Plant Physiol. (1980) 66, 750–753
0032-0889/80/66/0750/04/$00.50/0

Abscission of Citrus Leaf Explants

INTERRELATIONSHIPS OF ABSCISIC ACID, ETHYLENE, AND HYDROLYTIC ENZYMES

Received for publication November 16, 1979 and in revised form May 20, 1980

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ABSTRACT

The question whether abscisic acid (ABA) induces cellulase and polygalacturonase activity and, hence, abscission directly or whether its action is mediated by C_2H_4 was studied in citrus (Osbeck var. Shamouti) leaf explants using aminoethoxyvinyl glycine (AVG), an inhibitor of C_2H_4 biosynthesis. ABA in concentrations of 10 micromolar and higher induced C_2H_4 production and accelerated abscission. AVG inhibited C_2H_4 formation, activity of cellulase and polygalacturonase, and abscission in ABA-treated explants. AVG did not inhibit the increase in the activity of the cell-wall degrading enzymes or abscission in a saturating level of externally supplied C_2H_4. This indicates that the effect of AVG resulted from inhibition of the formation of endogenous ethylene. The data indicate that in citrus leaf explants the induction of the activity of cellulase and polygalacturonase and abscission by ABA is mediated by C_2H_4.

The abscission-accelerating effect of externally supplied ABA is well-documented (5, 20). Several types of evidence indicate that ABA acts as a primary inducer of abscission (7, 9, 25), but the fact that in many cases ABA is effective only in relatively high concentrations (20) raises the possibility that ABA induces abscission indirectly, possibly through its effect on C_2H_4 formation.

In many plant systems, ABA is capable of inducing C_2H_4 formation (1, 8, 9, 11, 12, 15, 17, 19, 25, 28). Moreover, in bean explants, no increase in abscission and cellulase activity above C_2H_4 treatment was detected when ABA and C_2H_4 were applied together (15). This favors the concept that the ABA effect is achieved via C_2H_4 production. On the other hand, several investigators have reported (10, 13, 18, 28) that ABA induces abscission without affecting C_2H_4 production. Support for the hypothesis that ABA induces abscission directly comes from experiments showing that ABA is active under hypobaric pressure conditions (7, 25), in the presence of CO_2 (9), and under saturating levels of C_2H_4 (9). ABA has also been reported to act directly on cellulase (9, 25). It has been concluded that one of the primary effects of ABA is to induce the activity of the cell-wall degrading enzymes which are involved in the abscission process (3, 14, 26, 27).

In the light of controversy on the role of ABA in abscission, the present study was undertaken to study whether ABA induces activity of cell-wall degrading enzymes, cellulase, and PG[1] and, hence, abscission directly or whether its action is mediated by C_2H_4.

MATERIALS AND METHODS

Plant Material and Treatments. One-year-old shade leaves from about 40-year-old Shamouti orange (*Citrus sinensis* L. Osbeck) trees were picked in the morning and processed immediately. Two-cm-long explants, each consisting of 1-cm petiolar and 1-cm midrib tissues, were cut from unifoliate leaves. When not otherwise stated, application of ABA and AVG was performed by placing 10 to 13 explants in 1.8-ml vials and dipping the basal ends of the petioles in 1 ml 50 mM K-phosphate (pH 6.8) containing the desired compounds. Incubation was carried out in the dark at 25 ± 1 C in a humid environment. Exogenous C_2H_4 was supplied in a continuous flow system as previously described (27).

Abscission was determined by counting the explants which had already abscised and those which abscised due to a gentle touch administered by forceps to the distal end.

Translocation and Metabolism of ABA. Explants were treated by the method described above with 0.5 ml 1 mM ABA containing 0.043 μCi [2-^{14}C]ABA (11.3 mCi mmol^{-1}, purchased from The Radiochemical Centre, Amersham, United Kingdom). Care was taken to avoid capillary movement of [^{14}C]ABA between vial sides and explants. At the end of the incubation (Table I), the explants were washed with distilled H_2O and cut into four even segments. Ten corresponding segments were ground with a mortar and pestle in a total volume of 4 ml 80% ethanol. The extract was centrifuged for 5 min at 10,000*g* and aliquots were taken for radioactivity determinations.

