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Induction of Lipid and Oleosin Biosynthesis by (+)-Abscisic Acid and Its Metabolites in Microspore-Derived Embryos of *Brassica napus* L. cv Reston[1]

Biological Responses in the Presence of 8′-Hydroxyabscisic Acid

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Microspore-derived (MD) embryos of *Brassica napus* L. cv Reston were used to test the effects of (+)-abscisic acid ([(+)-ABA]) and its metabolites, 8′-hydroxyabscisic acid (8′-OH ABA) and (−)-phaseic acid (PA), on the accumulation of very long-chain monounsaturated fatty acids (VLCMFAs) and induction of genes encoding a 19-kD oleosin protein and a Δ15 desaturase during embryogenesis. Developing early- to mid-cotyledonary MD embryos at 16 to 19 d in culture were treated with 10 μM hormone/metabolite for 4 d. At various times during incubation, embryos and medium were analyzed to determine levels of hormone/metabolite, VLCMFAs, and oleosin or Δ15 desaturase transcripts. The VLCMFAs, 20:1 and 22:1, primarily in triacylglycerols, increased by 200% after 72 h in the presence of (+)-ABA and 8′-OH ABA relative to the control. In contrast, treatment with PA for 72 h had little effect (20% increase) on the level of VLCMFAs. The first 24 to 72 h of (+)-ABA treatment were critical in the induction of VLCMFA biosynthesis, with 8′-OH ABA lagging slightly behind (+)-ABA in promoting this response. The accumulation of VLCMFAs was positively correlated with an increase in elongase activity. (+)-ABA and its 8′-OH ABA metabolite induced the accumulation of a 19-kD oleosin transcript within 2 to 4 h in culture. In addition, both (+)-ABA and 8′-OH ABA induced the same level of Δ15 desaturase transcript by 8 h. PA had no effect on the induction of either oleosin or Δ15 desaturase transcripts. To our knowledge, this is the first report of the biological activity of 8′-OH ABA and of stimulatory effects of (+)-ABA and 8′-OH ABA on lipid and oleosin biosynthesis.

The plant hormone (+)-ABA (Fig. 1) is involved in storage product deposition, maturation, and desiccation tolerance in developing seeds (Black, 1991; Thomas, 1993). (±)-ABA has been used to study the regulation of storage and oil body protein gene expression in MD and zygotic embryos of *Brassica napus* (Taylor et al., 1990b; Wilen et al., 1990; Holbrook et al., 1991; Taylor and Weber, 1994). We have shown developmental regulation of both VLCMFA and oleosin biosynthesis by (±)-ABA (Holbrook et al., 1992), and a link between (±)-ABA and induction of these processes may be inferred from other earlier studies (Vance and Huang, 1988; Finkelstein and Somerville, 1989; Hatzopoulos et al., 1990; Qu et al., 1990; Taylor et al., 1990b; Wilen et al., 1990).

The biological activity of (+)-ABA catabolites is less well understood, principally because the compounds have not been available for study. The catabolism of ABA occurs by both oxidation and conjugation (Zeevaart and Creelman, 1988). Conjugates are generally found to be inactive. (+)-ABA (Fig. 1) is oxidized at the 8′-methyl group to 8′-OH ABA (Fig. 1), which rearranges to PA (Fig. 1) by conjugate addition of the hydroxyl group to the enone of the ring. The 8′-hydroxylating enzyme exhibits characteristics of a Cyt P-450 monooxygenase, requiring O_2 and NADPH, and is inhibited by CO (Gillard and Walton, 1976). The isolation of 8′-OH ABA was once reported as "metabolite C" by Milborrow (1969), but since then, the chemical instability of this intermediate, which rapidly cyclizes to PA (Milborrow et al., 1988), has precluded a direct examination of its biological properties. However, the transient existence of 8′-OH-ABA has been inferred from GC-MS analyses of derivatized extracts from a number of plant tissues (Adesomoju et al., 1980; Fujioka et al., 1988; Yamane et al., 1988), and the 3-hydroxy-3-methylglutaryl conjugate of 8′-OH ABA, which yields PA following alkaline hydrolysis, has been isolated from the fruit of *Robinia pseudoacacia* (Hirai et al., 1978; Hirai and Koshimizu, 1981). Recently, it was shown that synthetic 8′-methoxy ABA possessed biological

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Abbreviations: 8′-OH-ABA, 8′-hydroxyabscisic acid; DPA, dihydrophaseic acid; DW, dry weight; FW, fresh weight; LC/MS, liquid chromatography/continuous flow secondary ion tandem MS; MD, microspore-derived; PA, natural (−)-phaseic acid; TLE, total lipid extract; VLCMFA, very long-chain monounsaturated (>18:1) fatty acid; 18:1, oleic acid, *cis*-Δ9-octadecenoic acid; 18:2, linoleic acid, *cis*-Δ9, *cis*-Δ12-octadecadienoic acid; 18:3, α-linolenic acid, *cis*-Δ9, *cis*-Δ12, *cis*-Δ15-octadecatrienoic acid; 20:1, *cis*-Δ11-eicosenoic acid; 22:1, erucic acid, *cis*-Δ13-docosenoic acid; 24:1, nervonic acid, *cis*-Δ15-tetracosenoic acid; all other fatty acyl groups are designated by numbers of carbons: number of double bonds.

