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Abscisic Acid Structure-Activity Relationships in Barley Aleurone Layers and Protoplasts[1]

Biological Activity of Optically Active, Oxygenated Abscisic Acid Analogs

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Optically active forms of abscisic acid (ABA) and their oxygenated metabolites were tested for their biological activity by examining the effects of the compounds on the reversal of gibberellic acid-induced α-amylase activity in barley (*Hordeum vulgare* cv Himalaya) aleurone layers and the induction of gene expression in barley aleurone protoplasts transformed with a chimeric construct containing the promoter region of an albumin storage protein gene. Promotion of the albumin storage protein gene response had a more strict stereochemical requirement for elicitation of an ABA response than inhibition of α-amylase gene expression. The naturally occurring stereoisomer of ABA and its metabolites were more effective at eliciting an ABA-like response. ABA showed the highest activity, followed by 7′-hydroxyABA, with phaseic acid being the least active. Racemic 8′-hydroxy-2′,3′-dihydroABA, an analog of 8′-hydroxyABA, was inactive, whereas racemic 2′,3′-dihydroABA was as effective as ABA. The differences in response of the same tissue to the ABA enantiomers lead us to conclude that there exists more than one type of ABA receptor and/or multiple signal transduction pathways in barley aleurone tissue.

The plant hormone ABA causes a number of distinct and varied physiological responses in plants, ranging from the control of stomatal aperture and enhancement of adaptation to various stresses, to an involvement in bud and seed dormancy and other general effects on growth (Zeevaart and Creelman, 1988). The response to the hormone can be relatively fast, as in the closure of stomata caused by changes in ion movement across the guard cell, or slow, as in the case of responses that require protein synthesis. These varying responses have led Milborrow (1980) to suggest that the receptor site for the two may differ. Recently, Allan et al. (1994) provided evidence to suggest that there may be two transduction pathways mediating ABA effects in guard cells.

Synthetic analogs of ABA frequently have been used to acquire information concerning the nature of the ABA receptor site (Walton, 1983). Many of the earlier studies of the structural requirements for biological response of the ABA molecule involved radical changes in the structure of the molecule. More recently, it has been possible to synthesize analogs that have been modified at a specific carbon atom to determine subtle effects of changes on activity (Churchill et al., 1992; Walker-Simmons et al., 1992). In this way, it was shown that the *trans* double bond of the dienoic side chain of ABA could be replaced with an acetylenic linkage with no change in biological activity and that saturation of the cyclohexenone ring double bond did not reduce the expression of some ABA responses. Of particular interest is the biological effectiveness of the two ABA stereoisomers. In some situations, such as the stomatal response (Kriedemann et al., 1972) or inhibition of root growth (Sondheimer et al., 1971), the natural form (+)-(*S*)-ABA is more effective than (−)-(*R*)-ABA. In other instances, such as inhibition of germination (Walker-Simmons et al., 1992) or inhibition of GA-stimulated synthesis and release of α-amylase in barley (*Hordeum vulgare* L.) half seeds (Sondheimer et al., 1971), no difference in response has been observed for the two stereoisomers. The spatial requirements at C-1′, therefore, appear to be a function of the biological response studied, suggesting the existence of more than one ABA receptor and/or multiple signal transduction pathways.

It is known that natural (+)-(*S*)-ABA is catabolized to 8′-hydroxyABA, followed by spontaneous cyclization to (−)-PA, which may then be reduced to dihydrophaseic acid (Walton, 1980). Recently, there have been reports on the bioconversion of (−)-(*R*)-ABA to the unnatural (+) form of PA (Okamoto and Nakazawa, 1993; Balsevich et al., 1994a, 1994b). (+)-7′-HydroxyABA was a minor and transient oxidation product when natural (+)-(*S*)-ABA was fed to cultured bromegrass cells, and (−)-7′-hydroxyABA was formed from the (−)-(*R*)-ABA component when racemic ABA was used in the feeding experiments (Hampson et al., 1992). Earlier, Boyer and Zeevaart (1986) showed that the 7′-hydroxyABA was produced when racemic ABA was fed to *Xanthium strumarium* and was likely derived from (−)-(*R*)-ABA. Racemic PA has been shown to be effective in the

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Abbreviation: PA, phaseic acid.

