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Importance of the Chiral Centers of Jasmonic Acid in the Responses of Plants[1]

Activities and Antagonism between Natural and Synthetic Analogs

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The importance of the two chiral centers at C-3 and C-7 in the molecular structure of jasmonic acid in plant responses was investigated. We separated methyl jasmonate (MeJA) into (3*R*)- and (3*S*)-isomers with a fixed stereochemistry at C-3, but epimerization at C-7 is possible. The four isomers of the nonepimerizable analog 7-methyl MeJA were synthesized. These six esters and their corresponding acids were tested in three bioassays: (a) senescence in sunflower (*Helianthus annuus*) cotyledons; (b) proteinase inhibitor II gene expression in transgenic tobacco (*Nicotiana tabacum*) with β-glucuronidase as a biochemical reporter; and (c) seed germination in *Brassica napus* and wheat (*Triticum aestivum*). The esters and acids had similar activities in the three assays, with the ester being more effective than its acid. The (3*R*)-stereochemistry was critical for jasmonate activity. Although activity was reduced after substituting the C-7 proton with a methyl group, the analogs with (3*R*,7*R*)- or (3*R*,7*S*)-stereochemistry were active in some of the assays. Although the four isomers of 7-methyl MeJA were inactive or only weakly active in the senescence assay, they could overcome the senescence-promoting effect of (3*R*)-MeJA. The strongest antagonistic effect was observed with the (3*R*,7*S*)-isomer.

There is increasing evidence for the involvement of JA and its ester, MeJA, in regulating a wide range of plant physiological processes, including senescence (Ueda and Kato, 1981; Weidhase et al., 1987a; Parthier, 1990; Porat et al., 1993; Emery and Reid, 1996), wound responses (Farmer et al., 1992; Farmer and Ryan, 1992; Peña-Cortés et al., 1993; Xu et al., 1994), accumulation of vegetative storage proteins (Staswick et al., 1991; Mason et al., 1993), embryo development (Holbrook et al., 1991; Wilen et al., 1991), and secondary metabolite biosynthesis (Gundlach et al., 1992; Aerts et al., 1994; Facchini et al., 1996). JA-induced alterations in gene expression appear to mediate many of these responses (Staswick, 1992; Sembdner and Parthier, 1993; Reinbothe et al., 1994).

The chemical structures of JA and MeJA contain two chiral centers at C-3 and C-7, each of which can have either the *R* or *S* absolute configuration (see Fig. 1). Jasmonates can therefore exist in four possible stereoisomeric forms. The (+)-(3*R*,7*S*)-isomer and its mirror image, the (−)-(3*S*,7*R*)-isomer, which are a pair of optical isomers with the two side chains in the *cis* arrangement with respect to the plane of the cyclopentane ring, are known as (+)- and (−)-epiJAs, respectively (Acree et al., 1985). A second pair of optical isomers, the (−)-(3*R*,7*R*)- and the (+)-(3*S*,7*S*)-forms, commonly called (−)- and (+)-JAs, respectively, have the two side chains in the *trans* arrangement. The epiJAs are thermodynamically less stable than the JAs due to higher steric hindrance between the *cis* side chains, and have a tendency to epimerize at C-7 to the *trans* arrangement through a keto-enol tautomerization involving the loss of the C-7 proton, which is rendered acidic by its neighboring electron-withdrawing ketone group. The stereochemistry at C-3 remains unchanged in this epimerization. The naturally occurring jasmonates from plants are reported to have the *R* stereochemistry at C-3 and either *S* or *R* at C-7 (Vick and Zimmerman, 1984). The (+)-(3*R*,7*S*)-isomer with the *cis* side chains, (+)-epiJA, is believed to be the initial 12-carbon product formed in the biosynthesis of jasmonates in plants. The (−)-(3*R*,7*R*)-isomer with the *trans* side chains, (−)-JA, is formed as a result of subsequent epimerization in planta or during isolation (Mueller and Brodschelm, 1994). Commercially available JA and MeJA usually used in biological studies are equilibrium mixtures containing approximately 5% each of the (3*R*,7*S*)- and (3*S*,7*R*)-*cis* isomers and 45% each of the (3*R*,7*R*)- and (3*S*,7*S*)-*trans* isomers.

Although the effects of JA have been investigated in many systems, there have been only a few studies in which the activities of pure stereoisomers of JA or MeJA have been compared. (3*R*,7*S*)-MeJA is reported to have the characteristic jasmine odor, the (3*R*,7*R*)-form is weakly fragrant,

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Abbreviations: ANOVA, analysis of variance; JA, jasmonic acid; MeJA, methyl jasmonate; MeOH, methanol; MS, Murashige-Skoog; pin II, proteinase inhibitor II.

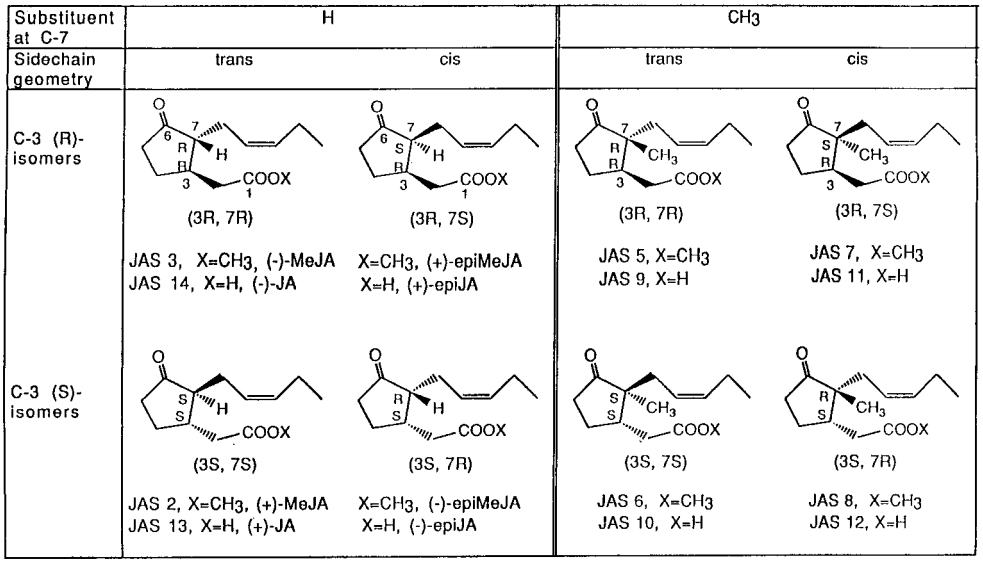

Figure 1. Stereoisomers of MeJA, JA, and their C-7 methyl-substituted analogs.

