Phytochemistry, 1981, Vol. 20, pp. 17–19. Pergamon Press Ltd. Printed in England. 0031-9422/81/0101-0017 $02.00/0

ABSCISIC ACID LEVELS IN THE ROOT TIPS OF SEVEN *ZEA MAYS* VARIETIES

L. RIVIER and P. E. PILET

Institute of Plant Biology and Physiology of the University, 6, Pl. Riponne, 1005 Lausanne, Switzerland

(*Revised received* 2 *April* 1980)

Key Word Index—*Zea mays*; Gramineae; endogenous abscisic acid; inhibitors; GC–MS; mass fragmentography; stable isotope dilution.

Abstract—Seven varieties of *Zea mays* were analysed for *cis*-abscisic acid (ABA) and *trans*-ABA levels in the primary root tips by a stable isotope dilution technique. Differences in ABA levels were observed between the first 1 mm apical segments of the different varieties. No *trans*-ABA could be detected. In the next 2 mm of the root segments, the amount of ABA did not vary very much from one variety to another. The levels of ABA might very well reflect the growth reactivity of the root tips towards geotropism and light.

INTRODUCTION

ABA has been unequivocally identified in the roots of only a few plants [1]. These include avocado [2], pea [3] and maize [4]. Root inhibitors appear to be produced in maize root caps in response to light [5–8], and exogenous ABA seems to have the same effects on root growth as the root-cap inhibitors [9, 10]. Mass fragmentographic (MF) determinations (also called selected ion monitoring or multiple ion detection) have shown that the ABA levels do not change significantly in the apical 5 mm of the roots kept either in the vertical or horizontal positions, but that the amounts found in this section are higher in the roots exposed for 1 hr to light than in those maintained in the dark [11].

The reactivity of the maize root segments has been shown to vary amongst the varieties examined: apical segments from roots of cv Anjou 210 and Kiowa developed a positive georesponse both in the dark and in light, while those from Orla 264 and Kelvedon 33 reacted only in the light [10]. The differences might reflect changes in the endogenous levels of inhibitors and auxins, as it has been demonstrated, in the same material, that both groups of endogenous regulators can interact with each other [7, 8, 12].

The aim of the present investigation sought to determine the endogenous levels of ABA and *trans*-ABA in the several varieties of *Z. mays* which were used in the georeaction experiments.

RESULTS AND DISCUSSION

The data calculated from MF determinations using a stable isotope dilution technique are reported in Table 1. It can be seen that ABA was detected in all varieties analysed. The section of the root tip between 1 and 3 mm (which corresponds mainly to the elongating section of the root [13]) displayed a very constant ABA level in all the varieties tested except for cv Kelvedon 33, the values for which were obtained in another set of experiments [4]. In contrast to this, the first 1 mm root tips (cap and meristems) showed a range of ABA levels from 0 to 3.9 ng per 100 segments. The same pattern is evident expressed on a ng per g fr. wt or ng per mg dry wt basis.

The *trans*-ABA levels are also reported in Table 1. Determination of this isomer was possible because its methyl ester has a different retention time to the methyl ester of the *cis*-isomer, though its mass spectrum is almost identical.

A suitable calculation procedure [14] shows that the coefficient of variation (standard deviation expressed as percentage of the mean) of machine-computed peak area determination is about 1%. The reproducibility, representing the variation within biological samples, of the present method using a deuterated internal standard is 8.3%. The variation between biological samples is 12.5%. This contrasts with the 25.3% variation in measurements derived solely from the m/z 194 peak areas from the same extracts. Quantitative GC–MS techniques require certain compromises: the necessity to identify fully the endogenous substance requires the scanning of a broad range of masses. This method is both insensitive and inaccurate when working at the nanogram level as is the case in this study. In contrast to this, MF has a higher sensitivity but at the expense of lowered specificity as only a few masses are monitored. As little as 3 pg of ABA can be detected as the methyl ester by single ion monitoring [11]. Consequently, a certain compromise must be made and the validity of the determination of the quantified substance must be evaluated [15].