For studying metabolism of ABA in explants, similar extracts were concentrated to a small volume on a rotary evaporator and applied to 10- × 20-cm plates coated with Silica Gel GF_{254}, 0.5 mm thick. The plates were activated at 105 C. Chromatography was performed for 15 cm with a chloroform/ethyl acetate/formic acid (5:4:1, v/v) solvent system. After drying, the plates were viewed under short UV light for location of standard ABA. The plates were separated into 10 R_F zones and scraped into scintillation vials for radioactivity determination. Radioactivity was measured with a Packard scintillation spectrometer, using Bray's scintillation fluid. Radioactivity data are expressed as dpm after quenching and efficiency corrections.

C_2H_4 Determination. For measuring the rate of C_2H_4 formation, the vials containing the explants were placed at different intervals into 14-ml flasks. The flasks were sealed with serum caps for 1 to 2 h and incubated in the dark at 25 C. Sampling of the atmosphere (2 ml), after injecting 0.5 ml air to prevent development of high hypobaric pressure, was carried out with a gas-tight hypodermic syringe. C_2H_4 was determined by means of a Packard gas chromatograph equipped with a flame ionization detector and alumina column held at 35 C. The vials containing the explants were removed from the flasks after each C_2H_4 determination for further incubation under the standard conditions.

Cellulase and PG Activity. Five-mm-long segments of the abscission zone tissues cut from 10 segments were ground with a mortar and pestle in 10 volumes/weight (ml/g) of 0.2 M K-phosphate (pH 7.5) containing 6% (w/v) NaCl and 0.05% (w/v) L-cysteine. Subsequent steps were carried out at 4 C. The homogenate was filtered through a fine nylon fabric and centrifuged for

[1] Abbreviations: PG, polygalacturonase; AVG, aminoethoxyvinyl glycine.

10 min at 10,000g. The supernatant was dialyzed overnight against the above solution except that the NaCl concentration was reduced to 0.5%. The dialysate was clarified by centrifugation as above and 0.5-ml samples were used for determination of cellulase and PG activity.

Cellulase activity was assayed by measuring the loss in viscosity of carboxymethylcellulose (sodium salt) (BDH Ltd., United Kingdom) (26). The standard reaction mixture in a total volume of 10.5 ml contained 0.5 ml enzyme solution and 1.2% (w/v) carboxymethylcellulose (sodium salt) in 20 mM K-phosphate (pH 6.5). The reaction mixtures were incubated for 18 h at 37 C and then viscosity was determined at 37 C using an Exax 300 viscosimeter. Activity is expressed as per cent loss of viscosity.

PG activity was assayed by measuring reducing groups liberation from sodium polypectate according to Riov (27). The routine 1-ml reaction mixture contained 0.5 ml enzyme solution, 0.25% (w/v) sodium polypectate (Sigma Chemical Co.) and 1 mM sodium hydrosulfite in 40 mM Na-acetate (pH 5.0). The reaction mixtures were incubated for 9 h at 37 C. The increase in reducing groups was measured with the dinitrosalicylic acid reagent (22), using D-galacturonic acid as a standard. One unit of activity is defined as the release of 1 μmol galacturonic acid in 18 h.

RESULTS

Effect of Application Methods. In preliminary experiments, citrus leaf explants were dipped for 10 min in 1 mM ABA solution and then placed with the basal ends in vials containing the hormonal solution. Under these conditions, almost no response to ABA was detected, *i.e.* rates of C_2H_4 evolution and abscission did not differ from the control. It was suspected that the slight response to ABA was due to the initial dipping of the whole explants. Therefore, the dipping was excluded and explants were placed in the treatment solution immediately after excision as explained above. When this modification in the application method was used, ABA induced significantly both C_2H_4 evolution and abscission (data not presented).

There are inconsistent reports concerning the movement of ABA through excised plant parts. Although several authors observed basipetal polarity, others reported that movement of ABA was not polar (21). It was, therefore, questionable whether ABA moves upwards in the explants when using the dipping method. Feeding experiments with labeled ABA (Table I) clearly showed that ABA moved acropetally in the explants, establishing a gradient of diminishing radioactivity. TLC analysis demonstrated that 50 to 70% of the radioactivity was present as free ABA when tested 44 h after the beginning of the experiment. No accumulation of labeled ABA was found to occur in the abscission zone.

ABA, C_2H_4, and Abscission. A study of the effect of different ABA concentrations on explants of mature leaves (Fig. 1) showed that both C_2H_4 evolution and abscission responses were concentration dependent. Significant effects were obtained with 10 μM ABA and above. In all cases, the increase in C_2H_4 evolution preceded the increase in abscission.