induction of synthesis of two ABA-inducible proteins in barley embryos and in blocking the GA_3-induced accumulation of α-amylase in aleurone layers (Robertson et al., 1994). The latter observation was also made by Walton (1983) using natural PA extracted from plant tissues. On the other hand, natural PA has been shown to be ineffective in inducing ABA-specific proteins in aleurone layers (Lin and Ho, 1986) and only weakly active in inhibiting the growth of cultured maize cells (Balsevich et al., 1994a, 1994b). Racemic PA was inactive in inducing freezing tolerance in bromegrass cultured cells (Robertson et al., 1994). (−)-7′-HydroxyABA was weakly active in inhibiting the growth of maize cells (Balsevich et al., 1994a, 1994b). The biological activity of (+)-7′-hydroxyABA has not been reported, whereas the biological activity of 8′-hydroxyABA is not known because the compound spontaneously cyclizes to PA in solution. The ABA analog racemic 2′,3′-dihydroABA has potent biological activity in the induction of freezing tolerance (Robertson et al., 1994) and in the reversal of GA_3-induced α-amylase activity (Hill et al., 1992). Furthermore, the (+)-optical isomer was a germination inhibitor in wheat embryos (Walker-Simmons et al., 1992). The racemic analog can be oxidized like ABA at the 8′-carbon atom by cultured bromegrass cells (Lamb et al., 1993), but the product 8′-hydroxy-2′,3′-dihydroABA cannot cyclize to PA because of the absence of the ring double bond. Since 2′,3′-dihydroABA retains ABA-like activity, it is possible to assess the effect of hydroxylation at the 8′ position on activity by using 8′-hydroxy-2′,3′-dihydroABA.

The objectives of the present study were to ascertain whether the biological activity observed for ABA could be attributable to its metabolites, to determine the effects of changes in orientation and structure at various carbon atoms on the activity of the molecule, and to assess the possibility that multiple ABA receptor sites might exist.

Two assays were used: the reversal of GA_3-induced α-amylase activity in barley aleurone layers and the appearance of GUS activity in transformed aleurone protoplasts using a chimeric construct containing the wheat *Em* gene promoter region (Marcotte et al., 1988). The *Em* gene is expressed abundantly in response to ABA in wheat embryos (Williamson et al., 1985). A homologous gene is expressed in barley embryonic tissue but is not expressed in aleurone layers (Espelund et al., 1992), whereas the reversal of GA-induced α-amylase activity by ABA occurs predominantly in aleurone layers (Jacobsen and Chandler, 1987).

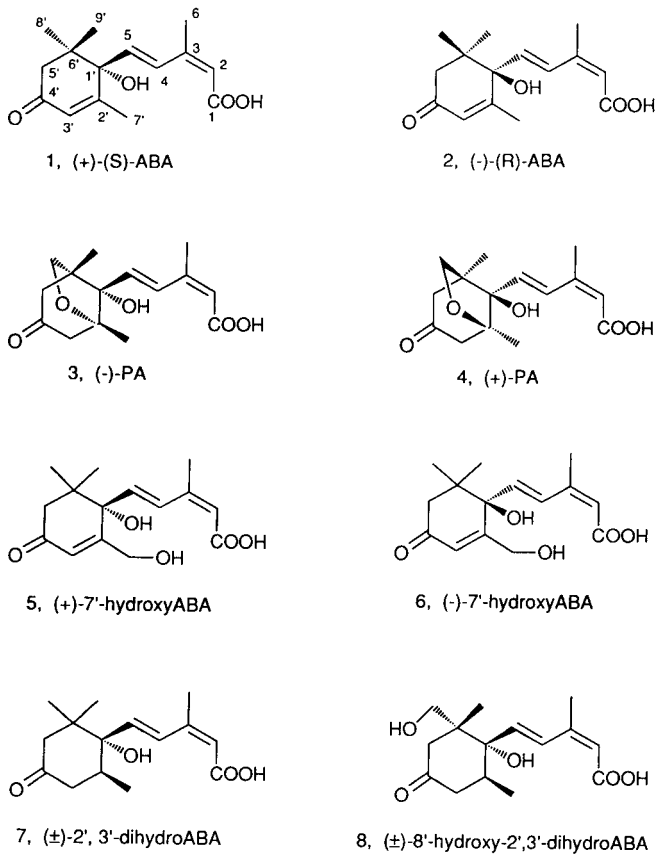

Figure 1. Structure of ABA and ABA metabolites used in this study. Compounds 1 to 6 are optically pure compounds. Among them 1, 3, and 5 are (+)-ABA like (natural), whereas 2, 4, and 6 are (−)-ABA like (unnatural). Compounds 1 and 2 are ABAs, 3 and 4 are PAs, and 5 and 6 are 7′-hydroxy-ABAs. Compound 7, (±)-2′3′-dihydroABA; 8, (±)-8′-hydroxy-2′3′-dihydroABA.

