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Role of the Ring Methyl Groups in Abscisic Acid Activity in Erucic Acid Accumulation in Oilseed Rape (*Brassica napus* L.)

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Abstract. Modification of the structure of abscisic acid (ABA) has been reported to result in modification of its physiologic activity. In this study we tested the effect of removing methyl groups from the ring and of chirality of ABA on activity in microspore-derived embryos of oilseed rape (*Brassica napus* L.). The natural (+)-ABA molecule induced growth inhibition and an increase in the amount of erucic acid accumulated in the oil at medium concentrations less than 1 μM. (−)-ABA showed similar effects. Removing the 7′-methyl group resulted in a dramatic decrease in activity: (+)-7′-demethyl-ABA retained some activity as a growth inhibitor; a 10–100 μM concentration of this compound was needed for a response, and (−)-7′-demethyl-ABA was almost completely inactive. Similar effects were observed with regard to elongase activity, which catalyzes erucic acid biosynthesis from oleic acid. Removal of the 8′- and 9′-methyl groups resulted in a more complex response. These compounds all showed intermediate activity; for growth inhibition, the presence of the 9′-methyl was the more important determinant, whereas chirality dominated the response on erucic acid accumulation, with the (+)-enantiomers being more active.

Key Words. *Brassica napus* L.—Abscisic acid analogs—Elongase—Erucic acid—Growth—Microspore-derived Embryos—Seed oil

Abbreviations: 18:1, oleic acid; 20:1, eicosenoic acid; 22:1, erucic acid; ABA, abscisic acid; (d)dm, (di)demethyl; FAME, fatty acid methylester; HPLC, high performance liquid chromatography; MDE(s), microspore-derived embryo(s); PGR, plant growth regulator; TLC, thin layer chromatography.

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In recent years a number of reports have been published on the activity of abscisic acid (ABA) derivatives in different plant systems. Activity was determined in combination with ABA application, in some cases resulting in competitive inhibition (Wilen et al. 1993), or separately to determine the relative activity compared with natural ABA. A number of different species and responses have been used to monitor ABA activity such as stomatal response and transpiration (Blake et al. 1990, Jung and Grossmann 1985), chilling tolerance (Churchill et al. 1992, Dorffling et al. 1989), dormancy and germination (Kim et al. 1995, Walker-Simmons et al. 1992, 1994), developmental processes (Abrams and Milborrow 1991, Suttle and Abrams 1993), and gene expression (Dong et al. 1994, Hays et al. 1996). Effects are expressed in relation to the activity of natural ABA in all of these studies, as the sensitivity to ABA varies with the system and the response studied. Also, the sensitivity to changes in the ABA structure depends upon the experimental system used.

It has been shown previously (Holbrook et al. 1992, Wilmer et al. 1997) that the addition of ABA can increase the amount of erucic acid (22:1) in oil of microspore-derived embryos (MDEs) of *Brassica napus* by about 10 mol %. In addition, the total amount of oil increases. In this study we have compared the effects of a number of optically pure (+)- and (−)-enantiomers of ABA analogs (Fig. 1), lacking one or two methyl groups from the ring (carbons 7′, 8′, or 9′), on oil accumulation and very long chain fatty acid production in MDEs. We compared our data with those obtained by Walker-Simmons et al. (1995), who tested the effects of the same dimethyl analogs on wheat germination, to extend the knowledge on the structural requirements of the ABA molecule for its interaction with receptors and physiologic activity.