Table I. *Distribution of [2-^{14}C]ABA in Citrus Leaf Explants*

Explants were placed upright in 1 mM ABA containing 0.043 μCi [^{14}C]ABA. After incubation, the explants were cut into four even segments and radioactivity was determined in each segment; A: basal segment; B and C: middle segments; D: apical segment. Standard errors for the data are shown.

Incubation Time	Total Uptake	Radioactivity in Segments			
		A	B	C	D
h	*dpm*	*% total uptake*			
22	15330 ± 680	43 ± 1.7	26 ± 1.3	20 ± 1.3	11 ± 1.2
44	17355 ± 840	55 ± 1.6	25 ± 1.3	12 ± 1.0	8 ± 0.9

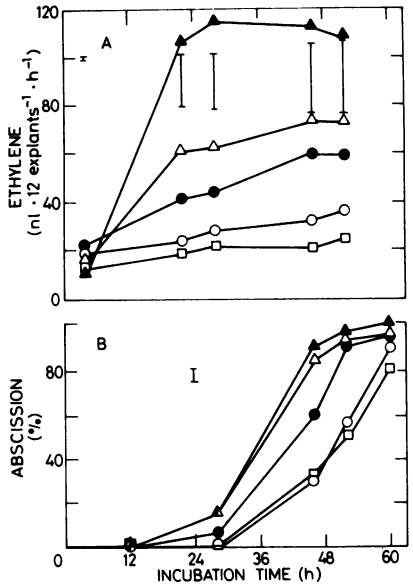

FIG. 1. Effect of ABA concentration on rate of C_2H_4 production (A) and abscission (B) of citrus leaf explants. ABA concentration: (□), 0; (○), 0.001 mM; (●), 0.01 mM; (△), 0.1 mM; (▲), 1 mM. Standard errors for the data are shown.

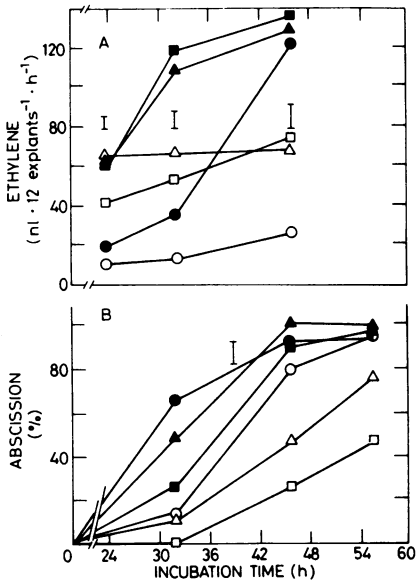

FIG. 2. Effect of ABA (0.5 mM) on rate of C_2H_4 production (A) and abscission (B) of explants of citrus leaves of different ages. Two-month-old leaves: (○), control; (●), ABA. Six-month-old leaves: (△), control; (▲), ABA. Twelve-month-old leaves: (□), control; (■), ABA. Standard errors for the data are shown.

Preliminary observations with citrus leaf explants (J. Wurzburger and R. Goren, unpublished) showed that the ABA effect is age-dependent. The effect of leaf age on the response to ABA was, therefore, studied (Fig. 2). Explants prepared from young leaves produced the least amount of C_2H_4 but were the most rapid in their abscission response both in control and ABA treatments.

Role of C_2H_4 in ABA-induced Abscission. The finding that ABA-induced abscission is accompanied by increased C_2H_4 formation (Fig. 1 and refs. 8, 9, 15, 25) raised the question whether the effect of ABA on abscission of citrus leaf explants is a primary one, or whether it is mediated by C_2H_4. An attempt was made to

study the effect of ABA on abscission in the presence of AVG, an inhibitor of C_2H_4 biosynthesis (23), and $AgNO_3$, an inhibitor of C_2H_4 action (6). In preliminary experiments using leaf explants incubated either under air or 4 $\mu l\ l^{-1}$ C_2H_4, it was found that $AgNO_3$, ranging between 50 to 500 mg l^{-1}, inhibited abscission only by 30 to 65%. AVG, on the other hand, almost completely inhibited the ABA-induced C_2H_4 formation and delayed abscission. Accordingly, the interaction between ABA and AVG in relation to abscission was studied further. Kinetic studies using AVG in concentrations of 0.03 to 0.24 mM showed a positive correlation between increasing concentrations of AVG and inhibition of ABA-induced C_2H_4 formation and abscission (results not presented). Such an experiment (Fig. 3), using 0.12 mM of AVG in combination with 0.5 mM ABA, shows this interaction. The ability of AVG to inhibit the ABA-induced C_2H_4 formation is emphasized by the finding that the level of the gas in the combined treatment was lower than the control and similar to that of AVG alone. It is clear from these data (Fig. 3) that the inhibition of ABA-induced C_2H_4 formation was reflected by a marked delay in abscission, the rate of which was lower than that of the control.

