Monoclonal antibodies for the detection and quantitation of the endogenous plant growth regulator, abscisic acid

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Monoclonal antibodies (mAB) have been produced which recognize the physiologically active 2-*cis*-(*S*)-form of the endogenous plant growth regulator, abscisic acid (ABA). Cross-reaction with the ABA-catabolites, phaseic and dihydrophaseic acid, is negligible, and (*R*)-ABA, 2-*trans*-ABA, the ABA-conjugate, ABA-β-D-glucopyranosyl ester, as well as the putative ABA precursor, xanthoxin, are totally unreactive. In addition to being very specific, the mAB exhibit high affinities for 2-*cis*-(*S*)-ABA: the *K* values were 7.9×10^9 l/mol and 3.7×10^9 l/mol for antibodies from two different clones. By mAB-radioimmunoassay (RIA), 4 pg of 2-*cis*-(*S*)-ABA (99.5% confidence level) can be detected. mAB-RIA can be used to quantitate ABA directly in unprocessed plant extracts.

Abscisic acid *Monoclonal antibody* *Monoclonal antibody radioimmunoassay* *Plant growth regulator*

1. INTRODUCTION

The endogenous plant growth regulator, abscisic acid (ABA), is thought to be involved in the regulation of a multitude of developmental processes such as growth inhibition, fruit and leaf abscission, bud and seed dormancy or senescence acceleration [1]. Furthermore, it appears to be a central factor in the defense to water stress in plants, in that it triggers stomatal closure [2]. The physiological functions of ABA, its intracellular distribution and its mode of action are under intensive investigation. The practical importance of ABA is amply shown by the fact that improvement of drought tolerance in major crop species is being attempted by altering the ABA economy of plants [2]. Crucial to such studies is the availability of an efficient tool for the detection and quantitation of ABA present in tissues in physiological concentrations (10^{-8}–10^{-6} M).

With the availability of radio-(RIA) and enzyme immunoassay (EIA) techniques based on polyclonal antibodies (pAB) [3–7], the analysis of ABA in plant material has been greatly simplified. However, the inevitable presence in these antisera of low-affinity antibodies and the resulting cross-reactivities preclude the use of pAB in studies of intracellular localization of ABA, and may limit the use of immunoassay for the quantitative analysis of ABA in the presence of high levels of ABA precursors, ABA catabolites or conjugates. These drawbacks of pAB should be overcome by the use of mAB. However, mAB exhibiting both high specificity and selectivity would be required; to date these have only rarely been obtained for low-M_r compounds [8–10].

Here, we report the production and characterization of mAB against ABA which exceed pAB in both affinity and selectivity. These results open

Abbreviations: ABA, abscisic acid; ABAG, ABA-β-D-glucopyranosyl ester; DP, dihydrophaseic acid; FCS, fetal calf serum; mAB, monoclonal antibodies; PA, phaseic acid, pAB, polyclonal antibodies; RIA, radioimmunoassay, TLC, thin layer chromatography

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new possibilities in the detection and determination of ABA in particular and other plant growth regulators and low-M_r plant constituents in general.

2. MATERIALS AND METHODS

2.1. *Reagents*

Cells were cultured in RPMI 1640 (Gibco) with 10% fetal calf serum (FCS) (Roth), 100 U penicillin/ml and 100 μg streptomycin/ml. To support growth of hybridoma cells, the medium was supplemented with MEM–non-essential amino acids (1×, Gibco) and 1 mM sodium pyruvate (Gibco).

2.2. *Antigen and immunization*

The antigen was synthesized by reacting 2-*cis*-(*R*,*S*)-ABA-4′-tyrosylhydrazone with *p*-aminohippuric acid-substituted bovine serum albumin [5]. The antigen (200 μg/animal) was dissolved in 0.2 ml PBS (0.1 M Na-phosphate, 0.15 M NaCl) and emulsified in an equal volume of complete Freund's adjuvant (Difco). Antigen was administered i.p. to 8-week-old female Balb/c mice. Booster injections with, per animal, 200 μg antigen in incomplete Freund's adjuvant were given i.p. 1, 2, 4, 5, 7 and 8 weeks after the first injection. A final booster injection (200 μg antigen in 0.3 ml PBS/animal) was given i.v./i.p. on week 13, 4 days prior to cell fusion. Controls of serum antibody titers were obtained by RIA, using blood taken from the retro-orbital system.