MATERIALS AND METHODS

Chemicals

GA_3 (K^+ salt, approximately 90%, G-7645) and racemic ABA (approximately 99%, A-2784) were purchased from Sigma.

ABA and the analogs used in the study are shown in Figure 1. (+)-(*S*)-ABA and (−)-(*R*)-ABA were obtained by preparative HPLC resolution of racemic methyl abscisate, followed by hydrolysis of the resolved esters, as previously described (Dunstan et al., 1992). (+)- and (−)-7′-HydroxyABA were prepared as previously reported (Nelson et al., 1991). (−)-PA was isolated from the medium of conifer or maize cell cultures that had been supplied with (+)-ABA (Dunstan et al., 1992; Balsevich et al., 1994a, 1994b). (+)-PA was synthesized by the method described by Abrams et al. (1990), except that a chiral starting material was used (Balsevich et al., 1994a, 1994b). Racemic 2′,3′-dihydroABA was synthesized by slight modification of the procedure by Oritani and Yamashita (1982) using racemic starting material. Racemic 8′-hydroxy-2′,3′-dihydroABA was synthesized as previously described (Lamb et al., 1993).

Preparation of Aleurone Layers

Aleurone layers and aleurone protoplasts were prepared from mature seeds of barley (*Hordeum vulgare* cv Himalaya). The isolation of aleurone layers was essentially as described earlier (Hill et al., 1992). Fourteen layers were incubated in a flask with 2 mL of treatment solutions. Treatments, in triplicate, included ABA or an ABA analog (0, 1, 2, 5, 10, and 20 μM) with or without 1 μM of GA_3.

After 18 h at 20°C in the dark, samples were ground in their own medium and then centrifuged at 12,000*g* for 10 min. The supernatants were assayed for α-amylase activity.

Preparation and Transformation of Aleurone Protoplasts

Plasmid pBM113Kp was kindly provided by R. Quatrano. This plasmid construct contains an ABA-responsive 646-bp segment from the wheat *Em* gene linked to the reporter gene GUS and a flanking region from the cauliflower mosaic virus promoter (Marcotte et al., 1988).

The procedure used to prepare and transform aleurone protoplasts was adapted from that of Jacobsen and Close (1991). De-embryonated barley grains (*H. vulgare*, cv Himalaya) were cut in half along the suture and sterilized in sodium hypochlorite (2.5 g/L) for 30 min. After the samples were washed four times in sterile, distilled water, they were allowed to imbibe with Arg solution (50 mM Arg hydrochloride, 20 mM $CaCl_2$, 150 μg/mL cefotaxime, 250 IU/mL nystatin, 1 mL/25 quarter seeds) for 44 h. The starchy endosperm was removed from the aleurone layer. The aleurone layers were incubated in the presence of isolation medium (Gamborg's B5 medium containing 2% Glc, 10 mM Arg hydrochloride, 4.5% Onazuka R10 cellulase, 1% PVP K25, 10 mM Mes, 0.35 M mannitol, 20 mM $CaCl_2$, 150 μg/mL cefotaxime, 250 IU/mL nystatin) at a ratio of 25 quarter layers per 1.5 mL of medium. The layers were incubated for 48 h at room temperature, with a change in isolation medium at 24 h. After the isolation medium was carefully removed, 1 mL of incubation medium (Gamborg's B5 with 0.67 M mannitol, 2% Glc, 10 mM Arg hydrochloride, 150 μg/mL cefotaxime, 250 IU/mL nystatin) was added and the flasks were gently swirled to release the protoplasts.