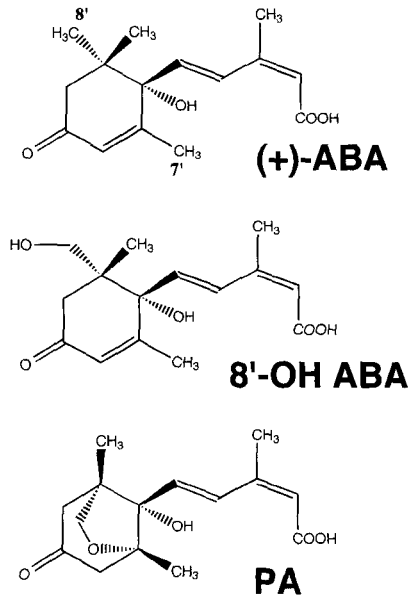

Figure 1. Structures of (+)-ABA, 8′-OH ABA, and PA supplied to developing MD embryos in culture.

activity in a number of bioassays, and it was suggested that this was due to a delayed metabolic conversion to PA (Todoroki et al., 1994). This raises the possibility that there might be biological activity exhibited by the short-lived 8′-OH ABA intermediate.

PA has been isolated from plant tissues fed with (±)-ABA (Milborrow, 1975). PA has been synthesized in racemic (Abrams et al., 1990) and optically pure forms (Kitahara et al., 1989; Balsevich et al., 1994a). Recently, (−)-PA was prepared by biotransformation of (+)-ABA by cultured cells of Black Mexican Sweet corn (Balsevich et al., 1994b). In that study, (−)-PA only weakly inhibited the growth of the corn cells. Similarly, (±)-PA has been shown to have little or no activity in ABA-induced processes such as inhibition of germination in cress seed (Gusta et al., 1992) and induction of freezing tolerance in cultured cells of bromegrass (Robertson et al., 1994). In barley, however, (±)-PA is active in inhibiting embryo germination and in inducing two ABA-responsive genes encoding barley germ agglutinin and an α-amylase inhibitor (Hill et al., 1992). In plants, the 4′-keto group of PA is reduced to two epimeric alcohols, giving DPA and *epi*-dihydrophaseic acid. A second, minor pathway of ABA catabolism is by oxidation of the 7′-methyl group (Hampson et al., 1992). Racemic 7′-OH ABA is inactive in inhibiting cress seed germination (Gusta et al., 1992), but (+)-7′-OH ABA did induce *Em* gene expression in barley aleurone protoplasts transformed with a chimeric construct containing the promoter region of the ABA-inducible *Em* gene (Hill et al., 1995).

Because of the availability of pure (−)-PA from biotransformation of (+)-ABA by cultured corn cells (Balsevich et al., 1994b), we were able to study the chemical stability of PA, the results of which will be reported elsewhere. We observed that in appropriate aqueous solutions PA exists in equilibrium with a small amount of its ring-opened form, 8′-OH ABA. Further investigation led to the development of a method to stabilize 8′-OH ABA in vitro and afforded the possibility of testing the biological activity of this normally transient intermediate.

Given the ease with which *B. napus* MD embryos can be manipulated and directly challenged with growth regulators at specific developmental stages in culture (Taylor et al., 1990b; Holbrook et al., 1992; Taylor and Weber, 1994) and the availability of optically pure (+)-ABA and its metabolites, we have undertaken, and report here, a study of the developmental association between lipid and oil body protein deposition and the induction of these processes by (+)-ABA and its metabolites in this gametophytic system.

MATERIALS AND METHODS

Preparation of (+)-ABA and Metabolites

The (+)-ABA (Fig. 1) was obtained by preparative resolution of the racemic methyl ester on a chiral column, followed by hydrolysis of the (+) enantiomer to the free acid, as described by Dunstan et al. (1992). (−)-PA, the naturally occurring enantiomer (Fig. 1), was obtained from the media of suspension cultures of corn (*Zea mays* L., cv Black Mexican Sweet) that had been supplied with (+)-ABA, according to the procedure of Balsevich et al. (1994b). 8′-OH ABA (Fig. 1) was prepared by heating PA (≤1 mg) with 1 molar equivalent of boric acid in 1 mL of glacial acetic acid for 1 h in a sealed glass autosampler vial at 100°C and then removing the solvent from the reaction mixture at room temperature with a stream of nitrogen. The residual borate complex was dissolved in ethanol for analysis by HPLC. The complex dissociates in water, liberating 8′-OH ABA, which is relatively stable in acidic solutions but cyclizes rapidly to PA under neutral or basic conditions. HPLC analysis under the conditions described below (H_2O:acetonitrile:CF_3COOH, mobile phase) indicated the ratio of 8′-OH ABA:PA in the hydrolysis mixture to be 9:1. Further details of the preparation and properties of 8′-OH ABA will be reported elsewhere.

Radiolabeled Substrates and Biochemicals

[1-^{14}C]Oleic acid (58 mCi/mmol) was purchased from Amersham Canada Ltd. (Oakville, ON) and converted to [1-^{14}C]oleoyl-CoA by an enzymatic method described previously (Taylor et al., 1990a). Unlabeled acyl-CoAs, polyvinylpolypyrrolidone, ATP, CoA, NADH, NADPH, and most other biochemicals were purchased from Sigma. HPLC-grade solvents (Omni-Solv, BDH Chemicals) were used throughout these studies.

Plant Material, Microspore Culture, and Control Versus (+)-ABA and Metabolite Treatments

Brassica napus L. cv Reston, a high erucic acid variety accumulating both C_{20} and C_{22} fatty acids in developing seeds, was acquired from the Department of Plant Science, University of Manitoba (Winnipeg, MA). Reston plants were grown in controlled growth chambers with a 16-h

photoperiod (photon flux density of 400 $\mu E\ m^{-2}\ s^{-1}$ [400–700 nm]), supplied by a mixture of fluorescent (CW 1500 cool-white, General Electric) and incandescent (60 W, Sylvania) lights and 20/15°C (day/night) for 6 weeks. The plants were then transferred to 10/5°C (day/night) using the same photoperiod conditions for an additional 4 to 6 weeks.

