Planta (1988) 173:330–339

Planta

A monoclonal antibody to (S)-abscisic acid: its characterisation and use in a radioimmunoassay for measuring abscisic acid in crude extracts of cereal and lupin leaves

S.A. Quarrie[1]*, P.N. Whitford[2], N.E.J. Appleford[2], T.L. Wang[3], S.K. Cook[3], I.E. Henson[4] and B.R. Loveys[5]

[1] AFRC-IPSR, Cambridge Laboratory, Maris Lane, Trumpington, Cambridge CB2 2LQ, UK
[2] Department of Agricultural Sciences, University of Bristol, Long Ashton Research Station, Long Ashton, Bristol BS18 9AF, UK
[3] AFRC-IPSR, John Innes Institute, Colney Lane, Norwich NR4 7UH, UK
[4] CSIRO Division of Plant Industry, Private Bag, P.O. Wembley, W.A. 6014, Australia
[5] CSIRO Division of Horticultural Research, Box 350, G.P.O. Adelaide, S.A. 5001, Australia

Abstract. A monoclonal antibody produced to abscisic acid (ABA) has been characterised and the development of a radioimmunoassay (RIA) for ABA using the antibody is described. The antibody had a high selectivity for the free acid of (S)-*cis,trans*-ABA. Using the antibody, ABA could be assayed reliably in the RIA over a range from 100 to 4000 pg (0.4 to 15 pmol) ABA per assay vial. As methanol and acetone affected ABA-antibody binding, water was used to extract ABA from leaves. Water was as effective as aqueous methanol and acetone in extracting the ABA present. Crude aqueous extracts of wheat, maize and lupin leaves could be analysed without serious interference from other immunoreactive material. This was shown by measuring the distribution of immunoreactivity in crude extracts separated by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), or by comparing the assay with physicochemical methods of analysis. Analysis of crude extracts by RIA and either, after TLC purification, by gas chromatography using an electron-capture detector or, after HPLC purification, by combined gas chromatography-mass spectrometry (GC-MS) gave very similar ABA concentrations in the initial leaf samples. However, RIA analysis of crude aqueous extracts of pea seeds resulted in considerable overestimation of the amount of ABA present. Determinations of ABA content by GC-MS and RIA were similar after pea seed extracts had been purified by HPLC. Although the RIA could not be used to analyse ABA in crude extracts of pea seeds, it is likely that crude extracts of leaves of several other species may be assayed successfully.

Key words: Abscisic acid (assay) – Cereals – Legumes – Monoclonal antibody – Radioimmunoassay (abscisic acid).

* To whom correspondence should be addressed, and from whom the antibody and a detailed protocol for its use in the RIA can be obtained

Abbreviations: ABA = abscisic acid; DW = dry weight; FW = fresh weight; GC-ECD = gas chromatography using an electron capture detector; GC-MS = combined gas chromatography-mass spectrometry; HPLC = high-performance liquid chromatography; McAb = monoclonal antibody; PVP = soluble polyvinylpyrrolidone; RIA = radioimmunoassay; TLC = thin-layer chromatography

Introduction

Abscisic acid (ABA) is a sesquiterpene with an α, β-unsaturated ketone in the ring and a conjugated diene side-chain. Consequently, it has both a high extinction coefficient in the ultraviolet region and strong electron-capturing properties. Physicochemical methods of analysis, such as high-performance liquid chromatography (HPLC), gas chromatography with an electron-capture detector (GC-ECD) or combined gas chromatography-mass spectrometry (GC-MS), have therefore been the mainstay of analytical techniques for quantifying ABA from plant extracts for the last decade (Sweetser and Vatvars 1976; Ciha et al. 1977; Little et al. 1978; Quarrie 1978). However, all these techniques have disadvantages which are associated with the need to purify plant extracts before reliable quantification can be achieved. These assays are therefore time consuming and not readily adaptable to handling large numbers of samples.

Recoveries of ABA are often low and variable, though they can be corrected for by using suitable internal standards.

Immunoassays offer certain advantages over physicochemical methods of analysis (for a review, see Weiler 1984). Since the first use of antibodies to measure ABA (Fuchs et al. 1972), several radioimmunoassay (RIA) and enzymeimmunoassay methods for analysing ABA have been reported using antibodies prepared to carboxyl-linked protein conjugates of ABA (Weiler 1979, 1982; Walton et al. 1979; Daie and Wyse 1982; Kannangara et al. 1984; Le Page-Degivry et al. 1984; Rosher et al. 1985; Zhou et al. 1985; Maldiney et al. 1986). However, all of these measure the total of free ABA and carboxyl-linked conjugates of ABA. There are only a few examples of the preparation and use in immunoassays of antibodies which are specific for the free acid of ABA (Weiler 1980; Mertens et al. 1983). The reports by Weiler (1980) and Mertens et al. (1983) have shown that it is possible to measure the free acid of ABA in crude plant extracts without the necessity for any purification.

The ability of monoclonal antibodies (McAbs) to recognise specific molecular structures may make immunoassays with McAbs less subject to interference from non-specific binding with impurities in crude plant extracts than immunoassays using antisera. Several McAbs to ABA have recently been produced (Mertens et al. 1983) and they have enabled ABA to be assayed in crude methanolic leaf extracts of at least one plant species (*Vicia faba*). The production and preliminary characterisation of another McAb (AFRC MAC 62) for ABA has recently been described (Quarrie and Galfre 1985). This McAb discriminates between the free acid and carboxyl-conjugated derivatives of ABA. In this paper we give further details of the specificity of the antibody for ABA and describe the development of an RIA for ABA using the antibody. The RIA has been validated by comparison with physicochemical methods of analysis.

Material and methods

Plant material. Wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) plants were grown in well-watered compost in controlled-environment cabinets (Henson and Quarrie 1981; Pekić and Quarrie 1987) to the stage of ligule emergence of the second (wheat) or third (maize) leaves. These leaves were then detached and frozen either immediately or after partial dehydration and incubation at 20° C for several hours to allow ABA to accumulate (Henson and Quarrie 1981).

