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Biological Activity of Optically Pure C-1 Altered Abscisic Acid Analogs in *Brassica napus* Microspore Embryos

D. B. Hays,[1] P. Rose,[2] S. R. Abrams,[2] and M. M. Moloney[1,*]

[1]Department of Biological Sciences, University of Calgary, Calgary, Alberta T2N 1N4; and [2]Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan S7N 0W9, Canada

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Abstract. We have examined the effects of stereochemically pure analogs of abscisic acid (ABA) on three responses in *Brassica napus* microspore embryos. The analogs used include modifications to natural (*S*-) (+)-ABA (=N-ABA) at the C-1 and C-1′ positions. At the C-1 position, the carboxylic acid function was replaced with an alcohol, aldehyde, or methyl ester functional group, and at the chiral C-1′ position both enantiomers were prepared. The rationale for choosing these particular analogs was that they had previously shown some potential as slow release forms of ABA (Gusta LV, Ewan B, Reaney MJT, Abrams SR (1992) Can J Bot. 70:1550–1555). The responsiveness of microspore-derived embryos of *B. napus* to these analogs was investigated. Three types of responses were evaluated: (1) the inhibition of precocious germination; (2) induction of oleosin gene expression; and (3) induction of napin gene expression. All of the structural analogs of ABA tested were effective in the three assays, regardless of functional group substitution or stereochemistry. However, the three assays showed differential sensitivity to the various analogs. The U-forms of abscisyl alcohol and abscisyl aldehyde were very effective in inhibiting precocious germination (greater than natural ABA). Oleosin mRNA accumulation responded most effectively to U-abscisyl alcohol, while the N-abscisyl aldehyde and ABA methyl ester were the most effective at inducing napin mRNA accumulation. This work highlights the distinct differences in activity which result from using stereochemically pure analogs. In addition, surprisingly potent responses are reported in one or more of the assays for abscisyl aldehyde and abscisyl alcohol.

Key Words. Abscisic acid—Gene expression—ABA analogs

The plant hormone abscisic acid (ABA) has been implicated as a key regulatory factor in many developmental and stress-related processes of higher plants. Some of the processes, such as the control of transpiration via the stomatal aperture (Mansfield et al. 1978), are believed to occur independently of alterations in gene expression (McAinish et al. 1991). Other processes appear to be dependent upon ABA-mediated alterations in gene expression (Hetherington and Quatrano 1991, Thomas et al. 1991). Pleiotropic effects of ABA are seen in seed development in flowering plants, some of which show tissue and organ specificity (Hetherington and Quatrano 1991).

Developing seeds accumulate and store large reserves of storage lipids and proteins which are utilized upon germination. This maturation process is mediated via alterations in gene expression regulated in part by ABA (Bray and Beachy 1985, Crouch et al. 1984). ABA-sensitive genes involved in seed development include storage protein genes in corn (Kriz et al. 1990), rapeseed (Finkelstein et al. 1985, Wilen et al. 1990), and soybean (Bray and Beachy 1985) and genes encoding oleosins (van Rooijen et al. 1992), proteins that provide structural stabilization to oilbodies (Huang 1992). ABA-regulated genes also play a role in the control of germination and dormancy. These include genes that block premature germination, such as the α-amylase inhibitor protein (Leah and Mundy 1989) and the late embryogenic abundant (LEA) class of genes, which is thought to impart desiccation tolerance on the dormant embryo and developing seedling (Dure et al. 1989).

Studies of ABA mutants support the above observations. The *vp5* mutant of maize is an ABA-deficient mu-

Abbreviations: ABA, abscisic acid; LEA, late embryogenic abundant; HPLC, high performance liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid; SDS, sodium dodecyl sulfate.

* Author for correspondence.

tant. Kriz et al. (1990) observed low levels of globulin-1 mRNA in isolated *vp5* embryos. Application of exogenous ABA to these embryos resulted in the accumulation of wild type levels of globulin-1 mRNA. Similar mutants have been observed in *Arabidopsis* (Finkelstein and Somerville 1990, Karssen et al. 1983).