Microspores were isolated and cultured as described previously (Taylor et al., 1990b, 1992; Holbrook et al., 1992). The culture medium was modified from Lichter (1982) and contained the NLN major salts (as described in table I of Huang and Keller, 1989) but reduced in concentration by 50%, the MS micronutrients minus KI, and 13% Suc. The pH of the medium was adjusted to 6.8. At 15 to 18 d in culture, MD embryo preparations (15–20 g FW) enriched in early-cotyledonary stages were obtained by filtration through sterile 500-μm nylon mesh and replated at a density of 0.2 to 0.25 g FW in 10 mL of medium in 100- × 10-mm Petri plates. After a 24-h equilibration period, embryo cultures were supplemented with either 10 μM (+)-ABA, 8′-OH ABA, or PA in 0.1% (v/v) ethanol (hormone treatment) or 0.1% (v/v) ethanol only (control treatment) and maintained in the dark at 25°C. Individual plates of embryos for each treatment were harvested after 0, 2, 4, 8, 24, 72, and 96 h. At each time, several thousand MD embryos (two to three Petri plates from each treatment) were harvested by suction filtration, the medium was saved for analysis, and FWs of the embryos were recorded. Embryo samples were immediately frozen in liquid nitrogen and stored at −70°C until analyzed. Portions of the embryo samples were used to prepare homogenates from which total protein, and total lipid content/composition were determined, as described previously by Holbrook et al. (1992). On the same samples, (+)-ABA content was estimated by MS as described below. Total homogenate protein was estimated by the method of Bradford (1976) using BSA as a standard.

Analysis of Culture Medium for (+)-ABA and Its Metabolites

Samples of culture media were filtered through 2-μm nylon filters directly into HPLC sample vials. Because of the lability of the 8′-OH ABA, samples were analyzed immediately or frozen and stored at −20°C overnight and analyzed the next morning. Filtrates were analyzed directly by injection (40 μL, autoinjector) into a Spectra Physics SP8800 HPLC fitted with a Supelco Hisep 15-cm × 4.6-mm 5-μm column preceded by a Supelco Hisep 2-cm × 4.6-mm guard column. The hormone and metabolites were eluted isocratically at 1.5 mL min^{-1} with 0.05% (v/v) CF_3COOH in H_2O:acetonitrile (87:13). The effluent was monitored at 262 nm with a Hewlett-Packard 1040A diode array detector. Retention times were: DPA, 2.1; PA, 2.2; 8′-OH ABA, 3.0; 7′-OH ABA, 3.1; and (+)-ABA, 4.1 min. Calibration curves were constructed of peak areas against appropriate ranges of known concentrations for ABA, 7′-OH ABA, and PA; the response curves for 8′-OH ABA and DPA were taken to be the same as those for 7′-OH ABA and PA, respectively.

Extraction and Quantitation of Endogenous (+)-ABA Levels in Treated Embryos

Homogenates were prepared from harvested embryos as described by Holbrook et al. (1992) and adjusted to give an FW equivalent of 100 mg FW/mL homogenate. Homogenates equivalent to 50 mg FW (500 μL) were lyophilized for 5 to 8 h. The residue was stirred at room temperature for 18 h with 1 mL of *iso*propanol:glacial acetic acid (95:5, v/v) per 10 mg FW equivalent, centrifuged at 2500*g* for 20 min to pellet undissolved solids, and the supernatant, containing the (+)-ABA and metabolites, was collected by aspiration. [$^2H_6$]ABA (230 ng) was added to each supernatant as an internal standard, and the solution was concentrated to dryness on a Speed-Vac concentrator (Savant Instruments, Farmingdale, NY).

(+)-ABA content was measured by two methods. In the first, the residue was methylated with diazomethane, dissolved in ethyl acetate, and analyzed by ammonia chemical ionization-selective ion monitoring by on-column injection into a Fisons 8000 gas chromatograph interfaced with a Fisons TRIO 2000 quadrupole mass spectrometer. Helium carrier gas flow was 4 mL s^{-1} measured at 150°C through the 60-m × 0.32-mm-i.d. DB 23 capillary column (J & W Scientific, Folsom, CA). The initial column temperature of 70°C was ramped at 20°C min^{-1} to 180°C, increased at 4°C min^{-1} to 240°C, and held. The GC interface and source were maintained at 250°C. Ions at *m/z* 296 $(M + NH_4)^+$, *m/z* 279 $(M + H)^+$, *m/z* 261 $([M + 1] - H_2O)^+$, and the corresponding ions for the [$^2H_6$]ABA internal standard were monitored using a dwell time of 80 ms and a settling time of 20 ms. This procedure detected (+)-ABA in all tissue extracts examined.

The second method, packed fused silica capillary LC-MS as described by Hogge et al. (1993), was used to analyze free (+)-ABA. The extract, containing the internal standard, was dissolved in acetonitrile (20 μL), and 1 μL of this solution was injected onto the column. The reactions $(M + 1)^+ \rightarrow ([M + 1] - 18)^+$, i.e. *m/z* 265 → *m/z* 247 for ABA and *m/z* 271 → *m/z* 253 for the internal standard [$^2H_6$]ABA, were monitored. The lower limit of detection with this method was about 0.3 ng μL^{-1} at a signal-to-noise ratio of 20:1. The two techniques, GC-MS of the methyl ester and LC-MS of the free acid, gave comparable estimates of the ABA level in the (+)-ABA-treated MD embryo samples (59 and 51 ng g^{-1} DW, respectively). The LC-MS method was not, however, deemed sufficiently sensitive for the reliable estimation of ABA in the extracts of tissue that had not been supplied with exogenous (+)-ABA.