and the two (3*S*)-isomers are odorless (Acree et al., 1985; Nishida et al., 1985). In assays to determine the effects of MeJA on cell division, tuberization, and senescence, varying degrees of activity were observed for the different isomers (Koda et al., 1992). For cell division, both the (3*R*,7*S*)- and (3*R*,7*R*)-isomers had strong inhibitory effects, whereas the two (3*S*)-isomers had very low activities, indicating that the (3*R*)-stereochemistry was significant. In contrast, the (7*S*)-configuration was more important than the (3*R*)-configuration for the tuber-inducing activity of MeJA. For the promotion of senescence, all four isomers were active, with the (3*R*,7*S*)-isomer being the most effective. In these assays it is not possible to determine which C-7 isomer was responsible for the observed activity due to the possibility of epimerization, which is facilitated by the exposure of the jasmonates to the cellular environment over an extended period (Mueller and Brodschelm, 1994).

We have undertaken to synthesize stereochemically fixed JA analogs in which epimerization is prevented by the replacement of the C-7 proton with a methyl group. Two laboratories have recently reported the synthesis of racemic 7-methyl analogs of MeJA, and one has reported the synthesis of an optically active 7-methyl analog of MeJA (see Fig. 1) (Ward and Beale, 1993; Taapken et al., 1994; Koda et al., 1995). The racemic 7-methyl compounds were found to be inactive in the assays employed. These included tendril coiling, elicitation of phytoalexins (Taapken et al., 1994) and tuber induction, senescence of oat leaves, and inhibition of kinetin-induced growth of soybean callus (Koda et al., 1995). Their negative results have led to the proposal that the steric bulk of the 7-methyl group interferes with the perception of the active jasmonates by the putative receptor.

In this study we prepared the four optically active isomers of 7-methyl MeJA (Fig. 1, JAS 5–8) and their corresponding acids (JAS 9–12). The stereochemistry at both the C-3 and C-7 positions of these synthetic analogs were determined. We also resolved commercially available MeJA into (3*R*,7*R*)-MeJA (JAS 3) and (3*S*,7*S*)-MeJA (JAS 2) (Okamoto and Nakazawa, 1992) and prepared the corresponding acids, JAS 14 and JAS 13, respectively. The 7-methyl analogs were tested alone and as antagonists in combination with JAS 3. The assays employed included induction of senescence in sunflower (*Helianthus annuus*) cotyledons, seed germination inhibition in *Brassica napus* and wheat (*Triticum aestivum*), and proteinase inhibitor gene promotor induction in transgenic tobacco (*Nicotiana tabacum*) using GUS as a biochemical reporter. The objective was to delineate which components of the JA molecule are active in influencing a number of bioassay systems in order to probe further the mechanism by which plant cells perceive and respond to jasmonates.

MATERIALS AND METHODS

Chemicals

JA and related compounds are identified by the JAS numbers used in our laboratories (see Fig. 1). Racemic MeJA (cyclopentaneacetic acid, 3-oxo-2-[2-pentenyl]-, methyl ester) was purchased from Bedoukian Research, Inc. (Danbury, CT). Analysis by GC and GC/MS showed that the sample contained 91% of the isomers with *trans* side chains (MeJA), 6% of the isomers with *cis* side chains (epiMeJA), and 3% of another unidentified isomer with the same molecular weight, possibly the (2*E*)-pentenyl isomer.

The four stereoisomers of MeJA were obtained by separating the commercial sample on a semipreparative HPLC column (10 × 250 mm, 10 μm; i-PrOH:hexane [1:9, v/v] at 2.0 mL min^{-1}, detection at 300 nm; Chiralpak AS, Chiral Technologies, Inc., Exton, PA) (Okamoto and Nakazawa, 1992). The JAS 2 obtained had $[\alpha]_D^{25}$ +91.3° (0.84 g 100 mL^{-1}, MeOH). The JAS 3 obtained contained some (3*R*,7*S*)-isomer (18:1 by GC) and had $[\alpha]_D^{25}$ −75.0° (0.88 g 100

mL^{-1}, MeOH). The (3*S*,7*R*)-isomer had $[\alpha]_D^{25}$ −47.2° (0.89 g 100 mL^{-1}, MeOH) (compare Nishida et al., 1985). The ^1H (500 MHz) and ^{13}C (125 MHz) NMR data obtained for JAS 2 and the (−)-(3*S*,7*R*)-isomer agree with data previously reported (Dathe et al., 1981; Nishida et al., 1985; Crombie and Mistry, 1991).

(3*S*,7*S*)-JA (JAS 13) and (3*R*,7*R*)-JA (JAS 14) were prepared by hydrolysis of JAS 2 and JAS 3, respectively, using porcine liver esterase under previously reported conditions (Lamb et al., 1996). The JAS 13 obtained had $[\alpha]_D^{25}$ +92.6° (1.00 g 100 mL^{-1}, MeOH). JAS 14 had $[\alpha]_D^{25}$ −84.7° (1.04 g 100 mL^{-1}, MeOH) (Aldridge et al., 1971; $[\alpha]_D^{25}$ −73° [0.1 g 100 mL^{-1}, MeOH]). The ^1H and ^{13}C NMR data of the optically active compounds agree with those of racemic JA obtained by hydrolysis of racemic MeJA under basic conditions.

(3*S*,7*S*)-7-Methyl MeJA (JAS 6) and (3*S*,7*R*)-7-methyl MeJA (JAS 8) ([1*S*,2*S*]- and [1*S*,2*R*]-2-methyl-3-oxo-2-[2*Z*-pentenyl]-cyclopentylacetic acid, methyl esters) were prepared by alkylating JAS 2 by a procedure similar to those previously reported (Ward and Beale, 1993; Taapken et al., 1994). The two analogs and unreacted JAS 2 were separated by column chromatography (silica gel, ether:hexane [1:3, v/v]), followed by further purification on a chiral HPLC column (10 × 250 mm, 10 μm; i-PrOH:hexane [1:9, v/v] at 2.0 mL min^{-1}, detection at 300 nm; Chiralcel OD, Daicel Chemical Industries Ltd., Japan). The 31% yield of C-7 methylated product is based on JAS 2 consumed (JAS 6:JAS 8, 1:2, v/v). JAS 6 had $[\alpha]_D^{25}$ +110.6° (0.88 g 100 mL^{-1}, MeOH), and JAS 8 had $[\alpha]_D^{25}$ +41.3° (0.96 g 100 mL^{-1}, MeOH). The ^1H and ^{13}C NMR data of both compounds agree with those previously reported for the racemic forms (Ward and Beale, 1993; Taapken et al., 1994).

