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RATIO OF (*S*)- TO (*R*)-ABSCISIC ACID FROM PLANT CELL CULTURES SUPPLIED WITH RACEMIC ABA

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Key Word Index—*Bromus inermis*; Gramineae; bromegrass; NMR; optical isomer; cyclodextrin; abscisic acid; abscisic acid analogues.

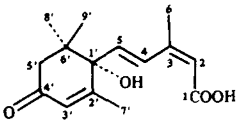
Abstract—Determinations of the ratios of (*S*)- to (*R*)-abscisic acid have been made by an NMR method employing γ-cyclodextrin as a chiral complexing agent with the sodium salt of abscisic acid. In cultures of bromegrass cells that have been fed synthetic racemic abscisic acid the natural isomer is depleted from the medium much more rapidly than the unnatural form. The NMR method is shown to be applicable to biologically active compounds related to abscisic acid.

INTRODUCTION

The plant hormone abscisic acid (ABA) has been implicated in the regulation of germination, growth, transpiration, senescence, and resistance to environmental stress [1, 2]. Naturally occurring (+)-ABA (**1**) is an optically active sesquiterpene with one asymmetric centre, a chiral carbon having the *S* configuration. In stomatal closure the stereochemical requirements for activity are stringent, and only the natural isomer is active [3]. Both the *R* and *S* enantiomers of ABA are active in many other assays however [3]; for example, both inhibit germination and α-amylase production in barley, and both retard growth in a number of growing plant tissues [2].

It is unusual for the enantiomer of a chiral biologically active molecule to have comparable biochemical activity. The high activity of (*R*)-ABA has been ascribed to the near symmetry of the molecule and the possibility that both enantiomers fit into some ABA receptor sites [4]. Racemic ABA has been used in most plant physiology and many metabolic studies because of its availability and considerably lower cost, but the activity of the 'unnatural' *R* enantiomer and the present uncertainty of the details of its metabolism have made it difficult or impossible to differentiate between the effects of natural ABA and those of its artificially introduced mirror image.

Racemic ABA has been demonstrated to induce increased resistance to freezing temperatures in plants and tissue cultures of some species of plants capable of cold hardening [5]. When smooth bromegrass (*Bromus inermis* Leyss) cell suspensions are treated for seven days at non-hardening temperatures with 75 μM racemic ABA, the cells subsequently survive slow cooling to −60°, whereas untreated cells survive only to −10° [6]. *De novo* synthesis of proteins is observed on this induction of cold tolerance with racemic ABA [7]. In the course of our continuing investigation of the mechanism of action of ABA in inducing freezing tolerance, ABA metabolites in the culture filtrates of cells supplied with ABA are being identified [8].



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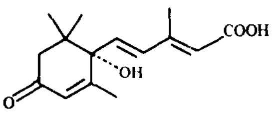

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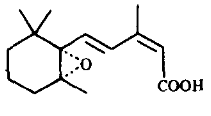

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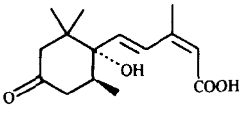

(±)-**4**

This report describes an investigation of the ratio of *R* to *S* enantiomers of ABA recovered from the filtrates of bromegrass cultures that had been supplied with racemic abscisic acid. Such a study provides information valuable in the elucidation of the relative rates of incorporation and metabolism of (*S*)- and (*R*)-ABA by the cell system.

A number of methods have been devised to determine ratios of the optical isomers of ABA [1]. Using HPLC Vaughan and Milborrow [4] were able to resolve the *cis*-diol produced on reduction of methyl abscisate with sodium borohydride, but not the concurrently formed *trans*-diol or methyl ABA itself. Other methods using immunoaffinity techniques [9, 10] and resolution on a chiral protein HPLC column [11] have been reported more recently. Railton [12] found that the methyl ester of ABA could be resolved on a chiral cellulose-based HPLC column. While this manuscript was in preparation, a report appeared describing the direct optical resolution of abscisic acid on the same cellulose tris(3,5-dimethylphenylcarbamate) column [13].

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Described here is a new NMR method that we have developed for directly determining the enantiomeric composition of abscisic acid using cyclodextrins as chiral complexing reagents. The method is applied to determining the ratio of (*R*)- to (*S*)-ABA recovered from the culture filtrates of bromegrass cells treated with racemic ABA. The results from the NMR analysis are compared with those obtained by the method of Railton [12].