Seeds of *Lupinus angustifolius* and *L. cosentinii* were inoculated with a *Rhizobium* preparation and sown in large plastic bins containing a sandy loam soil with added nutrients. Plants were grown in a glasshouse with day temperatures 22–26° C and night temperatures 12–15° C and a natural daylength of 11–12 h. From about 60 d after sowing, water stress was induced in some plants by withholding water from the bins. Between 60 and 70 d after sowing, upper, fully-expanded leaflets were taken daily at mid-day for ABA analysis. Leaflets were frozen in liquid nitrogen, freeze-dried and stored over silica gel in the dark at room temperature.

Pea plants were grown in John Innes No. 1 compost containing 30% grit in a glasshouse operating at 15° C/10° C (day/night temperatures) with a 16-h photoperiod. Seeds were sampled 14–17 d after fertilisation and stored at −20° C until required for ABA assay.

Extraction methods for RIA analyses. After thawing, wheat leaves were extracted into distilled and deionised water, aqueous methanol or aqueous acetone either by homogenisation for 5 min in an ice bath with an ultrasonic disintegrator (Soniprobe; Dawe Instruments, London, UK) fitted with a microtip, or by cooling the leaves in liquid nitrogen, crushing them to a powder with a glass rod and shaking them overnight at 2° C with solvent. Extraction ratios were usually 5:1, solvent volume (ml):leaf fresh weight (FW; g), unless stated otherwise.

Freeze-dried maize and lupin leaves were ground to a fine powder before being extracted overnight at 2–5° C with distilled, deionised water. Maize samples were usually extracted using a ratio of either 8:1 (solvent volume:leaf FW) or 20:1 (solvent volume:leaf dry weight, DW). Lupin leaves were extracted using a solvent volume:leaf DW ratio of 50:1. Aliquots of powdered maize leaves were extracted with 80% methanol or 80% acetone in the ratio 20:1 (solvent volume:leaf DW).

Pea seeds in duplicate batches of 10 (approx. 100 and 200 mg mean FW 14 and 17 d after flowering, respectively) were ground in liquid nitrogen and extracted for 1 h at 4° C in water or 80% methanol at a solvent volume:FW ratio of 5:1.

Antibody preparation. Ascitic fluid containing AFRC MAC 62 antibodies was collected over several days from a single rat and combined. The antibody was used either without purification or after partial purification by precipitation in the presence of increasing amounts of ammonium sulphate, collecting the fraction that precipitated between 40% and 45% saturation with ammonium sulphate. Partial purification did not improve the performance of the antibody.

Radioimmunoassay procedure. Several immunoassay protocols were developed in the different laboratories. All were based on phosphate-buffered saline (PBS) and used ammonium-sulphate solution to precipitate and wash the ABA-antibody complex. Incubation times for the various steps in the assay could be varied considerably without having a marked effect on the sensitivity of the assay. Although the majority of assays were carried out in the presence of soluble polyvinylpyrrolidone (PVP), the pH optimum for maximum binding (B_{max}) was determined in the absence of PVP. Polyvinylpyrrolidone significantly enhanced binding of the antibody to ABA (see *Results and discussion* section). An assay protocol giving a high value for B_{max} is detailed below.

Incubations were carried out in duplicate or triplicate in 1.5 ml or 2.0 ml polypropylene Eppendorf vials. A PBS buffer (50 mM sodium phosphate and 100 mM NaCl) was used at pH 6.0. [G-^{3}H]-(RS)-Abscisic acid (at approx. 2.5 TBq·mmol^{-1}, Amersham International) in ethanol was diluted 10-fold in water and frozen in aliquots. For assaying, this stock solution was diluted further to 4.8 µl·ml^{-1} in PBS containing 2.5 mg·ml^{-1} bovine γ-globulin (Sigma Chemical Co., Poole, Dorset,

UK) to act as a co-precipitant with the McAb. MAC 62 (either neat ascites or partially purified) was diluted 1:500 in PBS containing 5 mg·ml^{-1} bovine serum albumin (Sigma) and 4.5 mg·ml^{-1} soluble PVP (M_r 40000; Sigma). Samples and ABA standards were prepared in distilled, deionised water.

Solutions were added to assay vials in the following order: 200 µl 50% PBS, 10–50 µl sample, ABA standard or water (for B_{max} determination), 100 µl [^{3}H]ABA solution (typically 8500 cpm or 370 Bq) and 100 µl antibody solution. Non-specific binding (B_{min}) was determined by omitting antibody from the assay mixture. The volumes of samples and standards were usually the same and larger sample volumes could be tested by decreasing the volume of buffer added by an equivalent amount. The contents were mixed by shaking briefly and incubated at 2–5° C for 45 or 90 min. A saturated solution (500 µl) of ammonium sulphate (Sigma, Grade 1) was added and the mixture left at room temperature for 30 min. The precipitated antibodies were pelleted by centrifugation for 4 or 5 min at 8800·g (Eppendorf centrifuge). The supernatant was discarded and the pellet washed by resuspending it briefly in 50% saturated ammonium-sulphate solution (1 ml), centrifuging for 5 min and again discarding the supernatant. After removing any drops adhering to the inside of the vials, the pellets were dissolved in water (60 or 100 µl), 1.4 ml scintillation cocktail (Supersolve-X; Koch-Light Laboratories, Haverhill, Suffolk, UK) added and the samples counted in a liquid-scintillation counter (LS250; Beckman, High Wycombe, Bucks., UK or 1217; LKB, Bromma, Sweden).

Concentrations of ABA were calculated from the radioactivity (in either cpm or Bq) present in the pellets. A series of (S)-ABA standards (typically five, in twofold dilutions from 2000 to 125 pg per vial) was present in each batch of assays for constructing a calibration curve. This was usually linearised by substraction of B_{min} and plotting logit-transformation of the corrected data against the ln of unlabelled ABA present per vial, where

$$\text{logit}\,(B/B_{max}) = \ln\frac{B/B_{max}}{1-B/B_{max}}$$

and B is the corrected cpm or Bq bound in the presence of an ABA standard. Sample ABA concentrations were calculated from this line by interpolation.

Cross-reactivity tests. The percentage cross-reactivity of various compounds was calculated as

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

after correcting the data for B_{min}. Some of the substances tested, such as α-ionylidene acetic acid and α-ionone, were chemically synthesised and therefore would have been racemic mixtures. All of the assays to test cross-reactivity were carried out in the absence of PVP, and some also in the presence of PVP.