(3*R*,7*R*)-7-Methyl MeJA (JAS 5) and (3*R*,7*S*)-7-methyl MeJA (JAS 7) ([1*R*,2*R*]- and [1*R*,2*S*]-2-methyl-3-oxo-2-[2*Z*-pentenyl]-cyclopentylacetic acid, methyl esters) were prepared by alkylating JAS 3 by the same procedure. The C-7 methylated products were obtained in a 55% yield based on the starting material consumed. JAS 5 had $[\alpha]_D^{25}$ −105.9° (1.05 g 100 mL^{-1}, MeOH), and JAS 7 had $[\alpha]_D^{25}$ −43.4° (1.14 g 100 mL^{-1}, MeOH). The ^1H and ^{13}C NMR data of both compounds agree with those of their enantiomers. Under the HPLC parameters listed above, the following orders of elution were observed for the four isomers of the 7-methyl analog and the two MeJA enantiomers: on the Chiralcel OD column, JAS 5, JAS 6, and JAS 7 co-eluted with JAS 8, and JAS 3 was partially resolved from JAS 2; on the Chiralpak AS column, JAS 6 and JAS 5 co-eluted with JAS 7, and JAS 8 co-eluted with JAS 2 and JAS 3.

The four isomers of 7-methyl JA (JAS 9–12) ([1*R*,2*R*]-, [1*S*,2*S*]-, [1*R*,2*S*]-, and [1*S*,2*R*]-2-methyl-3-oxo-2-[2*Z*-pentenyl]-cyclopentylacetic acids) were prepared by hydrolysis of the corresponding methyl esters under basic conditions (1 M KOH:MeOH [1:1, v/v], room temperature, 15 h, 100% yield). JAS 9 had a melting point of 43 to 50°C, $[\alpha]_D^{25}$ −107.4° (0.81 g 100 mL^{-1}, MeOH). JAS 10 (recrystallized from hexane at 5°C) had a melting point of 50 to 54°C, $[\alpha]_D^{25}$ +105.7° (0.53 g 100 mL^{-1}, MeOH). JAS 11 was an oil, $[\alpha]_D^{25}$ −40.8° (0.76 g 100 mL^{-1}, MeOH). JAS 12 had $[\alpha]_D^{25}$ +36.5° (0.74 g 100 mL^{-1}, MeOH). The ^1H and ^{13}C NMR spectral data agree with those previously reported for the racemic forms of the compounds (Ward and Beale, 1993; Taapken et al., 1994).

All compounds were stored at −20°C in capped glass vials. They were solubilized in absolute ethanol prior to preparation of 100 mM aqueous solutions, and some were further diluted to 10 mM stocks. These stocks were also stored at −20°C. When used in assays, the equivalent volume of absolute ethanol was introduced into control samples. For senescence studies, the 100 mM stocks were used to minimize ethanol volumes in the medium.

Structure Determination

JAS 10 was recrystallized from hexane (5°C) and the structure was determined by x-ray crystallography. Knowing the absolute configuration of JAS 10 allowed the stereochemistry at the chiral centers of all four 7-methyl JA analogs to be deduced. A diffractometer with ω scans (CAD4, Enraf-Nonius, Bohemia, NY) was used for data collection. The data were as follows: $C_{13}O_3H_{20}$, M_r = 224.30, orthorhombic, space-group $P2_12_12_1$, colorless prism, 0.40 × 0.40 × 0.30 mm, a = 8.6719(6), b = 8.9408(7), c = 16.0303(15) Å^3, V = 1242.89(17) Å^3, Z = 4, D_x = 1.199 mg m^{-3}, and l(MoKa) = 0.71069 Å. Cell parameters obtained by least-squares fit of 25 reflections with 36.8 < 2Q < 43.0°, μ = 0.08 mm^{-1}, F(000) = 487.95, and T = 289K. Q_{max} = 52.5°, 0 ≤ h ≤ 10, 0 ≤ k ≤ 11, 0 ≤ l ≤ 19. Three intensity standards monitored at intervals of 120 min had no significant intensity variation. Three orientation standards were monitored after every 400 reflections. Merging R based on intensities was 0.005. A total of 1599 reflections was measured, of which 1468 were independent. A total of 1259 observed reflections (I > 2.0 s[I]) was used in the solution and refinement of the structure. No absorption correction was applied. The structure was solved by direct methods using NRCVAX (Gabe et al., 1989). The refinement of the structure was accomplished using full-matrix least squares with anisotropic thermal factors for nonhydrogen atoms. The function minimized was $w(F_o - F_c)^{2q}$ where $w = 1.0/(s^2[F] + 0.0016F^2)$. Refinement on F, R (F) = 0.032, wR (F) = 0.045, S = 1.00. Parameters refined = 145. Extinction correction = none. Atomic scattering factors and anomalous dispersion corrections were from a published source (International Union of Crystallography, 1974). All H atoms were located in DF maps. H atoms were placed by molecular geometry (C-H = 1.00 Å) and assigned U_{iso} = U_{eq} of the attached atom + 0.01. The H atom parameters were not refined. Structure diagrams were drawn using ORTEP plotting (Johnson, 1976).

Plant Material

Sunflower (*Helianthus annuus* L. cv Dahlgren 131) seeds were germinated in a soilless potting mixture and maintained in growth chambers at 22°C/18°C day/night temperatures, with a 16-h day at 300 μE m^{-2} s^{-1}. Six days after planting, cotyledons of a similar size were excised from the

young seedlings to be used for in vitro treatments. *Brassica napus* L. cv Westar seeds were originally obtained from Agriculture and Agri-Food Canada (Saskatoon). Wheat (*Triticum aestivum* cv Laura) for germination studies was provided by Alberta Wheat Pool (Calgary, Alberta, Canada). Transgenic tobacco (*Nicotiana tabacum* cv Xanthi) seeds were kindly provided by Dr. Robert Thornburg (Iowa State University, Ames).

The tobacco had been transformed with pRT210, a plasmid containing a tomato (*Lycopersicon esculentum*) pin II gene promoter of 980 bp fused with the gene encoding GUS as a biochemical reporter construct. These tobacco seeds were surface-sterilized with bleach and germinated on MS agar medium containing 200 μg/mL kanamycin. After about 3 weeks healthy seedlings were transferred onto plates containing MS medium with 2.5% Suc, 0.7% Phytagar (Gibco-BRL), pH 5.8, and no kanamycin. At approximately 5 weeks of age, seedlings were transferred into Magenta jars (Sigma), four in each one containing the same medium. Plants were allowed to recover from this transplanting and to grow for 2 weeks prior to the application of jasmonates. All seedlings were grown in a growth room at 22°C/18°C day/night temperatures with a 16-h day at 400 μE m^{-2} s^{-1}.

