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A Monoclonal Antibody for the Detection of Conjugated Forms of Abscisic Acid in Plant Tissues

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Abstract. A mouse monoclonal antibody against abscisic acid (ABA) was produced and characterized. It was raised using ABA conjugated to the carrier protein through the carboxyl (Cl) group as immunogen. It did not discriminate between free ABA or its ester derivatives. This antibody, which is the first monoclonal against Cl-conjugated ABA, shows interesting characteristics. It has high affinity (K_a = 1.5 × 10^9 L/mol) and specificity. Compounds structurally similar to ABA, such as phaseic acid, dihydrophaseic acid, and both the 2,*trans*-isomer and the (R)-enantiomer of ABA, are not reactive. The narrow linear range of the standard curve (0.018–1.8 pmol) ensures great precision of the assay. This monoclonal antibody has been used for the quantification of ABA conjugates in crude aqueous extracts of bean leaves by radioimmunoassay (RIA). The fractionation of the extracts by high-performance liquid chromatography (HPLC) confirmed the absence of cross-reacting compounds. Because of its affinity and specificity, in combination with antibodies against free ABA, this antibody should be a sound tool for studying the metabolism and immunolocalization of ABA in plant tissues.

Abscisic acid (ABA) conjugates, particularly the glucosyl ester, seem to be common in all plants investigated. Although the only endogenous conjugate of ABA to be fully characterized is the β-D-glucopyranosyl ester (ABAGE) (Koshimizu et al. 1968; Martin et al. 1982; Milborrow 1970; Zeevaart 1980), it appears that the so-called bound ABA may consist of more than one compound (Loveys 1979; Milborrow 1980). The techniques employed to quantify ABA conjugates are based mainly on the analysis of ABA released by alkaline hydrolysis of the aqueous phase after solvent partitioning. Immunoassays have also been used for the quantification of bound ABA, using polyclonal antibodies (Harris and Dugger 1986; Weiler 1979; Weiler 1980).

This is the first report on the production and characterization of a monoclonal antibody raised against ABA that recognizes the free acid molecule, as well as its conjugated forms. This antibody has been used to quantify conjugated ABA in crude aqueous extracts of *Phaseolus vulgaris* L. The absence of cross-reacting compounds was checked by high-pressure liquid chromatography (HPLC) fractionation of the extracts. Accuracy of the results obtained was confirmed by the use of another monoclonal antibody (clone C4-56/41, DBPA1), which binds only the free acid molecule of ABA and which has already been validated for bean leaves by combined gas chromatography–mass spectrometry analysis (Vernieri et al. 1989).

Materials and Methods

Synthesis of Immunogen

(S)-ABA was coupled to Keyhole Limpet Hemocyanine (KLH) through the carboxyl (Cl) group according to the method of Quarrie and Galfrè (1985). From the [^{14}C](R,S)-ABA incorporation, a coupling ratio of ABA:KLH of 14.4 mol/100,000 M_r was calculated.

Immunization Procedure

Eight-week-old male mice (Balb/C strain) were immunized according to the following schedule: day 1, 75 μg antigen in complete Freund's adjuvant intraperitoneally; days 7, 14, and 21, as day 1 but using incomplete Freund's adjuvant; days 28, 35, and 42, 75 μg antigen without adjuvant intraperitoneally; days 49, 50,

and 51, 75 μg antigen without adjuvant intravenously; and day 53, animals were sacrificed.

Cell Fusion and Cloning

Spleens were collected and splenocytes were fused with cells of the mouse myeloma line Sp2/0-Ag14 (splenocytes/myeloma cell ratio 1:1). Fusion was obtained adding a solution of polyethyleneglycol (final dilution 40%) to the cells during 2 min at 37°C. RPMI 1640 serum-free medium was then added slowly (5 min), and the cells were finally diluted in HT medium (50 ml) and distributed in 24-well cell trays. The next day, aminopterine (1% w/v) was added to the medium. When growth of hybrids was apparent, 100 μl supernatant was collected from the wells and assayed for ABA binding by radioimmunoassay (RIA). Cloning of positive hybrid cells was done by limiting dilution in 96-well trays with 0.7 cells per well. Ten days later 100 μl culture medium was collected from the wells containing growing clones and assayed as described above.