After the remaining seed coat/pericarp tissue was removed, the suspension was loaded on a Percoll gradient consisting of 5 mL each of 15 and 80% Percoll layers and spun at 4500 rpm for 15 min. The protoplast suspensions at the interface of the 15 and 80% Percoll layers were combined into a 50-mL Corning centrifuge tube, diluted to 50 mL with incubation medium, and centrifuged at 30*g* for 5 min. The supernatant was removed and the protoplasts were washed with mannitol-Mg solution (0.4 M mannitol, 15 mM $MgCl_2$, 0.1% Mes, pH 5.6) once. The washed protoplasts were then resuspended in mannitol-Mg solution. Aliquots of 1.4 mL of the suspension were dispensed into 50-mL Corning centrifuge tubes. To each tube were added 350 μg of calf thymus DNA (Sigma) and 70 μg of plasmid DNA. The contents of the tube were gently mixed for 1 min. Each tube then received 1.05 mL of PEG CMS 4000 and the contents were again gently swirled for 1 min. The tubes were then allowed to stand for 15 to 20 min. The concentration of PEG was then reduced by dilution of samples with 2.1 mL of 0.2 M $CaCl_2$, the samples were mixed for 1 min, and the protoplasts were allowed to settle for 30 min. The protoplasts were sedimented by centrifugation and resuspended in incubation medium containing 150 μg/mL cefotaxime and 500 IU/mL nystatin. Protoplasts were transferred to clean, sterile 30-mL Erlenmeyer flasks. GA, ABA, or an ABA analog was added. Flasks were allowed to stand on the bench for about 2 d before GUS activity was assayed.

For estimation of the effect of a compound on the reversal of GA_3-induced α-amylase activity, protoplasts were placed in incubation medium (see above) containing 150 μg/mL cefotaxime and 500 IU/mL nystatin, with the exception that the Ca^{2+} concentration was increased to 20 mM (Bush et al., 1986). GA_3 and the appropriate ABA analog were added and the flasks were incubated at room temperature for 48 h. The protoplast suspensions were vortexed, and the α-amylase activity of the supernatant was assayed.

α-Amylase Assay

The activity of the α-amylase from aleurone layer and protoplast extracts was measured using the Briggs' procedure (Briggs, 1961). Activity is expressed as iodine dextrin color units. One iodine dextrin color unit is defined as the amount of enzyme required to change the A_{540} of a β-limit dextrin solution from 0.6 to 0.4 in 100 min.

GUS Assay

Protoplast suspensions were transferred to microcentrifuge tubes, and the protoplasts were sedimented by low-speed centrifugation. The supernatant was removed and 0.5 mL of 0.25 M Tris-HCl buffer at pH 7.5 was added. The protoplasts were vortexed and the lysates were centrifuged. The supernatants were assayed for GUS activity by fluorometry (Jefferson, 1987).

RESULTS

Physiological Response to ABA Enantiomers

The two experimental systems used to test the metabolites gave different results for (+)-(*S*)- and (−)-(*R*)-ABA (Fig. 2). Both enantiomers inhibited GA_3-induced α-amylase gene expression (as measured by enzyme activity) to the same extent (Fig. 2A). α-Amylase activity was significantly reduced when either (+)-(*S*)- or (−)-(*R*)-ABA was added at equimolar concentrations to GA_3. The levels of activity approached those of untreated aleurone layers at molar ratios of ABA/GA_3 of about 5 to 10. The (+)-enantiomer, however, was significantly more effective than the (−)-enantiomer at inducing *Em* gene expression in transformed barley aleurone protoplasts (Fig. 2C). A comparison of the response of 0.1 μM (+)-(*S*)-ABA with 10 μM (−)-(*R*)-ABA suggests that the (+) form was, on a molar basis, approximately 100-fold more effective. The *Em*-gene response to the concentration of either stereoisomer was logarithmic.

The difference in response in the two assay systems could result from changes in the ABA response as a result of the protoplast preparation technique. To test this possibility, the ability of (+)-(*S*)- and (−)-(*R*)-ABA to reverse GA_3-induced α-amylase activity in protoplasts was examined. Both enantiomers were equally effective in reversing induced activity at all concentrations tested (Fig. 2B). The prepared protoplasts, compared to aleurone layers, con-