Analysis of Fatty Acyl Composition of Endogenous Embryo Lipids

Total acyl lipids were extracted immediately from duplicate samples of fresh MD embryo homogenate (0.4 mL, equivalent to 40–50 mg FW). To each sample, 1 mL of *iso*propanol was added, the tube was capped, and the mixture was placed in a boiling water bath for 5 min. The solution was cooled briefly, 0.5 mL of CH_2Cl_2 was added, and the mixture was allowed to sit for 30 min at room

temperature, with occasional vortexing. The organic and aqueous phases were then separated by the sequential addition of 2 mL of CH_2Cl_2 and of 2 mL of 1 M KCl in 0.2 M H_3PO_4. After the sample was centrifuged (500*g* for 5 min), the lower organic phase was saved and the aqueous phase was washed twice with 2 mL of CH_2Cl_2. The original organic phase was combined with the washes and dried under nitrogen to yield the TLE.

A portion of the TLE was transmethylated directly for assay of total acyl composition. An internal standard of 17:0 free fatty acid was added to the TLE to permit quantitative fatty acid analysis. The sample was transmethylated in the presence of 2 mL of 3 N methanolic HCl (Supelco Canada Ltd.) at 80°C for 1 h. Reaction mixtures were cooled on ice, 2 mL of 0.9% (w/v) NaCl were added, the mixture was extracted three times with 2 mL of hexane, and the hexane extracts were combined and taken to dryness on a cooling block at −10°C under nitrogen. The acyl composition was determined by GC of the fatty acid methyl esters on a Hewlett-Packard model 5880 gas chromatograph fitted with a DB-23 column (30 m × 0.25 mm; film thickness 0.25 μm; J & W Scientific). The GC conditions were: injector temperature and flame ionization detector temperature, 250°C; running temperature program: 180°C for 1 min, then increasing at 4°C/min to 240°C, and holding at this temperature for 10 min.

Assay for Biosynthesis of VLCMFAs (Elongation Activity)

Embryo homogenates were prepared as described previously (Holbrook et al., 1992) and used as the source of protein in elongase assays. In the standard reaction mixture, 0.2 to 0.25 mg of protein was incubated in open tubes in an oscillating water bath (100 rpm) at 30°C for 1 h at pH 7.2 with a solution containing 90 mM Hepes-NaOH, 1 mM ATP, 1 mM CoA, 0.5 mM NADH, 0.5 mM NADPH, 2 mM $MgCl_2$, 200 μM glycerol-3-phosphate, 1 mM malonyl-CoA, and 40 μM [1-^{14}C]18:1-CoA (12.1 nCi/nmol) in a final volume of 0.5 mL. Reactions were stopped by adding 3 mL of 5% (w/v) KOH in methanol, and the tubes were capped and heated at 80°C for 2 h to saponify the acyl lipids and acyl-CoA thioesters. Tubes were cooled on ice, and the contents were acidified with 1 mL of 6 N HCl and extracted three times with 2 mL of hexane. The hexane extract containing the ^{14}C-fatty acids was dried under nitrogen, and the fatty acids were transmethylated in the presence of 3 N methanolic HCl as described above. The ^{14}C-fatty acid methyl esters were then separated and quantified by radio-HPLC as described by Holbrook et al. (1992). Elongation reaction rates were calculated on the basis of the known specific activity of the [^{14}C]oleoyl-CoA substrate and expressed on a milligram of protein basis.

Oleosin and Δ15 Desaturase Probes

An oleosin gene fragment, pOB800, encoding a part of the 19-kD oleosin from *B. napus* was generously provided by Dr. Maurice M. Moloney (Department of Biological Sciences, University of Calgary). A plasmid, pBNDES3, carrying a cDNA clone of the *B. napus* homolog of the Arabidopsis *fad3* (Δ15 desaturase) gene was obtained from the Ohio State Biotechnology Center (Columbus, OH). cDNA inserts were excised, the DNA fragments were purified with the GENECLEAN II kit (Bio 101 Inc., La Jolla, CA), labeled with ^{32}P using the Gibco BRL Random Primers DNA Labeling System as described by the manufacturer (Gibco BRL Life Technologies, Inc.), and used as probes in northern analyses.

Northern Analyses for Induction of Oleosin and Δ15 Desaturase Gene Expression by (+)-ABA and Its Metabolites

Embryos, cultured in the presence of (+)-ABA, 8′-OH ABA, or PA or without hormone (control) were harvested at 2, 4, 8, 24, and 48 h, and extracted RNA was probed to determine the level of oleosin and Δ15 desaturase gene expression. Total RNA was extracted as described previously (Kawata and Cheung, 1990). RNA samples were denatured with glyoxal and separated on 1.2% agarose gels. About 5 μg of total RNA were loaded per lane. The amount of total RNA was also calibrated by the ethidium bromide-staining intensity of the rRNA bands. The RNA was transferred onto a Zeta Probe nylon membrane (Bio-Rad) and the hybridization with ^{32}P-labeled oleosin and Δ15 desaturase probes was performed under high-stringency conditions according to the manufacturer's protocol.

RESULTS AND DISCUSSION

Effect of Natural (+)-ABA and Metabolites on Embryo Growth and Development

To better characterize specific effects attributable to (+)-ABA and its metabolites, it was necessary to first examine the general effects of hormone treatment on MD embryogenesis. There were no visual differences in embryo morphology or stage of development observed in the different treatments during the course of the experiments. After a 72-h treatment with (+)-ABA, MD embryos were not significantly affected with respect to DW or protein content as compared to control embryos (Table I). Similarly, PA did not have a significant effect on embryo development as

Table I. *Effect of (+)-ABA and PA treatment on MD embryo growth parameters*

MD *B. napus* cv Reston embryos at 16 d in culture were transferred to medium supplemented with either 0.1% (v/v) ethanol only (control treatment), 10 μM (+)-ABA plus 0.1% (v/v) ethanol [(+)-ABA], or 10 μM PA plus 0.1% (v/v) ethanol (PA). After 72 h, embryos were harvested for assessment of FW/DW ratio and total protein (measured in a homogenate). Results of three to five independent experiments are shown as the average ± SE.