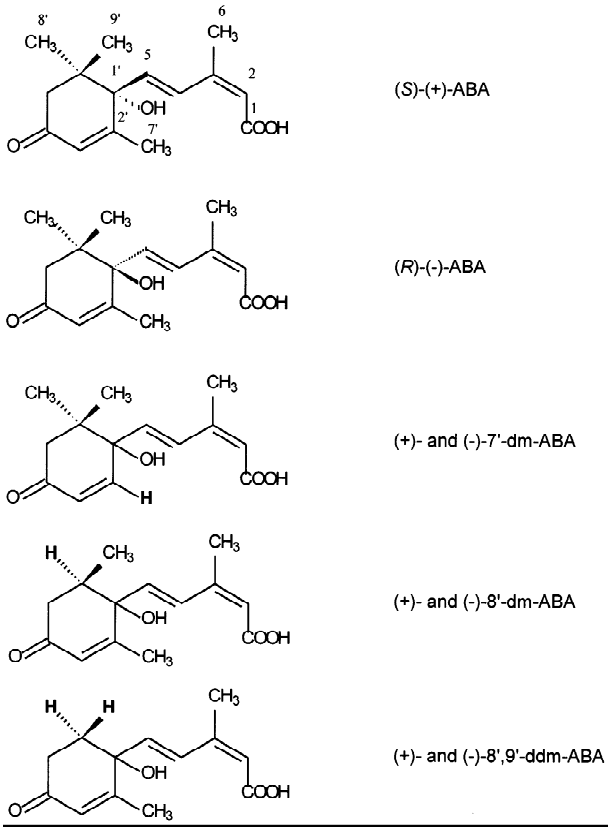

Fig. 1. Chemical structure of ABA and dm-ABA analogs. (+)- and (−)-enantiomers of dm analogs have the same chirality at C1′ as the corresponding form of the intact molecule.

Materials and Methods

Chemicals

All solvents used were HPLC or reagent grade. Radioactive malonyl-[2-^{14}C]CoA was purchased from Amersham. ABA, buffers, cofactors and FAME standards were purchased from Sigma and TLC plates from Merck.

Culture of Microspore-Derived Embryos

Plants of oilseed rape cultivar Reston, obtained from the University of Manitoba, were grown in the greenhouse at 20/17°C (day/night) at a photoperiod extended to 16 h with SON-T AGRO (Philips, Eindhoven, The Netherlands) to ensure induction of flowering. MDE culture was performed according to Wilmer et al. (1996). Briefly, racemes were collected at the onset of flowering, and buds of 3.4–3.6 mm were selected. The buds were sterilized in 2% NaClO and homogenized in a modified Lichter medium (NLN13). After filtration, microspores were collected by centrifugation, washed, diluted to 40,000 spores mL^{-1}, and 1-mL portions of suspension were plated in 35-mm Petri dishes. Embryogenesis was induced at 32°C for 3 days, then cultures were transferred to 25°C. Treatments were started at a temperature sum of 350°C day (14 days in culture). Upon reaching a temperature sum of 450°C day, embryos were rinsed with tap water and stored at −80°C until analysis for elongase activity. Cultures were allowed to grow until 800°C day (31 days in culture) for the analysis of fatty acids.

ABA and Analogs

Optically pure analogs were synthesized and purified as described elsewhere (Rose et al. 1996, Walker-Simmons et al. 1994). Compounds were at least 99% pure. In this study we used eight compounds (Fig. 1): both (+)- and (−)-enantiomers of natural ABA, of 7′-dimethyl-ABA (7′-dm-ABA), of 8′-dimethyl-ABA (8′-dm-ABA); and of 8′,9′-didemethyl-ABA (8′,9′-ddm-ABA). Treatments were started by replating the embryos in fresh medium containing ABA or its analogs, freshly diluted from a 0.1 M stock solution in methanol; controls were replated in medium containing 0.01% methanol.

Growth Inhibition

Growth inhibition was determined as the percent reduction in fresh weight at 800°C day compared with a control that contained no ABA or ABA analog. Data were calculated on this relative basis as the absolute size of the embryos varied in a batch-dependent way.

Enzyme Preparation and Elongase Assay

MDEs were ground in a buffer containing 80 mM HEPES, pH 6.8, 0.32 M sucrose, 10 mM β-mercaptoethanol, and 50 mg/mL polyvinylpolypyrrolidone at 5 mL of buffer gr of tissue. The homogenate was passed through two layers of Miracloth and centrifuged at 1,000 ×g for 5 min. The supernatant was recentrifuged at 15,000 ×g, for 25 min. The pellet was suspended in 80 mM HEPES, pH 6.8, 10 mM β-mercaptoethanol and centrifuged again at 15,000 ×g for 25 min.

Thirty μg of protein from the resuspended 15,000 ×g pellet was incubated at 30°C in a buffer containing 80 mM HEPES, pH 7.2, 0.5 mM NADH, 0.5 mM NADPH, 2 mM dithiothreitol, 1 mM $MgCl_2$, 150 μM Triton X-100, 17 μM [2-^{14}C]malony CoA (1.85 kBq), and 25 μM oleoyl-CoA in a final volume of 100 μL. After incubation for up to 30 min, the reaction was stopped by the addition of 100 μL of 5 N KOH, 10% methanol, and reaction products were saponified for 1.5 h at 70°C. Next, 100 μL of 5 M H_2SO_4, 10% malonic acid was added, and the mixture was extracted with 2 mL of chloroform. The organic phase was washed three times with 2 mL of water, evaporated under a stream of air, and radioactivity was counted.