Distribution of antibody-binding activity. Crude aqueous extracts of wheat leaves were fractionated by TLC on silica GF_{254}, 5·10 cm^2 plates (Merck, Darmstadt, FRG). After running the plates for approx. 7 cm in ethyl acetate saturated with water, the silica was divided into 10 zones equally spaced from the zone of application to the solvent front and collected (R_f of ABA = 0.6–0.7). Water, equal in volume to that of the extract initially applied, was added to the silica of each zone and the mixture shaken overnight at 2° C. After centrifugation, aliquots of the aqueous phase were assayed in duplicate.

An aqueous maize leaf extract was fractionated by HPLC (Laboratory Data Control). The crude extract was chromatographed on a column (250 mm long, 4.9 mm i.d.) of Spherisorb 5 ODS 2 using a linear gradient of increasing methanol in 2 mM acetic acid, from 28% methanol to 100% methanol over 40 min. The flow rate was 1 ml·min^{-1} and forty 1-ml fractions were collected directly into the RIA tubes. The solvents were removed under vacuum and the residues redissolved in water for RIA analysis.

Physicochemical analysis to validate the RIA. Crude aqueous extracts of wheat leaves were assayed on the same day by RIA and by the method of Quarrie (1978) which consisted of purification of the extract by thin-layer chromatography (TLC), esterification of the ABA eluted from the TLC plate with diazomethane and quantification by GC-ECD.

Crude aqueous extracts of maize leaves were assayed directly by RIA and aqueous, 80% methanol and 80% acetone extracts assayed by GC-MS as follows. The [$^2H_3$]ABA (labelled at the C-6 position) and [G-^{3}H]-(RS)-ABA were added to the crude extract as internal standards for GC-MS quantification and to monitor the recovery of ABA, respectively. The extract was evaporated to near dryness, resuspended in 50 µl of methanol and 5 ml of 2 mM acetic acid was added. This solution was adjusted to pH 3 and passed through a pre-equilibrated Sep-Pak C_{18} cartridge (Waters Associates, Milford, Mass., USA). After flushing the cartridge with equal volumes of 2 mM acetic acid and 10% methanol, ABA was recovered in a 50% methanol eluate. This fraction was evaporated to dryness, redissolved in 25% methanol containing 2 mM acetic acid and purified by HPLC as described for maize leaves above. The ABA fractions eluting from the HPLC after 21–22 min were combined, evaporated to dryness, redissolved in 50 µl of methanol and methylated with an ethereal solution of diazomethane. The methylated sample was evaporated to dryness and redissolved in methanol prior to quantification by GC-MS using a Hewlett-Packard (St. Louis, Mo., USA) 5890 gas chromatograph fitted with a fused-silica capillary column (25 m long, 0.2 mm i.d.) coated with a chemically-bonded non-polar phase (BP-1), which was coupled to a 5970B mass selective detector. Helium at 13 psi head pressure was used as carrier gas. Aliquots of 1 µl were injected at 60° C and after 30 s the split was opened and the oven was programmed from 60° C to 200° C in 7 min, then from 200° C to 270° C at 4° C·min^{-1}. The ions m/z 190 (methyl abscisate) and m/z 193 ([$^2H_3$]-methyl abscisate) were monitored and the ratio of the peak areas of these ions was compared with a standard calibration curve to quantify the ABA present in the initial extract. Concentrations of ABA were determined by RIA of the crude aqueous extract used for GC-MS analysis and also of crude aqueous extracts of two other aliquots of the freeze-dried and milled maize leaves.

Freeze-dried and powdered lupin leaves were extracted overnight at 2–5° C either in water and assayed for ABA in the crude extracts by RIA using MAC 62 and also McAb 16-1-C5 (Mertens et al. 1983) or in 80% methanol and assayed by GC-MS after HPLC purification. For GC-MS analysis, an aliquot of each leaf sample was extracted twice in 80% methanol and [$^2H_3$]ABA added to the centrifuged extract. Methanol was removed and the aqueous residue adjusted to pH 2.5 and extracted three times with ethyl acetate. The combined organic extracts were evaporated to dryness and the residues dissolved in 30% methanol for HPLC. The methanolic solutions were chromatographed on an RPC_{18} HPLC column (300 mm long, 4 mm i.d.) using a two-step methanol gradient (30 to 40% methanol in 5 min, then 40 to 70% methanol from 5 min to 15 min). A constant proportion (0.2%) of acetic acid was maintained throughout the gradient. A fraction containing ABA (9 to 9.6 min) was collected, evaporated to dryness and methylated with ethereal diazomethane. After removal of excess diazometh-

ane, ABA was quantified by GC-MS essentially as described for maize leaves, using a Packard (Caversham, Berks., UK) 427 gas chromatograph coupled to a Hewlett Packard 5970B mass selective detector.

Pea seeds, extracted in water or 80% methanol, were assayed by RIA without purification (methanol was removed from the 80% methanol extracts prior to RIA) and by RIA and GC-MS after purification. Samples for purification were spiked with [$^2H_6$]ABA for GC-MS quantification and [G-^{3}H]-(R)-ABA to monitor the recovery of ABA. Solvent partitioning, HPLC purification and GC-MS quantification were as described by Wang et al. (1984).

Results and discussion

Characterisation of MAC 62. A plot of the reciprocal of the unbound ABA versus the reciprocal of the bound ABA (Scatchard 1949) gave an affinity constant (K_a) of approx. $5 \cdot 10^8$ l·mol^{-1} (Quarrie and Galfre 1985) for ABA-antibody binding in the presence of PVP, using ammonium-sulphate precipitation to separate the bound and free antigen. Using equilibrium dialysis to separate free and unbound ABA, K_a was calculated to be $2.5 \cdot 10^8$ l·mol^{-1}, both in the presence and absence of PVP.

