Effects of Jasmonic Acid on Embryo-Specific Processes in *Brassica* and *Linum* Oilseeds

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ABSTRACT

A number of effects on embryogenesis of the putative phytohormone jasmonic acid (JA), and its methyl ester (MeJA), were investigated in two oilseed plants, rapeseed (*Brassica napus*) and flax (*Linum usitatissimum*). Results from treatments with JA and MeJA were compared with those of a known effector of several aspects of embryogenesis, abscisic acid (ABA). Jasmonic acid was identified by gas chromatography–mass spectrometry as a naturally occurring substance in both plant species during embryo development. Both JA and MeJA can prevent precocious germination of *B. napus* microspore embryos and of cultured zygotic embryos of both species at an exogenous concentration of >1 micromolar. This dose-response was comparable with results obtained with ABA. Inhibitory effects were also observed on seed germination with all three growth regulators in rapeseed and flax. A number of molecular aspects of embryogenesis were also investigated. Expression of the *B. napus* storage protein genes (napin and cruciferin) was induced in both microspore embryos and in developing embryos by the addition of 10 micromolar JA. The level of napin and cruciferin mRNA detected was similar to that observed when 10 micromolar ABA was applied to these embryos. For MeJA only slight increases in napin or cruciferin mRNA were observed at concentrations of 30 micromolar. Several oilbody-associated proteins were found to accumulate when the embryos were incubated with either JA or ABA in both species. The MeJA had little effect on oilbody protein synthesis. The implications of JA acting as a natural regulator of gene expression in zygotic embryogenesis are discussed.

Several factors are known to influence oilseed embryo development and seed maturation. Of particular importance are water potential and hormonal status of the developing seed and the surrounding maternal tissue (4, 6, 27). In *Brassica napus*, expression of genes encoding the storage proteins napin and cruciferin has been shown to be embryo specific (6), regulated by ABA (11), and inducible by osmotic stress (9, 27). In some plants, the production of oil-body proteins associated with storage of lipids or their compartmentation is known to be regulated by ABA (20, 23).

The relationship between osmotic stress and storage protein gene expression has not yet been fully elucidated. In some systems it seems probable that ABA may act as a mediator of the osmotic effect on gene expression (4, 27). In other systems there is evidence for separate control of storage protein gene expression by changes in water potential and by ABA. Independent transduction of the ABA and osmotic effects is supported by measurements of ABA levels in cultured zygotic embryos under water stress. Finkelstein and Crouch (9) found no major changes in ABA levels in these embryos as a result of osmotic stress. Fluoridone, an inhibitor of carotenoid metabolism, also inhibits ABA synthesis. When applied to osmotically stressed *Sinapis alba* embryos, fluoridone did not greatly reduce storage protein accumulation (12). These data were interpreted as supporting the independent action of osmoticum and phytohormones on storage protein gene expression. However, such a conclusion can only be drawn by assuming that ABA is the sole, or at least the major, hormonal regulator of these genes.

We therefore decided to investigate the requirement for ABA further and to determine if there were other hormonal regulators of storage protein gene expression in developing embryos. An obvious candidate for such investigation is JA. Jasmonic acid and 7′-iso-JA are synthesized in plants from linolenic acid (1, 25), which accumulates in large amounts in both flax and rapeseed during embryogenesis. Jasmonates have been proposed as naturally occurring growth regulators and are thought to be ubiquitous in higher plants (15). Jasmonic acid has several chemical (2) and biological (5, 7, 22, 28) similarities to ABA. However, there is not always reciprocity between ABA and JA effects (3, 7, 26). Early reports on JA and its methyl ester, MeJA, examined the effect of application of these compounds on a variety of physiological processes such as leaf abscission (7), leaf senescence (22), callus growth inhibition (21), or inhibition of seed germination (5). Recent studies have focused on the effects of JA and MeJA at the gene expression level. Jasmonic acid induces the accumulation of soybean vegetative storage proteins (3) in

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2 Abbreviations: JA, jasmonic acid; MeJA, methyl jasmonate; Rt, retention time; dd, distilled-deionized.
soybean suspension cultures, and in soybean leaves, JA provokes a rapid increase in mRNA encoding these vegetative storage proteins (14, 19). Because JA can act as a regulatory molecule in a manner similar to ABA, we wished to test the idea that it might be involved in embryo-specific processes. We have, therefore, investigated the effects of JA and MeJA on several developmental characteristics of flax and rapeseed embryogenesis, and we have compared the effects of JA to those obtained with ABA in developing zygotic embryos of flax and rapeseed and microspore-derived embryos of rapeseed.