Embryo Treatment	DW as Percent of FW	Total Protein
		mg (g DW)$^{-1}$
Control	22.5 ± 1.4	157.1 ± 11.4
(+)-ABA	24.1 ± 1.6 (107)[a]	150.0 ± 3.2 (95)
PA	22.4 ± 0.9 (99)	140.8 ± 2.1 (90)

[a] Values in parentheses express data for (+)-ABA or PA treatment as a percentage of control treatment.

measured by DW change. There was a slight decrease in the protein content of PA-treated embryos. These trends were identical in both early- and mid-cotyledonary MD embryos (data not shown). Thus, any effect on lipid-specific processes that one might attribute to treatments of MD embryos with (+)-ABA or metabolites would have to be significantly greater than the ±10% observed in general embryo growth parameters after a similar treatment. This level of significance is similar to that observed in a previous study with (±)-ABA (Holbrook et al., 1992). Whereas in the present study (+)-ABA and PA did not significantly inhibit MD embryo development, it has been reported recently that (+)-ABA could strongly inhibit the growth of maize suspension-cultured cells (Balsevich et al., 1994b). These differences may be due to the nature of the two culture systems: The maize cells are undifferentiated, whereas the MD embryos are composed of haploid, differentiating cells. Since (+)-ABA and its metabolites do not inhibit growth in early- to mid-cotyledonary MD embryos, this system is well suited for examining the effects of hormonal treatment on specific metabolic pathways, such as lipid bioassembly.

Persistence of (+)-ABA and Its Metabolites in the Culture Medium

The culture medium analyses were performed immediately to minimize the rearrangement of 8′-OH ABA to PA. The 8′-OH ABA proved to be surprisingly stable in the culture medium during incubation with MD embryos (Fig. 2). The pH of the medium ranged from 6.3 at time 0 to 5.8 at the end of the experiment. After 8 h in culture, about 50 to 60% of the (+)-ABA and 8′-OH ABA still remained, whereas about 70% of the PA was still present. By 48 h the (+)-ABA was no longer detectable in the culture medium, whereas about 10% of the 8′-OH ABA and about 50% of the PA remained. In all treatments, there was detectable DPA in the culture medium after 24 h (data not shown), and the amount of DPA continued to increase as the amount of PA decreased.

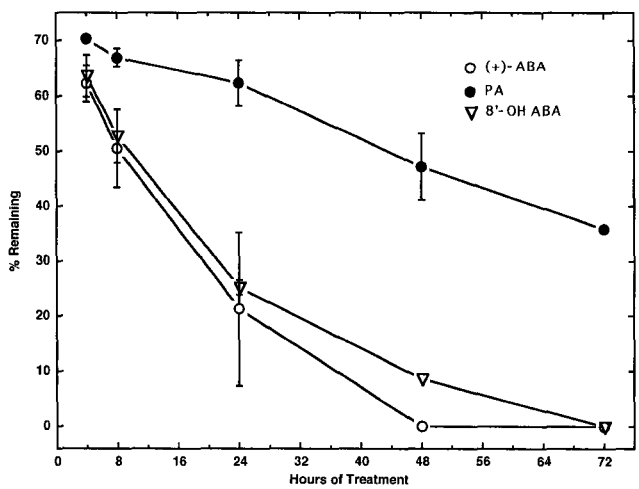

Figure 2. Time-course analyses of culture media for depletion of (+)-ABA, 8′-OH ABA, or PA supplied to developing MD embryos. Media were sampled at the times indicated and hormone levels were analyzed as described in "Materials and Methods." Values are the averages ± SD of duplicate determinations.

Effect of (+)-ABA and Its Metabolites on VLCMFA Biosynthesis

Early- to mid-cotyledonary Reston MD embryos have been shown to be ideally suited to the study of VLCMFA induction (Holbrook et al., 1992). After a 72-h treatment, the total lipid content in developing MD embryos at the early- to mid-cotyledonary stage was increased 14 to 17% and 23 to 34% in the presence of (+)-ABA and 8′-OH ABA, respectively, as compared to control embryos (Table II). There was no consistent trend in the response to PA (±7–12%). More importantly, dramatic changes were observed in the fatty acid composition of lipids extracted from MD embryos treated with (+)-ABA and 8′-OH ABA. In particular, there was a decrease in the levels of 18:1 and a concomitant increase in both the proportion and absolute amount of 20:1 and, especially, 22:1. In contrast, PA had only a small effect on the VLCMFA content. These trends were the same in both early-cotyledonary (Table II, experiment A) and mid-cotyledonary (Table II, experiment B) MD embryos. Although there was some variability in the overall lipid content (on a microgram per milligram of protein basis) between early- and mid-cotyledonary embryos, this did not invalidate the overall results since identical trends in VLCMFA content were observed regardless of differences in the absolute amount of lipid accumulated at a particular developmental stage.

Similar developmental differences during embryogenesis were documented previously (Holbrook et al., 1992; Taylor and Weber, 1994). The overall result of (+)-ABA and 8′-OH ABA treatment was an accumulation of VLCMFAs at a time earlier in development than observed in the control. The larger accumulation of 22:1 over its immediate precursor 20:1 is consistent with the known pathway of VLCMFA biosynthesis in most members of the Brassicaceae and Tropaeolaceae, in which 18:1 is elongated by successive condensations with malonyl-CoA to give 20:1-CoA and then 22:1-CoA, which are then incorporated into triacylglycerols via the Kennedy pathway (Agrawal and Stumpf, 1985; Fehling et al., 1990; Taylor et al., 1992; Taylor and Weber, 1994). In most members of the Brassicaceae, including *B. napus*, 22:1 is the VLCMFA that accumulates in triacylglycerols; in *Arabidopsis thaliana*, 20:1 is the elongation product that accumulates in the highest proportion (Kunst et al., 1992b).