As the antibody was produced in response to immunisation with (S)-ABA conjugated to a carrier protein through the ring ketone group (Quarrie and Galfre 1985), it was expected that MAC 62 would discriminate between (R)- and (S)-ABA and between free ABA and ester derivatives of the ABA carboxyl group. This was confirmed by the cross-reactivity tests (Table 1). For comparison, cross reactivities of one of the high-affinity McAbs to ABA produced by Mertens et al. (1983), namely McAb 16-1-C5, are also shown. Cross-reactivities for MAC 62 with ABA derivatives and metabolites that usually occur in plant extracts were in general very low and similar to those for McAb 16-1-C5. Although both 1′-deoxyabscisic acid and α-ionylidene acetic acid cross-reacted to an appreciable extent, neither of these substances has been identified with certainty in extracts of higher plants (Neill et al. 1984). Nevertheless MAC 62, and to a lesser extent 16-1-C5, did not readily distinguish between a hydrogen atom and a hydroxyl group at the ring 1′-position. The presence of PVP in the RIA altered the cross-reactivities with α-ionylidene acetic acid, 1′-deoxy ABA and (S)-2-*trans*-ABA. However, very little cross-reactivity of other plant metabolites was evident in RIA analysis of crude aqueous extracts in the presence of PVP, as shown by the distribution of immunoreactive material in leaf extracts described below.

Development of the RIA protocol. In the absence of PVP, antibody-antigen binding showed a broad pH optimum, with very similar values for B_{max} over a pH range from 5.5 to 7.0. For routine work pH 6.0 was chosen.

Although soluble PVP was initially added to alleviate any problems arising from the possible presence of phenols in the plant extracts (Rosher et al. 1985), it was found that even in the absence of any plant extract the binding of ABA to MAC 62 was markedly increased by PVP. The optimum concentration of PVP in the final assay mixture was about 0.1%. Concentrations of PVP higher than 0.2% resulted, after centrifugation, in a larger but more flocculent pellet which tended to be discarded with the supernatant. The presence of 0.1% PVP in the assay mixture increased B_{max} by approx. 100%, the effect being greatest if PVP was added to the antibody solution *before* being mixed with the other assay reagents. This effect of PVP was specific to MAC 62, as the binding of McAbs produced by other hybridoma cell lines (namely lines 1/7, 1/12, 2/33 and 2/43 of Quarrie and Galfre 1985) varied from being unaffected to being markedly inhibited by the presence of PVP (data not presented). Polyvinylpyrrolidone also slightly reduced B_{max} with McAb 16-1-C5 of Mertens et al. (1983) (data not shown). This effect of PVP in im-

Table 1. Specificities of monoclonal antibodies raised against (S)-2-*cis*-abscisic acid

Compound	Percentage cross-reactivity[a]	
	AFRC MAC 62 $K_a = 5 \cdot 10^8$ l·mol^{-1}	Clone 16-1-C5[b] $K_a = 3.7 \cdot 10^9$ l·mol^{-1}
(S)-2-*cis*-abscisic acid	100	100
(S)-2-*trans*-abscisic acid	<0.1 (0.9)	0
(RS)-2-*cis*-abscisic acid	49	50
(S)-2-*cis*-abscisic acid methyl ester	0.4	<0.1
(S)-2-*cis*-abscisic acid glucose ester	<0.1	0
Phaseic acid	<0.1	<0.1
Dihydrophaseic acid	<0.1	<0.1
1′-Deoxyabscisic acid	8 (58)	10.4[c]
cis-α-Ionylidene acetic acid	43 (19)	0.7[c]
α-Ionone	<0.1	–[d]
β-Ionone	<0.1	–[d]
Xanthoxin	<0.1	0
Dehydrovomifoliol	<0.1	–[d]

[a] Percentage cross-reactivities in ( ) are for compounds assayed in the presence of PVP
[b] One of two high-affinity antibodies produced by Mertens et al. (1983). Cross-reactivity data for McAb 16-1-C5 were taken from Mertens et al. (1983)
[c] Data obtained by us
[d] Not tested

munoassays does not appear to have been reported previously.

Assay characteristics. The maximum dilution of ascitic fluid of MAC 62 that could be used in the assay was only about 1 in 3000 in the final assay volume. This was a much higher final concentration of antibodies than those reported for RIA with other McAbs (Mertens et al. 1983, 1985). However, subsequent electrophoretic analysis of the supernatant of cells used to produce MAC 62 ascites showed heterogeneity in the immunoglobulin bands (G. Butcher, AFRC Monoclonal Antibody Centre, Babraham, Cambs., UK, personal communication) and the cell line was probably not pure at the time of injection into the rats.

The assay had a reliable working range from 100 to 4000 pg (0.4 to 15 pmol) (S)-ABA per vial, and a typical calibration curve with its linear transformation is shown in Fig. 1. Using the protocol detailed in *Materials and methods*, B_{max} was usually approx. 2000 cpm (approx. 88 Bq) i.e. about 50% of the total [^{3}H]-(S)-ABA was bound by MAC 62, and B_{min} was typically 50–60 cpm (2.2–2.6 Bq). For 40 batches of assays carried out over several months the 50% B_{max} figure (after adjusting for non-specific binding) ranged from 430 to 843 pg ABA per vial with a mean of 620 pg (2.35 pmol) ABA per vial. Duplicate assays of ABA standards or leaf extracts assayed in two batches of 40 vials gave cpm which differed between the two replicates on average by only 3.6%. Aliquots of three milled samples of maize leaves which were extracted and assayed in different laboratories using essentially the same methods gave very similar ABA results using the RIA: 303, 753 and 500 ng·g^{-1} DW in one laboratory and 304, 709 and 502 ng·g^{-1} DW, respectively, in another.

Binding of ABA to MAC 62 was significantly reduced by the presence in the assays of small quantities of acetone or methanol, solvents usually used for extracting ABA from plant tissue. Three percent methanol or acetone in the final assay volume reduced B_{max} by 20–25% and thus made the RIA of ABA in organic extracts less reliable. In contrast, Weiler (1979) found that ABA-antibody binding with an antiserum to ABA was unaffected by at least 2.2% methanol in the final assay volume. The efficiency with which ABA was extracted from plant tissues by water was therefore tested.