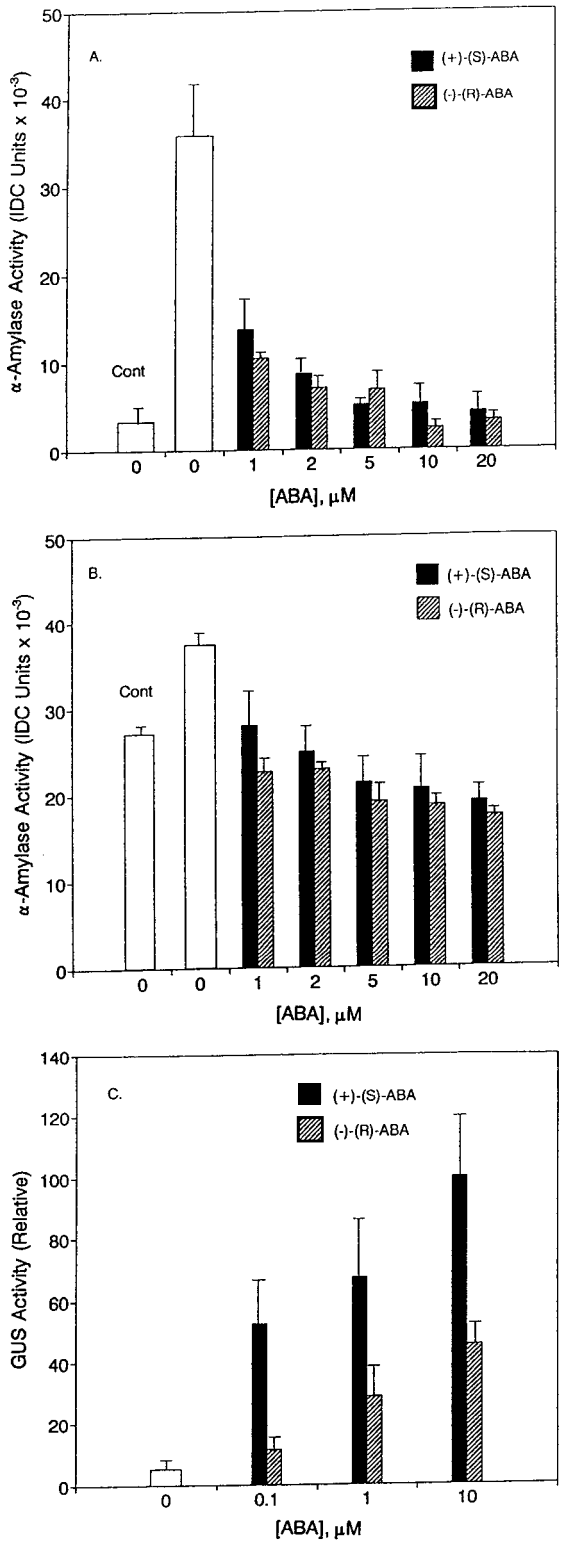

Figure 2. α-Amylase activity in barley aleurone layers (A) and protoplasts (B) and GUS activity in protoplasts (C) in the presence of optically pure ABA. A, Fourteen aleurone layers were incubated with 1 μM GA_3 and (+)-ABA or (−)-ABA at the concentrations noted for 18 h. The treatments labeled Cont contain no GA_3. The α-amylase activity values represent total activity in the medium plus that in the aleurone layers. B, Barley aleurone protoplasts were incubated at room temperature in the presence of 5 μM GA_3 and (+)- or (−)-ABA for 48 h. The treatments labeled Cont contain no GA_3. α-Amylase activity was determined as in A. C, Plasmid pBM113Kp was transformed into barley aleurone protoplasts via PEG. Protoplasts were incubated for 48 h in the presence of 0.1, 1, and 10 μM of (+)-ABA or (−)-ABA. Bars are the means ± SD (n = 3).

tained a high level of uninduced α-amylase activity that was not reversible with ABA.

Effect of ABA Metabolites on ABA-Responsive Events

Both (+)-(*S*)- and (−)-(*R*)-7′-hydroxyABA were moderately effective in reversing GA_3-induced α-amylase activity (Fig. 3A). The compounds significantly reduced the GA_3 response by about 55% at 10 μM, compared with an 80% reduction with an equivalent concentration of ABA. The difference in response between the (+) and (−) forms was not significant. Only (+)-7′-hydroxyABA, at a concentration of 10 μM, induced *Em* gene expression in the protoplast assay (Fig. 3B). The dose-response values for ABA in Figure 2C suggest that 10 μM (+)-7′-hydroxyABA has a response equivalent to a concentration of less than 0.1 μM ABA.

We have shown that 2′,3′-dihydroABA inhibits GA_3-induced α-amylase activity in barley aleurone layers (Hill et al., 1992). The compound was as effective as ABA at activating the *Em* promoter in the transformed protoplast assay (data not shown). Racemic 8′-hydroxy-2′,3′-dihydroABA, which is the analog of 8′-hydroxyABA, did not significantly reduce GA_3-induced α-amylase activity at concentrations as high as 20 μM (Fig. 4A), nor did it significantly induce *Em* gene expression in the protoplast assay (Fig. 4B) at concentrations up to 10 μM.

