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Competitive Inhibition of Abscisic Acid-Regulated Gene Expression by Stereoisomeric Acetylenic Analogs of Abscisic Acid[1]

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The properties of two enantiomeric synthetic acetylenic abscisic acid (ABA) analogs (PBI-51 and PBI-63) in relation to ABA-sensitive gene expression are reported. Using microspore-derived embryos of *Brassica napus* as the biological material and their responsiveness to ABA in the expression of genes encoding storage proteins as a quantitative bioassay, we measured the biological activity of PBI-51 and PBI-63. Assays to evaluate agonistic activity of either compound applied individually showed a dose-dependent increase in napin gene expression on application of PBI-63. Maximal activity of about 40 μM indicated that PBI-63 was an agonist, although somewhat weaker than ABA. PBI-63 has a similar stereochemistry to natural ABA at the junction of the ring and side chain. In contrast, PBI-51 showed no agonistic effects until applied at 40 to 50 μM. Even then, the response was fairly weak. PBI-51 has the opposite stereochemistry to natural ABA at the junction of the ring and side chain. When applied concurrently with ABA, PBI-63 and PBI-51 had distinctly different properties. PBI-63 (40 μM) and ABA (5 μM) combined gave results similar to the application of either compound separately with high levels of induction of napin expression. PBI-51 displayed a reversible antagonistic effect with ABA, shifting the typical ABA dose-response curve by a factor of 4 to 5. This antagonism was noted for the expression of two ABA-sensitive genes, napin and oleosin. To test whether this antagonism was at the level of ABA recognition or uptake, ABA uptake was monitored in the presence of PBI-51 or PBI-63. Neither compound decreased ABA uptake. Treatments with either PBI-51 or PBI-63 showed an effect on endogenous ABA pools by permitting increases of 5- to 7-fold. It is hypothesized that this increase occurs because of competition for ABA catabolic enzymes by both compounds. The fact that ABA pools did not decrease in the presence of PBI-51 suggests that PBI-51 must exert its antagonistic properties through direct competition with ABA at a hormone-recognition site.

The plant hormone ABA has been implicated in a variety of developmental and stress-related responses in higher plants. These responses include relatively rapid effects on ion fluxes across plant plasma membranes (Kasamo, 1981; MacRobbie, 1981), which are thought to occur independently of altered gene expression (McAinsh et al., 1991) and other longer term effects that involve modulation of expression of a variety of genes, many of which show tissue or organ specificity (Hetherington and Quatrano, 1991; Bray, 1991).

ABA is believed to be involved in a number of aspects of seed development and maturation including the regulation of expression of storage protein genes in soybean (Bray and Beachy, 1985), rapeseed (Finkelstein et al., 1985; Wilen et al., 1990), and corn (Kriz et al., 1990). Apart from storage protein genes, several other genes involved in seed development appear to respond to ABA. These include the late embryogenesis-abundant genes (Dure et al., 1989), genes encoding inhibitors of germination enzymes such as the α-amylase inhibitor protein (Leah and Mundy, 1989), and genes specifying major oil-body proteins (van Rooijen et al., 1992). Many of these seed-specific responses to applied ABA are also mimicked by osmotic stress (Zeevaart and Creelman, 1988). However, the relationship between osmotic stress and ABA in the regulation of gene expression is not clear. Studies of the expression of β-conglycinin in soybean suggested that the effect of osmoticum was mediated by elevated ABA content of the embryos (Bray and Beachy, 1985). In rapeseed and mustard, Finkelstein and Crouch (1986) and Fischer et al. (1987), respectively, reported results consistent with the independent action of osmoticum and ABA on storage protein gene expression. These divergent conclusions were all obtained from experiments based on the blockage of ABA biosynthesis using the carotenoid biosynthetic inhibitor fluridone.

Alternative approaches involve the use of mutants in ABA biosynthesis or action (Koornneef et al., 1989). These mutants have not yet yielded a resolution to the above dichotomy, although it is clear that ABA-deficient mutants show reduced storage protein accumulation but not complete loss of this property.

We reasoned that many of these questions might be resolved if we had a reversible, competitive inhibitor of ABA action. This would enable us to block the contribution of ABA to gene expression provoked by high osmoticum and

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Abbreviation: NLN, Lichter medium.