**METHODS AND MATERIALS**

**Plant Material**

*Brassica napus* cv Topas and *Linum usitatissimum* cv McGregor plants (Agriculture Canada, Saskatoon) used for zygotic embryo culture were grown at 20°C/15°C day/night temperatures with a 16 h photoperiod (400 μmol·m⁻²·s⁻¹). Plants for microspore culture were raised as above for 5 weeks and then transferred to 15°C/10°C (day/night) until buds were harvested. Seed germination studies were conducted as outlined in Corbineau et al. (5).

**Embryo Culture**

The microspore embryos were cultured as previously described (20, 27). Zygotic embryos were obtained from selfed plants (at indicated days post anthesis). The rapeseed siliques were surface sterilized with a 30% commercial bleach solution (Javex) for 10 min. The developing seeds were then excised under aseptic conditions, placed in a 30% ethanol solution for 10 to 15 s, and rinsed three times with sterile ddH₂O. For RNA studies the excised seeds were then cultured on germination medium (Murashige-Skoog [16] salts, 2.5% sucrose, 0.7% phytagar (Gibco)) with or without various concentrations of the three growth regulators. After 48 h embryos were excised from the seed, quick frozen in liquid N₂, and stored at −70°C until required. For oilbody protein studies and precocious germination studies the embryos were excised after surface sterilization and placed on the above described media or labeled using [³⁵S]methionine (370 kBq per mL of culture medium; specific activity 43.4 TBq/mmoll, Amersham) as previously described (20). Torpedo stage microspore embryos for precocious germination studies were individually selected and transferred to the above media. Flax seed capsules were surface-sterilized as described above. After sterilization, one end of the capsule was cut, and the embryos were then squeezed out of the capsules and transferred to the appropriate medium, which was the same as previously described except that it contained 12% sucrose. All embryos were cultured at 25°C with a 16 h photoperiod. Mixed isomers of ABA (99% pure; Sigma, (±) JA and (±) MeJA (90% pure; Apex Organics, STEP Centre, Osney Mead, Oxford, OX2 OES, UK) were used for treating embryos and seeds. The ABA, JA, and MeJA were equimolar racemic mixtures.

**Jasmonic Acid Extraction and Identification by GC-MS**

Extracts were made from torpedo stage zygotic and microspore-derived embryos from rapeseed and from cotyledonary stage zygotic embryos of flax using the method previously described for ABA (27). To confirm the retention times in crude extracts and standard, [³H]ABA (1670 Bq; 394 GBq mmol⁻¹ Amersham) was added to both the JA standard (300 ng) and the crude extracts prior to HPLC purification. The samples were then run on a 5 μm N(CH₃)₂ (Nucleosil) column, 150 x 4.6 mm (Alltech Associates). The samples were readily separated using isocratic elution using 99.9% methanol and 0.1% acetic acid at a flow rate of 1 mL min⁻¹. Fractions were collected at 30 s intervals and the Rₜ of the internal [³H]ABA standard checked to ensure that no alteration in Rₜ occurred in either standard or crude extracts. After reducing fractions to dryness in vacuo, the JA standard fractions were checked by TLC to determine the retention time of JA. TLC analysis was conducted using aluminum silica gel 60 plates (Merck) with a hexane:chloroform (95:5) solvent system. The fractions which ran between 9 to 10 min were found to contain the standard. The samples from the embryo extracts which were eluted at this time were then methylated with ethereal diazomethane at room temperature for GC-MS analysis. The methylated samples were analyzed by gas chromatography-mass spectrometry. Aliquots were injected directly onto a DB1701–15N column (J & W Scientific) installed in a Hewlett-Packard 5790 GC with a capillary direct interface to a HP 5970B mass selective detector. The GC temperature program was 60°C to 165°C at 15°C min⁻¹, with a second ramp to 230°C at 5°C min⁻¹. The gas flow rate was 1 mL min⁻¹. Mass spectra were acquired at 1 scan s⁻¹ under control of a HP 300 series computer. The full mass spectra from the JA standard and embryo extracts were compared using the probability-based matching algorithm of the Hewlett Packard library.