(−)-PA, the metabolite of natural (+)-ABA, was capable of inhibiting the increase in aleurone α-amylase activity because of GA_3 (Fig. 5A). The unnatural enantiomer, (+)-PA, had no significant effect on GA_3-stimulated α-amylase activity. A concentration ratio of greater than 10 was required to reduce the activity by about 50%, whereas a similar ratio of ABA to GA_3 completely eliminated the GA_3 effect (Fig. 2A). (−)-PA was incapable of inducing *Em* gene expression in transformed protoplasts at concentrations up to 10 μM (Fig. 5B).

DISCUSSION

The results with the enantiomers of ABA demonstrate that there are differences in the stereochemical requirements for the biological response to ABA in the two assays. Thus, they permit examination of the possible range of responses that might be expected from ABA metabolites or analogs.

It is assumed that treatment of aleurone layers to remove the cell wall does not alter the response to the elicitor. Jacobsen and Beach (1985) have demonstrated that barley aleurone protoplasts respond normally to GA_3 and ABA. The observation that aleurone protoplasts respond to (+)-(*S*)-ABA and (−)-(*R*)-ABA (Fig. 2) in a similar fashion to

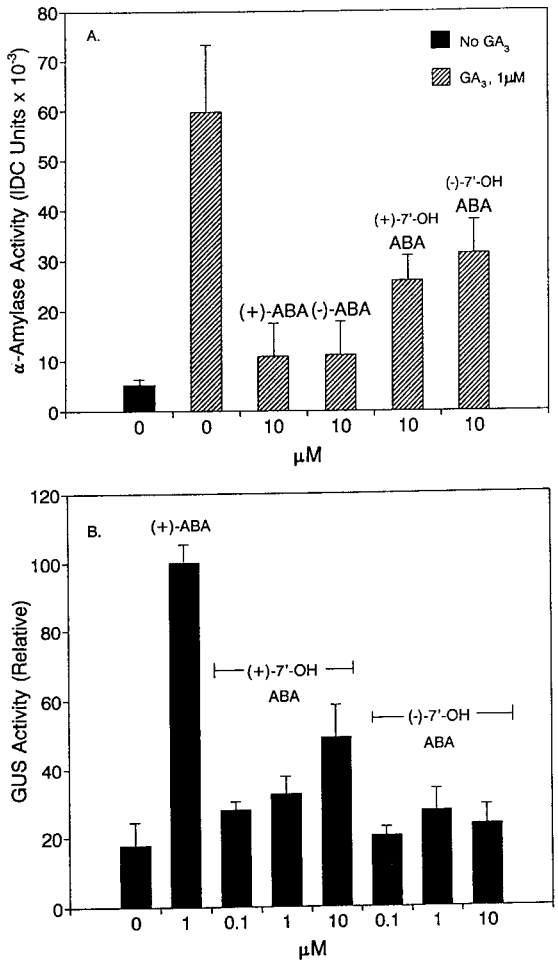

Figure 3. α-Amylase activity in barley aleurone layers (A) and GUS activity in protoplasts (B) in the presence of (+)-7′-hydroxyl-ABA and (−)-7′-hydroxyl-ABA. Incubation conditions for aleurone layers and protoplasts are described in Figure 2. The response to ABA is included as a reference to the activity of the given preparation. Bars are the means ± SD (n = 3).

aleurone layers, with respect to the reversal of GA_3-induced α-amylase activity, suggests that there has been no modification of the ABA response during protoplast preparation. Thus, the differential response that is observed between the two systems is possibly due to one or both of the following: the response of unique ABA receptors or varying signal transduction mechanisms as a result of differing binding constants.

Differing responses to the enantiomers of ABA have been previously demonstrated for a number of systems. (−)-(*R*)-ABA has been reported to be as effective as the (+)-(*S*)-enantiomer at inhibiting GA-stimulated development of α-amylase activity in barley half seeds, although presentation of data was limited (Sondheimer et al., 1971). In the same paper, it was noted that both forms were effective at inhibiting shoot and root growth of germinating barley, although the (−)-(*R*)-enantiomer was less effective at inhibiting root growth. Walker-Simmons et al. (1992) found no difference in the effectiveness of the two forms at inhibiting wheat embryo germination and inducing *lea* gene expression, but they found the (*R*) form less effective at eliciting *Em* gene expression. Sondheimer et al. (1971) found that (*S*)-ABA was twice as effective as the racemic mixture at closing stomates. In conjunction with the present experiments, this would suggest that more than one type of ABA receptor must exist and/or the signal transduction pathways must be sensitive to the binding affinity of the hormone or its analog.