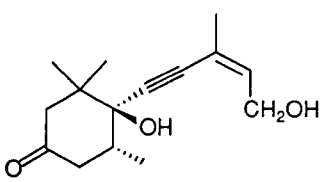
thus might reveal the role, if any, of ABA in vivo. A group of ABA analogs has been identified with physiological effects agonistic (Reaney et al., 1990) or antagonistic to ABA in a variety of biological systems (Abrams and Gusta, 1989; Reaney et al., 1990; Walker-Simmons et al., 1992). Of particular interest was the activity of acetylenic analogs of ABA, which reversed the inhibitory effects of ABA on cress seed germination (Reaney et al., 1990).

In this report, we discuss the properties of two acetylenic ABA analogs (PBI-51 and PBI-63) that appear to act as an antagonist and agonist of ABA, respectively, and their potential for use in the understanding of ABA action. To assist in this analysis, we have exploited the properties of microspore-derived embryos of *Brassica napus*, which give well-defined responses in embryo-specific gene expression to applied ABA, osmoticum, and jasmonic acid (Taylor et al., 1990; Wilen et al., 1990, 1991).

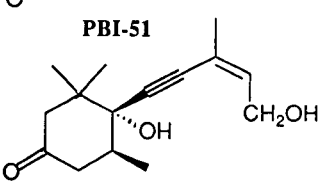
MATERIALS AND METHODS

Chemical Synthesis

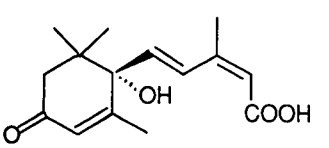
PBI-51 [(−)-4(Z)-(4*S*,5*R*)-4-hydroxy-4-(5-hydroxy-3-methylpent-3-en-1-ynyl)-3,3,5-trimethylcyclohexanone] and PBI-63 [(+)-4(Z)-(4*R*,5*S*)-4-hydroxy-4-(5-hydroxy-3-methylpent-3-en-1-ynyl)-3,3,5-trimethylcyclohexanone] were synthesized by the method of Lamb and Abrams (1990). The structures of these two compounds, both of which exhibit a *cis* configuration between the methyl at C-2′ and the carbon side chain, are shown in Figure 1. The stereochemistry at C-1′ of PBI-63 is the same as natural (*S*)-ABA. PBI-51 is similarly related to (*R*)-ABA. The resolution of (*R*)- and (*S*)-enantiomers of ABA was performed as described by Dunstan et al. (1992).



PBI-51



PBI-63



(S)-ABA

Figure 1. Structures of PBI-51, PBI-63, and natural (*S*)-ABA, showing the stereochemistry around the chiral centers. PBI-63 is stereochemically similar to (*S*)-ABA, and PBI-51 is similar to unnatural (*R*)-ABA.

Plant Material

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

Microspore Culture

Buds (2–3 mm long) were collected from the cold-treated plants, and the microspores were isolated by grinding in NLN (Lichter, 1982) with 13% Suc, washed, pelleted, and then suspended in 40% Percoll containing 13% Suc. After the tubes were overlaid with a hormone-free NLN, they were spun (220*g*, 10 min), and the cells at the NLN/Percoll interface were collected. After these cells were washed, they were pelleted and resuspended in the NLN, and 10 mL were plated at a density of 3×10^4 cells mL^{-1} in Petri dishes (100 × 15 mm). The microspores were incubated in the dark for 4 d at 30°C and then transferred to 25°C. They were subcultured after 7 d with fresh medium with a 4-fold dilution. For harvesting, the embryos were sieved through various size nylon meshes to isolate the morphological stages. After isolation, treatment with (*RS*)-ABA (except where noted in "Results") was performed by the addition of 10 μL of a 10 mM stock solution of (*RS*)-ABA (Sigma) dissolved in 70% ethanol to 10 mL of culture medium containing the embryos. Control embryos were treated with 10 μL of 70% ethanol. Treatment with sorbitol was performed by plating isolated embryos directly in NLN containing both 13% Suc and 12.5% sorbitol. In the ABA inhibition experiments using PBI-51 and PBI-63, the microspore-derived embryos were pretreated with the PBI compounds. This pretreatment involved adding the appropriate concentration of PBI compounds to the NLN medium with the microspore-derived embryos and incubating the embryos with the chemical for 2 to 3 h. The appropriate concentration of ABA was then added to the medium that already contained the PBI compounds.

RNA Extractions

Total RNA was extracted by the method of Natesan et al. (1989). The RNA was quantitated by A_{260} measurements and by ethidium bromide staining intensity and then stored as an ethanol precipitate at −20°C until use.