The increased accumulation of VLCMFAs (20:1 plus 22:1) observed after 72 h in the presence of (+)-ABA and 8′-OH ABA (200 and 195%, respectively) was positively correlated (r = 0.990) with the induction of elongase activity measured in vitro at 48 h (Fig. 3). A similar temporal correlation between induction of elongase activity and subsequent VLCMFA accumulation was observed previously in a study using (±)-ABA (Holbrook et al., 1992). As expected, the effect of PA on elongase activity was slight and this lower activity was correlated with the small but measur-

Table II. *Effect of hormone treatment on fatty acid composition of TLE*

MD *B. napus* cv Reston embryos at 16 d (experiment A; early cotyledonary stage) or 19 d (experiment B; mid-cotyledonary stage) in culture were transferred to medium supplemented with either 0.1% (v/v) ethanol only (Control treatment), 10 μM (+)-ABA plus 0.1% (v/v) ethanol [(+)-ABA], 10 μM 8′-OH ABA plus 0.1% (v/v) ethanol (8′-OH ABA), or 10 μM PA plus 0.1% (v/v) ethanol (PA). After 72 h, embryos were harvested and the total lipids were extracted, transmethylated, and analyzed by GC as described in "Materials and Methods."

Experiment	Treatment	Fatty Acid Content								Total	Percent of Control
		16:0	18:0	18:1	18:2	18:3	20:1	22:1	Others[a]		
		wt %								μg/mg protein	
Experiment A	Control	6.7	4.5	47.7	17.1	5.9	7.2	5.0	5.9	2550	
	(+)-ABA	6.5	2.9	39.7	17.9	5.5	9.8	12.1	5.7	2915	114
	8′-OH ABA	6.4	3.7	41.3	16.7	6.5	10.1	10.7	4.6	3135	123
	PA	8.2	3.6	42.3	20.4	6.3	7.7	6.7	4.7	2245	88
Experiment B	Control	6.8	2.7	36.9	21.6	9.5	9.5	10.1	3.1	1380	
	(+)-ABA	5.4	2.0	28.8	17.2	10.1	11.5	21.7	3.3	1610	117
	8′-OH ABA	5.8	2.1	32.2	18.3	10.0	12.3	16.4	2.9	1850	134
	PA	6.4	2.3	34.8	20.4	9.5	10.9	12.8	2.9	1470	107

[a] Includes 20:0, 22:0, 24:0, 24:1, and 18:1-*cis 11* isomer.

able (25%) increase in VLCMFA content observed after 72 h in the presence of PA compared to control treatment.

In time-course studies (Fig. 4) there was a difference in the response of the VLCMFA biosynthesis pathway to (+)-ABA and its metabolites. Within the first 24 h, there was a significant increase in VLCMFA content in the presence of (+)-ABA relative to the control, and this trend increased during the 24- to 48-h period. Although the VLCMFAs also showed significant responses to 8′-OH ABA and to a much lesser extent PA in the 24- to 48-h period, they lagged behind (+)-ABA. However, by the 72-h point the increase in VLCMFA content was essentially identical in the presence of (+)-ABA and 8′-OH ABA (about 200% compared to the control treatment). In contrast, the 72-h PA treatment showed a VLCMFA content only 28% higher than the control treatment. By 96 h, the (+)-ABA response had leveled off, whereas on a milligram of protein basis, there was a decrease in VLCMFA content in the 8′-OH ABA treatment. The latter may reflect the relative rates of conversion of (+)-ABA and 8′-OH ABA to the relatively inactive PA.

Collectively these data indicate that there is a rapid stimulation of the VLCMFA biosynthetic pathway induced by treatment of the MD embryos with both (+)-ABA and 8′-OH ABA. However, it is not yet clear whether there is a requirement for conversion of (+)-ABA to 8′-OH ABA within the embryos for this induction process. Applied 8′-OH ABA itself can produce an (+)-ABA-like response. This finding is consistent with the observed activity of 8′-methoxy ABA (Todoroki et al., 1994) and the ability of many structural analogs of (+)-ABA to produce (+)-ABA-

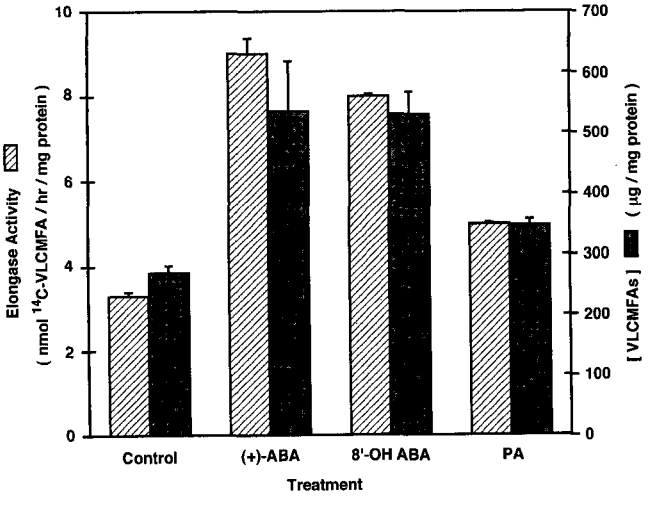

Figure 3. Correlation ($r = 0.990$) between VLCMFA accumulation at 72 h and induction of elongase activity at 48 h by treatment of Reston MD embryos with (+)-ABA and its metabolites. Reston embryos were harvested at 48 h, and homogenates were prepared and assayed for elongase activity in vitro as described in "Materials and Methods." At 72 h in culture, embryos were harvested and VLCMFA content was measured. Values are the averages ± SE, $n = 3$.