7′-HydroxyABA had only limited efficacy in promoting ABA responses, indicating that the 7′-methyl of the ABA molecule is involved in recognition at the receptor site. Hampson et al. (1992) have shown that (+)-7′-hydroxyABA can be identified as a product of (+)-(*S*)-ABA metabolism in bromegrass cultures, but it is present in low abundance relative to other metabolites. It is, therefore, possible that the observed lower effect in promoting ABA responses may represent a more rapid turnover of the compound, relative to ABA, by aleurone tissue. The retention of some biological activity as a result of oxidation of

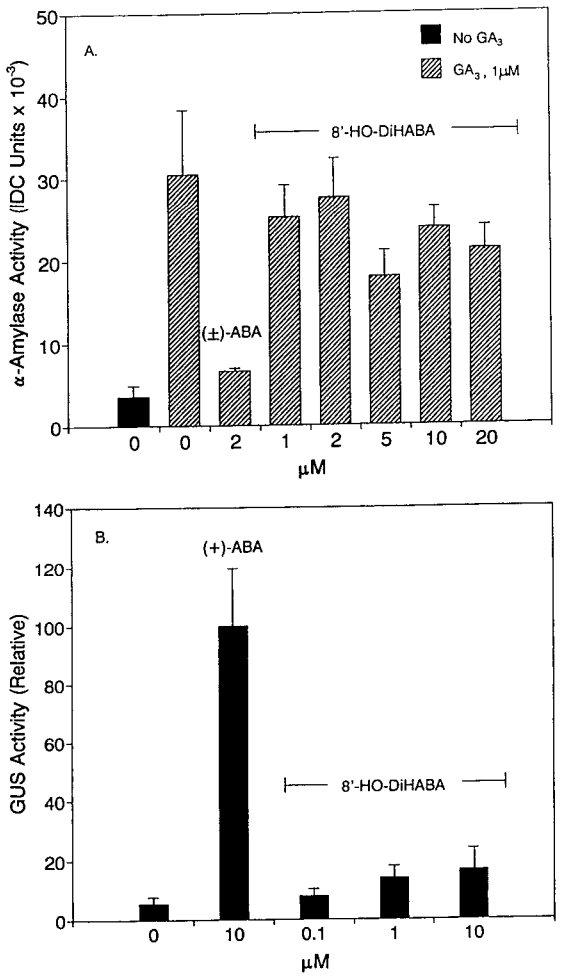

Figure 4. α-Amylase activity in barley aleurone layers (A) and GUS activity in protoplasts (B) in the presence of 8′-hydroxy-2′,3′-dihydroABA (8′-HO-DiHABA). Incubation conditions are described in Figure 2. The response to ABA is included as a reference to the activity of the given preparation. Bars are the means ± SD (n = 3).

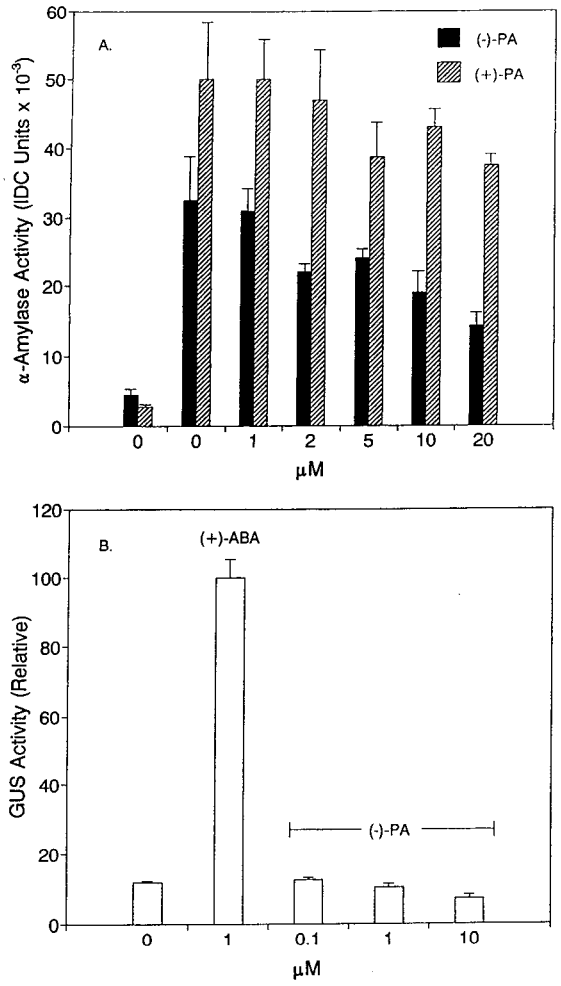

Figure 5. α-Amylase activity in barley aleurone layers (A) and GUS activity in protoplasts (B) in the presence of optically pure PA. Incubation conditions for aleurone layers and protoplasts are described in Figure 2. The response to ABA is included as a reference to the activity of the given preparation. Bars are the means ± SD (n = 3).