Northern and RNA Dot Blot Hybridization

Total RNAs were separated by electrophoresis on formaldehyde gels (6% formaldehyde, 1 × Mops, 1.2% agarose) by running at 80 V for 3 to 4 h. The RNA was transferred to Gene Screen Plus (New England Nuclear-Dupont) membranes by capillary blotting with 20× standard sodium citrate (1× standard sodium citrate = 150 mM sodium chloride, 15 mM sodium citrate) for 24 h. The RNA was fixed onto the membranes by UV irradiation for 5 min and stored in Seal-a-Meal bags at −20°C. The filters were prehybridized in 25 mL of hybridization solution (50% formamide, 5× SSPE [1× SSPE = 150 mM sodium chloride, 10 mM sodium phosphate,

1 mM EDTA], 1% SDS, 10% dextran sulfate, 5× Denhardt's solution) with 5 mg of yeast tRNA at 43°C for at least 6 h. Hybridization was carried out in fresh hybridization solution under the same conditions for at least 16 h with 5 mg of denatured yeast tRNA and 50 ng of a radiolabeled napin cDNA probe (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}. The membranes were then washed twice in 2× SSPE, 2% SDS at room temperature for 10 min per wash, followed by two washes with 0.1× SSPE, 0.1% SDS at 65°C for 15 min per wash. The filters were exposed to XAR 5 film at −70°C for varying times.

Dot Blots and Densitometry

Total RNA samples were prepared as described for northern analysis. After the samples were denatured, 5-μL aliquots were spotted directly onto Gene Screen Plus nylon membranes, UV irradiated, and air dried. The membranes were hybridized as described above. Densitometry was performed using a scanning densitometer (Hoefer Scientific) linked to an integrator (Hewlett-Packard). Exposed x-ray films were passed through the beam three times, and an average reading was taken. Densitometry values were obtained by scanning the autoradiograms from the membranes that had been hybridized with the napin probe. These values were then normalized by dividing by the values obtained from scans of the same membranes hybridized with the constitutive clone pGS43 (Harada et al., 1989) to account for any discrepancies in RNA loadings. To calculate relative intensities, the highest reading from an ABA-treated sample in each series was arbitrarily assigned a value of 100%. The remaining points in each series were normalized with respect to the 100% value. Where conflicting values were obtained between the same samples analyzed by dot blots and northern blots, the value for the northern blot was taken preferentially. The reason for this choice was that nonspecific binding of the labeled probe to the rRNAs occasionally occurred. This type of nonspecific binding could not be distinguished from the binding of the probe to transcripts in dot blots but was readily distinguishable in northern analysis.

ABA Extraction and GC-MS Analysis

Microspore-derived embryos were lyophilized for 3 to 5 d before extraction. The weighed tissue was powdered in liquid N_2 and extracted with cold 80% aqueous methanol. [^{3}H](*RS*)-ABA (100,000 dpm; specific activity, 69 Ci mmol^{-1}; Amersham) and [$^2H_6$](*RS*)-ABA (50 ng; Martial Saugy and Laurent Rivier, Université de Lausanne, Lausanne, Switzerland) were added to the methanol extract. The extract was filtered through a Whatman No. 1 filter and reduced in vacuo for 24 h. The dried residue was dissolved in 500 μL of 1% acetic acid (pH 3.0) and then partitioned twice with equal volumes of ethyl acetate:hexane (95:5) saturated with formic acid. After the residue was dried with N_2, it was dissolved in 10% methanol and separated on a reversed-phase μBondapak C_{18} HPLC column (Koshioka et al., 1983). The radioactive fraction and the fraction running just before the labeled ABA were collected to account for the differential chromatography of ABA and [$^2H_6$]ABA (Wodzicki et al., 1987) combined, and solvent was removed completely in vacuo. The samples were redissolved in 10 μL of 100% methanol and methylated with 500 μL of diazomethane at room temperature. The samples were then analyzed by GC-MS-selected ion monitoring using the procedure of Ross et al. (1987).

ABA Uptake Experiments

Approximately 4000 torpedo-stage microspore-derived embryos were suspended in 5 mL of NLN without or supplemented with 10 or 40 μM PBI-51 or PBI-63 in 5-cm diameter Petri plates. After a 2-h preincubation to equilibrate the embryos, 100,000 dpm of hexatritiated (*RS*)-ABA (6 Ci mol^{-1}) were added to each plate. The embryos were then incubated for an additional 24 h at 24°C with gentle agitation (30 rpm) on a reciprocal shaker. After this period, the embryos were separated from the medium through coarse nylon sieves (approximately 350 μm) and then washed three times with NLN medium to remove surface radiolabel. This washing procedure was optimized to ensure that only insignificant amounts of contamination, due to adsorption of radiolabel to the embryo surface, remained. The washed embryos were immediately lyophilized, weighed, and extracted four times by grinding in 80% methanol. Radioactivity in aliquots of the methanolic filtrate was then estimated by liquid scintillation counting.