The increase in endogenous ABA prior to the programmed dehydration of the seed not only directs reserve deposition and desiccation tolerance acquisition, but also prevents the immature embryo from germinating precociously. Evidence for ABA regulation of this process comes from studies on isolated embryos (Ackerson 1984, Finkelstein et al. 1985, Wilen et al. 1990), embryos exposed to the ABA biosynthesis inhibitor, fluridone (Fong et al. 1983), and studies of ABA-deficient and -insensitive mutants (Groot and Karssen 1992, Koorneef et al. 1989, Neill et al. 1987).

We reasoned that ABA analogs could be useful for exploring how ABA regulates embryo maturation and germination. ABA analogs have been used previously to probe the structural requirements of ABA receptors in a number of biologic processes: in freezing tolerance in cultured bromegrass cells (Churchill et al. 1992, Robertson et al. 1994), and in expression of ABA-inducible genes in barley aleurone protoplasts (Van der Meulen et al. 1993), and in germination inhibition of wheat embryos (Walker-Simmons et al. 1994). In an earlier study in wheat embryos, optically active ABA analogs that inhibited germination induced elevated levels of mRNA of a subset of ABA-inducible genes, indicating which genes were related to the biologic effect (Walker-Simmons et al. 1992). A study on the influence of analogs including racemic forms of ABA metabolites on germination of cress seed showed that ABA and its precursors had high biologic activity (Gusta et al. 1992). In cress seed, natural (*S*)-(+)-ABA was a strong germination inhibitor, whereas the nonnatural form (*R*)-(−)-ABA was much less active. This difference in activity between ABA enantiomers was also observed in induction of the mRNA of oleosin and storage protein genes in *Brassica napus* microspore-derived embryos (Wilen et al. 1993).

In the present investigation, we undertook to study the effects of the optically active forms of abscisyl alcohol, aldehyde, abscisic acid, and methyl ester, all of which had shown biologic activity as racemic mixtures when applied to cress seeds. We studied two processes in *B. napus* microspore-derived embryos: germination and induction of mRNA of ABA-inducible genes oleosin and napin. Microspore-derived embryos of *B. napus* give rapid responses to applied ABA at the level of embryo-specific gene expression and inhibition of germination (Taylor et al. 1990, Wilen et al. 1990, 1991). In this paper we have quantified and compared the relative activities of various ABA analogs and their resolved optical isomers.

Materials and Methods

Source of Chemicals

Racemic ABA was obtained from Sigma. Methyl ABA was resolved by HPLC using a preparative chiral column and the separated enantiomers hydrolyzed to the acids as described previously (Dunstan et al. 1992). The chiral ABA alcohols and aldehydes were synthesized according to published methods (Rose et al. 1992). Optically active ABA analogs were synthesized.

Plant Material

B. napus cv. Topas plants (Agriculture Canada, Saskatoon) were raised in growth chambers at 20°C daytime temperature (16-h photoperiod, 400 mmol m^{-2} s^{-1}) and 15°C nighttime temperature. Plants used for microspore isolation were transferred after 6 weeks to growth chambers with a 15°C day/10°C nighttime temperature regime until flower buds were harvested.

Microspore Culture and Germination Assays

Flower buds (2–3 mm long) were collected from cold-treated plants. Microspores were isolated by grinding buds in NLN medium (Lichter 1982) with 13% sucrose (pH 6.0) and then washing and pelleting the microspores in the same. The microspores were then suspended in 40% Percoll containing 13% sucrose, overlaid with NLN medium, and spun at 220 × *g* for 10 min. Microspores at the Percoll-medium interface were collected, washed, pelleted, and resuspended in fresh NLN. Ten ml of cell suspension was plated in Petri dishes at a density of 3×10^4 cells ml^{-1}. Microspores were incubated in the dark for 4 days at 30°C, transferred to 25°C, and subculture after 7 days with fresh NLN with a fourfold dilution.

Treatment of embryos with ABA analogs was performed by adding 10 μl of analog from 10–100 mM stock solutions dissolved in 70% ethanol. Control embryos were treated with 10 μl of 70% ethanol. Embryos were harvested after 48 h and sieved through 250-μm nylon membranes to harvest torpedo-staged embryos. For precocious germination studies torpedo-staged microspore-derived embryos were individually selected and placed on germination medium [salts (Murashige and Skoog 1962), 2.5% sucrose, 0.7% phytoagar pH 6.0] with or without various concentrations of (N)-ABA or ABA analogs. All embryos were cultured at 25°C for 3 days after which embryos were scored for percentage germination.