Measurement of Conductivity and Chlorophyll Degradation

Excised, 6-d-old sunflower cotyledons were surface-sterilized in 5% (v/v) commercial bleach for 20 min, followed by extensive rinsing with sterile, distilled water. Three cotyledons were then randomly transferred to each sterile Petri dish containing 5 mL of either sterile, double-distilled water as a control or 200 μM aqueous solutions of the series of JA analogs. Preliminary testing of jasmonates from 10 to 500 μM revealed that 200 μM was consistently highly effective. For measurement of conductivity, the cotyledons were pretreated with distilled water or 10 mM ACC for 1.5 h before adding jasmonates. The Petri plates were sealed with Parafilm (American National Can, Neenah, WI), wrapped in aluminum foil, and stored in the dark at 26 ± 1°C for either 6 d (conductivity) or 7 d (chlorophyll) before measurements were taken. Conductivity, as a measure of ionic leakage due to membrane degradation, was done on d 7 by bringing the solution bathing the three cotyledons up to 6 mL in total volume. Conductivity of this solution as micromhos per centimeter was then measured on a conductance meter (model 32, YSI, Yellow Springs, OH). Chlorophyll content was measured on cotyledons after 7 d in the dark. Each cotyledon was extracted in 6 mL of 80% ethanol at 70°C. The cooled extract was then measured at both A_{663} and A_{645} in a spectrophotometer (Spectronic 2000, Bausch and Lomb, Rochester, NY). The chlorophyll content of the treated cotyledons was calculated as milligrams per gram fresh weight.

Measurement of Ethylene

The experiments were conducted following the method of Chou and Kao (1992). Sunflower cotyledons of a similar size from 6-d-old seedlings were excised and pretreated with or without 10 mM ACC for 1.5 h. The ACC solution was poured off and the cotyledons were treated with solutions of 200 μM jasmonate for 4 h in darkness.

The amount of ethylene produced after 4 h of treatment was analyzed as described by DeWit et al. (1990). Ethylene content was measured on a gas chromatograph (model 3700, Varian, San Fernando, CA) with a flame ionization detector and a stainless-steel 2-mm × 2-mm i.d. column containing Poropak Q, 80 to 100 mesh, using nitrogen as a carrier gas. The column temperature was 40°C and the detector temperature was 120°C. Each cotyledon was weighed, placed in a 10-mL gastight syringe connected to a three-way valve, and left for 40 min. After the incubation, the connection to the second syringe was opened and 1 mL of head space was extracted and injected into the gas chromatograph for ethylene measurement.

For all of the senescence studies each experiment was performed four times with three replicates per experimental sample. Each replicate contained three cotyledons.

pin II-GUS Expression

Transgenic tobacco plants were grown from the original seed provided by Dr. Robert Thornburg. These were selfed and the seed was kept separate from each plant. Seed from one plant was germinated on MS medium containing 200 μg/mL kanamycin. All seedlings were green and had similar GUS activities as measured by the method of Jefferson (1987). Seed from this plant was therefore used for all further studies.

After growing for 2 weeks on solid MS medium without kanamycin, the tobacco seedlings were at the seven- to eight-leaf stage. A 10-μL drop of 200 μM jasmonate or plain ethanol as a control was applied to the upper leaf of each plant (four per jar). Plants were then returned to the growth room for 24 h. Each treated leaf was quickly cut off the plant and then homogenized in GUS extraction buffer in 1.5-mL Eppendorf tubes with a motor-driven pestle. GUS activity was measured over time at 37°C using 4-methylumbelliferyl-β-D-glucuronide as the substrate. Fluorescence was measured on a fluorescence spectrophotometer (model F-2000, Hitachi, Danbury, CT) with a programmed standard curve calibrated with 4-methylumbelliferone. Protein was determined by the method of Bradford (1976) using BSA as a standard. Each analog was tested on four separate seedlings per jar, and these were replicated three times. ANOVA was performed on the data for all of the compounds (six acids and six esters) and the control, and differences between treatments were determined using an LSD test at the 5% level of significance. ANOVA was also performed on the data for the control and the ester-type compounds.

Inhibition of Germination

B. napus seeds were surface-sterilized with a 20% (v/v) solution of commercial bleach for 20 min, followed by extensive rinsing with sterile, distilled water. Wheat seed was first treated with a 0.025% mercuric chloride solution

before a similar bleach treatment. Both types of seeds were then plated out on Petri dishes containing MS medium with 2.5% Suc, 0.7% Phytagar, pH 5.8, and 50 μM of the individual jasmonates. Controls contained the appropriate volume of ethanol. Each plate had 20 seeds and treatments were replicated three times.

Plates were placed in a growth room at 22/18°C day/night temperatures and 16-h days for 7 d. The percentage of germination, average radicle length, average hypocotyl length, and fresh and dry weights were determined and recorded after 7 d. ANOVA was performed for each variable and differences between jasmonate treatments were determined using a LSD test carried out at the 5% level of significance. The data from all treatments (control and 12 JA-related compounds comprising 6 acids and 6 esters) were compared. ANOVA was also performed on the set of data from the control and treatments with the 6 esters.

RESULTS

Synthesis and Stereochemistry of Jasmonate Analogs

Preparative chromatographic separation of racemic MeJA using a HPLC column with a chiral stationary phase afforded gram quantities of (3*S*,7*S*)- and (3*R*,7*R*)-MeJA, JAS 2, and JAS 3. The JAS 3 sample contained about 5% (3*R*,7*S*)-MeJA due to partial overlapping of the chromatography peaks. The C-3 position is nonepimerizable, whereas C-7, being next to the ketone on the cyclopentanone ring, is susceptible to epimerization during storage or bioassays. Some epimerization at C-7 was observed after the initially pure (3*S*,7*R*)-MeJA had been stored at 4°C for several months. It is therefore more appropriate to consider JAS 2 and JAS 3 as (3*S*)- and (3*R*)-MeJAs, respectively. The esters were converted to the acids JAS 13 and JAS 14 by enzymatic hydrolysis to minimize epimerization, which is catalyzed under acidic and basic conditions.