RESULTS

Effects of (*R*)- and (*S*)-ABA on Storage Protein Gene Expression

Cultures of microspore-derived embryos were evaluated in a napin gene expression assay to determine the effects of natural (*S*)- and unnatural (*R*)-ABA. Embryos were treated for 48 h in the absence of exogenous ABA, in the presence of racemic ABA (10 μM) or in the presence of (*R*)- or (*S*)-ABA at three different concentrations (1, 10, 30 μM). RNA was extracted from these embryos and analyzed by northern blots using napin cDNA as a probe. The results of this experiment are depicted in Figure 2. From this figure it is clear that natural (*S*)-ABA and unnatural (*R*)-ABA are both agonistic in this system. However, there is a noticeable difference between (*R*)- and (*S*)-ABA in the dose response. (*R*)-ABA is a much weaker agonist than (*S*)-ABA, with significant stimulation of napin gene expression occurring only in 30 μM treatments. Conversely, (*S*)-ABA is promotive even in treatments of 1 μM. The effect of 10 μM (*S*)-ABA is quite similar to that for the racemic mixture. Given this observation and the relatively small amounts of resolved ABA that were available to us at the initiation of these experiments, we conducted the remaining experiments on ABA antagonism with racemic ABA.

Effects of PBI-51 and PBI-63 on Storage Protein Gene Expression

PBI-51 and PBI-63 (Fig. 1) are optically pure mirror-image forms. Given the antagonism of a racemic mixture of these

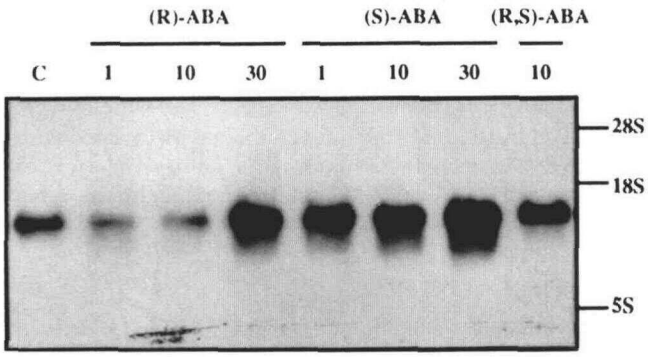


Figure 2. Effect of (*R*)-, (*S*)-, and (*RS*)-ABA on the accumulation of napin transcripts in torpedo-stage microspore-derived embryos of *B. napus*. Northern analysis of total RNA (30 μg/lane) using a [^{32}P]-dCTP-labeled napin cDNA clone as a probe. Lane designations represent the concentrations (in μM) of the compound used. Lane C represents control (untreated) embryos. All treatments were for 48 h.

two ABA analogs to a variety of ABA responses (Reaney et al., 1990; R.W. Wilen, S.R. Abrams, M.M. Moloney, unpublished data), it was suspected that at least one of the isomers might be an ABA antagonist. Furthermore, consistent with the results shown in Figure 2, it had been noted by Gusta et al. (1992) that natural (*S*)-ABA acted as a strong inhibitor of cress seed germination, whereas (*R*)-ABA was only weakly agonistic, indicating the importance of the stereochemistry of these isomers. Therefore, we evaluated the abilities of the enantiomers PBI-51 and PBI-63 to stimulate or inhibit expression of ABA-sensitive genes in the microspore-derived embryos. To do this, torpedo-stage embryos were treated with different concentrations of either PBI-51 or PBI-63 for 48 h. After this time, total RNA was isolated and used to obtain northern blots corresponding to each treatment. These were then probed with radiolabeled napin cDNA. The results of these experiments are shown in Figure 3. It is clear that the two enantiomers show very distinct differences in their activity. PBI-63, which is of similar stereochemistry to natural

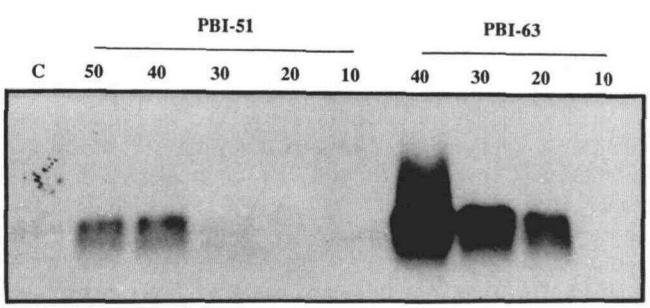


Figure 3. Effect of PBI-51 and PBI-63 on the accumulation of napin transcripts in torpedo-stage microspore-derived embryos of *B. napus*. Northern analysis of total RNA (30 μg/lane) using a [^{32}P]dCTP-labeled napin cDNA clone as a probe. Lane designations represent the concentrations (in μM) of PBI-51 or PBI-63 used to treat microspore-derived embryos. Lane C, Microspore-derived embryos cultured on basal medium. All treatments were for 48 h.