RESULTS AND DISCUSSION

As reasonably large quantities of ABA (2–5 mg/l) could be recovered from the culture filtrates after the incubation times employed in this study, it seemed feasible to attempt to determine the ratios of the optical isomers by means of ^{1}H NMR in the presence of a chiral shift reagent. Such an approach has the advantage that all the proton signals from the sample are recorded, and thus both the purity of the ABA and its enantiomeric composition are assessed simultaneously. Initial NMR experiments conducted with deuterochloroform solutions of racemic methyl abscisate and chiral lanthanide shift reagents proved unsuccessful and resulted only in line broadening. In contrast, the antipodes of racemic ABA in deuterium oxide could be resolved spectroscopically using γ-cyclodextrin as the chiral complexing agent.

The cyclodextrins are naturally occurring water-soluble torus-shaped cyclic structures of α-1,4-linked D-glucopyranose units. They are designated as α, β, or γ depending on whether the ring is comprised of six, seven, or eight glucose units respectively. Cyclodextrins have central hydrophilic cavities, the size of which depend on the number of glucose units in the cycle; these recesses allow the formation of inclusion complexes with small organic molecules [14]. Complexation of a cyclodextrin with a binary enantiomeric mixture of suitably sized substrate molecules gives rise to two diastereomeric complexes: in one the dextrorotatory cyclodextrin has included the (+)-enantiomer of the substrate; on the other the (−)-substrate antipode is the guest molecule. Diastereomeric pairs do not in general display identical NMR spectra, and thus it is possible in principle to distinguish spectroscopically between the two substrate enantiomers while they are held within the chiral cyclodextrin cavities [15–17]. In the first example reported for proton NMR, racemic propanolol hydrochloride was shown to form 1:1 inclusion complexes with β-cyclodextrin, and a doubling of one set of signals was observed [16]. Recently, an NMR study of a number of racemic low-M_r chiral antihistaminic and analgesic agents complexed with cyclodextrins demonstrated doubling of signals [17].

In NMR experiments with ABA, no doubling of peaks was observed with α- or β-cyclodextrin. However, the spectrum of racemic ABA and the γ form showed doubling of the doublet due to the axial methylene proton on C-5′, while the multiplicity of the remaining signals were unaffected (Fig. 1). A spectrum of natural (*S*)-ABA obtained under the same conditions showed only one doublet. None of the signals due to ABA were obscured by those of the cyclodextrin complexing agent. Higher concentrations of complexes were obtained using the sodium salt of ABA; the NMR spectrum showed doubling of many more signals, and the increased signal-to-noise ratio enabled more accurate determination by signal area integration of the ratio of enantiomers. Both ring methylene protons, the two vinyl methyl groups, and the vinyl protons on C-4 and C-5 all showed doubling of their resonance patterns. Substitution of lithium and potassium for sodium, and variation of the mol ratio of γ-cyclodextrin to the ABA salt were employed to optimize the resolution of the doubled peaks. A 2:1 molar ratio of γ-cyclodextrin to sodium abscisate gave the best separation and baseline resolution of the signals. The doublet of the C-4 vinyl proton, which was well separated from the other signals in the spectrum, proved the most useful

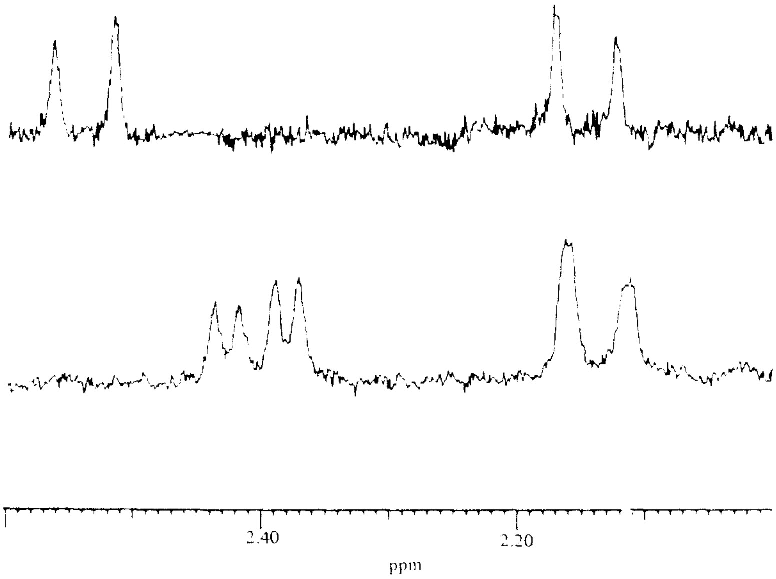

Fig. 1. Partial ^{1}H NMR spectrum (δ 2.0–2.6) showing resonances of C-5′ protons of racemic abscisic acid [(±)-ABA] in deuterium oxide. The upper trace shows signals observed for (±)-ABA alone; the lower trace shows the signals for (±)-ABA and the γ-cyclodextrin complex, with doubling of the axial methylene signal.