The stereochemically fixed 7-methyl MeJAs (Fig. 1, JAS 5–8) were synthesized by alkylation of the C-3 resolved MeJAs using methods similar to those reported previously (Ward and Beale, 1993; Taapken et al., 1994). The compounds were purified by conventional chromatography and HPLC, and were free of JAS 2 and JAS 3, as determined by GC and HPLC analyses. Hydrolysis of the 7-methyl MeJAs to their corresponding acids (Fig. 1, JAS 9–12) was achieved under basic conditions.

The absolute stereochemistry at C-7 of the 7-methyl MeJAs was established by x-ray crystallography of the acid JAS 10. The compound was determined to be (3*S*,7*S*)-7-MeJA, as shown in Figure 2. Since both JAS 6 (the ester of JAS 10) and JAS 8 were obtained from the alkylation of JAS 2, JAS 8 (and its acid, JAS 12) should then have the (3*S*,7*R*)-absolute stereochemistry. It can also be deduced that the absolute configurations of the analogs JAS 5 and JAS 7, which were prepared from the alkylation of JAS 3, are (3*R*,7*R*)- and (3*R*,7*S*)-, respectively (see Fig. 1).

Twelve JA-related compounds made up of 6 esters and 6 acids, all with known C-3 stereochemistry and some with fixed C-7 stereochemistry, were obtained. The set of esters can be subdivided into two groups according to the C-3 stereochemistry, the (3*R*)-isomers, JAS 3, JAS 5, and JAS 7, and the (3*S*)-isomers, JAS 2, JAS 6, and JAS 8. The 6 acids can be subdivided in a similar fashion (see Fig. 1).

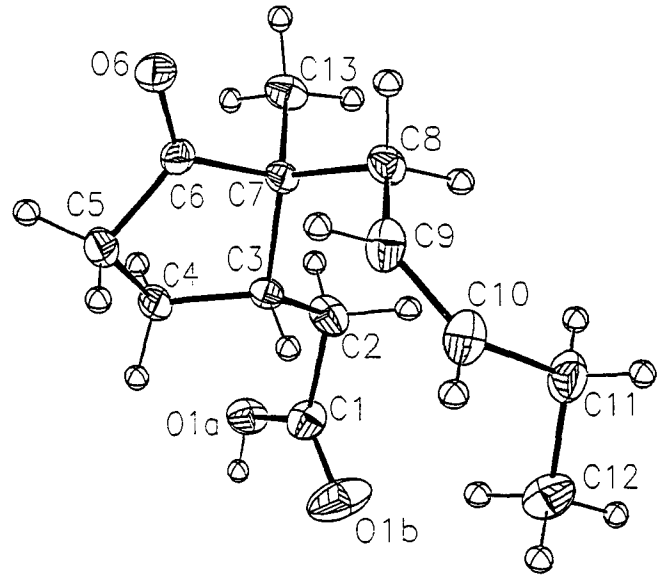

Figure 2. Ortep diagram (Johnson, 1976) of JAS 10, (3*S*,7*S*)-7-methyl jasmonic acid. Displacement ellipsoids are plotted at the 30% probability level.

In each of the bioassays employed in this study, the following structure-activity comparisons were made: (a) esters versus the corresponding acids; (b) (3*R*)-stereoisomers versus the corresponding (3*S*)-stereoisomers; (c) compounds with H at C-7 versus the corresponding C-7 methyl analogs; and (d) (7*R*)- versus the corresponding (7*S*)-isomers for the C-7 methyl analogs.

For most of the assays described below very similar activity was observed for each ester/acid pair, and only the data obtained for the treatments with esters will be shown. Any significant difference between the effects of an ester and its acid will be noted.

Effects on Senescence Conductivity Measurements

In this study changes in membrane breakdown, chlorophyll loss, and ethylene production were measured in excised sunflower cotyledons incubated for 6 d in aqueous solutions of the JA-related compounds. The visible effect of this treatment was that control cotyledons remained green in color and firm in texture, whereas senescing cotyledons turned black and became much softer. The more active the compound was in senescence stimulation, the earlier the black coloration was observed.

A measure of membrane degradation during jasmonate-induced senescence is the conductivity of the medium bathing the cotyledons. The results of testing the JA-related compounds at a concentration of 200 μM is shown in Figure 3, where only the data for the six esters are graphed. In these experiments the newly excised cotyledons had all been pretreated for 1.5 h with 10 mM ACC before 6 d of jasmonate exposure. Strikingly increased conductivity in the medium (Fig. 3, top) was observed on treatment with

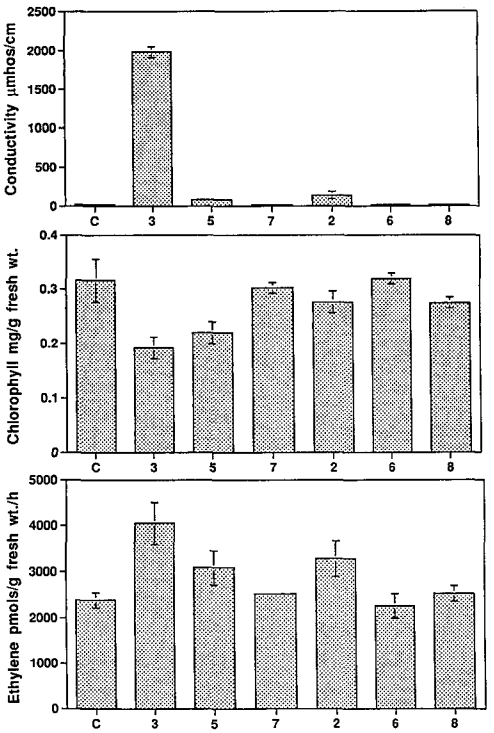

Figure 3. The effect of 200 μM jasmonate esters (JAS 3, 5, 7, 2, 6, and 8) and control (C) on sunflower cotyledon senescence. Top, Ionic conductivity of the bathing medium. Middle, Extracted chlorophyll. Bottom, ethylene produced after 4 h in the dark. Values are means ± SE. Fresh wt., Fresh weight.

JAS 3, i.e. (3*R*)-MeJA. In contrast, material not pretreated with ACC did not show obvious induction of senescence. The (3*S*)-isomers JAS 2 and JAS 13 (not shown) were relatively ineffective, as were all of the C-7 methyl-substituted esters (JAS 5–8) and acids (JAS 9–12, not shown), although JAS 9, a (3*R*,7*R*)-isomer, did reveal some slightly increased conductance above the control. Treatment with JAS 3 not only gave the highest final measure of conductivity, but also caused earlier visible darkening. The cotyledons treated with JAS 3 were turning black by d 4 of treatment, whereas those treated with the corresponding acid, JAS 14, turned black after 5 to 6 d.