ABA, shows a dose-dependent stimulation of napin mRNA accumulation. This is very similar to the response found with ABA in an equivalent concentration range (Wilen et al., 1990). PBI-51, on the other hand, shows no activity in this gene expression assay until 40 μM, and then the stimulation observed is very weak by comparison with PBI-63 at the equivalent concentration.

Interactions of PBI-63 and PBI-51 with ABA-Stimulated Gene Expression

The differential activity of PBI-51 and PBI-63 on napin gene expression suggested that they might also have different effects on embryos in combined treatments with ABA. Therefore, we treated the embryos with 40 μM PBI-51 or PBI-63, and in a series of treatments, we added progressively higher concentrations of ABA to the medium. The effect of these mixed treatments on napin gene expression was monitored by northern blotting. The results are shown in Figure 4. Again, the difference in effect between the two enantiomers is evident. In both the presence and absence of ABA, PBI-63 is stimulatory to napin gene expression consistent with the results shown in Figure 3. However, the presence of PBI-51 caused a shift in the response of the embryos to applied ABA so that at 5 μM ABA in the presence of PBI-51, there was no detectable increase in signal above control levels, whereas at 5 μM ABA alone there was a strong stimulation of napin

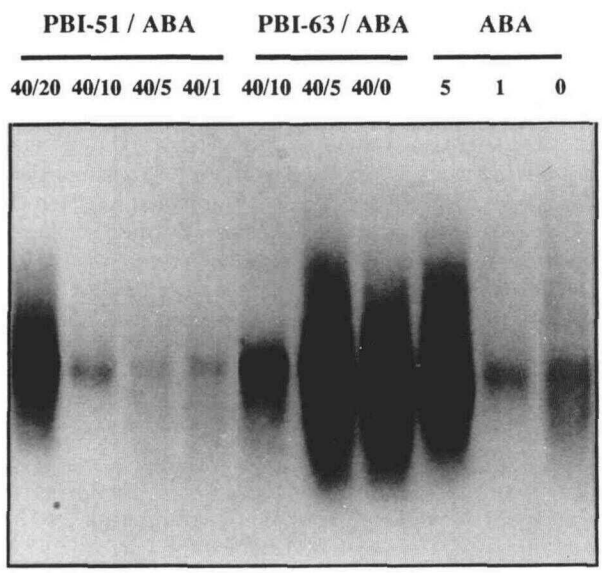


Figure 4. Interactive effects of combined ABA and PBI analog treatments on napin transcript accumulation in torpedo-stage microspore-derived embryos of *B. napus*. Northern analysis of total RNA (30 μg/lane) using a [^{32}P]dCTP-labeled napin cDNA clone as a probe. Lane designations represent the concentrations of each component of the treatment (in μM). In the case of combined treatments, the first number represents the concentration of the PBI analog, and the second number represents the ABA concentration. Lanes marked ABA correspond to control embryos that received only ABA but no PBI analogs. All treatments were for 48 h (after a 2-h preincubation with the PBI analogs; see "Materials and Methods").