**RNA Extractions and Northern Blotting**

Total RNA was extracted according to the procedure of Verwoerd et al. (24) and quantitated by absorbance readings at 260 nm and ethidium bromide staining. Northern blotting and hybridizations were carried out as previously described (27) with the following modifications. No dextran sulfate was added to the prehybridization solution and following the prehybridization the [³²P]dCTP labeled probe and 2 mg of yeast tRNA were added directly to the prehybridization solution in a sealable plastic bag (Fischer Scientific). The membrane was then incubated overnight at 43°C. The napin and cruciferin cDNA clones were kindly provided by Dr. M. L. Crouch (Indiana University).

**Oilbody Isolation and Protein Extractions**

Oil bodies were isolated from developing microspore embryos or zygotic embryos. The developing embryos were frozen in liquid nitrogen and ground to a fine powder using mortar and pestle. All further operations were carried out at 4°C. The ground tissue was added to the extraction buffer A (0.15 M Tricine-KOH [pH 7.5], 10 mM KCl, 1 mM MgCl₂, and 1 mM EDTA) to which sucrose 0.6 M and PMSF 1 mM were added prior to use. After regrinding with mortar and pestle...
the homogenate was centrifuged at 5,000g for 5 min. The floating pad was resuspended in buffer A containing 0.1 M sucrose and overlaid with an equal volume of buffer A containing 0.1 M sucrose and centrifuged at 18,000g for 20 min. This procedure was repeated twice with the floating fat pad. The cleared 18,000g supernatants and the fat pad in 0.1 M sucrose were again overlaid with 0.1 M sucrose and centrifuged at 120,000g for 90 min. The floating fat pad consisting of washed oil bodies was transferred into the SDS-buffer. All samples in the SDS buffer were boiled for 3 min and centrifuged for 10 min at 14,000g before PAGE and Coomassie brilliant blue staining, fluorography, and/or Western blotting. The gels were run on a 7 to 15% acrylamide gradient for fluorography, or on a 12% acrylamide gel for Western blotting.

Flax Antibody Production and Western Blotting

Oilbody proteins were separated by preparative SDS-PAGE. After staining with Coomassie brilliant blue R and destaining, the gel was dried on 3MM paper. After drying the gels, the 19 and 21 kD polypeptide bands were cut out and released from the 3MM paper by adding a few drops of water on the back side of the paper. The bands were frozen in liquid nitrogen and ground into a fine powder. The powder was suspended into 1.5 mL PBS (140 mM NaCl, 28 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4 [pH 7.0]) and mixed with an equal volume of Freud’s complete adjuvant (Sigma) and injected into female New Zealand white rabbits. At week 2 the rabbits were re-injected with the protein suspension and an equal volume of Freud’s incomplete adjuvant. After 6 weeks total blood was collected and stored at 4°C overnight, then centrifuged at 5000g for 30 min. The serum was stored at −60°C in 500 μL aliquots. Protein samples are separated by 15% SDS-PAGE. The proteins were transferred to Gene Screen Plus (NEN Dupont) membrane by electrophoretic transfer for 18 h at 100 mA in 25 mM Tris-Cl, 192 mM glycine, 0.05% SDS. The rest of the procedure was as previously described (20). For the primary antibody incubation a 1:2000 dilution was used and for the secondary antibody (goat anti-rabbit alkaline phosphatase linked) incubation at 1:3000 dilution was used.

RESULTS

Identification of Jasmonic Acid as a Natural Product in Linum and Brassica Embryos

Jasmonic acid was identified as a natural product in developing embryos of both Brassica napus (Fig. 1) and Linum usitatissimum by full spectrum GC-MS. The derivatized compounds isolated from the embryo extracts had identical retention times to methylated JA and gave very similar mass spectra (Fig. 1). When the spectrum of putative JA in the flax extract was compared with the JA standard, the library algorithm calculated a probability of greater than 95% that the spectra were of identical compounds. For the corresponding rapeseed embryo extract, it was found greater than 80% probable that the mass spectrum obtained was for JA. Absolute JA concentrations in the tissues were not measured due to the lack of a stable isotope labelled JA internal standard.

However, comparisons of peak area and spectrum quality for JA and ABA in the same hormone extracts suggested that the relative concentrations were similar (data not shown).