In the present study, proof of identification of endogenous ABA is based on the detection of the base peak of its mass spectrum at the correct retention time relative to the internal standard. This is good evidence, since the blanks do not show any peaks at the same retention time. Furthermore, the control of homogeneity of the detector responses is of utmost importance in chromatography [16] for an accurate and precise measurement. According to the technique discussed here, MF determinations were performed before and after an additional TLC step of purification on the same extracts:

Table 1. ABA content—obtained by GC–MS—of the root tip prepared from primary roots of several maize varieties. The calculations were obtained from a calibration curve of 6 points whose equation was: $Y = 0.0166X + 0.04132$ with a correlation factor of 0.9969

Varieties	Root tip section (mm)	ABA (ng/100 sections) ± standard error	ABA (ng/g fr. wt)	ABA (ng/mg dry wt)	*trans*-ABA (ng/100 sections)
LG 11	0–1	3.9 ± 0.4	43.1	0.23	0.4
	1–3	2.9 ± 0.4	8.7	0.07	0.1
LG 5	0–1	1.4 ± 0.5	13.6	0.09	n.d.
	1–3	2.5 ± 0.4	9.1	0.06	n.d.
Inra 258	0–1	0.5 ± 0.5	5.9	0.03	n.d.
	1–3	3.7 ± 0.4	13.0	0.09	0.2
Orla 264	0–1	0.0 ± 0.5	0.0	0.0	n.d.
	1–3	1.9 ± 0.5	6.4	0.06	0.2
Kiowa	0–1	0.2 ± 0.5	2.3	0.03	n.d.
	1–3	1.6 ± 0.5	5.7	0.05	n.d.
Anjou 210	0–1	2.3 ± 0.4	28.5	0.41	n.d.
	1–3	2.8 ± 0.4	8.8	0.08	0.3
Kelvedon 33*	0–1	3.4 ± 0.9	53.8	0.37	n.d.
	1–3	10.3 ± 0.4	33.3	0.25	n.d.

n.d.: Not detected within the limits of the technique used.
* Values calculated from data in [4].

since the ratios of the areas of both channels were the same, it could be concluded that the responses measured were due to the endogenous ABA and the IS only. Enough information was also obtained [16] to give confidence in the identification of ABA: solubility, derivatization, behaviour during thin-layer and gas chromatography, and mass spectrometric fragmentation pattern.

When dealing with very small quantities of starting material, it is mandatory to develop specific techniques of extraction and detection when the substance being analysed is present in trace amounts. For this purpose, an isotope dilution method for quantitative analysis of endogenous ABA has been set up and successfully used in our Institute for several years [4]. This technique requires the use of hexadeuterated ABA (ABA–D_6) as an internal standard. This acts as a carrier and standard in all steps of the analytical procedure, except that of detection itself. In our hands, it was not possible to rely on the results obtained without using an IS because the recovery of the trace amounts of endogenous ABA from the tissues was erratic. The reasons for this are presumably due to adsorption to glass walls and precipitation with other endogenous substances. On the contrary, the accuracy of the MF determination depends upon the precision of the quantity of IS added and on the instrumentation. The most accurate determinations are obtained when the amount of IS is close to the endogenous ABA levels. In the present study, 50 ng of ABA–D_6 was added, representing about a 30-fold excess in order to get the appropriate carrier effect. In view of this, the isotope purity of ABA–D_6 should obviously be considered. The isotope composition of ABA–D_6 calculated from data in the m/z 194 region is 1.95% for m/z 190 [4]. However, due to the use of standard curves established for each series of determinations, the true endogenous ABA quantities can be measured. The usefulness of the deuterated internal standard is further demonstrated when considering the standard variations obtained in this study.