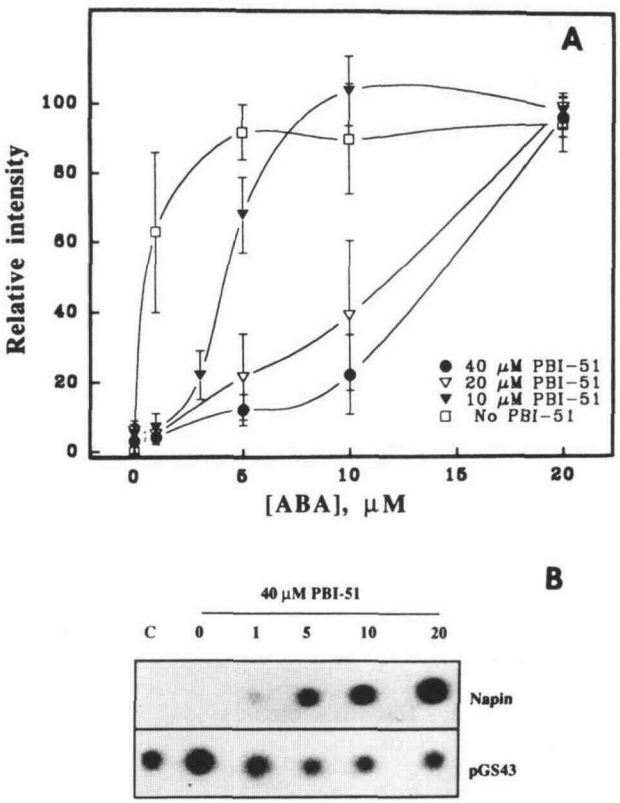


Figure 5. Effect of various concentrations of PBI-51 on ABA-induced napin transcript accumulation in torpedo-stage microspore-derived embryos of *B. napus*. A, Relative intensities of densitometry scans of autoradiographs from dot blots of total RNA (5 μg/dot) probed with a [^{32}P]dCTP-labeled napin cDNA clone are plotted. Error bars represent the SD in the 40 μM PBI-51-treated embryos. In the 20 and 10 μM PBI-51-treated embryos, the error bars represent either the range (duplicate assays) or the SD (triplicate assays), depending on the data point. B, Representative dot blot from which the graph in A was constructed. The top row of RNA dots (approximately 5 μg) was probed with napin. The bottom row is the same set of RNA dots reprobed with pGS43 (Harada et al., 1989), a gene expressed constitutively in developing embryos. The signal obtained from this hybridization was used to correct for slight variations in RNA loadings.

mRNA accumulation. As the concentration of applied ABA is increased, the stimulation of napin gene expression is reestablished, suggesting that PBI-51 is exerting its effects in a reversible manner.

Reversible Inhibition of ABA Action by PBI-51

The apparent reversibility of PBI-51-dependent inhibition of ABA action required further characterization. We decided to estimate the concentration dependency of this inhibition using several combinations of ABA and PBI-51 concentrations. This was done for convenience using dot blots rather than northern blots. To account for relative RNA loadings on these dot blots, the plasmid pGS43 was used as a constitutive probe (Harada et al., 1988). Multiple dot blots were used for quantitative autoradiography. Relative intensities of hybridization were measured using a scanning densitometer.

Graphs depicting these results for napin expression are shown in Figure 5A. A representative dot blot used to obtain this graph is also depicted in Figure 5B. As can be seen, for this ABA-sensitive gene, there is a concentration-dependent reversible inhibition of expression in the presence of PBI-51. In embryos treated with ABA alone, maximal expression of napin is achieved at concentrations of 5 μM or higher. In the presence of 10, 20, or 40 μM PBI-51, this ABA effect is drastically reduced. The inhibition by PBI-51 is not, however, irreversible. Increases in ABA concentration reverse the inhibition so that at 20 μM ABA, even in the presence of 40 μM PBI-51, the napin mRNA accumulation returns to the maximal value. This experiment was also repeated for a second ABA-sensitive gene that encodes the major oil-body protein (oleosin) of rapeseed with very similar results (Fig. 6). These