Inhibitory Effects of JA, MeJA, and ABA on Embryo and Seed Germination

The effects of JA, MeJA and ABA on precocious germination (Fig. 2) and seed germination (Table I) were investigated. All three growth regulators inhibited precocious germination of rapeseed microspore-derived embryos and cultured zygotic embryos of both flax and rapeseed. Partial inhibition of germination was observed when the embryos were plated on
Effects of JA, MeJA, and ABA on Storage Proteins in Rapeseed

The regulation of storage protein gene expression by JA and MeJA in B. napus embryos was investigated. Figure 3A shows the induction of napin mRNA in microspore-derived embryos by application of 10 µM ABA and 10 µM and 30 µM JA. The level of mRNA was comparable with both hormone treatments and much greater than was found in the untreated embryos. Cruciferin mRNA has also been shown to be similarly induced by 10 µM JA (Fig. 3B). The induction of napin mRNA in cultured zygotic embryos excised at 20 DAF using various concentrations of JA is shown in Figure 4. Little induction of napin mRNA was detected until the embryos were plated on medium containing 10 µM JA and the level of napin transcripts remained steady in the presence of 30 µM JA. Little induction of storage protein transcripts was detected after application of 30 µM MeJA to rapeseed zygotic embryos (Fig. 4, lane MJ). A similar result was found using rapeseed microspore-derived embryos (data not shown).

Effects of JA and ABA on Oilbody Protein Accumulation in Rapeseed and Flax

Oilbody protein accumulation appears to be a characteristic of embryo maturation in oilseeds (17). We have previously shown that several of these polypeptides in B. napus are increased in both torpedo stage microspore-derived and cultured zygotic embryos in response to exogenous ABA (20). When JA was applied to B. napus torpedo stage microspore-derived embryos or to cultured zygotic embryos (excised 18 DAF), increased accumulation of these oilbody proteins was noted. Dramatic increases in several of these proteins, previously detected after treatment with ABA (20), were observed when JA was applied at 30 to 50 µM. Figure 5 demonstrates the effect on these polypeptides when microspore-derived embryos are treated with either JA or ABA.

In flax there were two major oilbody proteins (19 and 21 kD) which accumulated in 11 DAF cultured zygotic embryos. The accumulation of these proteins in response to ABA and

Table 1. Percent Germination of Rapeseed and Flax Seeds in the Presence of Various Concentrations of Jasmonic Acid, Methyl Jasmonate and Abscisic Acid

<table>
<thead>
<tr>
<th>Days after Treatment</th>
<th>Rapeseed</th>
<th>Flax</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 3 5</td>
<td>1 3 5</td>
</tr>
<tr>
<td>Control</td>
<td>47.9 (3.5)*</td>
<td>90.0 (2.1)</td>
</tr>
<tr>
<td>JA (1 µM)</td>
<td>33.0 (3.4)</td>
<td>56.0 (3.7)</td>
</tr>
<tr>
<td>JA (10 µM)</td>
<td>24.0 (1.4)</td>
<td>60.6 (5.7)</td>
</tr>
<tr>
<td>JA (30 µM)</td>
<td>11.4 (2.3)</td>
<td>13.3 (2.2)</td>
</tr>
<tr>
<td>MeJA (1 µM)</td>
<td>25.0 (3.8)</td>
<td>60.0 (4.5)</td>
</tr>
<tr>
<td>MeJA (10 µM)</td>
<td>23.0 (4.3)</td>
<td>52.1 (4.7)</td>
</tr>
<tr>
<td>MeJA (30 µM)</td>
<td>24.8 (4.1)</td>
<td>25.8 (9.3)</td>
</tr>
<tr>
<td>ABA (1 µM)</td>
<td>29.0 (3.9)</td>
<td>64.0 (3.8)</td>
</tr>
<tr>
<td>ABA (10 µM)</td>
<td>18.0 (1.7)</td>
<td>35.3 (5.1)</td>
</tr>
<tr>
<td>ABA (30 µM)</td>
<td>11.6 (1.3)</td>
<td>20.0 (2.8)</td>
</tr>
</tbody>
</table>

* SE.
JA is shown in Figure 6. The 19 kD protein appears to be more sensitive than the 21 kD polypeptide to the application of either growth regulator. Oilbody protein accumulation in flax embryos responded equally to JA or ABA applied at a concentration of 10 μM. This differed from the rapeseed microspore embryos or zygotically cultured embryos where increased accumulation of oilbody proteins was not observed after treatment with 10 μM JA (data not shown). In rapeseed we have noted accumulation of these polypeptides only when applied JA concentrations are equal to or greater than 30 μM.