Chlorophyll Loss Measurements

Loss of chlorophyll and thus yellowing of leaves or darkening of cotyledons, perhaps due to loss of integrity of chloroplast membranes (Weidhase et al., 1987a), is a visible determinant of senescence in plants. The concentration of chlorophyll was measured in sunflower cotyledons after 7 d of treatment with 200 μM jasmonate. Here the control level would be expected to be higher than in the senescing tissues. As can be seen in Figure 3 (middle), the most active ester analogs in this assay were JAS 3 and JAS 5, both with (3*R*)-stereochemistry. As with the conductivity assay, the (3*S*)-isomers JAS 2 and JAS 13 (not shown) were ineffective. Activity was also observed for JAS 9 (not shown), which was more effective than its ester, JAS 5.

Evolution of Ethylene

Ethylene production is associated with plant senescence and has been correlated with JA induction in some instances (Saniewski and Czapski, 1985; Saniewski et al., 1987; Nowacki et al., 1990; Emery and Reid, 1996). This was tested with the sunflower cotyledons after treatment for 4 h with 200 μM of the 12 compounds, and the results with the esters are presented in Figure 3 (bottom). Here the effects are more variable than those of the assays described above, but showed similar trends. The greatest stimulation of ethylene production was again by treatment with JAS 3. In contrast to conductivity measurement and chlorophyll loss, activity was observed for JAS 2, i.e. (3*S*)-MeJA. Similar to the loss of chlorophyll, the (3*R*,7*R*)- analogs JAS 5 and JAS 9 (not shown) were also effective in stimulating ethylene production. The 7-methyl analogs JAS 6 to 8 and their acids (JAS 10–12, not shown) did not differ from the controls.

Effects of Combined Treatments of JA Analogs

With the exception of JAS 5 and JAS 9, most of the C-7 methyl-substituted analogs were relatively ineffective in our senescence assays. However, it was still possible that they might be interacting with putative receptor sites but not effectively producing measurable results when tested on their own. One way to test this was to apply the individual ester or acid analogs in combination with a strongly active natural compound such as JAS 3 (i.e. [3*R*]-MeJA) in the bioassays. When this was performed on our conductivity assay, striking interference with the JAS 3 activity was

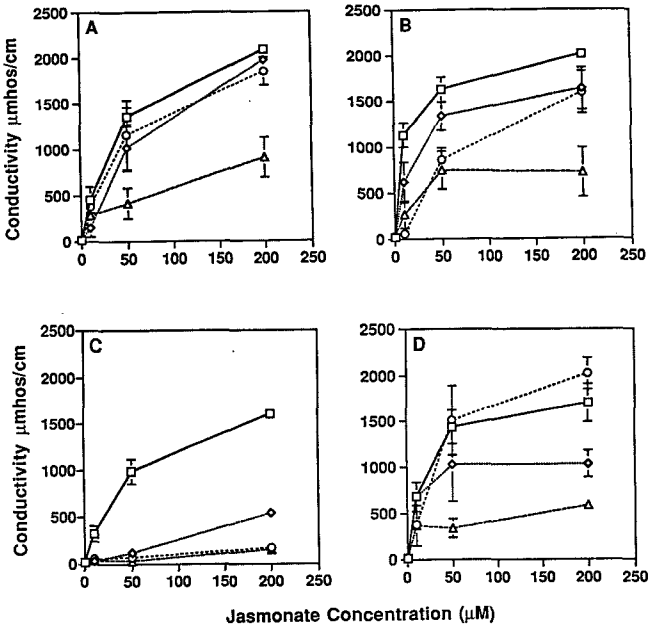

Figure 4. Conductivity in the medium bathing sunflower cotyledons when JAS 3 is applied in combination with the C-7 methyl MeJAs. A, JAS 5; B, JAS 6; C, JAS 7; and D, JAS 8. The concentrations of JAS 3 are shown on the horizontal axis of each panel. The analog concentrations are: □, 0 μM; ◇, 10 μM; ○, 50 μM; and △, 200 μM. Values are means ± SE.

observed. Representative results of combining JAS 3 with the C-7 methyl analogs JAS 5 to 8 are shown in Figure 4. These are conductivity measurements of the medium after a 1.5-h ACC pretreatment, followed by 6 d of cotyledon incubation with mixtures containing various concentrations of JAS 3 (10, 50, and 200 μM) and an individual analog (0, 10, 50, and 200 μM). Treatment with 200 μM JAS 3 alone (0 μM analog) led to consistently high ionic leakage.

With increasing relative concentrations of each of the four C-7 methyl-substituted esters at 10, 50, and 200 μM, there was usually an increased inhibitory action up to the equimolar samples, where there was an extensive inhibition of this senescence measure. There were differences among the four analogs in their effective apparent antagonism to JAS 3, with JAS 7 being qualitatively the most effective, even at the lowest concentration of 10 μM, and JAS 5 having little effect until the 200 μM level was reached. A photographic representation is shown in Figure 5, which reveals that JAS 7 concentrations of 50 or 200 μM are equally antagonistic to 200 μM JAS 3. This photograph also demonstrates the brown coloration of the medium with JAS 3, which relates to the high-conductivity measurements. The senescing cotyledons are distinctly blackened, whereas those that are antagonized retain a normal green color. Thus, combining the C-7 methyl-substituted esters with the demonstrably active JAS 3 clearly revealed an effect not predicted by their relative inactivity when tested alone.

Effects on pin II-GUS Gene Expression

It is expected that the proteinase inhibitor promoter would be induced by the action of exogenously applied JA or MeJA (Farmer and Ryan, 1990; Farmer et al., 1992; Hildmann et al., 1992; Peña-Cortés et al., 1993). This is particularly evident in Solanaceae species, and has been found to be associated with wounding. Our particular construct, pRT210 in tobacco, has a pin II promoter that had previously been used to drive chloramphenicol acetyltransferase (Thornburg et al., 1987). Kanamycin-selected seedlings were established and selfed, and the jasmonate dose-response was investigated. The effects of the six esters on the expression of this tobacco GUS gene as measured fluorometrically are shown in Figure 6.

