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Sites of Abscisic Acid Synthesis and Metabolism in *Ricinus communis* L.[1]

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ABSTRACT

The sites of abscisic acid (ABA) synthesis and metabolism in *Ricinus communis* L. were investigated by analyzing the levels of ABA and its two metabolites phaseic acid (PA) and dihydrophaseic acid (DPA) in the shoot tips, mature leaves, and phloem sap of stressed and nonstressed plants.

Water stress increased the concentration of ABA, PA, and DPA in phloem exudate and also increased the levels of all three compounds in mature leaves and in shoot tips. The latter had a very high DPA content (18.7 μg/g fresh weight) even in plants not subjected to water stress. When young and mature leaves were excised and allowed to wilt, the level of ABA increased in both, demonstrating that leaves at an early stage of development have the capacity to produce ABA.

These results have been interpreted to mean that in mature leaves of nonstressed *Ricinus* plants, ABA is synthesized and metabolized, and that ABA itself, as well as its metabolites, are translocated in the phloem to the shoot tips (sinks). Since DPA, but not ABA, accumulates in the shoot tips, it follows that ABA is metabolized rapidly in the apical region. To what extent ABA present in young leaves of nonstressed plants is the consequence of synthesis *in situ* and of import from older leaves remains to be determined.

Young, rapidly expanding leaves and apices of plants usually have a much higher ABA content than mature leaves (*e.g.* 4, 5, 13, 14). *A priori* this could be the consequence of (*a*) a higher rate of ABA synthesis in young leaves than in mature ones; (*b*) a low rate of ABA metabolism in young leaves; (*c*) translocation of ABA with the assimilates from mature leaves to the importing young leaves; or (*d*) a combination of these three factors.

So far, most investigators concerned with ABA levels have focused their attention on actual amounts present in tissues or organs, and only in recent work has the role of transport (4) and metabolism (3) been considered. In the present paper, I report the results of experiments on the question as to which organs are the sites of ABA synthesis and metabolism in *Ricinus* plants. The investigation was conducted by studying the distribution of ABA and its metabolites PA[2] and DPA in the phloem sap and in leaves of different ages, and how the levels of these three compounds are affected by water stress.

MATERIALS AND METHODS

Plant Material. Seeds of *Ricinus communis* L. cv. gibsonii, purchased from Thompson and Morgan, Somerdale, N.J., were planted in a greenhouse maintained at a minimum temperature of 22 C. Natural light was supplemented with light from Sylvania Gro-Lux/WS fluorescent lamps (irradiance about 40 μw cm^{-2} at plant level) to give a photoperiod of 20 hr. The plants were grown in a mixture of vermiculite and gravel in 7.6-liter plastic containers with holes in the bottom for drainage. They were used for experimentation when 8 to 12 weeks old. Watering was done once per day with half-strength Hoagland nutrient solution, and once per day with deionized H_2O.

Prior to the start of an experiment, the plants were transferred to a growth chamber for at least 1 week. The temperature in the growth chamber was maintained at 23 C throughout and the relative humidity around 60%. A 12-hr period of high intensity light from fluorescent tubes (Sylvania Cool White FR96T12/CW/VHO/135) and incandescent lamps (irradiance 13 mw cm^{-2}) was followed by an 8-hr period of light from the incandescent lamps only (irradiance 2 mw cm^{-2}). Water stress was induced by withholding water for 3 to 4 days and was terminated by flooding the pots with deionized H_2O.

For extractions, the leaves without the petioles and shoot tips were harvested. *Ricinus communis* cv. gibsonii is a day-neutral plant, and under the experimental conditions produced eight to 11 leaves preceding the terminal inflorescence (unpublished observations). The shoot tips included therefore the youngest one or two not yet unfolded leaves as well as the young inflorescence.

Detached wilted material (see Table III) was prepared by excising young, just unfolded leaves or the youngest fully expanded leaves and exposing them to a stream of air from a fan until they had lost 12% of their fresh weight. The wilted leaves were kept in polyethylene bags in darkness at 22 C for various periods of time until harvest as indicated under "Results" (see Table III). Harvested plant material was immediately frozen in liquid N_2 and pulverized.

Phloem Exudate. Plants to be used for collection of phloem sap were massaged two to three times daily as described by Milburn (11, 12), starting 3 or 4 days prior to the actual collection. Water-stressed plants were watered and allowed to recover briefly before collection of exudate was started. Phloem content was collected as an exudate emanating from diagonal incisions made in the stems with a razor blade (11, 12). The exudate was collected in capillary pipettes and bulked in 12-ml centrifuge tubes kept in ice. Each sample of exudate was centrifuged at low speed and the small amount of precipitate discarded. The exudate was then frozen and lyophilized for determination of the dry weight.