RNA Extraction

Total RNA was extracted according to the method of Verwoerd et al. (1989). The RNA was quantified by A_{260} measurements and stored as an ethanol precipitate at −20°C until use.

Northern Blotting and Densitometry

Total RNA was separated by electrophoresis on formaldehyde gels (6% formaldehyde, 1 × MOPS [1 × MOPS = 20 mM MOPS, 5 mM sodium acetate, 0.1 mM EDTA), 1.2% agarose]. RNA was transferred to GeneScreen Plus (DuPont NEN) membranes by capillary blotting with 20 × standard sodium citrate (1 × SSC = 150 mM sodium chloride, 15 mM sodium citrate) for 24 h. The RNA was fixed to the membrane by exposure to UV irradiation for 5 min. Prehybridization was carried out

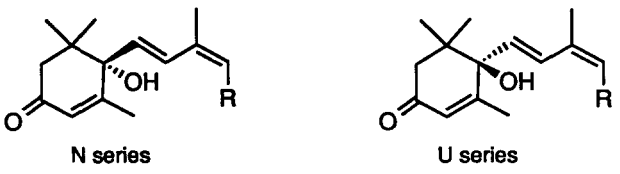

Fig. 1. Structural formula of natural N-series and unnatural U-series analogs, showing the stereochemistry around the chiral centers. The alterations made to N-ABA or U-ABA were to the carboxylic acid functional group at the C-1 position were R = CHO, CH_2OH, or CO_2CH_3 for both N- and U-series analogs.

in Seal-a-Meal bags with 25 ml of hybridization solution [50% formamide, 5 × SSPE (1 × SSPE = 150 mM sodium chloride, 10 mM sodium phosphate, 1 mM EDTA), 1% SDS, and 5 × Denhardt's solution] with 5 mg of yeast tRNA at 43°C for 12 h. Hybridization was carried out in fresh hybridization solution for 16 h with 5 mg of yeast tRNA and 50 mg of radiolabeled oleosin or napin cDNA (kindly provided by Dr. M. L. Crouch, Indiana University). The DNA was labeled by the random oligonucleotide-priming method (Feinberg and Vogelstein 1984) to a specific activity of $>5 \times 10^9$ dpm μg^{-1}. Membranes were washed twice in 2 × SSPE, 0.1% SDS at room temperature for 20 min and twice in 0.2 × SSPE, 0.1% SDS at 65°C for 20 min. Filters were used to expose Kodak XAR-5 film at −70°C for varying times to ensure that exposure was in linear range of the densitometer.

Densitometry was performed on exposed X-ray films of the Northern blots described above using a scanning densitometer (Hoefer Scientific) linked to an integrator (Hewlett-Packard). Signals on exposed X-ray films were passed through the beam three times, and the average of the readings was determined. A standard curve was incorporated into each membrane as described by Wygant and Nelson (1993) to account for the nonlinear response of the film. Total RNA was loaded evenly according to the intensity of ethidium bromide-stained genomic ribosomal RNA bands. To calculate the relative intensities, the readings from samples treated with 10 μM (N)-ABA were arbitrarily assigned a value of 100%. The remaining values were normalized with respect to the 100% value.

Results

Structural Analogs of ABA

The structural analogs used are depicted in Figure 1. The analogs comprise (*S*-) (+)-ABA (natural form = N-ABA) and (*R*-)(−)-ABA (unnatural form = U-ABA), the N- and U-enantiomers of abscisyl aldehyde (R = CHO), and the N- and U-enantiomers of abscisyl alcohol (R = CH_2OH) and the N- and U-enantiomers of methyl abscisate (R = CO_2CH_3). Each of the analogs was used in the germination assay as well as the gene expression assays. Depending on the assay concentrations in the range 0.05–30 μM were used. The highest concentration (30 μM) was chosen as the upper limit from previous studies which suggested the "saturation" of any gene expression effects at that concentration (Holbrook et al. 1991, Wilen et al. 1990).