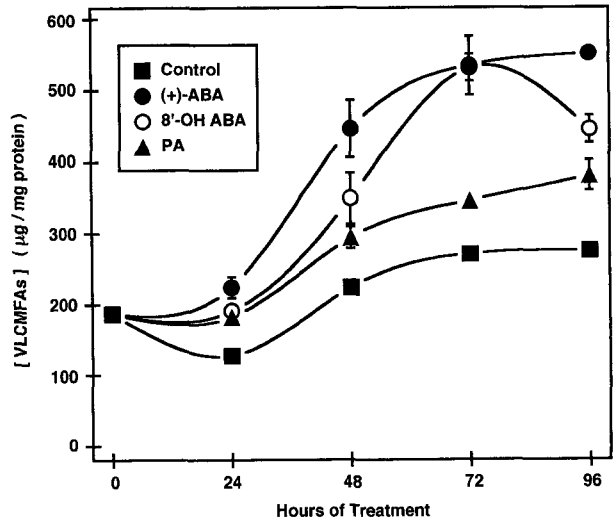

Figure 4. Time course of VLCMFA accumulation in Reston MD embryos cultured in the presence of (+)-ABA and its metabolites. At each point, embryos were harvested and VLCMFA content was measured. Values are the averages ± SE, $n = 3$.

like effects (Churchill et al., 1992; Walker-Simmons et al., 1992; Balsevich et al., 1994b). Furthermore, 8′-OH ABA is a metabolite of (+)-ABA, and the large amount of free energy released in the catalysis would suggest little likelihood of the reaction being reversible.

Given the high level of induction observed in the presence of (+)-ABA and 8′-OH ABA, it is difficult to attribute strong significance to the slight stimulatory effect observed in the PA treatment. It may be that the data are biased by expression of VLCMFA content on a per milligram of protein basis. When taken into consideration, the small but measurable decrease in total protein observed in the presence of PA (Table I) reduces even further the already minor apparent stimulation of VLCMFA biosynthesis observed in the PA-treated embryos. The relatively weak response exhibited by PA compared to (+)-ABA found in the current context of VLCMFA biosynthesis has also been observed in studies of other biological responses in other model systems. In a study utilizing maize cell-suspension cultures (Balsevich et al., 1994b), cellular growth was inhibited by more than 50% in the presence of 1 μM (+)-ABA, whereas a similar degree of growth inhibition required a PA concentration of 50 μM in the culture medium.

Although the current results strongly demonstrate a stimulatory effect of (+)-ABA and 8′-OH ABA at the protein level (elongase activity) that is correlated with an accumulation of VLCMFAs in embryo storage lipids, an examination of induction effects at the gene level must await the cloning of genes encoding the component proteins of the extraplastidic fatty acid elongase complex. In this regard, there is interest in cloning the gene found at the *FAE1* locus in *A.* thaliana using the technique of chromosome walking (Kunst et al., 1992a, 1992b). A mutation in this gene has been shown to severely disrupt VLCMFA biosynthesis. Recently, Lassner et al. (1994) reported the cloning of a jojoba (*Simmondsia chinensis*) gene encoding β-keto-acyl-CoA synthase, the rate-limiting initial condensing enzyme in the microsomal fatty acyl-CoA elongation complex. Clearly, such molecular tools would be invaluable in the context of the current study and when available will allow an examination of the (+)-ABA-responsiveness of elongase genes.

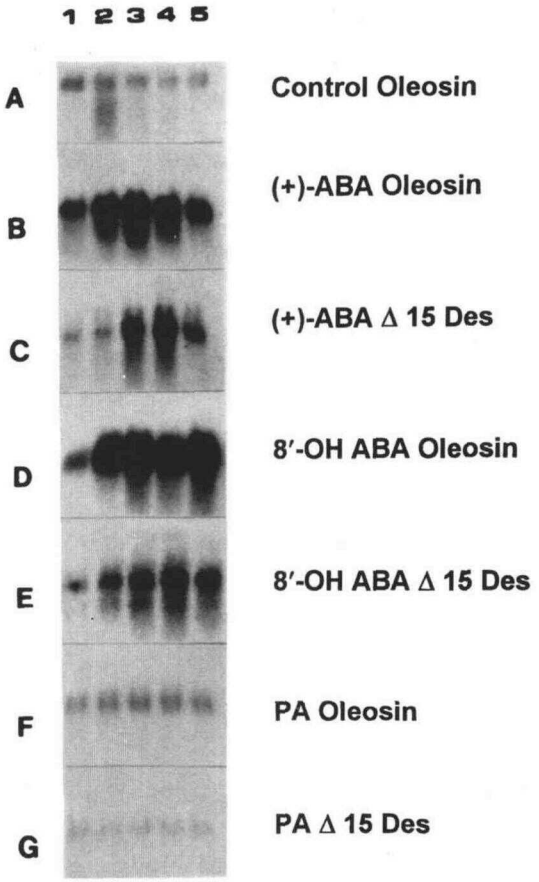

Figure 5. Northern analyses of transcripts for a 19-kD oleosin and Δ15 desaturase induced by treatment of Reston MD embryos with (+)-ABA and its metabolites. Control or hormone-treated embryos were harvested after 2 h (lane 1), 4 h (lane 2), 8 h (lane 3), 24 h (lane 4), or 48 h (lane 5) in culture. RNA extractions and northern analyses were performed as described in "Materials and Methods." A, RNA from control embryos, probed with 19-kD oleosin; B, RNA from (+)-ABA-treated embryos, probed with 19-kD oleosin; C, RNA from (+)-ABA-treated embryos, probed with Δ15 desaturase; D, RNA from 8′-OH ABA-treated embryos, probed with 19-kD oleosin; E, RNA from 8′-OH ABA-treated embryos, probed with Δ15 desaturase; F, RNA from PA-treated embryos, probed with 19-kD oleosin; G, RNA from PA-treated embryos, probed with Δ15 desaturase.