Lipid Extraction and Analysis of Fatty Acid Composition

Oil was extracted from mature MDEs, and fatty acid composition was determined as fatty acid methylesters as described previously (Wilmer et al. 1996). Briefly, embryos were homogenized in 5 mL of a mixture of methanol:chloroform:0.01 N hydrochloric acid (2:1:0.8, v/v/v) containing trimargarin as an internal standard. After shaking, chloroform and 0.01 N hydrochloric acid were added to obtain a mixture with a ratio of 2:2:1.8 by volume. The suspension was centrifuged to achieve phase separation. The aqueous phase was washed once with 1.5 mL of chloroform. Aliquots of organic fractions were applied to TLC plates, which were developed in hexane:diethyl ether:acetic acid (70:30:1, v/v/v), and lipids were visualized with iodine vapor. Areas containing triglycerides were scraped off, and the powder was transferred to tubes. Three mL of 5% sulfuric acid in methanol was added, and tubes were

Table 1. Relative growth inhibition and 22:1 increase by ABA derivatives and classification of concentrations resulting in half-maximum reduction in fresh weight or half-maximum increase in 22:1 ($[PGR]_{50}$).

	Growth inhibition		Increase in fraction 22:1	
	% at 10 μM[a]	$[PGR]_{50}$ (μM)	% at 10 μM[b]	$[PGR]_{50}$ (μM)
(+)-ABA	63 ± 15	<1	29 ± 10	<1
(−)-ABA	47 ± 27	<1	28 ± 6	<1
(+)-7′-dm-ABA	−1 ± 44	1–10	14 ± 5	10–100
(−)-7′-dm-ABA	−21 ± 21	10–100	−1 ± 8	>100
(+)-8′-dm-ABA	27 ± 27	1–10	22 ± 13	1–10
(−)-8′-dm-ABA	24 ± 25	1–10	11 ± 5	10–100
(+)-8′,9′-ddm-ABA	41 ± 20	<1	26 ± 11	1–10
(−)-8′,9′-ddm-ABA	49 ± 20	<1	11 ± 13	10–100

[a] Control values (no ABA): 356–935 mg/100 embryos; 45–60% growth inhibition at saturating concentrations.
[b] Control values: 19–31 mol % 22:1; maximum 22:1 increase 25–30% at saturated response.
Note. Values are means ± S.D. from three experiments.

capped and incubated at 70°C for 3 h. Subsequently, FAMEs were extracted with hexane.

FAMEs were analyzed using a capillary Chrompack CP9000 gas chromatograph equipped with a CPwax 52 CB column and flame ionization detection. The oven temperature was programmed from 190°C (4.1 min) to 230°C (4.4 min) at 20°C min^{-1}. FAMEs were identified on the basis of retention time and quantified by electronic integration with reference to methyl margarate, derived from the internal standard trimargarin.

Calculation of $[PGR]_{50}$ Values

Dose-response curves were constructed for the different ABA analogs over the range of 10 nM–100 μM, with the exception of (+)-ABA, where a range of 1 nM–10 μM was used. The concentrations of plant growth regulator resulting in half-maximum response ($[PGR]_{50}$ values) were calculated from sigmoidal data fits on these dose-resonse curves, reflecting the transitional shape of the response. Because of variation among experiments and the limited resolution of the concentration range, $[PGR]_{50}$ values are given in range classes.

Results

Effects of Dimethyl Analogs on Embryo Growth

The capacity of dm analogs of ABA to inhibit growth of MDEs is shown in Table 1. Because of large variability in the experimental system (see footnotes to Table 1), no proper statistical analysis could be performed, but the order of activity for the analogs was the same over three independent experiments. The (+)– and (−)-enantiomers of ABA and 8′,9′-ddm-ABA were equally active in inhibiting embryo growth at 10 μM. Both 8′-dimethyl enantiomers showed reduced inhibitory activity (about 50% in comparison with (+)-ABA), and (−)-7′-dm-ABA was even less active. The extremely large S.D. on the value for (+)-7′-dm-ABA indicates that the threshold concentration for activity of this compound was about 10 μM. The $[PGR]_{50}$ values for these compounds indicated a similar ranking in inhibitor activity, which was illustrated most clearly by the high concentrations of (−)7′-dm-ABA required for half-maximum activity (Table 1), again indicating the impact of the 7′-methyl group.