Antibody Characterization

Percentage cross-reactivity of the antibody with compounds structurally similar to ABA was calculated as follows:

$$\frac{\text{Concentration of (S)-ABA giving 50\% } B_{max}}{\text{Concentration of competitor giving 50\% } B_{max}} \times 100$$

where B_{max} is the binding of [^{3}H](S)-ABA in the absence of unlabeled antigen. These data were corrected for unspecific binding.

Scatchard plot (Scatchard 1949) was used to calculate the affinity constant of the antibody. The immunological subclass was determined by the Ouchterlony method (Ouchterlony 1958).

RIA Procedure

Incubations were carried out in 1.5-ml polypropylene Eppendorf vials. Reagents were added in the following order: 100 μl PBS buffer (50 mM Na-phosphate, 75 mM NaCl, pH 7) containing 0.5% bovine gamma-globulins and approximately 150 Bq [^{3}H](R,S)-ABA; 50 μl ABA standard solution or sample; 50 μl antibody solution (ammonium sulphate purified hybridoma culture supernatant, diluted 1:300 in PBS buffer containing 0.5% bovine gamma-globulins). This mixture was incubated for 30 min at 4°C in the dark. Saturated ammonium sulphate (200 μl) was then added and precipitation allowed to occur for 30 min at 25°C. After centrifugation, the supernatants were discarded and the pellets washed once with 400 μl of 45% saturated ammonium sulphate and then recentrifuged. The washed pellets were then dissolved in 100 μl water and, after adding 1 ml scintillation cocktail, counted for 5 min.

Solid-phase RIA was performed using DBPA1 as previously described by Vernieri et al. 1989.

Plant Material

Phaseolus vulgaris L. cv. "Mondragone" seedlings were grown in pots in a greenhouse. For ABA feedings experiments, stems were cut at ground level and cuttings were dipped in a solution of (R,S)-ABA until 5 μg ABA were taken up (3 days at 25°C, 16-h photoperiod).

Leaf samples were collected, frozen in liquid nitrogen, and immediately extracted.

Extraction of Plant Material

Plant material was powdered in a mortar filled with liquid nitrogen and extracted with distilled water (water:tissue ratio 5:1) for 16 h at 4°C in the dark. The extracts were centrifuged (13,000 *g*, 10 min) and the supernatants, suitably diluted, used for RIA analysis.

Solvent Partitioning

Extracts were partitioned four times against diethyl ether after adjusting the pH to 2.5 with 1 N HCl. Both aqueous and ethereal phases were taken to dryness and resuspended in PBS buffer, pH 7 before RIA. Alkaline hydrolysis of the aqueous phase was performed according to Milborrow (1970).

HPLC Fractionation

Extracts were fractionated using an HPLC instrument (LDC) equipped with an UV absorbance detector operating at 254 nm. A column (15 cm × 1/4 in. O.D.) packed with LiChrosorb RP 18, 10 μm was used. The solvent flow rate was 1 ml/min. The column was eluted as follows: 30% methanol in water (0.05 M acetic acid) for 6 min; a linear gradient 30–50% methanol for 20 min; 50% methanol for 6 min; a linear gradient 50–100% methanol for 15 min.

RIA Data Processing

Standard curves were linearized by plotting the logit–log transformation of data against the ln of unlabeled ABA added. Coefficients of variability and standard errors for three experiments were calculated. All RIA data were processed using a computer program.

Chemicals

All reagents were purchased from Sigma with the following exceptions: KLH (Calbiochem); (S)-ABA and (R,S)-ABA (Fluka); ABAGE (Apex Organix); cell culture media (Gibco); cell culture trays (Costar); [^{3}H](R,S)-ABA (4.25 TBq/mmol) and [^{14}C](R,S)-ABA (204 MBq/mmol) (Amersham); subclass anti-Ig antibodies (Meloy); scintillation cocktail (Opti-fluor, Packard). PA and DPA were obtained feeding *Phaseolus vulgaris* L. seedlings with (R,S)-ABA as described by Pierce and Raschke (1981). These compounds were extensively purified to homogeneity, characterized, and quantified by combined gas chromatography–mass spectrometry. 2,*trans*-(R,S)-ABA was prepared by irradiating (R,S)-ABA for 4 h under UV light and purified by HPLC.

