 20 μM MeJA for 1, 4, 12, 26, and 36 h.

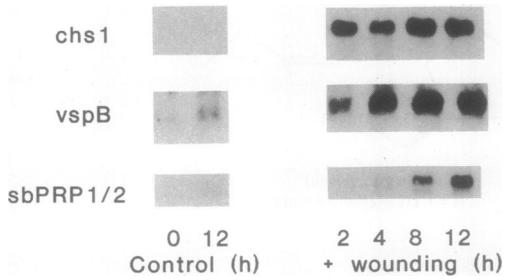

FIG. 5. Effect of wounding on chs1, vspB, and sbPRP1/2 mRNA abundance in wounded mature (chs1 and vspB) or elongating (sbPRP1/2) soybean hypocotyl tissue. Total nucleic acid for Northern blot analysis was harvested from unwounded intact seedlings at 0 and 12 h or from wounded elongating or mature hypocotyl tissue at 2, 4, 8 and 12 h.

product may function in the pathway between JA/MeJA perception and gene expression.

In wounded tissue, the increase in vspB and sbPRP1 mRNA levels follows JA/MeJA accumulation (Figs. 3 and 5). These kinetics are consistent with a role for JA/MeJA in modulating the expression of these genes in wounded tissue. However, chs1 mRNA accumulation in wounded tissue was as fast as or faster than JA/MeJA accumulation. This result suggests that other factors (such as cell wall fragments; refs. 40–44) are involved in modulating *chs1* gene expression. Although increased levels of JA/MeJA may not be responsible for the rapid induction of *chs1* gene expression in wounded tissue, JA/MeJA may play a role in maintaining elevated expression several hours after wounding has occurred (40–44). Similarly, in unwounded tissue other factors must regulate expression of *chs1* and *sbPRP1*. In unwounded hypocotyl tissue, levels of MeJA are highest in the elongating region and lowest in the mature region of the hypocotyl (45). In the elongating region of hypocotyls, *sbPRP1* gene expression is low and increases dramatically in the mature region (27). Expression of the *chs1* gene occurs primarily in root tissue (27). In contrast, expression of the *vspB* gene is tightly correlated with MeJA levels in soybean hypocotyls (45).

Previous studies showed that chs1, sbPRP1, and vspB mRNA levels increase when plant tissues are wounded. We now describe that JA/MeJA levels increase in wounded soybean stem tissue. Also, the addition of MeJA at physiological concentrations to soybean suspension cultures causes accumulation of chs1, vspB, and sbPRP1 mRNA. The kinetics of vspB and sbPRP1 mRNA accumulation in wounded tissue are consistent with a role for wound-induced JA/MeJA in modulating these genes. These results confirm the suspected role of JA/MeJA in mediating some plant wound responses. In addition, the mRNA levels of a cell wall protein gene (*sbPRP1*) and a gene in the phenylpropanoid pathway (*chs1*) can be modulated by MeJA. These genes were previously reported to be induced by wounding or by addition of cell wall fragments or purified oligosaccharides to plants (40–44). The data described here may indicate that cell wall fragments released by wounding could directly or indirectly activate *chs1* and *sbPRP1* gene expression by stimulating JA/MeJA accumulation.

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