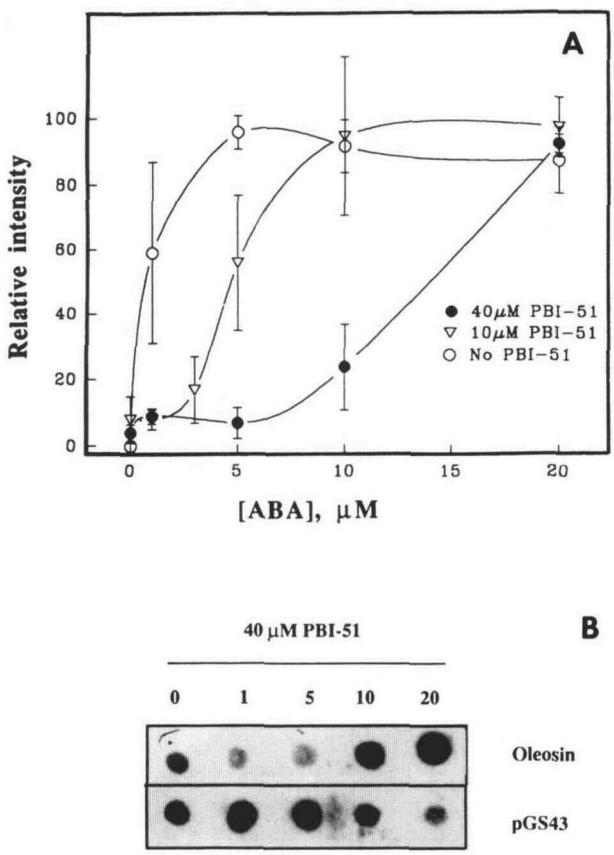


Figure 6. Effect of various concentrations of PBI-51 on ABA-induced oleosin transcript accumulation in torpedo-stage microspore-derived embryos of *B. napus*. A, Relative intensities of densitometry scans of autoradiographs from dot blots of total RNA (5 μg/dot) probed with a [^{32}P]dCTP-labeled oleosin cDNA clone are plotted. In 10 μM PBI-51-treated embryos, the error bars represent either the range (duplicate assays) or the SD (triplicate assays), depending on the data point. B, Representative dot blot from which the graph in A was constructed. The top row of RNA dots (approximately 5 μg) was probed with oleosin. The bottom row is the same set of RNA dots reprobed with pGS43 (Harada et al., 1989), a gene expressed constitutively in developing embryos. The signal obtained from this hybridization was used to correct for slight variations in RNA loadings.

Table I. *The effect of PBI-51 and -63 on uptake of ABA into microspore-derived embryos of B. napus*

Numbers represent the mean fraction of the applied radiolabel (100,000 dpm) extracted from washed embryos after a 24-h treatment. The means ± SE were calculated from triplicate assays. Because some differential growth occurs in the embryos during the treatments, the mean fraction of label was also expressed normalized to the final dry weight of the embryos using the mean final weight of the treatment as the point of normalization. Expressing these data in this way does not alter the trend found in the previous column.

Treatment	Fraction of Label in Embryos	Fraction of Label in Embryos (Normalized by Dry Wt)
Control (no analog)	0.39 ± 0.05	0.33 ± 0.04
PBI-63 (10 μM)	0.54 ± 0.02	0.43 ± 0.02
PBI-63 (40 μM)	0.47 ± 0.02	0.48 ± 0.02
PBI-51 (10 μM)	0.45 ± 0.02	0.55 ± 0.025
PBI-51 (40 μM)	0.35 ± 0.08	0.46 ± 0.1

data were obtained by reprobing the membranes used to obtain hybridization results shown in Figure 5 using a cDNA clone for *B. napus* oleosin (van Rooijen et al., 1992).

PBI-51 and PBI-63 Do Not Act Primarily on ABA Transport into Cells

Because the above experiments all involve an exogenous supply of (*RS*)-ABA and a PBI compound, it was considered important to distinguish between competition for uptake rather than competition at the site of action of ABA. To test this possibility, PBI-51 and PBI-63 were used in uptake experiments to determine any effects by these compounds on ABA influx to the embryos. Embryos were treated with 1 μCi of hexatritiated (*RS*)-ABA (specific activity, 2.6 Ci mol^{-1}) prepared by the method of Bonnafous et al. (1971) in the presence (10 or 40 μM) or absence of PBI-51 or PBI-63. Embryos were incubated for 24 h, and the percentage of total label taken up (normalized for weight of embryos) was estimated. The results of these experiments are summarized in Table I. From these experiments it is clear that there is no inhibition of ABA uptake in the presence of 10 or 40 μM concentrations of these analogs. There is a small, but statistically significant, increase in ABA uptake in the presence of micromolar concentrations of the analogs, but the amount of ABA involved would be unlikely to have any effects on gene expression (Wilen et al. 1990).

Effects of PBI-51 and PBI-63 on Endogenous ABA Pools

One further possible explanation for the inhibitory effect of PBI-51 on ABA responses that could not be excluded from the above data was that PBI-51 somehow promoted the degradation of applied or endogenous ABA to less active metabolites such as phaseic and dihydrophaseic acids, thus reducing the overall response to applied ABA. To determine whether these analogs have any effect on ABA levels, we measured endogenous ABA pools, using GC-MS, in microspore-derived embryos treated with either PBI-51 or PBI-63. The results of these measurements are summarized in Table II. It is clear from these results that there is a similar effect on ABA pools in these embryos after treatment with either PBI-51 or PBI-63. In both cases there was a significant increase in the pool of endogenous ABA in the presence of these compounds.