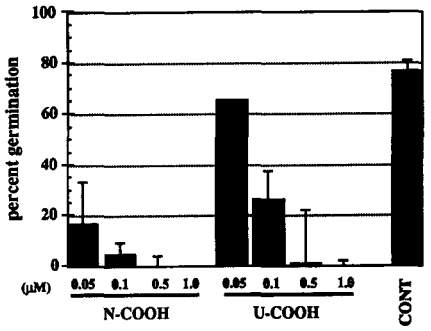

Fig. 2. Biologic activity of N-ABA and U-ABA as inhibitors of germination. Effects of various concentrations on the inhibition of germination are shown. Data are the mean ± S.E. of three replicates. In all cases 30 embryos were scored per treatment. Values represent the percentage of embryos that germinated in the presence of the various analogs. Embryos were treated with 0.05, 0.1, 0.5, or 1.0 mM N-ABA or U-ABA. *CONT*, a control treatment in which 30 embryos were treated with 10 μl of 70% ethanol in place of analog solution (this was the solvent for all analogs and the maximum volume used for a treatment).

Effect of (N)-ABA and ABA Analogs on Inhibition of Germination

The biologic activity of seven structural analogs of natural ABA was analyzed to determine each analog's ability to mimic N-ABA and inhibit germination. Microspore-derived embryos of *B. napus*, which respond to exogenous ABA in germination assays in a manner comparable with zygotic embryos (Wilen et al. 1991), were used as the biologic material. All structural analogs of ABA, regardless of their stereochemistry, affected precocious germination of microspore-derived embryos of *B. napus*. In the absence of growth regulators, within 4 days of plating on basal medium, 77% of the embryos turned green and developed roots. Application of a 1 μM concentration of any structural analog of ABA resulted in complete inhibition of precocious germination (Figs. 2 and 3). When intermediate concentrations were examined, N-ABA was more potent than U-ABA in inhibiting germination (Fig. 2). Methyl abscisate is thought to act as a slow release form of ABA (Walton 1983). Because of this characteristic we tested its ability to inhibit germination (Fig. 3). The two methyl ester analogs tested showed the same pattern as the acid enantiomers (Fig. 3). The N-enantiomer of the methyl ester was more active than the U-enantiomer; however, both were slightly weaker than their corresponding carboxylic acids (compare Fig. 3 with Fig. 2). The greater potency of acid over ester is in agreement with the results obtained by

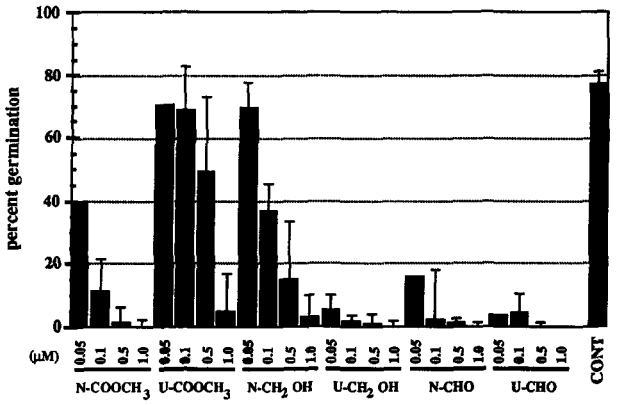

Fig. 3. Biologic activity of analogs of ABA as inhibitors of germination. Effects of various concentrations of the analogs are shown. Data are the mean ± S.E. of three replicates. Values represent the percentage of embryos that germinated in the presence of the various analogs. Embryos were treated with 0.05, 0.1, 05, or 1.0 mM analogs.