ABA and the Increase in Hydrolytic Enzymes. Rasmussen (25) provided evidence that in citrus fruit explants ABA is capable of inducing increased activity of cellulase in the calyx abscission zone without the mediation of C_2H_4. In experiments which tested this concept in citrus leaf explants, we found that the ABA-induced

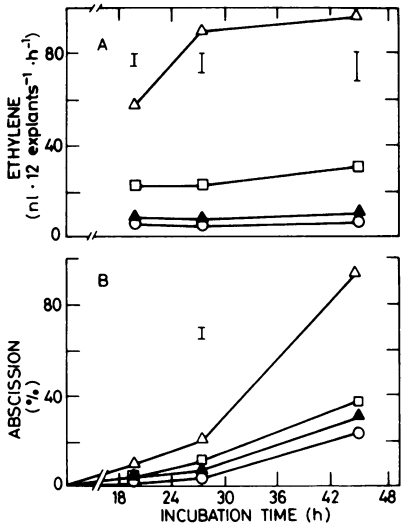

FIG. 3. Inhibition of ABA-induced C_2H_4 formation (A) and abscission (B) of citrus leaf explants by AVG. ABA and AVG concentrations were 0.5 and 0.12 mM, respectively. (□), Control; (○), AVG; (△), ABA; (▲), ABA + AVG. Standard errors for the data are shown.

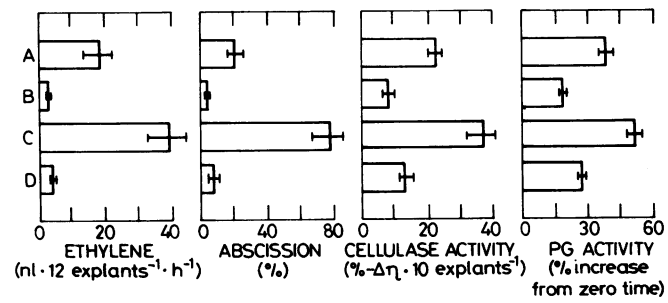

FIG. 4. Inhibition of ABA-induced C_2H_4 formation, abscission, and cellulase and PG activity of citrus leaf explants by AVG 28 h after excision. ABA and AVG concentrations were 1 and 0.24 mM, respectively. PG activity at zero time was 1.14 units/10 explants. A, Control; B, AVG; C, ABA; D, ABA + AVG. Standard errors for the data are shown.

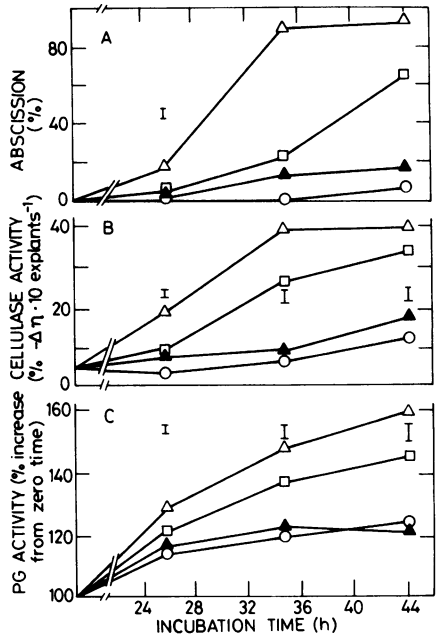

FIG. 5. Time course of the inhibition of ABA-induced abscission (A) and cellulase (B) and PG (C) activity of citrus leaf explants by AVG. ABA and AVG concentrations were as in Figure 4. PG activity at zero time was 1.43 units/10 explants. (□), Control; (○), AVG; (△), ABA; (▲), ABA + AVG. Standard errors for the data are shown.