DISCUSSION

In this study we measured the effects of two enantiomers of an acetylenic ABA analog. We found that one of these analogs (PBI-63) acts as an ABA agonist in the napin and oleosin gene expression assay. PBI-63 has a similar stereochemical configuration to natural ABA. It is, therefore, reasonable to propose that PBI-63 acts at the site of action of ABA. The enantiomer PBI-51, which has the opposite stereochemistry to natural ABA, showed no activity in the equivalent assay except at higher concentrations, and even then the response was rather weak.

Given the critical role of stereochemistry in these responses, we evaluated the potency of natural (*S*)- and unnatural (*R*)-ABA in the napin gene expression assay (Fig 2.). It is noteworthy that both ABA enantiomers are agonistic in this assay; however, (*S*)-ABA is clearly more active at lower concentrations (1–10 μM) than (*R*)-ABA. It is interesting to note that in treatments of 1 and 10 μM (*R*)-ABA there is a noticeable depression in the basal level of napin expression, suggesting that (*R*)-ABA, although a weak agonist, may be an effective competitor at the site of action of natural ABA.

In studies using the acetylenic ABA analogs, PBI-51 displayed low potency as an agonist. Nevertheless, PBI-51 is not devoid of biological activity. When PBI-51-pretreated (2 h) embryos were challenged with ABA at concentrations that would normally lead to maximum stimulation of napin mRNA accumulation, there was a much reduced level of gene expression. However, this inhibition could be overcome by the application of higher concentrations of ABA. The graphs depicted in Figures 5A and 6A, using two ABA-sensitive gene-expression systems, show dose-response shifts typical of reversible, competitive inhibition. It is possible that the small stimulation seen at the higher concentrations of PBI-51 is due to the "sparing" effect on endogenous ABA pools shown in Table II. As endogenous ABA is allowed to

Table II. *Effect of PBI-51 or PBI-63 on endogenous ABA content in microspore-derived embryos of B. napus*

Treatments at the concentrations indicated were conducted for 24 h after which the embryos were frozen, lyophilized, extracted, and quantitated by GC-MS as described in "Materials and Methods." Treatments were performed in triplicate (except for PBI-51 at 10 μM for which one replicate was lost).

Treatment	Mean ABA Content ± SE
	ng g^{-1} dry wt
Control (no analog)	162 ± 38
PBI-63 (10 μM)	410 ± 136
PBI-63 (40 μM)	677 ± 70
PBI-51 (10 μM)	755 ± 113
PBI-51 (40 μM)	937 ± 64

accumulate, presumably due to decreased turnover, this may provide enough endogenous ABA to compete with the applied PBI-51 and thus reestablish a small ABA effect. Alternatively, PBI-51 may simply be a very weak agonist compared with ABA but one that has a fairly high affinity for the ABA site of action. Thus, at lower concentrations it competes with ABA for this site but does not itself promote a great deal of gene activation. At saturating concentrations, however, it may produce a real, albeit weak, agonistic response. This interpretation is consistent with the results reported in Figure 2 when the comparison between the activity of (*R*)- and (*S*)-ABA is made.

The possibility that the inhibition of ABA action by PBI-51 is the result of competition at a site of uptake rather than site of action was investigated. The results from Table I indicate no inhibition of ABA uptake by either PBI-51 or PBI-63. At higher concentrations of the analogs a small increase in uptake was found, but it is unlikely that this could have a major physiological effect because generally the dose-response to ABA in this system is logarithmic (Wilen et al., 1990). These results are also in agreement with the view that ABA uptake is not mediated by a surface carrier saturable in the micromolar range.

The antagonistic analog PBI-51 does not act by means of an increase in ABA degradation in these cells (Table II). Thus, the apparent antagonism of PBI-51 against ABA action is most probably explained at the level of competition for an ABA-binding protein. It is noteworthy that both PBI-51 (antagonist) and PBI-63 (agonist) had the same effect on endogenous ABA pool size, causing a 5- to 7-fold increase in endogenous ABA. This may be indicative of competition with ABA at the active site of a degradative enzyme, although this remains to be established.

In this report, we have provided direct evidence that PBI-51 acts as a reversible, competitive inhibitor of ABA action. The use of a specific competitor such as PBI-51 may help to resolve some of the ambiguities that arise from the use of carotenoid biosynthetic inhibitors, such as fluridone, which inhibit other processes in addition to ABA biosynthesis (Fong et al., 1983). We are using PBI-51 and other ABA homologs to help to distinguish the role of ABA during seed development from that of other natural products known to affect gene expression in seeds, such as jasmonic acid (Wilen et al., 1991). Furthermore, we expect that these analogs will find additional uses in studies on ABA receptors or ABA signal transduction.

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