2.3. *Cell fusion and cloning*

For the cell fusions, the 8-azaguanine resistant Balb/c MOPC 21 derived myeloma line P3-NS1-AG4-1 (NS1) [11], provided by V. Fantl, London Imperial Cancer Research Fund, was used. The protocol for cell fusion is based on [12]. After removal of the spleen from a sacrificed animal, 7×10^7 spleen cells and 1.8×10^7 myeloma cells were fused in the presence of polyethylene glycol 1500 (Roth, 50% w/v, at 37°C, 1.5 min, 1 ml). After fusion, the cells were distributed in 24-well Costar trays (Nunc) and cultured in RPMI 1640 containing HAT (H, hypoxanthine; A, aminopterine; I, thymidine) and 20% FCS. To support the growth of hybrids, each well had been incubated with 4×10^4 peritoneal cells from non-immunized Balb/c mice the day before. The incubation medium was not changed until growth of cell clones became apparent. Aliquots of the supernatants were removed and examined for the presence of antibodies against ABA by RIA. Positive clones were, then, supplemented with fresh medium containing HT, every 3–4 days until the bottom of the wells was nearly covered with cells. These were, then, transferred to 50 ml culture flasks.

Cloning of hybrid cells was done twice by limiting dilution [12] in 96-well Costar trays with 0.75 cells/well. After cloning, cells were adapted to grow in the absence of HT, harvested, frozen in RPMI 1640 with 20% FCS and 10% dimethylsulfoxide and stored in liquid nitrogen. Ascites fluid was produced by injecting Balb/c mice with 0.5 ml pristane (Aldrich) i.p. 6 days prior to the i.p. injection of 1.5×10^6 hybridoma cells.

2.4. *Double immunodiffusion*

The Ouchterlony technique [13] was used to determine the immunoglobulin class of the secreted mAB, using rabbit anti-mouse Fab (Nordic), IgG1, IgG2a, IgG2b, IgA and IgM (Bionetics) as probes.

2.5. *Radioimmunoassay*

Antibody titration, cross-reactivity determinations and ABA quantitation were performed by RIA as in [5], with 2-*cis*-(*R*,*S*)-[^{3}H]ABA (39 Ci/mmol, Amersham) as radiotracer.

3. RESULTS

3.1. *Cell fusion and cloning*

Growth of cell hybrids was detected 8 days after fusion. At this time, microscopic examination showed growing cells in every well (1–5 colonies/well). Supernatants from 16 wells (8.3%) initially showed ABA binding capacity which was later lost in 10 wells. From the remaining, 4 stable cell lines were derived by cloning. Ascites fluid was produced from 2 of the lines. These clones (16-I-A4 and 16-I-C5) were characterized further. By double immunodiffusion [13], 16-I-C5 was shown to secrete IgG_1, whereas 16-I-A4 secretes IgA.

3.2. *Antibody affinity, specificity and sensitivity of mAB-RIA*

Antibody affinity for 2-*cis*-(*S*)-ABA was calculated from Langmuir plots: $K = 7.88 \times 10^9$ l/mol (16-I-A4) and $K = 3.69 \times 10^9$ l/mol (16-I-C5). Titers of mAB in ascites fluids were 1:396000

(16-I-A4) and 1:450000 (16-I-C5) (final dilutions binding 30% of 0.2 pmol 2-*cis*-(*S*)-[^{3}H]ABA). Standard curves (fig.1) for 2-*cis*-(*S*)-ABA defined a measuring range of mAB-RIA between 5–1000 pg (16-I-A4), the detection limit being 4 pg (15 fmol) at the 99.5% confidence level.

A cross-reactivity study of both mAB (table 1) revealed the high degree of selectivity for the physiologically active [14] 2-*cis*-(*S*)-ABA. Both the *R*-enantiomers and the 2-*trans* isomer were non-immunoreactive. The predominant ABA-conjugate in plants, ABA-*β*-D-glucopyranosyl ester (ABAG), the ABA catabolites, phaseic (PA) and dihydrophaseic (DPA) acid and the putative ABA precursor, xanthoxin, were either non-immunoreactive (ABAG, xanthoxin) or over 1000-fold less reactive (PA, DPA). In TLC fractions of unpurified plant extracts, only the 2-*cis*-ABA zone showed immunoreactivity which excludes unknown interfering material and documents that 2-*cis*-(*S*)-ABA can be determined directly in unpurified plant extracts (fig.2).