Table 1. Determination of composition of (*S*) and (*R*) ABA mixtures

Nominal composition *R*:*S*	HPLC of methyl esters (average of three runs)	NMR of sodium salts plus γ-cyclodextrin
50:50	50.0:50.0	49.7:50.3
25:75	26.2:73.8	29.4:70.6
10:90	11.2:88.8	9.2:91.8
5:95	5.7:94.3	5.5:94.5
2:98	2.2:97.8	3.7:96.3

for determining the ratio of the optical isomers (Fig. 2). While the coupling constant ($J = 16$ Hz) did not change, the chemical shift of the doublet for the C-4 proton was shifted downfield from δ 7.13 to 7.18 for the signal of (*R*)-ABA and to 7.15 for (*S*)-ABA. No attempts were made to determine the structural details of the cyclodextrin–ABA complexes.

Mixtures of varying enantiomeric composition were prepared from natural *S*- and racemic ABA, and the ratios of the enantiomers determined both by the NMR method with the salt, and by HPLC of the methyl esters on a Chiralcel-OD column according to Railton [12] (Table 1). Integrations of the areas under the two HPLC peaks were compared with the integrals of the C-4 proton doublet signals in the NMR spectra. For the mixture containing 2% (*R*)-ABA comparison of the peak heights of the major and minor NMR signals gave a more accurate representation of the composition than simple integration because of overlapping peaks.

Filtrates from bromegrass cell suspension cultures that had been supplied with racemic ABA were examined at intervals of up to 21 days. ABA was isolated from the acidic fractions by reversed-phase HPLC and the enantiomeric ratios were determined by the NMR and HPLC methods (Fig. 3). By day 21 the composition of the residual ABA was found to be 93% (*R*)- and 7% (*S*)-abscisic acid. The residual (*S*)-ABA was likely from the original exogenously supplied material. In another experiment in which hexadeuterated racemic ABA was supplied to the culture, only hexadeuterated ABA was recovered.

It is demonstrated in this paper that bromegrass plant cells capable of cold acclimation, metabolize natural ABA

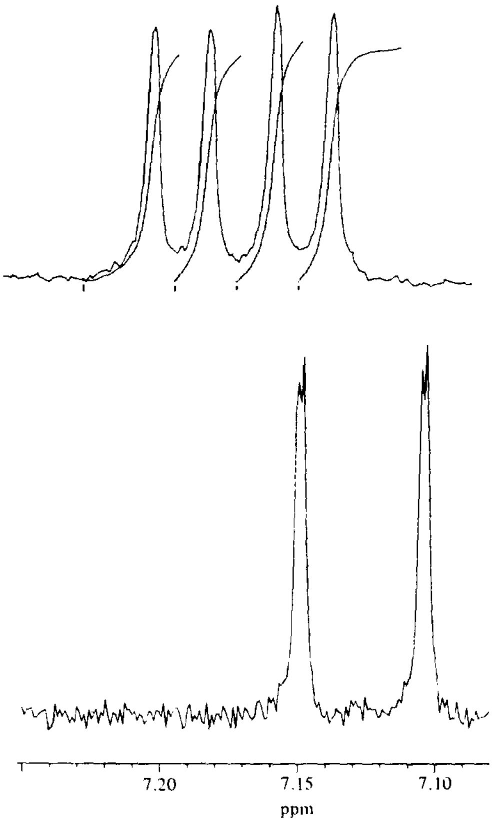

Fig. 2. Partial ^{1}H NMR spectrum (δ 7.09–7.25) showing resonances of C-4 proton of the sodium salt of (±)-ABA in deuterium oxide. The lower trace shows the spectrum obtained for (±)-ABA sodium salt alone; the upper trace shows the doubling of the signals in the spectrum of (±)-ABA sodium salt and γ-cyclodextrin complex.