DISCUSSION

Studies on the regulation of gene expression by phytomolecules during seed development have mainly concentrated on the role of ABA, and it is clear that ABA is capable of regulating accumulation of storage products including sucrose (18), lipids (10) and proteins (4, 11). For storage proteins the regulation is at least in part transcriptional (8). Studies to elucidate the relationship between osmoticum, ABA and storage product accumulation have not produced a definite conclusion (4, 11, 12, 27). One major outstanding question is the relationship between osmotically and hormonally regulated storage protein gene expression. Furthermore, it is important to know how many and which factors, environmental or chemical, can regulate these genes in developing seeds. Previous studies have shown that jasmonic acid may regulate the accumulation of vegetative storage proteins (2, 14, 19). However, no reports have investigated effects by jasmonates on seed-specific gene expression.

First, we have established that in flax zygotic embryos and
rapeseed zygotic and microspore-derived embryos, JA is a natural product. While it has been shown that several dicot seeds accumulate JA, the assays were always performed on embryos that had been in contact with the maternal plant (15). In such material it is difficult to determine whether JA came from the vegetative organs or was synthesized in the embryos directly. In the rapeseed microspore-derived embryo system, detection of JA indicates that it is indeed synthesized in the developing embryo as these cultures are derived from single cells. Concentrations of JA in these embryos are similar to those of ABA, although lack of a stable isotope labelled JA for an internal standard precludes a precise comparison.

The effects of JA as an inhibitor of precocious germination in these species suggest similar dose responses for JA and ABA. ABA is slightly more effective at seed germination inhibition at higher concentrations than JA, but their effects on this process are similar up to concentrations of 30 μM. MeJA is also active in these bioassays. Thus, JA and MeJA mimic the classical effects of ABA on embryo and seed dormancy.

Under the conditions used JA, but not MeJA, was also effective in eliciting the typical responses of ABA on storage product gene expression in both microspore-derived and zygotic embryos of rapeseed. One hypothesis to be investigated is that JA acts as an ABA analog at the site of ABA recognition. Although this cannot be excluded on the basis of structure, there are systems in which there are significant divergences in the effects of JA and ABA implying separate receptors or transduction chains. For example, in soybean JA, but not ABA, can induce the accumulation of certain vegetative storage proteins (2, 3) and their mRNAs (14, 19). Similarly, in senescing barley leaves, JA and MeJA may induce polypeptides which are unresponsive to ABA (26). It is interesting to note that in both the barley and soybean leaf systems, the polypeptides which accumulate in response to JA treatment are also osmotically sensitive (3, 19, 26). In zygotic embryos, we have shown that responses to JA closely parallel those found with applied ABA. Thus, it may be possible that in reproductive tissues JA and ABA have common sites of action, whereas in certain vegetative tissues and organs their modes of action are segregated. Although JA has been reported in reproductive organs of several species (15), our report appears to be the first to demonstrate an effect on embryo-specific and/or seed-specific gene regulation by JA. The question of separate or common sites of action of these regulators remains to be clarified.

If JA is indeed a regulator of seed development, maturation or dormancy, this may help to reconcile some of the conflicting results relating to hormonal regulation of seed-specific processes. For example, it has been shown that ABA deficient mutants of Arabidopsis are still capable of storage protein accumulation, albeit at reduced levels (13). Equally, in embryos of Sinapis alba it was shown that storage protein accumulation was unaffected by fluorodone treatment which blocks carotenoid biosynthesis and presumably ABA biosynthesis (12). If a molecule such as JA is physiologically active in embryos, as is suggested by our data, the suppression of ABA accumulation genetically or by the use of inhibitors may be insufficient to eliminate hormonally mediated effects particularly if JA originates from linolenic acid (25), a metabolic pathway different from ABA biosynthesis. It is possible that ABA and JA might share one common biosynthetic step, the enzyme lipoxygenase. However, none of the ABA-deficient mutants used in studies on embryo-specific gene expression (13) are known to be lipoxygenase-deficient. Thus, even in the absence of endogenous ABA it is still possible that osmotic effects on storage product accumulation could be mediated by means of a phytohormone such as JA.

The relative importance of JA and ABA in these systems remains to be determined. To this end we are attempting to obtain precise estimates of the levels and modulation of JA during embryogenesis. The use of ABA analogues and ABA insensitive mutants may help elucidate whether JA and ABA act at the same receptor/transduction chain or whether they act independently in developing embryos.

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LITERATURE CITED