Comparison of aqueous and organic extracts. Abscisic acid was extracted from sub-samples of large batches of finely cut wheat and maize leaves into water, aqueous acetone or aqueous methanol and

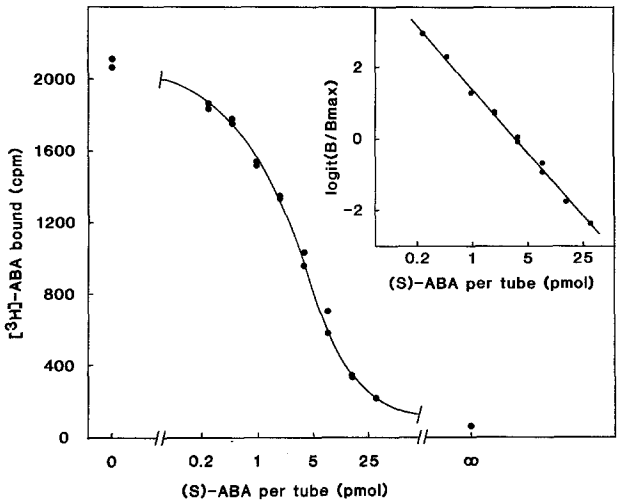

Fig. 1. Standard curve for the RIA with (S)-ABA and its linear transformation, showing the results for duplicate RIA tubes of each ABA concentration

Table 2A, B. Comparison of the extraction of abscisic acid into water, aqueous methanol and aqueous acetone from aliquots of a single batch of wheat (**A**) and maize (**B**) leaves. Wheat leaf extracts were assayed by GC-ECD after purification by TLC and maize leaf extracts by GC-MS after HPLC purification

(A) Solvent	Leaf ABA content (ng·g^{-1} FW)				
	Replicate tissue aliquots				Mean±SE
Water	123	144	144	117	132± 7
80% methanol	100	123	100		108± 8
90% acetone	155	121			138±17

(B) Solvent	Extract ABA content (ng per 2 ml)±SE	
	Recovered in the initial extract[a]	Recovered after reextracting the residue with 80% acetone[b]
Water	109.7±2.0	0.70±0.22
80% methanol	104.2±2.2	–
80% acetone	113.9±0.4	–

[a] SE based on two replicate injections into the GC-MS of a single extract
[b] SE based on three replicate injections into the GC-MS of a single extract

the extracts assayed for ABA by TLC/GC-ECD (wheat) or HPLC/GC-MS (maize) as detailed in *Materials and methods*. Water and 80 or 90% acetone extracted ABA with equal efficiency from wheat and maize leaves (Table 2). Aqueous acetone and water extracted slightly more ABA from both wheat and maize leaves than did 80% methanol. Aliquots of another batch of wheat leaves were

extracted at a solvent volume:tissue FW ratio of 10:1 by ultrasonic disintegration using a range of water:acetone and water:methanol solvent combinations from 9:1 to 2:8 either after fresh leaf sections were frozen and thawed or after leaf sections had been freeze-dried and milled. The concentration of ABA in these extracts varied from 1.17 to 1.49 ng·(100 µl)$^{-1}$ with the highest concentrations occurring in the extracts with high water contents (75:25 and 90:10, water:methanol).

Not only was water just as efficient as organic solvents at extracting ABA from leaf tissue, but shaking freeze-dried and milled tissue with water overnight at 2–4° C was also very efficient at dissolving the extractable ABA in leaves. Reextracting the residue from an 80:1 (water:leaf DW) extraction of maize leaves with 80% acetone recovered only 0.6% more ABA (Table 2).

Enzymatic activity originating from the tissue might have affected levels of ABA during overnight extraction of the leaves at 2–4° C by, for example, releasing free ABA from ABA conjugates. This was tested by passing aliquots of an extract, obtained by homogenisation at approx. 0° C of drought-stressed wheat leaves for 5 min in water, through ultrafiltration membranes with molecular weight cut-offs of either 10 kDa or 20 kDa (Centrisart 1 membrane ultrafilters; Sartorius, Göttingen, FRG). Immediately after homogenisation and membrane filtration the ABA concentration in the extract, determined by RIA, was 6.0 ng·(100 µl)$^{-1}$. After 3 d at 2–4° C the ABA concentrations in unfiltered and membrane filtered aliquots were very similar (6.0, 6.8 and 5.7 ng·(100 µl)$^{-1}$ in unfiltered, 10-kDa and 20-kDa filtered aliquots, respectively). Thus, the levels of ABA in aqueous extracts of wheat leaves were unlikely to have been affected by any enzymatic activity that may have been present in the water under the extraction conditions normally used (up to 24 h at 2–4° C).

This conclusion is supported by the data on ABA concentrations in aqueous and organic extracts of both wheat and maize leaves (Table 2), assuming that enzymatic activity would be very low in organic solvents. Nevertheless, it is possible that in some tissues hydrolysis of ABA conjugates may take place during extraction in water, particularly if the temperature of the extract is allowed to increase at any time. Aqueous leaf extracts from several other species hydrolysed from 0% (sunflower and avocado) to approx. 30% (citrus and mango) of added [G-^{3}H]-(RS)-ABA glucose ester to free ABA during incubation in the dark at 18° C for 5 h (data not shown).

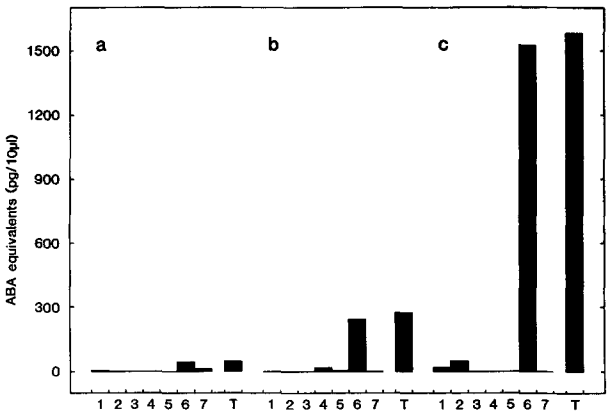

Fig. 2. Distribution of antibody-binding activity (in ABA equivalents) in unpurified aqueous extracts of three wheat leaves differing in ABA content, separated by TLC into 10 equally-spaced zones. Zone 1 is the zone of application. No antibody-binding activity was ever found in zones 8 to 10 and these zones are, therefore, not shown. *T* Indicates the total, unpurified extract; *a* = unstressed-leaf extract; *b*, *c* = stressed-leaf extracts

Distribution of antibody-binding activity in aqueous extracts. As expected from the specificity of MAC 62 for the free acid of ABA, the large majority of antibody-binding activity in aqueous extracts of both wheat and maize leaves was associated with the ABA zones of TLC plates or ABA fractions of HPLC separations. Figure 2 shows the distribution of MAC 62-binding activity in an unstressed wheat leaf (Fig. 2a) and in water-stressed leaves of two other varieties of wheat (Fig. 2b, c). No antibody binding activity was ever found in zones 8 to 10 from the thin-layer chromatograms. Over 92% of the total activity was in the ABA zone from each extract. In ABA equivalents, the sum of immunoreactive material from each TLC plate was essentially the same as that found in the corresponding crude extract assayed by RIA without purification. Water was therefore highly efficient in eluting all the immunoreactive material from the silica. Maize leaf extracts separated by HPLC showed a similar distribution of immunoreactive components, with over 95% of the total antibody-binding activity in the ABA fractions (fractions 20–22). Fractions 7 and 30 also contained some immunoreactive material.