Table 1

Specific of mAB raised against 2-*cis*-(*S*)-ABA

Compound	Clone 16-I-A4	Clone 16-I-C5
2-*cis*-(*S*)-ABA	100	100
2-*cis*-(*S*)-ABA methyl ester	<0.1	<0.1
2-*cis*-(*R*)-ABA	0	0
2-*trans*-(*S*)-ABA	0	0
2-*cis*-(*S*)-ABA–*β*-D-glucopyranosyl ester	0	0
2-*cis*-(*S*)-ABA–*cis*-diol	0	0
Phaseic acid	<0.1	<0.1
Dihydrophaseic acid	<0.1	<0.1
Xanthoxin	0	0
All-*trans*-Farnesol	0	0

The data are cross-reactions in % on molar basis. They were calculated at 50% displacement from radiotracer-displacement curves run for each compound, assigning 2-*cis*-(*S*)-ABA the relative value of 100%

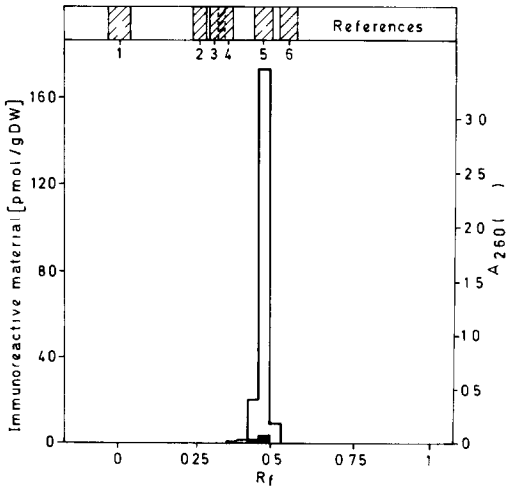

Fig.1. Typical standard-curve for the mAB-RIA of 2-*cis*-(*S*)-ABA. Assays were performed according to [5]. Bars represent ±SD of triplicate standards. *B* = binding of 2-*cis*-(*S*)-[^{3}H]ABA in the presence of unlabeled antigen (B_0, binding in the absence of unlabeled antigen). The insert shows the linearized logit/log plot of the data (logit $B/B_0 = \ln[B/B_0/(100 - B/B_0)]$).

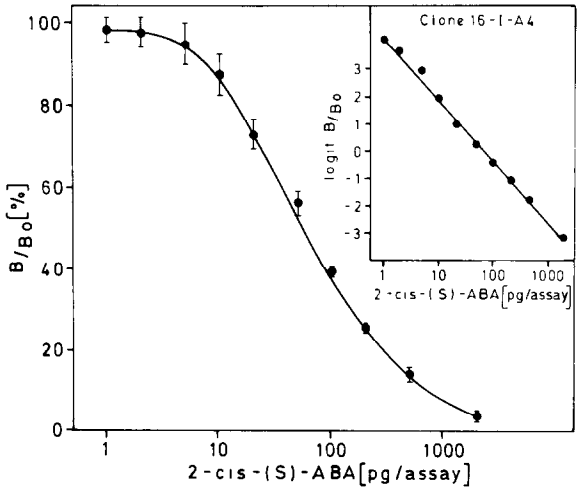

Fig.2. Distribution of immunoreactive material on thin-layer chromatograms (TLC) of methanolic extracts of *Vicia faba* L. leaves (black bars, unstressed; open bars, water-stressed by allowing detached leaves to wilt for 24 h at ambient temperature). Leaf tissue (0.7 g) was extracted and processed as in [5] (TLC, silgel; solvent, toluene–ethyl acetate–acetic acid = 50:30:4, by vol.). To indicate the degree of impurity of the extracts, A_{260} was recorded in all fractions (···). DW, dry weight; 1, ABAG and conjugates of PA, DPA; 2, DPA; 3, xanthoxin; 4, PA; 5, 2-*cis*-ABA; 6, 2-*trans*-ABA.

4. DISCUSSION

A critical factor in developing mAB for low-M_r antigens is the selection of hybridoma clones combining a high degree of specificity with high affinity. The mAB producing clones reported here fulfill these requirements and are superior to the pAB hitherto available for ABA [3–7]. The binding area of the mAB to ABA includes all positions relevant for physiological activity of the molecule. As becomes evident from table 1, structurally and biogenetically related compounds, the 2-*trans* isomer of ABA and even the (*R*)-enantiomer show very little or no cross-reactivity. These compounds would thus not interfere with ABA determination, even in situations of increased ABA-metabolism (fig.2), and even if they occur in large excess. mAB–RIA now allows a very convenient quantitation of low levels of the physiologically active form of ABA in unprocessed plant extracts. This renders mAB–RIA a powerful tool in studies on general ABA-physiology as well as in attempts to improve drought resistance of crop plants by genetically or chemically altering their ABA-economy. The mAB now available should also be extremely useful to study the intracellular localization of ABA and should provide a convenient means of resolution or racemic mixtures of synthetic radiolabeled ABA for metabolic studies.

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