Accumulation of Erucic Acid

Accumulation of erucic acid can be calculated in two ways, as a fraction of the oil (percent of total fatty acids) or as the absolute amount accumulated (μmol 22:1/100 embryos). To induce effects on oil composition, high concentrations of some analogs were required, which resulted in a strong reduction in embryo growth (see above). When the effects of ABA analogs were calculated as changes in the absolute amount of 22:1, increases induced by the addition of the compound tested were at least partially masked by a general reduction in growth, as illustrated for (+)-8′-dm-ABA (Fig. 2). (−)-7′-dm-ABA showed no effect, whereas the other analogs all appeared to have at least some impact on 22:1 accumulation, with (+)-ABA as the most active compound (data not shown).

Changes in the fraction 22:1 in the oil caused by application of ABA analogs are more easily interpreted (Table 1). Again, both enantiomers of intact ABA had reached maximum activity, at 10 μM, as was also observed for (+)-8′,9′-ddm-ABA and (+)-8′-dm-ABA. The (−)-enantiomers of these analogs showed intermediate activity as did (+)-7′-dm-ABA. The (−)-7′-dm analog

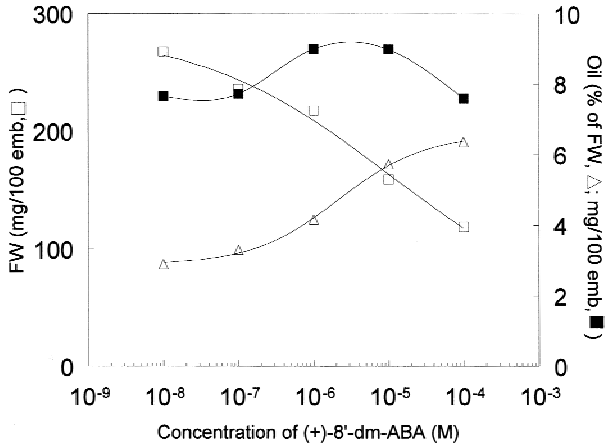

Fig. 2. Effect of (+)-8′-dm-ABA concentration in the medium on fresh weight (□), absolute amount of oil accumulated (■), and on oil accumulation as percent of fresh weight (△) in microspore-derived embryos of *B. napus* L. cv. Reston.

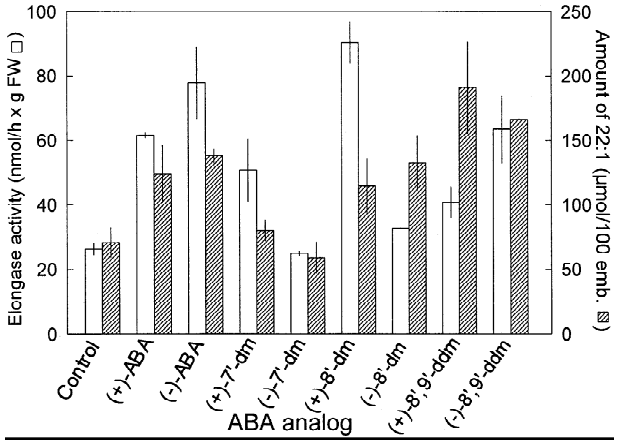

Fig. 3. Elongase activity at 450°C day and amount of 22:1 at 800°C day after treatment of MDEs with a 10 μM concentration of each ABA analog. Data are means ± SD from the same experiment. *Open bars*, elongase activity (nmol/h/g fresh weight; 3,000 embryos ~ 1 g fresh weight at this stage), *hatched bars*, amount of 22:1 (μmol/100 embryos).

was completely inactive. The $[PGR]_{50}$ values with respect to 22:1 accumulation showed a similar order of activity, although (+)-8′-dm- and (+)-8′9′-ddm-ABAs were somewhat less active than (+)- and (−)-ABA (Table 1).

Elongase Activity

Because of the large differences among batches of MDEs and limited batch size we only determined elongase activity at 10 μM for each analog. The maximum effect induced by (+)-8′-dm-ABA was a 3.5-fold increase, whereas adding of (+)-ABA only doubled elongase activity (Fig. 3). The effects of (+)-ABA, (−)-ABA, and (−)-8′,9′-ddm-ABA were similar, and (−)-7′-dm-ABA was completely inactive in this assay. The remaining compounds showed intermediate activity, resulting in about a 50% increase in elongase activity.