Table 1. Percentage of cross-reactivity (molar basis) of various compounds structurally related to ABA with DBPA3 monoclonal antibody and several sera raised in rabbits.

	Antibody									
Compound	DBPA3	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
(S)-ABA	100	—	—	100	—	100	100	9	100	100
(R,S)-ABA	50	100	100	—	100	—	50	—	90	48.6
(R)-ABA	—	—	—	0.8[a]	—	0.2	1.16	—	—	0.7
2,*trans*-ABA	2[a]	—	8.3[a]	—	<2	0	0.31[b]	—	2[b]	<0.05
ABA-Me	105[a]	182	133[b]	55[a]	181	270[a]	85.8[b]	100[a]	160[b]	—
ABAGE	167[a]	—	—	53[a]	—	100[a]	50[b]	—	34[b]	28.5[b]
PA	0.3	0.16	3.6	0.53	1.7	0	—	—	—	<0.05
DPA	0.01	0.12	0.6	0.08	2	0	—	—	—	<0.05
2,*cis* + 2,*trans* ABA (1:1 mixture)	—	47	—	51	55	—	—	—	—	—

(1) Weiler 1979; (2) Walton et al. 1979; (3) Weiler 1980; (4) Daie and Wyse 1982; (5) Weiler 1982; (6) Le Page-Degivry et al. 1979; (7) Leroux et al. 1985; (8) Rosher et al. 1985; (9) Harris and Dugger 1986.

[a] Racemic form used for assay. Percent cross-reactivity calculated for the S enantiomer only (R enantiomer is not reactive).

[b] R,S racemic form.

Results

Antibody Characterization

Several clones were positive for ABA binding in the RIA test. Some of these were further characterized and among these clone C1-35/27, DBPA3 (immunological subclass IgG1) was chosen for its specificity properties and its high affinity constant.

Specificity characteristics of DBPA3, compared with those of the sera raised against the same antigen, are reported in Table 1. The data from Table 1 indicate that DBPA3 has a strong cross-reaction only with ABA conjugates. Moreover, the saturation curve obtained using ABAGE parallels the standard curve (unpublished observations). This allows the quantification of ABAGE by the combined use of DBPA1 and DBPA3 monoclonal antibodies.

Moles of ABAGE can be calculated using the equation suggested by Weiler (1980):

$$\text{ABAGE} = (T - F)/1.67$$

where T is moles of total ABA as determined with DBPA3, F is moles of free ABA as determined with DBPA1, and 1.67 is cross-reaction of ABAGE (on molar basis).

Nevertheless, it is interesting to note that if the cross-reaction of ABAGE with DBPA3 is calculated on a weight basis, the result is 100%. Therefore, if the standard curve is constructed using picograms instead of picomoles, no calculation on the basis of cross-reaction is needed, and ABAGE can be measured by simple subtraction of free ABA from the total ABA equivalent detected. DBPA3 shows negligible cross-reactivity with phaseic and dihydrophaseic acids, which are the main ABA metabolites. Therefore, levels of ABA conjugates can be measured also in extracts from stressed plants without interference due to the high level of these metabolites, which are known to increase during stress conditions.

The high affinity constant of DBPA3 (1.5×10^9 L/mol) allows the detection of as little as 5–10 pg antigen. A typical standard curve is shown in Fig. 1: the working range is 0.018–1.8 pmol; 50% B_{max} ranges from 50–100 pg antigen. Coefficients of variation for triplicate analyses of standards and samples typically range between 2–6%.