A small amount of activity was usually found at the base line of the TLC plates (Fig. 2). This was probably caused by the presence of very polar derivatives of ABA which cross-reacted with MAC 62. In view of the lack of specificity of MAC 62 for functional groups in the ring 1′-position of ABA (Table 1), some of the very polar cross-reacting material could have been the 1′-glucopyranoside

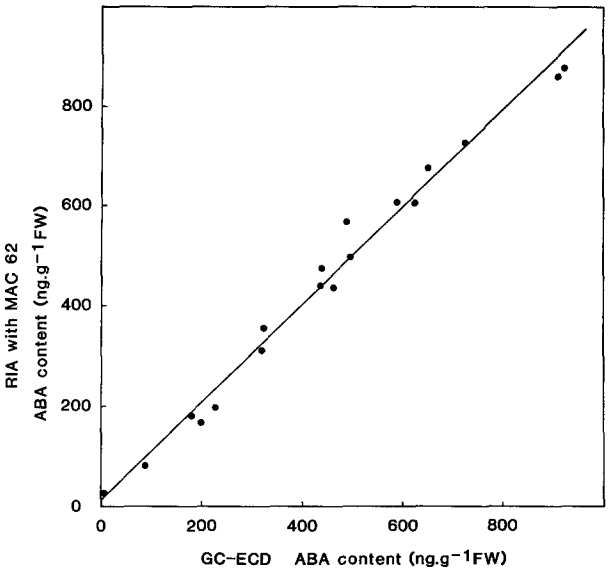

Fig. 3. Comparison of estimates of ABA concentrations in wheat leaves from simultaneous assays of the same extracts by RIA (without purification) and GC-ECD (after purification). The regression line is shown

of ABA which is known to occur in some plants (Loveys and Milborrow 1984). Weiler (1980) also found a small amount of cross-reaction with very polar material from crude plant extracts when using his C-4′ antiserum which was otherwise specific for free-ABA.

Wheat leaf extracts in 80% methanol or 90% acetone showed the same distribution of immunoreactive material as aqueous extracts when they were fractionated by TLC and the zones of silica eluted with water. The ABA zone accounted for at least 89% and 91% of the total immunoreactivity on the plates for extracts in 80% methanol and 90% acetone, respectively.

Validation by GC-ECD and GC-MS of the RIA with crude extracts. The small amounts of immunoreactive material present outside the ABA fractions of TLC and HPLC separations indicated that crude aqueous extracts of wheat and maize leaves could be used in the RIA without any need to purify the extracts. This was confirmed for wheat leaf extracts by GC-ECD (Fig. 3) and for maize leaf extracts by GC-MS (Table 3A). Aqueous extracts of leaves of 18 different wheat varieties, water-stressed to varying extents gave essentially the same results whether assayed by RIA without purification or by GC-ECD after TLC purification. The regression of RIA data on GC-ECD data had a correlation coefficient of 0.99 with a slope of 0.97. The aqueous maize leaf extract also gave

Table 3A, B. Comparison of quantification of ABA present in aliquots of maize leaves (**A**) and pea seeds (**B**) by RIA and GC-MS. Aqueous maize leaf extracts were assayed by RIA without purification and by GC-MS after HPLC purification. Pea seed extracts in either water or 80% methanol were assayed without purification by RIA and after solvent partitioning and HPLC purification by both RIA and GC-MS

(A)	Leaf ABA content ($ng \cdot g^{-1}$ DW)	
	RIA[a]	GC-MS[b]
	4519	4297
	4477	4386
	4561	–
Mean	4519	4341

(B) Solvent	Seed ABA content ($ng \cdot mg^{-1}$ FW)		
	RIA		GC-MS
	Unpurified extract	Purified extract	Purified extract
Water	3.2	0.03	0.05
	4.0	0.23	0.28
80% methanol	3.4	0.19	0.23
	3.9	1.30	0.90

[a] Data for three aliquots of a single sample of freeze-dried and milled maize leaves
[b] Data for two aliquots of the milled maize leaves

the same result by RIA and, after HPLC purification, by GC-MS (Table 3A).

In addition to these cereal leaves, lupin leaves were examined by RIA and GC-MS (Fig. 4). Even in lupin, a genus that can produce large quantities of ABA-conjugates, particularly as the leaves age (Weiler 1980), there was good overall agreement between RIA data for crude aqueous extracts and GC-MS data for methanolic extracts of aliquots of the same finely ground leaf samples after they were purified by HPLC. Only at very high leaf ABA concentrations did the RIA results deviate from the 1:1 line (Fig. 4). However, these very high ABA concentrations occurred only in severely drought-stressed leaves. The regression had a correlation coefficient of 0.99, and when the data points for the two severely stressed leaf samples were omitted, had a slope of 1.03.

Analyses of lupin leaves by RIA using MAC 62 antibody were also compared with RIA analyses using one of the highly specific McAbs to ABA produced by Mertens et al. (1983), viz. clone 16-1-C5 (Fig. 5). The correlation between analyses of the same extracts with the two McAbs was also highly significant (correlation coefficient 0.99) with a slope for the regression of MAC 62 data on