Exudate from leaves was obtained by treating the cut surface of the petioles for 1 hr with 20 mM EDTA (6). After rinsing with distilled H_2O, the petioles were placed in vials with distilled H_2O in darkness at 27 C and high relative humidity for 21 hr. The exudate was frozen and lyophilized to determine the dry weight.

Sugar Analysis. Phloem exudate was serially diluted and 2 μl spots applied to precoated silica gel plates (Merck), 0.25 mm thick. Spots of several standard sugars were also applied. The

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[2] Abbreviations: PA: phaseic acid; DPA: dihydrophaseic acid; EC: electron capture.

plate was developed four times with ethyl acetate-acetic acid-water (10:5:2) as solvent (2). Sugar spots were visualized by spraying with *p*-anisaldehyde-sulfuric acid-acetic acid (1:2:100) and heating at 100 C (7).

Extraction and Purification of ABA, PA, and DPA. Lyophilized exudate was dissolved in a small volume of water and the pH adjusted to 2.5 with 6 N HCl. The acidic fraction was prepared by partitioning six times with equal volumes of ethyl acetate.

Frozen samples of leaves and shoot tips were extracted several times at 4 C with methanol containing a small amount of glacial acetic acid (9), and 2,6-di-*tert*-butyl-4-methylphenol as an antioxidant (17). The acidic fractions were prepared as described in reference 17.

Acidic fractions prepared from exudates, leaves, or shoot tips were applied to Brinkmann precoated Silica Gel F_{254} plates (200 × 200 × 0.25 mm) and developed twice to 12 cm in toluene-ethyl acetate-acetic acid (50:30:4). The zones co-chromatographing with authentic ABA, PA, and DPA were scraped off and eluted with a mixture of ethanol and acetone (1:1). The fractions thus prepared were methylated with ethereal diazomethane and rechromatographed on Silica Gel F_{254} plates in hexane-ethyl acetate using the ratio 2:1 for Me-ABA, 1:1 for Me-PA, and 1:2 for Me-DPA. Zones corresponding to R_F values of authentic standards were scraped off and eluted as before.

Determination of ABA, PA, and DPA. The amounts of methylated ABA, PA, and DPA present in samples purified by TLC were determined with a Hewlett-Packard model 402B gas chromatograph equipped with a ^{63}Ni EC detector, and a U-shaped glass column (183 × 0.3 cm) packed with 1% XE-60 on 100/120 Gas-chrom Q. The carrier gas was argon-methane (95:5) at a flow rate of 65 ml/min. The oven temperature was 200, 208, and 204 C for the methyl esters of ABA, PA, and DPA, respectively. The detector temperature was 240 C.

Absolute amounts were determined by comparing peak heights from unknown samples with those obtained from known amounts of the various standards. The amount present in each standard was measured by UV spectrophotometry using λ_{max} = 265 nm and ϵ = 20,900 for Me-ABA (10), λ_{max} = 263 nm and ϵ = 16,900 for Me-Pa (3), and λ_{max} = 267 nm and ϵ = 19,900 for Me-DPA (3).

Combined GLC-MS. Mass spectra were obtained with an LKB-9000 and a column of 3% SP-2100 (183 × 0.3 cm) equipped with a data acquisition system. The temperature was programed from 200 to 240 C at 5 C/min, and a He gas flow of 25 ml/min. All spectra were obtained at 70 ev.

RESULTS

Composition of Exudate. Previous work has shown that phloem exudate can be obtained from *Ricinus* with relative ease (2, 4, 11, 12). In the present experiments, several ml of exudate were collected from two plants over a 3-hr period (Table I). Stressed and subsequently watered plants also yielded exudate in good quantities. The data in Table I indicate that the per cent dry weight (w/v) of exudate collected from nonstressed plants was consistently higher than that from plants that had been subjected to water stress. In agreement with earlier work (2), sucrose was the only sugar detected by TLC in the exudate. The pH of exudate from nonstressed plants ranged from 7.4 to 8 while that collected from stressed plants was always 0.2 to 0.5 pH units lower.

As analyzed by GLC with EC detector ABA, as well as its metabolites PA and DPA, were present in *Ricinus* phloem exudate (Table I). The concentration of all three compounds increased several times following a period of water stress. After a 30-hr recovery period (Table I, expt. 2) the ABA concentration had declined as compared to a 5-hr period, while that of PA and DPA had increased further.