Quantification of ABA Conjugates in Plant Tissues

As described in Materials and Methods, plant material was extracted in distilled water. This method has been reported to show the same extraction efficiency as organic solvents for ABA in several plant tissues (Loveys and van Dijk 1988; Quarrie et al. 1988; Vernieri et al. 1989). Since ABA conjugates are more polar than the free acid molecule, it seemed reasonable to assume that no losses would arise from the extraction method. Moreover, in the [^{14}C]ABA feeding experiments (see below), more than 99% of the total radioactivity present in the tissue was recovered in the aqueous extracts.

Preliminary experiments carried out on aqueous crude extracts of bean leaves using DBPA3 and DBPA1 [which binds only free (S)-ABA] indicated, for the former, a higher amount of immunoreactive compounds. Since DBPA1 has already been validated for this tissue, the higher immunoreactivity shown by DBPA3 indicated the presence of com-

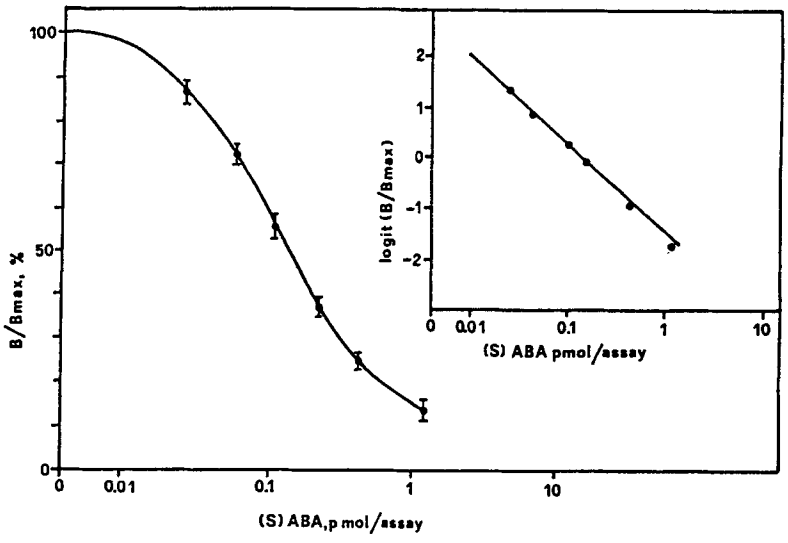

Fig. 1. Typical standard curve obtained using DBPA3. Inset shows logit–log linearization of the curve. Bars indicate SE (n = 10).

pounds other than ABA. These compounds, when the extracts were partitioned against diethyl ether, were found in the aqueous phase (Table 2). An aliquot of the aqueous phase was then subjected to alkaline hydrolysis and analyzed by RIA using DBPA1 to detect the presence of free ABA released from conjugated forms. As shown in Table 2, free ABA was effectively released by alkaline hydrolysis.

Crude aqueous extracts of bean leaves were then fractionated by HPLC in order to confirm these positive results. All the fractions were assayed by RIA, and the results indicated that the only two immunoreactive fractions had the same elution volumes as ABA and ABAGE, respectively (unpublished observations).

To investigate if the presence of high levels of ABA metabolites could affect the accuracy of the estimation and if ABA metabolism could lead to the production of conjugated forms other than ABAGE, bean seedlings were fed with 5 μg (R,S)-ABA. Leaves were collected and the extracts fractionated by HPLC. Results (Fig. 2) confirmed the presence of only two immunoreactive fractions corresponding to ABA and ABAGE using DBPA3 (Fig. 2B), while, of course, only ABA was detected by DBPA1 (Fig. 2C). The fraction showing the same elution volume as ABAGE, subjected to alkaline hydrolysis, released ABA which was detected by DBPA1 (not shown). The efficiency of the hydrolysis treatment was calculated to be approximately 80% on the basis of previous experiments carried out with the ABAGE standard.

To investigate the whole pattern of ABA metabolites formed during the feeding treatment, some cuttings were fed 5 μg (R,S)-ABA plus 8000 Bq

Table 2. Quantification of ABA in aqueous crude extracts before and after solvent partitioning and alkaline hydrolysis of the aqueous phase using DBPA1 and DBPA3 monoclonal antibodies.