Correlation of Elongase Activity and 22:1 Accumulation

In previous studies we found that elongase activity at 450°C day, when no differences in growth patterns induced by ABA were yet visible, correlated closely with the amount of 22:1 formed at maturity (Wilmer et al. 1998). In this study no significant correlation ($r^2 \sim 0.1$) was observed between elongase activity and the amount of 22:1 accumulated for the full complement of ABA analogs. When only the data obtained with (+)- and (−)-ABA enantiomers and their 7′-dm analogs were considered, together with the control, the correlation was much higher ($r^2 \sim 0.9$). This selection coincides with the group of compounds that give consistent rankings over both growth and oil accumulation patterns. A similar conclusion can be drawn for the correlation of elongase activity with the fraction 22:1 in oil ($r^2 \sim 0.3$ and ~0.8, respectively).

Discussion

Our results indicate that the role of the three ring methyl groups in ABA activity on growth and 22:1 accumulation in MDEs of oilseed rape is similar to the effects described for wheat germination (Walker-Simmons et al. 1994).

Removal of the 7′-methyl group results in a reduction in ABA activity in both species. Only at high concentrations, with a $[PGR]_{50}$ of 10 μM or higher, (+)-7′-dm-ABA, with the same chirality as natural ABA, has limited activity both in inhibiting growth and in increasing 22:1. However, the response at 100 μM is similar to that obtained with (+)-ABA. The compound with the opposite chirality, (−)-7′-dm-ABA, only showed an effect on growth at the highest concentration and had no effect on 22:1 accumulation. In wheat germination studies both 7′-dm enantiomers were completely inactive (Walker-Simmons et al. 1994). Both enantiomers had effects on elongase activity similar to those on 22:1 accumulation; (+)-7′-dm-ABA induced a small increase in activity, and its (−)-isomer was completely inactive. If we combine these data and determine correlations as was done for elongase activity and absolute amount 22:1 in the oil,

consistent effects are observed. This suggests that either one ABA uptake and perception system is involved in transmitting hormonal signals in MDEs of oilseed rape or, if more than one system is involved, all require a methyl group in the 7′-position for recognition.

All 8′- and 8′,9′-demethyl analogs of ABA showed activity both with regard to growth inhibition and erucic acid level (Table 1). For growth inhibition the ranking of activities was similar to that found by Walker-Simmons et al. (1994) using the wheat cultivar Brevor; the 8′,9′-ddm analogs were somewhat more active than the 8′-dm analogs, but no differences can be found between chiral forms. The effects on the level of 22:1 suggested a clear effect of chirality but little effect of removing the second methyl group; the effects at 10 μM were identical, and $[PGR]_{50}$ values fell in the same range. Elongase activity data are more difficult to interpret: (−)-8′,9′-ddm-ABA was more active in inducing elongase activity than its (+)-isomer, whereas (+)-8′-dm-ABA was more effective than its (−)-enantiomer; in fact it was the most active compound in enhancing elongase activity, but it caused only intermediate increases in the absolute level of 22:1. This lack of relation between elongase activity and 22:1 accumulation suggests that the effects on elongase activity cannot be assessed properly at 450°C day as far as these analogs are concerned (Wilmer et al. 1998). Whether this is the result of an interaction between induction of oil accumulation and inhibition of growth as demonstrated in Fig 2 or a shift in response timing or developmental rate caused by the removal of the 8′- and 9′-methyl group in the ABA analogs cannot be concluded from the present data.

Reduced metabolism of the 8′-dm and 8′,9′-ddm analogs compared with the intact molecule might also explain some of the effects observed on elongase activity and 22:1 accumulation, where (−)-8′-dm and (+)-8′,9′-ddm-ABA gave high levels of 22:1 with limited increases in elongase activity; but these effects were not observed for all compounds lacking the 8′-methyl group. Thus a role of reduced metabolism of these compounds remains unclear. Also, at least for 8′9′-ddm-ABA, uptake was similar to that of the intact molecule as can be inferred from the low $[PGR]_{50}$ values for these compounds in growth inhibition, excluding differences in uptake as a cause for observed differences in activity.

Thus the fact that the effects on growth and on 22:1 accumulation are different suggests the presence of at least two uptake and perception systems for ABA in MDEs: one involved in regulation (or termination) of growth of the embryo and the other involved in regulation of oil composition and accumulation. The combination of the separate signal transduction pathways originating from these two systems then results in the observed differential effects.

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