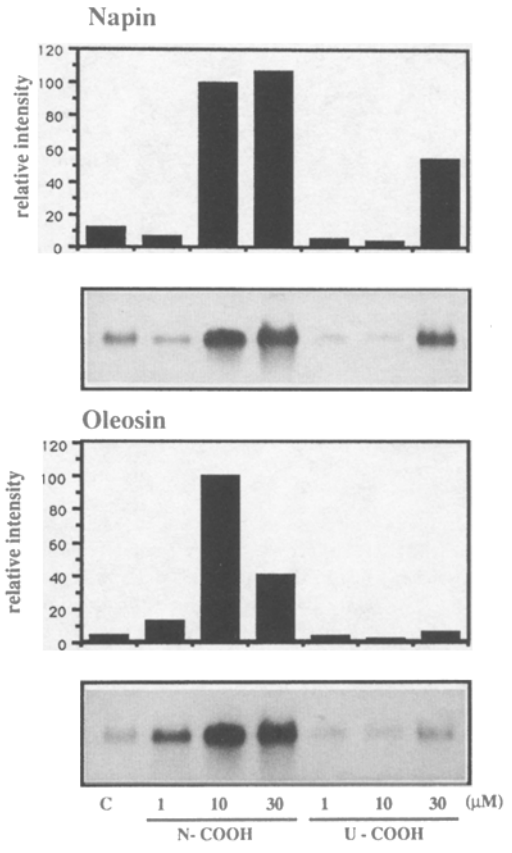

Fig. 4. Comparison of the effects of natural N-ABA or U-ABA at a concentration of 1, 10, or 30 mM on napin and oleosin mRNA accumulation in torpedo-staged microspore-derived embryos of *B. napus*. Transcript levels were quantified by scanning the densitometry of autoradiographs from Northern blots (25 mg of total RNA) probed with either a [^{32}P]dCTP-labeled napin or oleosin cDNA clone; relative intensities of the bands are shown. Total RNA was loaded evenly based on the intensity of ethidium bromide-stained genomic ribosomal bands. The graphs are representative of the results obtained in three independent experiments.

Walker-Simmons et al. (1992) on wheat embryos, although in that study both enantiomers were equally active. In contrast, the analogs in which the carboxylic acid function was replaced with an alcohol functional group showed the opposite pattern of inhibition (Fig. 3). Replacement with an aldehyde functional group yielded inhibitors of germination in both N- and U-forms. At a concentration of 0.05 μM *N*-abscisyl alcohol, 70% of the embryos germinated, whereas at the same concentration of the U-enantiomer, 5% of the embryos germinated. Of all of the analogs of ABA tested here, the aldehydes were uniformly the best inhibitors of germination at the lowest concentration used. When compared with natural N-ABA both the U-enantiomers of the aldehyde and alcohol were more effective than N-ABA in the germination inhibition assay (Figs. 2 and 3).

Effect of ABA Analogs on Embryo-specific ABA-responsive Gene Expression

The effect of the structural analogs of ABA on ABA-regulated gene expression was also examined. Their effect on napin and oleosin gene expression in microspore-derived embryos of *B. napus* was used in a semiquantitative biologic assay. Transcripts encoding both of these genes have been shown to respond to exogenous ABA in a dose-dependent manner (van Rooijen et al. 1992, Wilen et al. 1990, 1991). Microspore embryo cultures were treated with no analog or with varying concentrations (1, 10, 30 μM) of N- or U-ABA or one of the seven analogs. Total RNA was extracted from these embryos and analyzed by probing with either napin and oleosin cDNAs on northern blots. Signals from Northern blots were used to compare the expression levels using densitometry. The results of these experiments are depicted in Figures 4–6. As expected, N-ABA is more effective than U-ABA in inducing the expression of napin and oleosin genes (Fig. 4). From Figures 4–6 it is clear that all analogs, regardless of stereochemistry or functional group substitution, are agonistic. However, there are noticeable differences between their activities. In most instances for both napin and oleosin, the N-enantiomers that are similar to natural N-ABA at the junction of the ring and side chain yield a stronger response. The analogs that substitute an alcohol functional group for the carboxylic acid deviate from this rule with both N- and U-enantiomers showing strong agonism at 10 μM treatments (Fig. 6). There were minor differences in the responses of oleosin and napin transcription to these analogs. However, with regard to which ABA enantiomer is the most effective agonist of oleosin and napin gene expression, clearly