	ABA equivalent (pg/assay)	
	DBPA1	DBPA3
Extract before solvent partitioning	67 ± 4	152 ± 6
After solvent partitioning		
Diethylether phase	64 ± 3	71 ± 3
Aqueous phase	8 ± 2	75 ± 5
Aqueous phase after alkaline hydrolysis	57 ± 7	

Values are ± SE, n = 3.

[^{14}C](R,S)-ABA. The leaves were extracted as above, and the extracts fractionated by HPLC. The results (Fig. 3) showed that, despite the high number of ABA metabolites formed during the feeding experiment (probably PA, DPA, and their conjugation products), only the fractions with an elution volume corresponding to ABA and ABAGE contained immunoreactive material, thus confirming the absence of cross-reacting compounds in bean crude extracts.

It must be taken into account that preferential conjugation of (R)-ABA can occur during feeding experiments with labeled (R,S)-ABA (Milborrow 1972). This may explain the apparently higher proportion of ABAGE present by relative radioactivity in Fig. 3 as compared to the estimation in the immunological method (Fig. 2).

Discussion

DBPA3 is a monoclonal antibody with high affinity

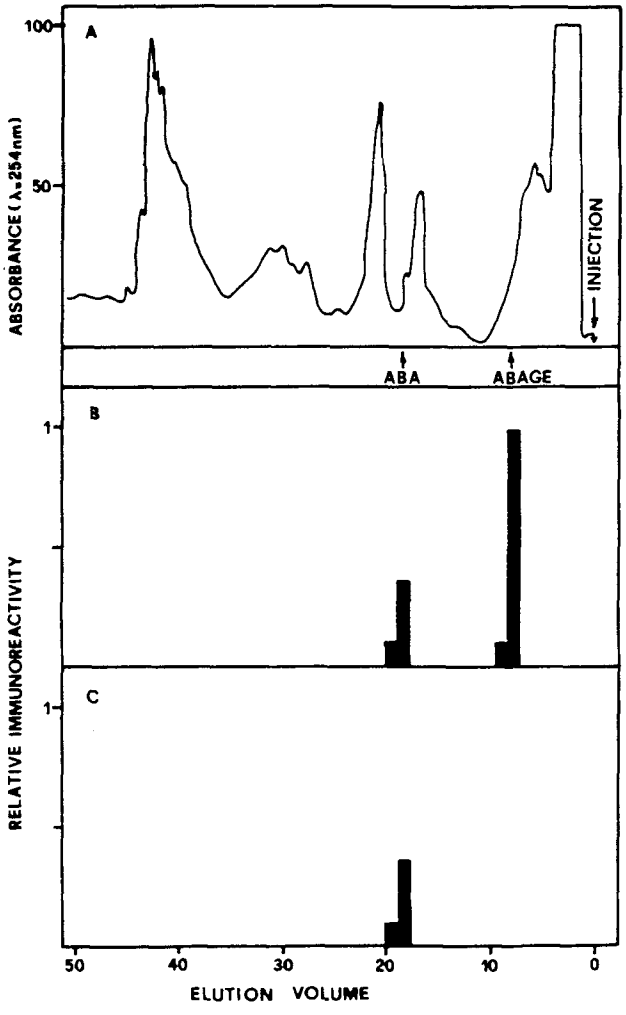

Fig. 2. HPLC fractionation of bean aqueous crude extract (A) and distribution of immunoreactivity in the fractions collected as measured using DBPA3 (B) and DBPA1 (C).