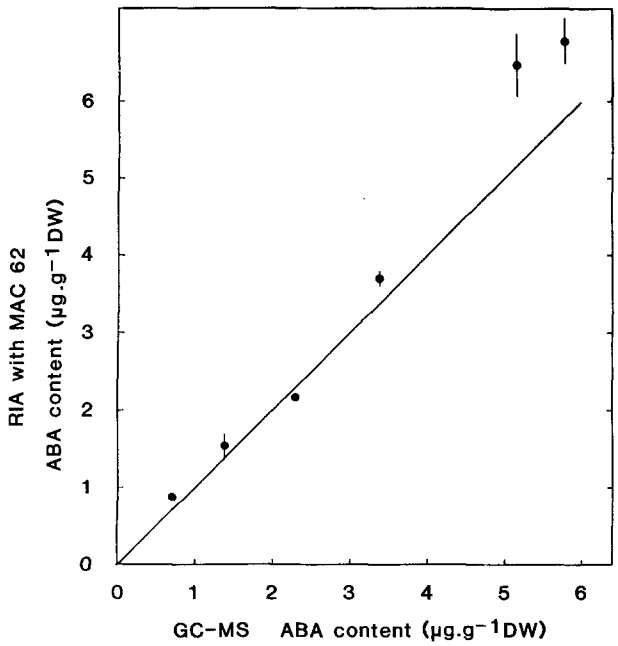

Fig. 4. Comparison of estimates of ABA concentrations in lupin (*Lupinus cosentinii*) leaves using aqueous extracts assayed by RIA (without purification) and methanolic extracts assayed by GC-MS (after purification). Each freeze-dried and powdered leaf sample was sub-sampled for three RIA and one GC-MS determination. SE bars for means of RIA determinations are shown where they exceed the size of the symbols, together with the 1:1 line

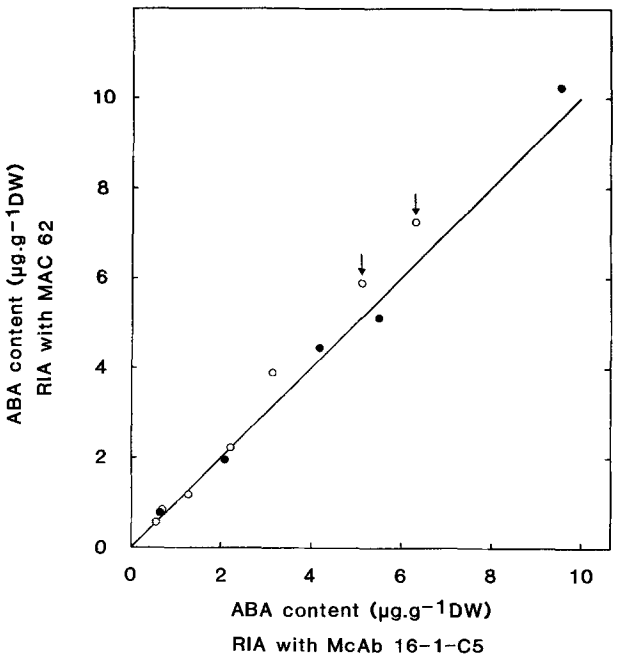

Fig. 5. Comparison of estimates of ABA concentrations in aqueous extracts of lupin leaves from RIA of the crude extracts with both MAC 62 and 16-1-C5. The same extract was assayed by RIA with both McAbs. The 1:1 line is shown. ● = *L. angustifolius*; ○ = *L. cosentinii*. For explanation of arrows see text

16-1-C5 data of 1.08, showing that again at high leaf ABA concentrations MAC 62 tended to give higher estimates of the ABA present in lupin leaves. The two severely stressed leaf samples which gave, in Fig. 4, an overestimate by approx. 20% of the ABA content when determined by RIA with MAC 62, gave ABA estimates using McAb 16-1-C5 (Fig. 4) which were very close to the GC-MS estimates of the ABA present (arrowed data in Fig. 5). This overestimation using MAC 62 was probably a reflection of the slightly greater selectivity of 16-1-C5 antibody (Table 1). As the same aqueous extracts were used for RIA with both McAbs it is unlikely that the overestimation with MAC 62 could have been the consequence of hydrolysis of ABA conjugates to the free acid during aqueous extraction of the severely stressed leaves.

In contrast, RIA with MAC 62 of crude aqueous extracts of pea seeds greatly overestimated the ABA present in the extracts (Table 3B). Only after HPLC purification did RIA and GC-MS analyses give similar estimates of the ABA present. The source of interference could not be established, as it failed to show up as a separate immunoreactive fraction in HPLC separations of 80% methanolic extracts of pea seeds which had been evaporated to dryness and taken up in 50% methanol in 0.1 M acetic acid for reverse-phase chromatography. Analyses by HPLC of such pea seed preparations showed all the immunoreactivity to be confined to the ABA fractions (Fig. 4b of Wang et al. 1986). The presence of contamination in the crude pea seed extracts was also evident when a range of extract dilutions was assayed with different amounts of added (S)-ABA. The resulting slopes of linear regressions of the ABA found on the ABA added were not parallel to the 1:1 line obtained for ABA standards assayed in the absence of any extract (Fig. 4a of Wang et al. 1986). However, pea seed extracts could be sufficiently purified, so that extract dilutions given increasing amounts of (S)-ABA produced lines parallel to the 1:1 standard line, by passing them through Sep-Pak C_{18} cartridges (Waters Associates) (data not shown). Similar problems of gross overestimation of ABA in crude extracts analysed by RIA with either highly specific antisera or McAbs have also been found with other tissues (E.W. Weiler, Lehrstuhl für Pflanzenphysiologie, Universität Osnabrück, FRG, personal communication.

Conclusions

MAC 62 is a McAb that is specific for the free acid of ABA, and it has an affinity constant high

enough for the antibody to be used in a sensitive RIA for ABA. Although the affinity constant is not as high as those reported for two McAbs produced by Mertens et al. (1983) or for antisera specific to the free acid prepared by Weiler (1980), as little as 100 pg of ABA can be measured reliably by MAC 62. For most plant tissues (i.e. those containing at least 20 ng ABA $\cdot g^{-1}$ FW) this would be equivalent to no more than 5 mg tissue FW per assay.

We have demonstrated that water is an excellent solvent for extracting ABA from leaf tissues and for all the leaf samples tested, the crude aqueous extracts could be assayed without serious interference from other immunoreactive material. However, as demonstrated by the results with extracts of pea seeds, unpurified tissue extracts could not always be used with the assay. Consequently, any other tissue samples tested using our assay would initially need careful examination to determine how much purification of the extracts was necessary before the RIA could be used to give reliable ABA data. Nevertheless, it seems likely from the tissue types that we have already tested that cereal leaves and leaves of at least some dicotyledonous species could be assayed in aqueous extracts without the necessity for any purification beforehand. This has subsequently been confirmed for rice leaves as well as for barley grains and embryos (data not shown). However, to avoid the possibility of any hydrolysis of ABA conjugates to the free acid during extraction in water, such aqueous extracts should be kept near 0° C.