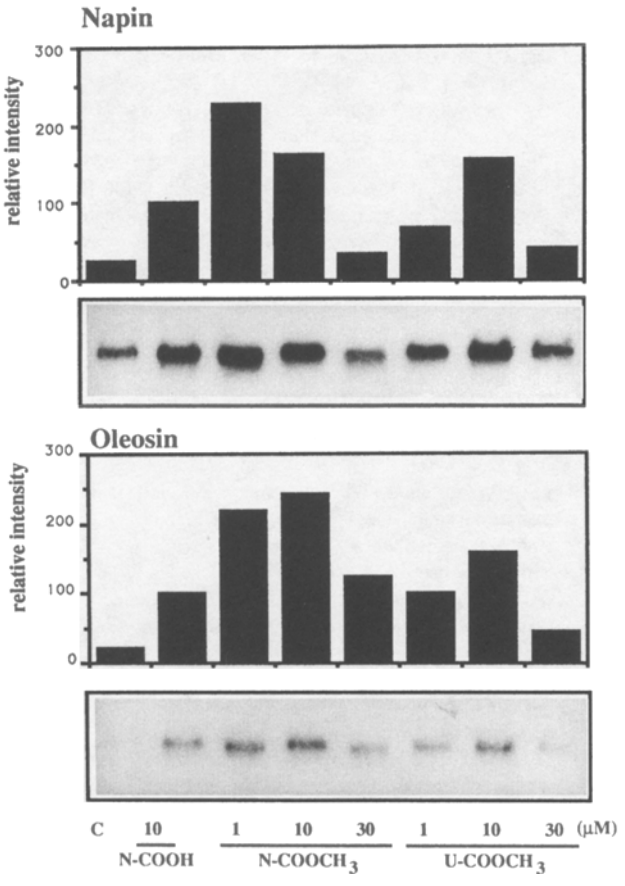

Fig. 5. Comparison of the effects of both the N- and U-stereoisomer of methyl abscisate (-$COOCH_3$) at a concentration of 1, 10, or 30 μM on napin and oleosin mRNA accumulation in torpedo-staged microspore-derived embryos of *B. napus*. *C*, a control treatment in which 30 embryos were treated with 10 μl of 70% ethanol in place of analog solution (this was the solvent for all analogs and the maximum volume used for a treatment). N-COOH is a positive control showing the effect of 10 μM N-ABA on the same batch of embryos. Transcript levels were quantified by scanning the densitometry of autoradiographs from Northern blots (25 mg of total RNA) probed with either a [^{32}P]dCTP-labeled napin or oleosin cDNA clone; relative intensities of the bands are shown. Total RNA was loaded evenly based on the intensity of ethidium bromide-stained genomic ribosomal bands. The graphs are scans of blots shown below them but are representative of the results obtained in three independent experiments.

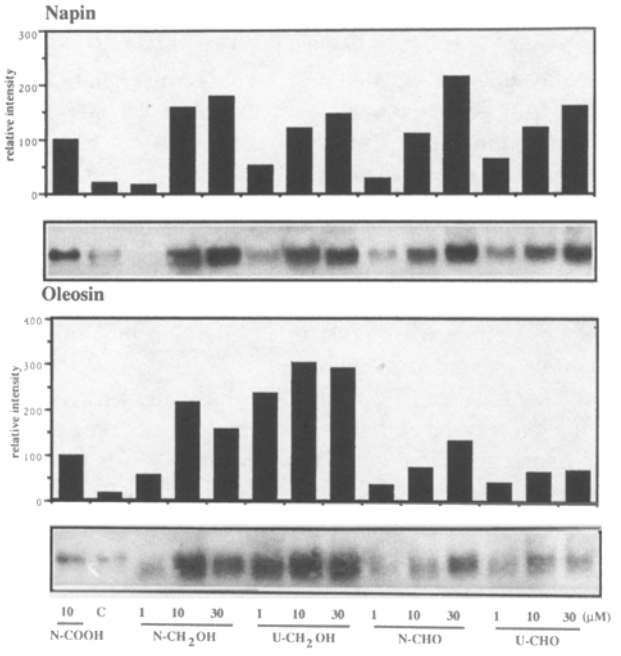

Fig. 6. Comparison of the effects of both the N- and U-stereoisomer of abscisyl alcohol and abscisyl aldehyde at a concentration of 1, 10, or 30 mM on napin and oleosin mRNA accumulation in torpedo-staged, microspore-derived embryos of *B. napus*. *C*, a control treatment in which 30 embryos were treated with 10 μl of 70% ethanol in place of analog solution (this was the solvent for all analogs and the maximum volume used for a treatment). N-COOH is a positive control showing the effect of 10 μM N-ABA on the same batch of embryos. Transcript levels were quantified by scanning the densitometry of autoradiographs from Northern blots (25 mg of total RNA) probed with either a [^{32}P]dCTP-labeled napin or oleosin cDNA clone; relative intensities of the bands are shown. Total RNA was loaded evenly based on the intensity of ethidium bromide-stained genomic ribosomal bands. The graphs are representative of the results obtained in three independent experiments.