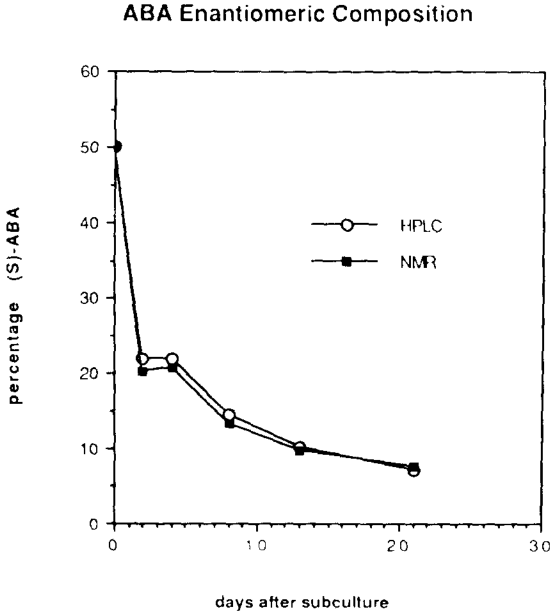

Fig. 3. Composition of abscisic acid recovered from filtrates of bromegrass cell suspension cultures that had been supplied with racemic ABA.

Table 2. Detection of optical isomers of ABA and analogues by HPLC and NMR

Compound	NMR (Na salt) separation of signals (Hz)	HPLC (Me ester) emergence time (min)
±1	H-4 8.0 H-5 2.7 H-5'ax overlapped	6.5, 9.3
±2	H-4 2.9 H-5 2.0	7.8
±3	H-4 2.4 H-5 4.1	4.3, 5.3
±4	H-5'ax 5.1 H-4 overlapped H-5 overlapped	7.6, 9.5

more rapidly than the unnatural form. It is not yet known if (*S*)-ABA alone mediates the increased tolerance to freezing stress. It is also not yet known if the metabolites of (*S*)-ABA, phaseic acid and dihydrophaseic acid, are involved in this process. These questions are being addressed currently in our laboratories.

Plant extracts commonly contain 2-*trans*-ABA (**2**), the photoisomerization product of ABA. The methyl ester of (±)-*trans*-ABA was not resolved by the Chiralcel-OD column, but in the NMR spectrum of the sodium salt of racemic **2** complexed with α-cyclodextrin the signals for the vinyl protons at C-4 and C-5 both appeared as doublets of doublets.

Two biologically active acidic ABA analogues could be resolved by both HPLC of their methyl esters and by NMR of their complexed sodium salts (Table 2). Epoxy-β-ionylideneacetic acid (**3**) has been demonstrated to be biotransformed by plants into ABA [3]. The (+)-isomer, which has the same absolute configuration at C-1' as does (+)-*S*-ABA, has been found to be 1000 times more active than the (−)-enantiomer [18]. In the NMR spectrum of the complexed sodium salt of racemic **3** the *trans* double bond protons appeared as doubled doublets. Similarly, the two optical isomers of the 2-*cis*-4-*trans*-2',3'-dihydroabscisic acid (**4**), the racemic mixture with the two alkyl groups on the same face of the cyclohexanone ring, [19] were analytically resolved in the NMR spectrum of their complexed salts. In this instance the signals due to the axial protons at C-5' were doubled.

EXPERIMENTAL

Cell cultures. Stock cell suspension cultures of smooth bromegrass (*Bromus inermis* Leyss. culture BG970) were grown according to Gamborg and Wetter (E1) in a modified Eriksson's medium containing 3% sucrose, B5 micronutrients and B5 hormone supplements. The medium was adjusted to pH 5.8 and autoclaved for 20 min at 121° before inoculation. The cultures (initial inoculum of 1.00 g cells) were grown at 25° in darkness in 250 ml Erlenmeyer flasks containing 50 ml liquid medium and shaken at 100 rpm. Stock cultures were pooled 6–8 days after previous subculture and filtered over nylon mesh to remove spent medium.

For the metabolism experiments culture flasks were charged with 45 ml of Eriksson's medium at pH 5.8, and the whole sterilized by autoclaving. To each flask was added 5 ml of a filter sterilized (0.22 μm) 750 μM aq. soln of (±)-ABA that had been adjusted to pH 5.8, and the resulting medium, approximately 75 uM in racemic ABA, was inoculated with 1.00 g of bromegrass cells. The cultures were incubated at 25° in the dark with rotary agitation as before.