It is known that *trans*-ABA can arise from isomerization by light during the work-up procedure [15]. Using the procedure described, such an artefact did not occur because special care was taken to avoid strong illumination of the extracts, specially after esterification, though it was possible to monitor each extract and if necessary correct them for isomerization by following the behaviour of the IS. Data reported in Table 1 show that *trans*-ABA levels were very low compared to those of *cis*-ABA. No *trans*-ABA could be detected in the first 1 mm root tips of all the varieties tested. The amounts found in the next 2 mm were constant. *Trans*-ABA seems to play no important physiological role as its biological activity is known to be much weaker than that of the *cis*-isomer [1].

The quantification of ABA presents additional difficulties when plant material has to be manipulated before freeze-drying. It is recognized that water stress, water logging, cold treatments and some pathological conditions rapidly stimulate ABA production [1]. In this study, the roots were not manipulated at all before they were cut on an ice-cold glass plate and freeze-dried.

From the data presented here, it is clear that ABA is present in all the maize varieties analysed. A certain pattern of distribution is discernible between the apical 1 mm of the root tip and the section between 1 and 3 mm. Although it is difficult at the moment to prove a direct relation between these ABA levels and the particular behaviour of each of the varieties, the results presented here suggest that the differences in endogenous ABA levels may reflect differences observed in the georeacti-

vities of the root tips from the maize varieties tested. However, additional data are still necessary, especially in regard to the metabolism and the redistribution of ABA, and in conjunction with endogenous IAA, before a more definitive statement can be made.

EXPERIMENTAL

Reagents and solvents. ABA was obtained from Fluka. ABA–D_6 was synthesized earlier [4]. *N*-Nitroso-*N*-methyl-*p*-toluolsulfonamide for diazomethane was from Fluka. All other solvents were of the grade 'for the analyses of pesticide residue' or 'for analyses' from E. Merk.

Extraction procedure. The complete extraction procedure has been previously described [11]. Similar results were obtained before and after the use of TLC, thus permitting a simpler extraction procedure to be used routinely. Briefly, for each extract, 100 root segments (corresponding to 100–300 mg fr. wt or 15–45 mg dry wt) were used and 50 ng of ABA–D_6 in buffer were added at the beginning of the extraction procedure. The plant material was homogenized in 5 ml of Na acetate–acetic acid buffer (0.2 M, pH = 4.0) and the sample was allowed to equilibrate for 5 min. This was then extracted with 5 ml $CHCl_3$ for 20 min. The organic layer was transferred to a 10 ml conical glass centrifuge tube and evapd to dryness (vacuum, 30). The residue was treated with 1 ml cold ethereal diazomethane solution (0.3 M) for 10 min with 100 μl of methanol. After the evapn of the reagents (N_2, 22), the residue was taken up in 50 μl of *n*-hexane and spotted between markers of authentic ABA–Me at each side of a 0.25 mm F-245 20 × 20 cm precoated thin-layer silica gel plate (Woelm Pharm., Eschwege D) and developed in EtOAc–*n*-hexane (1:1) mixture over 15 cm. Upon detection of the markers only with UV (R_f 0.3), 1 cm^2 area of the silica gel of each chromatographed extract was collected and poured into a Pasteur pipette previously plugged with a small piece of cotton. The silica gel was eluted with 1.5 ml EtOAc–*n*-hexane mixture into a 2 ml glass tube. The eluate was evaporated (N_2, 22) and the residue dissolved in 50 μl *n*-hexane.

GC–MS. A Hewlett–Packard Model 5985A GC–MS–COM apparatus equipped with a 10 m WCOT glass capillary column coated with SP 2100 was used and run under the same conditions already given [11] except that the oven temperature was programmed at 100 for 1 min, heated then at 25°/min up to 240 . Under those conditions, the retention time of ABA–Me was 6.7 min; *trans*-ABA emerged 0.7 min later. For the MF determinations, ions at *m/z* 190.2 (for ABA–Me) and *m/z* 194.2 (for ABA–D_6–Me) were monitored for 150 and 50 millisec, respectively. The MS parameters were obtained by the *Autotune* standard program and were used routinely without modifications: electron energy, 70 eV; emission, 300 μA and source temperature, 200 .