the 7′-methyl group indicates that the site does have some tolerance for modification at this carbon. Previous studies have shown that the 7′-carbon atom is needed for activity in rice seedling growth and wheat embryo germination, in that analogs missing the methyl group were inactive in these systems (Nanzyo et al., 1977). In our experiments, reversal of GA_3-induced α-amylase activity by the metabolite, as with ABA, was insensitive to the stereochemistry at C-1′. This might be expected since the general stereochemistry of the molecule remains unchanged by hydroxylation at the 7′ position.

The results with 2′,3′-dihydroABA are in agreement with earlier work that the double bond in the cyclohexenone ring of ABA is not essential for biological activity. Walker-Simmons et al. (1992) found that (+)-2′,3′-dihydroABA had little effect on the expression of the *Em* gene in isolated, mature wheat embryos, whereas the compound effectively inhibited embryo germination. Our results with transformed aleurone protoplasts, that the *Em* gene promoter is equally sensitive to both ABA and racemic 2′,3′-dihydroABA, provide support for the view (Walker-Simmons et al., 1992) that uptake of the derivative may be slower than ABA in tissues in which the cell wall remains intact. The (+) form dihydro analog was active in a conifer protoplast assay using the same construct (Dong et al., 1994).

The lack of response to racemic 8′-hydroxy-2′,3′-dihydroABA in both the reversal of the aleurone GA_3 response and the induction of *Em* gene expression indicates that the 8′-methyl group is an important determinant of ABA function. 8′-Hydroxy-2′,3′-dihydroABA was also shown to be inactive at inducing freezing tolerance (Lamb et al., 1993). Recently, an 8′-methyl ether has been reported to be highly active (Todoroki et al., 1994). Whereas 8′-hydroxy-2′,3′-dihydroABA failed to give a significant ABA response, the cyclized version of 8′-hydroxyABA, (−)-PA, retained the ability to inhibit GA_3-induced α-amylase activity, although requiring higher concentrations than ABA to elicit a response. There was, however, no evidence of an *Em* gene response. The retention of some hormonal response in PA suggests that either the masking of the 8′-hydroxyl group or the distortion due to cyclization restores some ABA function to the molecule.

In the two assays used in this study, the natural forms of all ABA metabolites elicited some significant hormonal response. For the unnatural enantiomers, the (−)-(*R*) form of ABA induced *Em* gene expression, and (−)-7′-hydroxyABA was moderately effective in reversing GA_3-induced α-amylase activity. (+)-PA had no significant effect on GA_3-stimulated α-amylase. The configuration at C-1′ appears, therefore, to have a greater impact on hormonal response under conditions involving direct control of ABA-induced gene expression, which is essentially in agreement with the results of Walker-Simmons et al. (1992).

Interference by ABA of GA_3-stimulated gene expression may operate via a different response mechanism than those that involve a direct induction of gene expression by ABA. Milborrow (1980) has suggested that the receptor sites that elicit fast (stomatal closure) and slow (RNA and protein synthesis) responses to ABA may differ. Walker-Simmons et al. (1992) have evidence to suggest that there may even be differential effects on ABA-responsive gene expression. *Em* gene expression in wheat embryos was found to have a stricter stereochemical requirement than the expression of wheat *lea* proteins or a wheat dehydrin protein.

We have demonstrated that the oxygenated metabolites of ABA have some limited biological activity in two, distinct processes effected by the hormone. Since the two assays utilize the same cell type, a number of the interpretative problems of examining responses in different organs are minimized. Induction of *Em* gene expression in transformed protoplasts by ABA has a stricter stereochemical and structural requirement than the reversal of GA_3-stimulated α-amylase activity. The results suggest that a single transduction pathway for ABA hormone action is unlikely and that the selection of systems for examining these pathways may have an influence on the type of results obtained.

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