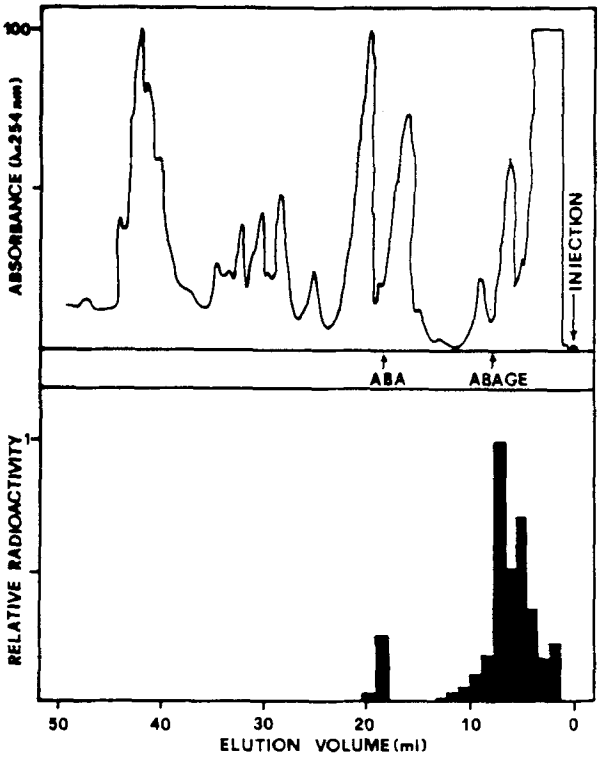

Fig. 3. Distribution of radioactivity in HPLC fractionated aqueous crude extract of bean plants fed with [^{14}C](R,S)-ABA.

and specificity for ABA. Moreover, the linear range of the standard curve is quite narrow (0.018–1.8 pmol). These characteristics ensure high sensitivity and precision of the immunoassay.

Many reports on the levels of ABA conjugates in plant extracts are based on the measurement of free ABA released by alkaline hydrolysis of the aqueous residue after solvent partitioning. This method is time consuming with some rearrangement of the products being produced during hydrolysis (Neill et al. 1983), which leads to errors in the quantification. The combination of DBPA1 and DBPA3 allowed the direct and differential analysis of free and conjugated ABA in crude aqueous extracts, in a short time, and with great precision, in bean extracts. Results of HPLC fractionation demonstrate the ability of DBPA3 to discriminate among ABA metabolites and to bind only ABA or ABA conjugates even in the presence of very high levels of ABA metabolites.

It is important to point out that the results obtained using immunoassays need to be validated for accuracy. In our experiments HPLC fractionation indicated the presence of only two immunoreactive fractions with the same elution volumes as ABA and ABAGE. Moreover, in the ABA fraction both DBPA1 and DBPA3 gave similar quantities of free ABA and, therefore, since DBPA1 has already been validated for bean leaves by physicochemical methods (Vernieri et al. 1989), we can assume that the amount of ABA estimated by DBPA3 is correct. The results of alkaline hydroysis of the ABAGE fraction also confirmed the reliability of the results for the quantitation of this metabolite.

In our experiments, levels of ABAGE were found to be higher than previously reported for bean plants (Neill et al. 1983), while it was confirmed that only a small proportion of total ABA is metabolized to the glucosyl-ester (Zeevaart and Creelman 1988).

Our results also indicate in bean plants the absence of so-called "polar metabolites" formed during ABA metabolism in plants (Dewdney and McWha 1978; Loveys 1979; Tietz et al. 1979; Weiler 1980), since ABAGE appears to be the only conjugation product of ABA in radiotracer experiments.

This seems to confirm that some of these polar compounds may represent rearrangement products of ABAGE (Neill et al. 1983). However, because of its specificity, DBPA3 should be successfully used for investigating the occurrence of unknown ABA conjugates by combining ABA feeding experiments (also using radioactive ABA) with immunopurification techniques (e.g., immunoaffinity columns).

The high sensitivity of an antibody against ABA is of course important for quantitative analysis, since phytohormones occur in low concentrations in plant tissues; but it is essential when very small amounts of ABA have to be detected, such as in immunolocalization experiments. For this technique, only antibodies to ABA conjugated to protein through the Cl-carboxyl can be used, since the antibodies to ABA conjugated to the carrier through the C4′-carbonyl do not recognize the Cl-conjugates that are produced by the carbodiimide method of ABA fixation. Moreover, the use of highly specific monoclonal antibodies should greatly reduce the occurrence of unspecific binding due to low specificity antibodies present in the antisera (Skene et al. 1987).

Therefore, due to its high affinity and specificity, DBPA3 should also prove to be a very powerful tool for the immunolocalization of ABA in plant tissues.

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