ABA, PA, and DPA Contents as Related to Leaf Age. Data on ABA, PA, and DPA levels in nonstressed *Ricinus* leaves of different ages are presented in Figure 1. The ABA content was highest in the young leaves and decreased with leaf age; the shoot tips were also low in ABA. The levels of DPA were always higher than those of ABA and showed a sharper decline with leaf age than the latter (Fig. 1B). The content of DPA, unlike that of ABA and PA, was always highest in the shoot tips. The PA content peaked in the youngest mature leaf (Fig. 1B) and decreased with further increase in leaf age.

Effect of Water Stress on ABA, PA, and DPA Content of Source and Sink Regions, and of Phloem Sap. In the following

Table I. *Concentrations of ABA, PA, and DPA in Phloem Exudate from Ricinus Plants and from Plants Subjected to Water Stress*

"Non-stressed" exudates obtained from two plants, "stressed" from 4 plants, except in expt. 2 where only two plants were used.

Treatments	Recovery Period	Exudate collected	% dry wt	ABA	PA	DPA
	hr	ml	w/v	µg/ml		
Expt. 1						
Non-stressed		5.0	18.4	1.0	0.4	0.4
Stressed	5	6.8	14.0	1.7	1.4	3.0
Expt. 2						
Non-stressed		2.7	24.1	1.4	0.3	0.4
Stressed	5	2.3	14.0	2.6	0.7	1.1
Stressed	30	6.0	16.0	2.2	2.7	2.1
Expt. 3						
Non-stressed		4.7	22.5	0.6	1.0	0.3
Stressed	6	5.7	17.4	5.2	5.8	2.0

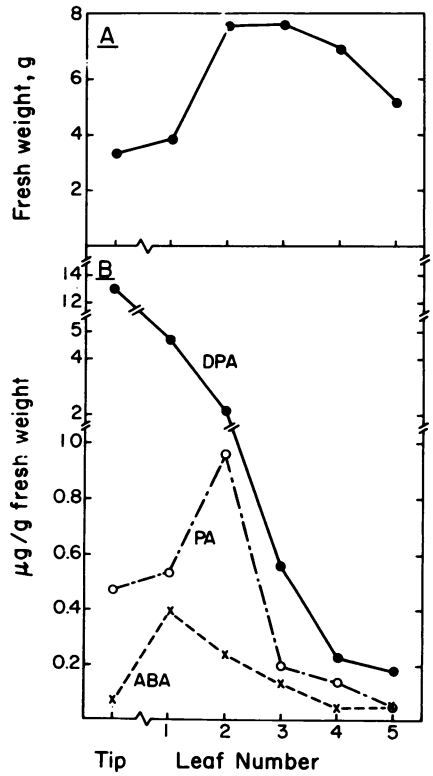

FIG. 1. Changes in ABA, PA, and DPA content of *Ricinus* leaves with age. A: Fresh weight of shoot tip and leaves of different ages; B: ABA, PA, and DPA content of shoot tip and leaves expressed per unit fresh weight.

experiment, the effect of water stress on the content of ABA and its metabolites was not only determined in the phloem exudate, but also in the youngest fully expanded leaves (source leaves) and in the shoot tips (sinks). The results (Table II) confirm that a period of water stress resulted in an increase in the concentration of ABA, PA, and DPA in the phloem exudate. The levels of all three compounds, particularly that of ABA, also increased in the mature leaves. The increase in ABA in the shoot tips (four times) was smaller than that in the leaves (nine times) and in the exudate (11.5 times). On the other hand, DPA was already present in very high levels in the shoot tips (18.7 μg/g) and following water stress the content increased only about two times to 34.8 μg DPA/g fresh weight. Thus, this compound appears to accumulate in the shoot tips (sinks) of nonstressed and stressed plants as the end product of the metabolic pathway ABA→PA→DPA.

Effect of Wilting on ABA, PA, and DPA Levels in Young and Mature Leaves. In order to investigate the ability of leaves of different ages to produce ABA in response to water stress while avoiding complications caused by translocation from attached leaves, young or fully mature leaf blades were detached and wilted. Control blades were frozen immediately. As shown in Table III, young leaves responded with a more rapid and greater increase in ABA than did the mature blades. PA and DPA also increased during the wilting period, thus establishing that in *Ricinus*, as in excised primary leaves of *Phaseolus* (3), both the synthesis and metabolism of ABA are elevated during a period of wilting.