Northern Analyses of Transcripts for a 19-kD Oleosin and Δ15 Desaturase: Gene Induction Studies

Northern analyses were performed to study the effects of (+)-ABA and its metabolites on genes encoding a 19-kD oleosin (oil body protein), and a Δ15 desaturase, responsible for the desaturation of 18:2 to 18:3. In time-course studies, both (+)-ABA and 8′-OH ABA were able to strongly induce transcripts for both genes as compared to untreated control embryos and those treated with PA, although there were differences in the temporal response (Fig. 5). Compared to the control treatment (Fig. 5A), the induction of oleosin message was strongly evident in as little as 2 to 4 h and remained so throughout the 48-h sampling period in the presence of both (+)-ABA and its 8′-OH ABA metabolite (Fig. 5, B and D, respectively). In contrast, there was no significant oleosin induction by PA (Fig. 5F). Transcripts for the Δ15 desaturase were induced somewhat later, by 8 h, in both the (+)-ABA- and 8′-OH ABA-treated embryos (Fig. 5, C and E, respectively), whereas there was no induction in the PA-treated embryos (Fig. 5G) or untreated control embryos (data not shown). Again, the induction signal remained strong throughout the 48-h sampling period for the (+)-ABA- and 8′-OH ABA-treated embryos.

Thus, the present study has demonstrated that genes encoding both the 19-kD oleosin and Δ15 desaturase are responsive to exogenous treatment with (+)-ABA and its 8′-OH ABA metabolite. Biosynthesis of the 19-kD oleosin was previously shown to be responsive to (±)-ABA (Holbrook et al., 1992); therefore, the strong induction of oleosin transcript by (+)-ABA was, perhaps, expected. Others have

examined the competitive inhibition of ABA-regulated napin gene expression by stereoisomeric acetylenic analogs of ABA, but again, (±)-ABA was utilized in the competition studies (Wilen et al., 1993). In the present study, the oleosin transcript was strongly induced by (+)-ABA or 8′-OH ABA in as little as 2 h, and the signal remained strong throughout the sampling period (48 h).

Although the effects of (+)-ABA and 8′-OH ABA on induction of Δ15 desaturase are documented here, a Δ9 desaturase transcript in zygotic *B. napus* was shown to be responsive to (±)-ABA in the 1- to 10-μM range and there was evidence from time-course studies of regulation at the level of transcription (Slocombe et al., 1994). It is interesting to note that in the present study the strong induction of Δ15 desaturase transcript observed at 8 to 48 h by both (+)-ABA and 8′-OH ABA was followed by only slight (20%) increases in 18:3 content measured at 48 to 96 h (data not shown), whereas the VLCMFA content increased dramatically during this period. These results would suggest that the transcript level is not limiting and that the expression of Δ15 desaturase and the accumulation of its product, 18:3, are regulated beyond the mRNA level. Thus, the roles of (+)-ABA and its metabolites in regulating Δ15 desaturase activity clearly require further study at both the gene and protein levels.

Endogenous Levels of (+)-ABA in Cultured MD Embryos

Since the strong induction of oleosin and Δ15 desaturase transcripts occurred within 8 h in response to treatment with both exogenous (+)-ABA and 8′-OH ABA (Fig. 5), this time was chosen to examine the effects of the hormone treatments on endogenous (+)-ABA levels in the MD embryos. Only the embryos treated with exogenous 10 μM (+)-ABA had endogenous (+)-ABA levels (50–60 $\mu g\ g^{-1}$ DW) that were elevated beyond the low endogenous level observed in control embryos (0.6 $\mu g\ g^{-1}$ DW). The control sample estimate of endogenous (+)-ABA content agrees favorably with that reported in a previous study of MD embryos by Wilen et al. (1993), in which, after 24 h, the endogenous (+)-ABA present in control treatments was reported to be 0.16 $\mu g\ g^{-1}$ DW. In the present study, the up to 100-fold increase in the (+)-ABA-treated sample after 8 h of treatment indicates that exogenous (+)-ABA is indeed taken up by the MD embryos. Perhaps more important, it establishes that the rapid induction of oleosin and Δ15 transcripts observed in the presence of 8′-OH ABA is not due to a stimulation of (+)-ABA biosynthesis in the MD embryos but is, rather, a direct result of treatment with 8′-OH ABA.

In summary, the present study has shown that genes and processes related to lipid bioassembly during embryogenesis in *B. napus* are responsive to both (+)-ABA and its metabolite 8′-OH ABA, whereas PA elicits little or no response. This would seem to suggest that during embryogenesis the induction of the lipid pathway is regulated by (+)-ABA or close structural analogs either directly or, more probably, at the receptor/signal transduction level.

Clearly, there is more study required to elucidate the role of the (+)-ABA metabolite 8′-OH ABA in the responses cited herein. For example, the availability of radiolabeled 8′-OH ABA would allow an examination of the kinetics of uptake and metabolism. Such studies are the focus of future work, which will no doubt add to our understanding of this (+)-ABA metabolite. The rapid induction of lipid and oleosin biosynthesis in the MD embryo system could be exploited as a bioassay tool in screening (+)-ABA analogs and their metabolites for biological activity and to clarify the responses at the gene transcription and translation levels. Certainly, the use of this embryo culture system has been instrumental in helping to identify a biological response following challenge with the (+)-ABA metabolite 8′-OH ABA. In a practical sense, one may be able to exploit the stimulatory effect of (+)-ABA, its analogs, or metabolites on oilseed embryogenesis and lipid-related processes to harness the regulatory elements controlling oil quantity or quality (e.g. VLCMFA content).

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