In speed, and simplicity, when crude aqueous extracts can be assayed, this RIA is far superior to any of the current physicochemical methods of analysing ABA.

We are grateful to S. Pekić, P.G. Lister, J.M. Burke and H. van Dijk for help with the ABA analyses. We are also grateful to Dr. R. Mertens (Lehrstuhl für Pflanzenphysiologie, Ruhr-Universität Bochum, FRG) and Dr. E.W. Weiler for their generous gift of McAb 16-1-C5, and to Dr. R. Horgan (Department of Botany and Microbiology, University College of Wales, Aberystwyth) for supplying deuterated ABA standards and several of the substances tested for cross reactivity with the antibodies. This work was supported by the Agricultural and Food Research Council via a grant-in-aid to the IPSR, John Innes Institute. The IPSR, Cambridge Laboratory and Long Ashton Research Station are funded through the Agricultural and Food Research Council.

References

Ciha, A.J., Brenner, M.L., Brun, W.A. (1977) Rapid separation and quantification of abscisic acid from plant tissues using high performance liquid chromatography. Plant Physiol. **59**, 821–826

Daie, J., Wyse, R. (1982) Adaptation of the enzyme-linked immunosorbent assay (ELISA) to the quantitative analysis of abscisic acid. Anal. Biochem. **119**, 365–371

Fuchs, Y., Mayak, S., Fuchs, S. (1972) Detection and quantitative determination of abscisic acid by immunological assay. Planta **103**, 117–125

Henson, I.E., Quarrie, S.A. (1981) Abscisic acid accumulation in detached cereal leaves in response to water stress I. Effects of incubation time and severity of stress. Z. Pflanzenphysiol. **101**, 431–438

Kannangara, T., Simpson, G.M., Rajkumar, K., Murphy, B.D. (1984) Analysis of abscisic acid in wheat leaves by a combination of high-performance liquid chromatography and radioimmunoassay. J. Chromatogr. **283**, 425–430

Le Page-Degivry, M.Th., Duval, D., Bulard, C., Delaage, M. (1984) A radioimmunoassay for abscisic acid. J. Immunol. Meth. **67**, 119–128

Little, C.H.A., Heald, J.K., Browning, G. (1978) Identification and measurement of indoleacetic and abscisic acids in the cambial region of *Picea sitchensis* (Bong.) Carr. by combined gas chromatography – mass spectrometry. Planta **139**, 133–138

Loveys, B.R., Milborrow, B.V. (1984) Metabolism of abscisic acid. In: The biosynthesis and metabolism of plant hormones, pp. 71–104, Crozier, A., Hillman, J.R., eds. Cambridge University Press, Cambridge

Maldiney, R., Leroux, B., Sabbagh, I., Sotta, B., Sossountzov, L., Miginiac, E. (1986) A biotin-avidin-based enzyme immunoassay to quantify three phytohormones: auxin, abscisic acid and zeatin-riboside. J. Immunol. Meth. **90**, 151–158

Mertens, R., Deus-Neumann, B., Weiler, E.W. (1983) Monoclonal antibodies for the detection and quantitation of the endogenous plant growth regulator, abscisic acid. FEBS Lett. **160**, 269–272

Mertens, R., Eberle, J., Arnscheidt, A., Ledebur, A., Weiler, E.W. (1985) Monoclonal antibodies to plant growth regulators. II. Indole-3-acetic acid. Planta **166**, 389–393

Neill, S.J., Horgan, R., Walton, D.C. (1984) Biosynthesis of abscisic acid. In: The biosynthesis and metabolism of plant hormones, pp. 43–70, Crozier, A., Hillman, J.R., eds. Cambridge University Press, Cambridge

Pekić, S., Quarrie, S.A. (1987) Abscisic acid accumulation in lines of maize differing in drought resistance: a comparison of intact and detached leaves. J. Plant Physiol. **127**, 203–217

Quarrie, S.A. (1978) A rapid and sensitive assay for abscisic acid using ethyl abscisate as an internal standard. Anal. Biochem. **87**, 148–156

Quarrie, S.A., Galfre, G. (1985) Use of different hapten-protein conjugates immobilized on nitrocellulose to screen monoclonal antibodies to abscisic acid. Anal. Biochem. **151**, 389–399

Rosher, P.H., Jones, H.G., Hedden, P. (1985) Validation of a radioimmunoassay for (+)-abscisic acid in extracts of apple and sweet-pepper tissue using high-pressure liquid chromatography and combined gas chromatography – mass spectrometry. Planta **165**, 91–99

Scatchard, G. (1949) The attraction of proteins for small molecules and ions. An. N.Y. Acad. Sci. **51**, 660–672

Sweetser, P.B., Vatvars, A. (1976) High-performance liquid chromatographic analysis of abscisic acid in plant extracts. Anal. Biochem. **71**, 68–78

Walton, D.C., Dashek, W., Galson, E. (1979) A radioimmunoassay for abscisic acid. Planta **146**, 139–145

Wang, T.L., Donkin, M.E., Martin, E.S. (1984) The physiology of a wilty pea: abscisic acid production under water stress. J. Exp. Bot. **35**, 1222–1232

Wang, T.L., Griggs, P., Cook, S. (1986) Immunoassays for plant growth regulators – a help or a hindrance? In: Plant growth substances 1985, pp. 26–34, Bopp, M., ed. Springer, Berlin Heidelberg

Weiler, E.W. (1979) Radioimmunoassay for the determination of free and conjugated abscisic acid. Planta **144**, 255–263

Weiler, E.W. (1980) Radioimmunoassays for the differential and direct analysis of free and conjugated abscisic acid in plant extracts. Planta **148**, 262–272

Weiler, E.W. (1982) An enzyme-immunoassay for *cis*−(+)−abscisic acid. Physiol. Plant. **54**, 510–514

Weiler, E.W. (1984) Immunoassay of plant growth regulators. Annu. Rev. Plant Physiol. **35**, 85–95

Zhou, X., Xu, Y.-J., Chen, W.-F. (1985) A radioimmunoassay for abscisic acid. J. Nanjing Agric. Univ. **1**, 89–91

Received 14 April; accepted 18 August 1987