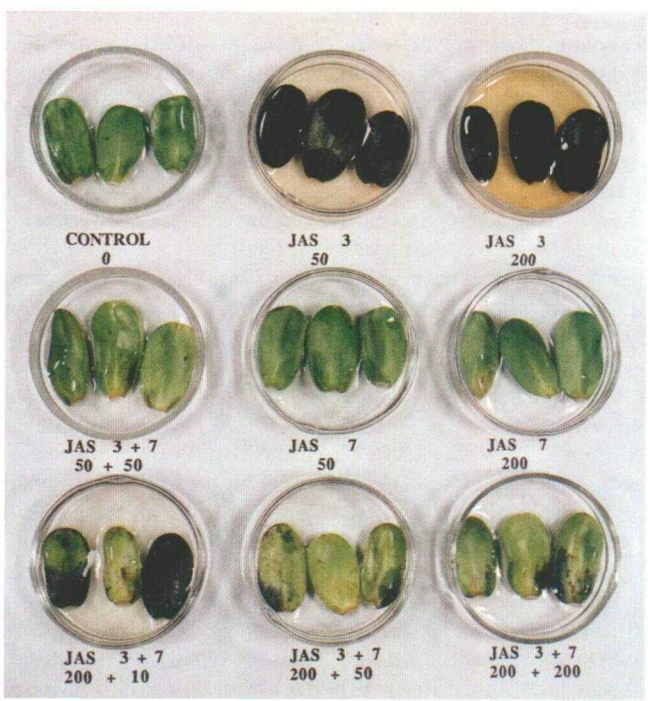

Figure 5. *Sunflower cotyledons after 7 d of treatment with JAS 3 and JAS 7 in various relative concentrations. The molar combinations are indicated below each plate.*

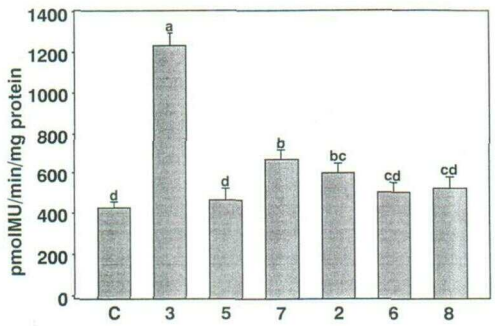

Figure 6. The effect of 200 μM jasmonate esters (3, 5, 7, 2, 6, and 8) and control (C), 24 h after application as a 10-μL droplet onto the adaxial surface of an upper leaf of pin II-GUS transgenic tobacco plantlets at the seven- to eight-leaf stage. The bars show GUS specific activity plus SE. Letters above each bar indicate significant difference from control as determined by ANOVA for the six esters and control. MU, 4-Methylumbelliferone.

A number of methods for applying JA to the leaves were attempted, including floating leaf discs, intact leaf blades with the petiole immersed, or whole seedlings with root immersion in the JA solutions. Since not all of the analogs, especially the acids, could be considered equally volatile and the logistics of spraying so many compounds was problematic, applying JA droplets to the leaf surface gave the most consistent results. As described in "Materials and Methods," 10-μL droplets of 200 μM aqueous solutions of individual JA analogs were applied directly onto single, sterile plantlet leaves and left for 24 h. Treated leaves were excised and processed for determination of GUS activity. Replicate pairs of samples were processed over 3 consecutive d and the ANOVA results indicated that the active jasmonates were highly significant ($P > 0.001$), whereas the day-to-day variation within the experiment was not significant ($P = 0.345$).

Similar to the results from the senescence assays, (3*R*)-MeJA (JAS 3) and its acid (JAS 14, data not shown) were the most active in stimulation of the pin II-GUS fusion reporter. Treatment with either compound resulted in GUS activity that was approximately 2.5 times that of the control.

In contrast to the results from the senescence assays, treatment with (3*S*)-MeJA (JAS 2) also led to GUS activity that was significantly above that of control, although its acid (JAS 13) was control-like (not shown). Among the C-7 methyl-substituted analogs (JAS 5–12), the (3*R*,7*S*)-isomer

(JAS-7) was the only compound that had any activity in stimulating the pin II-GUS reporter.

Inhibition of Germination by JA Analogs

The effects of the 12 JA-related compounds on the seed germination of a monocot (wheat) and a dicot (*B. napus*) were examined. The seeds were plated on agar medium in the presence of 50 μM of each compound. The effects of the six esters on percentage of germination (as measured by protrusion of radicle and shoot) and seedling growth (as measured by fresh weight) after 7 d are shown in Figures 7 and 8, respectively.

For *B. napus*, (3*R*)-MeJA (JAS 3) and its acid (JAS 14, not shown) were both strong inhibitors of seed germination and seedling growth, with the acid being slightly more active than the ester. Neither (3*S*)-MeJA (JAS 2) nor its acid (JAS 13, not shown) had much activity. The (3*R*,7*R*)-analog JAS 5 had little effect on percentage of germination, but stimulated seedling growth, whereas its acid (JAS 9, not shown) and the other C-7 methyl analogs were control-like by both means of measurement. A similar pattern of activity was observed for wheat-seed germination, with the exception that JAS 3 was slightly more inhibitory than its acid, JAS 14.

The results shown in Figures 7 and 8 are the measurements at the end of a 7-d growth period. It is interesting to note that, although not quantitated, differences in the rates of growth among the various treatments, especially those that were not inhibitory, were observed during the course of the experiment.

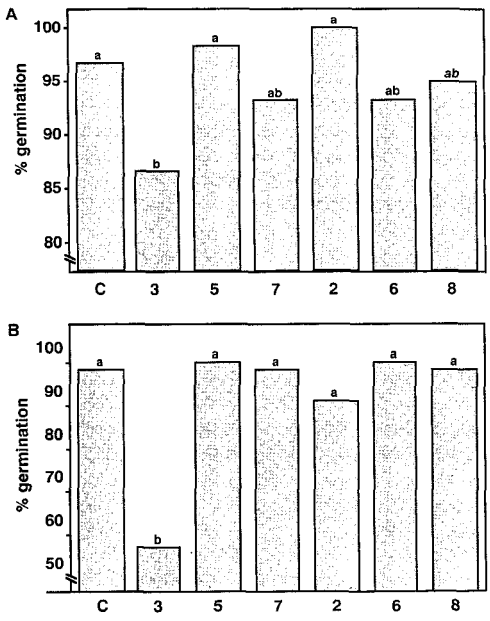

Figure 7. The effect of 50 μM jasmonate esters (3, 5, 7, 2, 6, and 8) and control (C) on seed germination of *B. napus* (A) and wheat (B). Germination was counted as any root or shoot protrusion of more than 2 mm. There were 20 seeds per plate in triplicate. Letters above each bar indicate significant difference from control as determined by ANOVA for the six esters.