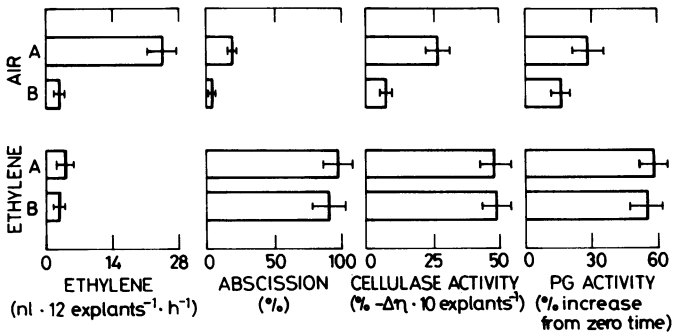

FIG. 6. Specificity of the inhibitory effects of AVG in citrus leaf explants. Explants were treated with buffer or with 0.24 mM AVG and incubated either under air or 8 $\mu l\ l^{-1}$ C_2H_4 for 28 h. PG activity at zero time was 1.64 units/10 explants. A, Control; B, AVG. Standard errors for the data are shown.

increase in the activity of cellulase, PG, and abscission was markedly inhibited by AVG (Fig. 4). At the same time, AVG almost completely inhibited the ABA-induced C_2H_4 formation. The inhibitory effect of AVG on the activity of the hydrolytic enzymes and abscission could be followed during the whole experimental period (Fig. 5).

The specificity of the effects of AVG was tested by incubating explants treated with the inhibitor either in air or C_2H_4 (Fig. 6). In air-treated explants, AVG inhibited, as expected (Figs. 3–5), C_2H_4 production, the increase in cellulase and PG activity, and abscission. AVG did not interfere with the inducing effects of exogenously supplied C_2H_4 on the activity of the hydrolytic enzymes and abscission. In C_2H_4-treated explants, formation of endogenous C_2H_4 was inhibited.

DISCUSSION

The present study clearly shows that citrus leaf explants respond to ABA treatment by accelerated abscission. The response to ABA

is achieved in a relatively high concentration range as reported earlier for herbaceous plants (9, 15). We have found that, in citrus leaf explants, 30 to 50% of the applied ABA is metabolized within 44 h from excision. This may indicate that a constant supply of exogenous ABA is needed to induce the hormonal effect and this is partly achieved by an initial application of a relatively high dose.

The data presented (Figs. 1 and 2) show clearly that, in all cases in which ABA induces abscission, increased formation of C_2H_4 is also observed. This suggests that ABA-induced abscission is mediated by C_2H_4. Careful examination of Figure 2 reveals, however, that in relation to leaf age there is, at least during the first part of the incubation period, a negative correlation between C_2H_4 production and abscission. This may indicate age-dependent sensitivity of citrus leaf explants to C_2H_4. Similar results have also been reported for leaf explants of various other plants (24).

A more direct support for the idea that ABA-induced abscission is mediated by C_2H_4 comes from the experiments with AVG (Figs. 3 and 4), in which we have shown that inhibition of ABA-induced C_2H_4 formation markedly delays abscission. In contrast to previous studies (9, 25), our results (Figs. 4 and 5) indicate that ABA does not have a direct effect on the hydrolytic enzymes which play a role in the abscission process. It is suggested that, in citrus leaf explants, the following sequence of events occurs when ABA is inducing abscission: ABA $\rightarrow$ C_2H_4 formation $\rightarrow$ induction of cellulase and PG $\rightarrow$ cell-wall degradation $\rightarrow$ abscission.

Previous studies in which it was shown that ABA has, at least in part, a direct effect on the activity of the hydrolytic enzymes and abscission were based mainly on the use of CO_2 (9) or hypobaric conditions (25). There are cases in which CO_2 was unable to reverse completely C_2H_4 effects (2, 4) and recent unpublished data (H. Brisker and R. Goren) show that, even under hypobaric pressure conditions, there is an increase in the accumulation of endogenous C_2H_4 in the internal atmosphere of citrus fruits, a system similar to that used by Rasmussen (25) for studying the effect of ABA on cellulase activity and abscission of citrus fruits. The use of AVG as an inhibitor of C_2H_4 biosynthesis provides a more precise experimental tool for studying the direct role of a given plant hormone since it eliminates the formation of C_2H_4 in the experimental system. The data in Figure 6 indicate that, in citrus leaf explants, AVG specifically inhibited C_2H_4 formation. In the presence of exogenously supplied C_2H_4, AVG was unable to inhibit the C_2H_4-induced activity of cellulase and PG as well as abscission. We concluded that the presence of C_2H_4 is obligatory for the induction of these enzymes and abscission.

The precise relationship between ABA treatment and C_2H_4 formation remains unclear (16). Some investigators suggest that ABA has a primary effect on C_2H_4 formation (19), whereas others (15) suggest that it has a secondary effect, resulting from a primary effect on senescence.

Acknowledgment—The authors wish to thank Hoffman La Roche Inc., Nutley, N. J., for the gift of AVG.

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