Isolation of ABA. 2, 4, 8, 13.5 and 21 days after inoculation the filtrates from 8–20 cultures were combined, satd with $(NH_4)_2SO_4$ at room temp., chilled for 2 days, and the pptd proteins removed by suction filtration trhough a pad of H_2O-washed filter aid (Hyflo Supercel). The filtrates were acidified to pH 2.5 with concd HCl, and extracted × 5 with EtOAc. The combined organic phases were washed 4 × with satd aq. $NaHCO_3$, and the combined bicarbonate washes brought to pH 2.5 by cautious addition of conc. HCl. The acidified aq. mixtures were extracted × 5 with EtOAc, and the combined organic extracts were washed twice with satd aq. NaCl, then dried over Na_2SO_4. Filtration followed by evapn of the solvent from the filtrates under red. pres. (temp. ≤ 30°) gave mixtures of acidic metabolites that included the desired ABA.

The acidic mixtures were dissolved in 25% MeCN–H_2O and sepd by prep. reversed-phase HPLC over a Whatman M9-25 cm Partisil 10 ODS-2 column preceded by a Whatman CSK1 Co:Pell ODS guard. Elution at 2.5 ml/min was employed using a gradient prepared from 1% HOAc in H_2O (Solvent A) and MeCN (solvent B). The solvent composition was increased linearly from 25% B to 35% B over the first 5 min, increased to 40% B over the next 5 min, and then held at 40% B for 6 min. Progress of the separation was monitored at 262 nm. The solvent eluted between 12.8 and 14.4 min after each *ca* 50-μl injection contained the ABA, and was collected and pooled. 21 to 23 injections were required to separate each of the acidic metabolite samples.

The pooled HPLC fractions were reduced by *ca* one-half by evapn under red. pres. (temp. ≤ 30°) and the aq. residue then acidified to pH 2.5 with HCl and extracted × 5 with EtOAc. The combined organic phases were washed twice with satd aq. NaCl, dried over Na_2SO_4, and filtered. Evapn of the organic solvent under red. pres. gave 0.6–3.0 mg of solid ABA.

Preparation of analogues. Racemic *trans,trans*-abscisic acid was obtained by repeated injections of a mixture of 2-*trans*- and 2-*cis*-ABA (Sigma, mixed isomers, 90%) on the Partisil-10 ODS-2 column, using isocratic elution with MeCN–H_2O–HOAc (35:65:1) at 3.5 ml/min and monitoring at 262 nm. From 3.7 mg of the mixed geometrical isomers was obtained 1.8 mg of 2-*trans*-4-*trans*-ABA. Racemic 2-*cis*-4-*trans*-epoxy-β-ionylideneacetic acid and its methyl ester were prepared according to ref. [20]. Racemic 2-*cis*-4-*trans*-2′,3′-dihydroabscisic acid was prepared according to Oritani and Yamashita [19].

NMR analysis of enantiomeric mixtures. ^{1}H NMR spectra were recorded at 360 MHz employing D_2O as the solvent and as ref. and with supression of the water peak. A 10 mM stock soln of γ-cyclodextrin in D_2O was prepared by first exchanging hydroxyl hydrogens for deuterons by freeze-drying γ-cyclodextrin (62 mg, 0.05 mmol) with 10 ml D_2O ($\times 2$), then dissolving the residue in 5.0 ml D_2O. D_2O solutions of the salts of the acids were prepared by dissolving the acid (1.8 μmol) in 0.25 ml of a D_2O soln that was 7.5 mM in NaOD. NMR samples were prepared by adding 0.4 ml (4 μmol) of the γ-cyclodextrin soln to the soln of the salt.

HPLC resolution of enantiomeric mixtures. Analytical resolutions were performed using a Daicel Chiralcel OD 0.46 × 25 cm column preceded by a Whatman CSK1 HC Pellosil guard, eluted with hexane–*i*-PrOH (9:1) at a nominal flow rate of 1 ml/min, and monitored at 262 nm. Injections size was 4 μg for each of the methylated (CH_2N_2) acids. Racemic methyl 2-*trans*-4-*trans*-abscisic acid was analysed as a component of a 1:1 mixture of the diastereomeric 2-*cis*-4-*trans*- and 2-*trans*-4-*trans* ABA's.

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