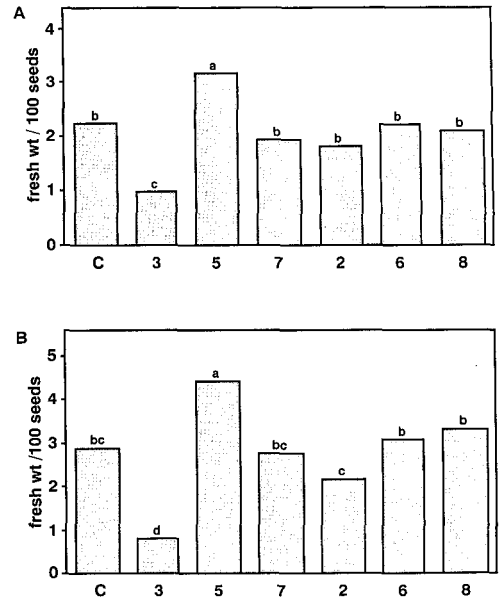

Figure 8. The effect of 50 μM jasmonate esters (3, 5, 7, 2, 6, and 8) and control (C) on seedling fresh weight of *B. napus* (A) and wheat (B). After 7 d of growth, the total fresh weight of germinating seedling tissue for 100 seeds was determined. There were 20 seeds per plate. Letters above each bar indicate significant difference from control as determined by ANOVA for the six esters. Fresh wt, Fresh weight.

DISCUSSION

The racemic forms and the (3*R*,7*S*)-isomer of 7-methyl MeJA have previously been synthesized and were found to be inactive in a number of bioassays (Ward and Beale, 1993; Taapken et al., 1994; Koda et al., 1995). Our results also indicate that overall activity was reduced on substituting the C-7 proton with a methyl group; however, the analogs JAS 5 and JAS 7, which most closely resemble the MeJA isomers of plant origin (i.e. with [3*R*,7*R*]- or [3*R*,7*S*]-stereochemistry, respectively), had activity in some of our assays. The most significant result, however, is that although JAS 7 by itself was completely inactive in all three parameters measured in the senescence assay, it overcame the senescencè-promoting effect of JAS 3 monitored by conductivity when the two compounds were applied together in an equimolar ratio. Similar but weaker antagonistic effects were observed when the other analogs, JAS 5 (3*R*,7*R*), JAS 6 (3*S*,7*S*), or JAS 8 (3*S*,7*R*), were applied in combination with JAS 3. Thus, the results from our competition experiment on senescence contradict the proposal of Koda et al. (1995) that the steric bulk of the methyl substituent at C-7 prevented the analogs from interacting with the putative JA receptors.

Promotion of senescence by jasmonates has been widely studied (Ueda and Kato, 1980, 1981; Weidhase et al., 1987a, 1987b; Parthier, 1990, 1991; Koda, 1992; Emery and Reid, 1996). It is associated with degradative activities (Reinbothe et al., 1994), and in cotyledon senescence can also be seen to mobilize metabolites. There are reports of an ethylene-MeJA interaction and synergism in the stimula-

tion of senescence (Porat et al., 1993; Xu et al., 1994). Membrane hydrolysis is a part of senescence, so MeJA and JA may promote their own synthesis through the release of linolenic acid from membranes. The membrane breakdown may also alter the accessibility of the ethylene precursors ACC to ACC oxidase, leading to the synergism of these two growth regulators (Emery and Reid, 1996). In the present study we carried out three senescence-related bioassays on sunflower cotyledons. The parameters measured increase in intensity as senescence advances. All three assays showed that (3*R*)-MeJA (JAS 3) was slightly more effective than the corresponding acid (JAS 14) in stimulating these three senescence-related phenomena, whereas (3*S*)-MeJA (JAS 2), the (3*R*,7*R*)-analog JAS 5, and its acid, JAS 9, were generally less active.

The induction of proteinase inhibitors in plants by wounding and insect feeding has been firmly established as a jasmonate-inducible response (Farmer and Ryan, 1990; Farmer et al., 1992; Hildmann et al., 1992). Also, a structure-activity study of the effects of JA derivatives on two potato proteins, cathepsin D inhibitor and pin II, has been reported (Ishikawa et al., 1994). Tobacco transformed with a 980-bp tomato pin II promoter driving a GUS gene proved to be a sensitive reporter plant. Like the senescence assays, (3*R*)-MeJA (JAS 3) and (3*R*)-JA (JAS 14) were the most effective inducers of this pin II promoter. Relatively weak activities were observed for (3*S*)-MeJA (JAS 2) and the analog (3*R*,7*S*)-7-methyl MeJA (JAS 7).

Racemic JA and MeJA inhibit the germination of seeds (Corbineau et al., 1988) and precocious germination in both zygotic and microspore-derived embryos (Wilen et al., 1991). In the experiments reported here the expected inhibition of seed germination and seedling growth in both *B. napus* and wheat was observed. One difference was that (3*R*)-MeJA (JAS 3) was more inhibitory than its acid, (3*R*)-JA (JAS 14), in *B. napus*, whereas the reverse was seen with wheat. In contrast to other assays, the analog (3*R*,7*R*)-7-methyl MeJA (JAS 5), although identical to controls in germination inhibition, stimulated the gain in seedling fresh weight in both *B. napus* and wheat. This may be an example of our previous observation that low concentrations of some jasmonates can promote certain processes, whereas higher concentrations have the opposite effect (Emery and Reid, 1996).

The results from the three bioassays employed in this study indicate and reinforce the view that the C-3 (*R*)-stereochemistry is critical for the activities of the JA-type compounds (Yamane et al., 1981; Acree et al., 1985; Koda et al., 1992). Among the 12 compounds tested, (3*R*)-MeJA (JAS 3) and (3*R*)-JA (JAS 14) were the most effective. Activities were also observed for the analogs (3*R*,7*R*)- and (3*R*,7*S*)-7-methyl MeJA (JAS 5 and JAS 7). Among the compounds with (3*S*)-stereochemistry, only (3*S*)-MeJA (JAS 2) showed weak activities in promoting ethylene production in senescing sunflower cotyledons and in inducing pin II-GUS expression in tobacco.

Further studies on binding, competition, and metabolism may clarify the mode of action of these compounds. Antagonism to JAS-3 by the nonepimerizable C-7 methyl analogs, especially over a number of days in culture (which perhaps suggests stability), is an observation that requires further study. If any of these C-7 methyl-substituted analogs are true antagonists, they might be useful for testing endogenous JA function by blocking natural JA responses, and could even be useful as anti-senescence agents.

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