Composition of Leaf Exudate after EDTA Treatment. Exudate was obtained from a total of 31 mature, stressed leaves. The dry weight of the lyophilized material was 132 mg. Assuming a dry weight of 18% (w/v), this corresponds to a volume of 0.7 ml. Calculated on this basis, the exudate contained per ml 0.5 μg ABA, 0.4 μg PA, and 7.2 μg DPA. The values for ABA and PA were lower and the one for DPA higher than those measured in exudates from stressed plants (Table I). However, leaf exudate was collected later, *viz.* 33 to 54 hr after stress conditions had been terminated, than was exudate from stems.

Identification of ABA, PA, and DPA by Combined GLC-MS. Representative samples of all three compounds prepared from phloem exudate, leaves, or shoot tips were analyzed by GLC-MS for unequivocal identification. In each case, the mass spectrum of the putative substance was identical to that of the methy ester of the authentic compound (*cf.* 17).

Table II. *Levels of ABA, PA, and DPA in Phloem Exudate, Shoot Tips and Mature Leaves of Stressed and Non-Stressed Ricinus Plants*

Exudate collected 21 to 24 hr after end of stress period; tips and leaves harvested after 24 hr.

Treatments	ABA	PA	DPA
Phloem Exudate		μg/ml	
Non-Stressed	0.2	0.3	0.4
Stressed	2.3	0.8	3.6
Shoot Tips		μg/g fresh wt	
Non-Stressed	0.1	0.5	18.7
Stressed	0.4	0.8	34.8
Leaves			
Non-Stressed	0.3	0.2	1.2
Stressed	2.7	0.7	2.8

Table III. *Effect of Water Stress on ABA, PA, and DPA Levels in Detached Young and Mature Leaf Blades of Ricinus*

Young leaves: 8 leaves per sample; average 0.5 g/leaf.
Mature leaves: 4 leaves per sample; average 9.8 g/leaf.

Treatments	ABA	PA	DPA
Young leaves		μg/g fresh wt	
Control, 0 hr	0.2	1.6	8.8
Wilted, 8 hr	2.5	2.6	9.1
Wilted, 24 hr	3.3	6.9	13.5
Mature leaves			
Control, 0 hr	0.2	0.2	0.7
Wilted, 8 hr	0.3	0.3	1.2
Wilted, 24 hr	1.4	0.9	1.3

DISCUSSION

It is generally accepted (2, 4, 11, 12) that the exudate collected from incisions in the bark of *Ricinus* represents the contents of sieve tubes. The presence of ABA, PA, and DPA in this exudate (Tables I and II) establishes that these compounds are translocated in the phloem.

Following a period of water stress, the concentrations of ABA, PA, and DPA in the exudate increased. All three compounds accumulated in excised wilted leaves (Table III), and exuded from the petioles of detached leaves. As a whole, these results demonstrate therefore that (*a*) both ABA synthesis and metabolism take place in mature leaves; and (*b*) ABA itself as well as its two metabolites are exported from mature leaves.

As for the young organs, it follows from the results in Table III that leaves at a very early stage of development are capable of producing ABA in response to water stress. Apices of water-stressed intact plants also showed an increase in ABA, although this increase was less than that in mature leaves and in the phloem exudate of such plants (Table II). Thus, ABA, whether synthesized *in situ* or imported from elsewhere, did not accumulate to a large extent in the growing region, but was apparently metabolized via PA to DPA which accumulated to very high levels.

Although detached young leaves were capable of producing ABA in response to stress (Table III), it does not follow that these leaves *in situ* were also sites of ABA synthesis. While mature leaves of water-stressed plants always exhibited wilting symptoms, the youngest leaves remained turgid, *i.e.* they probably did not reach the point of zero turgor. In mature leaves of sorghum and maize (1), and of two *Ambrosia* species (16), the ABA levels increased abruptly when the leaf water potential reached a threshold value of around -10 bars. Without similar data for *Ricinus* leaves of different ages, it is impossible to conclude whether an increased ABA content in young leaves of stressed plants is at least partly based on synthesis *in situ*.

The distribution of DPA in *Ricinus* (Fig. 1) is similar to that reported for the alkaloid ricinine (15), *viz.* highest in young tissue. In the course of the present investigation, ricinine was found in exudate at a concentration of approximately 20 μg/ml (unpublished results), indicating that this compound is also translocated in the phloem.

At present, no physiological role of PA or DPA is known in *Ricinus*, but recent work by Kriedemann *et al.* (8) with grapevine (*Vitis*) has raised the interesting possibility that PA acts as an inhibitor of photosynthesis. If this proves to be generally correct, it is obvious that in future work on water stress in plants not only the role of ABA, but also those of the metabolites PA and DPA deserve attention.

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