N-ABA is more effective (Fig. 4). Among the synthetic analogs 10 μM N-methyl abscisate and 10 μM U-abscisyl alcohol most effectively induced oleosin mRNA accumulation (Figs. 5 and 6). Conversely, 1 μM N-methyl abscisate and 30 μM N-abscisyl aldehyde were the most effective at inducing napin mRNA accumulation (Figs. 5 and 6).

Discussion

Optically pure analogs of ABA (with respect to the C-1′ position) which have been modified at the C-1 position were used to determine the structural requirements of the ABA receptor(s) to elicit the various responses. All analogs, regardless of functional group substitution or stereochemistry, were effective at inhibiting precocious germination and stimulating the accumulation of oleosin and napin transcripts. In both assays U-ABA was active, although it is active only at higher concentrations than the N-enantiomer. It is possible that it acts directly on the ABA receptor or acts indirectly by increasing the endogenous pool of ABA at the site of inactivation. In *B. napus* we showed previously that the U-enantiomer of an acetylenic ABA analog caused a sevenfold increase in the endogenous pool of ABA (Wilen et al. 1993).

The high activity of the N-abscisyl alcohol and N-abscisyl aldehyde is intriguing given that they are natural intermediates in the biosynthesis of N-ABA (Taylor et al. 1988). Furthermore, in previous studies with racemic ABA alcohol and aldehyde, these compounds were both more potent than ABA in inhibiting germination of cress seed (Gusta et al. 1992). Abscisyl aldehyde is thought to be the immediate precursor to N-ABA, whereas abscisyl

alcohol is converted to N-ABA via a minor pathway (Rock et al. 1991). Whether the U-isomers of abscisyl alcohol and abscisyl aldehyde are also converted to U- or N-ABA is not yet known. With respect to oleosin mRNA accumulation, the U-enantiomers of abscisyl alcohol were more inductive than the N-enantiomer or natural ABA. The U-abscisyl alcohol was also extremely effective in inhibiting germination. The observation that in all of the assays performed, the aldehyde and alcohol are more effective may be a reflection of their increased uptake. Both are less polar than ABA and thus may be more permeable to membranes.

It has not been determined if the methyl esters are being hydrolyzed to the active acids or whether the esters are interacting directly with the receptor. When an ABA methyl ester was used as a growth inhibitor in *Phaseolus*, the conversion of methyl abscisate to N-ABA accounted for all of the activity of methyl abscisate (Walton and Sondheimer 1972). In wheat embryo germination, methyl abscisate was one tenth as active as ABA, suggesting that hydrolysis was relatively slow over the experiment (Walker-Simmons et al. 1992).

It is intriguing to note that there are some differences in the sensitivity of napin and oleosin genes to the analogs. For example, while the U-analogs were all somewhat effective in the gene expression assays, oleosin was particularly sensitive to the U-abscisyl alcohol even at concentrations of 1 μM. When the same blot was reprobed with a napin cDNA, it revealed a much lower level of response than did oleosin. In contrast, oleosin transcript accumulation was less sensitive to the abscisyl aldehyde series than that of napin. These differences are less noticeable at higher concentrations when, presumably, the effects are close to saturation. Recent work by Plant et al. (1994) indicated that the oleosin promoter was structurally very different from storage protein promoters in the Brassicaceae. It is also possible that the differences in response to the analogs are an indication of their differential effects on the endogenous pool of ABA. In that case, the observed difference in gene expression might be an indication of their different levels of sensitivity to the endogenous concentration of N-ABA. However, using an immunoassay system, Walker-Simmons et al. (1992) observed that three structural analogs of ABA, including U-ABA, had little effect on the endogenous pool of N-ABA in wheat. Whether this also holds true in *B. napus* embryos has not been determined, although other U-ABA analogs can cause an increase in the pool of N-ABA in *Brassica* (Wilen et al. 1993).

Overall, it is clear from this work that resolved optical isomers of ABA and its analogs can result in dramatically different activities in a variety of assays. The potential for the compounds studied in this report for use as agonists or antagonists in whole plants is now under investigation.

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