Quantitative calculations. For each series of analyses, a standard curve was established: various amounts of ABA ranging from 0 to 50 ng per tube were processed exactly as the plant material together with a constant amount of int. standard (50 ng of ABA–D_6). Duplicate samples were prepared for each concentration. After MF analyses, the peak areas of *m/z* 190.2 and *m/z* 194.2 were measured by the computer at the retention time of the corresponding reference substances. The areas ratio was plotted graphically against the amount of ABA added to the sample. The curve obtained, which appeared to be a straight line within the concentrations tested, was used to calculate the amounts of ABA in the biological material.

Reproducibility and variation of the technique. The reproducibility of the present assay was calculated from data obtained from 1000 root tips (4 ± 0.2 mm) of *Zea mays* cv LG 11 to which 500 ng of ABA–D_6 were added. The 1000 segments were homogenized, extracted and the $CHCl_3$ layer obtained. The organic phase was then divided into 10 identical vols of $CHCl_3$ which were worked up in parallel and analysed by MF separately. For the determination of the variation between samples, 500 root segments (5 ± 0.2 mm long) of the same variety were divided into 5 lots of 100 segments each and extracted through the entire procedure with 50 ng of ABA–D_6.

Plant material. Seven varieties of *Zea mays* L. were used: Anjou 210 (Hodée, La Menitrée, F), Inra 258, Kiowa, LG 5 and LG 11 (Assoc. Suisse des Sélectionneurs, Lausanne, CH) and Kelvedon 33 (Hurst Gunsons, London, GB). All seeds originated from the 1978 harvest and the caryopses had been graded by the suppliers. The conditions for germinating the maize caryopses, in order to obtain linear primary roots of the different varieties reaching 15 ± 3 mm long after 3 days in the dark, have been previously described [6]. For each determination, 100 apical segments were prepared, freeze-dried and stored in the dark at −30° until analysed.

REFERENCES

1. Milborrow, B. V. (1978) in *Phytohormones and Related Compounds—A Comprehensive Treatise* (Letham, D. S., Goodwin, P. B. and Higgins, T. J. V., eds.), Vol. 1, p. 295. Elsevier, Amsterdam.
2. Wright, S. T. C. (1975) *J. Exp. Bot.* **26,** 161.
3. Tietz, A. (1974) *Biochem. Physiol. Pflanzen* **165,** 387.
4. Rivier, L., Milon, H. and Pilet, P. E. (1977) *Planta* **134,** 23.
5. Wilkins, H. and Wain, R. L. (1974) *Planta* **121,** 1.
6. Pilet, P. E. (1975) *Physiol. Plantarum* **33,** 94.
7. Pilet, P. E. (1976) *Planta* **130,** 245.
8. Pilet, P. E. (1979) *Planta* **145,** 403.
9. Pilet, P. E. (1975) *Planta* **122,** 299.
10. Pilet, P. E. (1978) *Z. Pflanzenphysiol.* **86,** 411.
11. Pilet, P. E. and Rivier, L. (1980) *Plant Sci. Letters,* **18,** 201.
12. Pilet, P. E., Elliott, M. C. and Moloney, M. M. (1979) *Planta* **146,** 405.
13. Pilet, P. E. (1977) in *Plant Growth Regulation* (Pilet, P. E., ed.), p. 115. Springer, Berlin.
14. Colby, B. N. and McCaman, M. W. (1979) *Biomed. Mass Spec.* **6,** 225.
15. Saunders, P. F. (1978) in *Isolation of Plant Growth Substances,* (Hillman, J. R., ed.), p. 115. Cambridge University Press, Cambridge.
16. Reeve, D. R. and Crozier, A. (1980) in *Molecular and Subcellular Aspects of Hormonal Regulation* (MacMillan, J., ed.). Encyclopedia of Plant Physiology, New Series. Springer, Berlin, (in press).