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Abscisic Acid Metabolism by Source and Sink Tissues of Sugar Beet[1]

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ABSTRACT

The fate of exogenously applied, labeled abscisic acid (±)-(ABA) was followed in source leaves and taproot sink tissues of sugar beet (*Beta vulgaris* cv AH-11). The objective was to determine if differential pathways for ABA metabolism exist in source and sink tissues. Tissue discs were incubated for up to 13 hours in a medium containing 1 micromolar labeled ABA. At various time intervals, samples were taken for metabolite determination by reverse-phase high performance liquid chromatography. The labeled metabolites were identified by retention times using an online scintillation counter.

Dihydrophaseic acid (DPA) aldopyranoside, DPA, phaseic acid (PA), ABA glucose ester (ABA-GE), and two unidentified compounds were recovered from both tissues. An additional unidentified metabolite was also present in root tissue. Leaf tissue discs exhibited a higher capacity for ABA conjugation, and root discs showed a greater preference for ABA catabolism to PA and DPA. After 4 to 5 hours, ABA incorporation into the various metabolites was proportional to the external ABA concentration in both tissues. But the internal ABA pool size was independent of external concentrations below 10^{-6} molar. These results suggested that rates of ABA metabolism was proportional to the rates of uptake in both tissues.

The importance of ABA in a wide range of physiological responses is well recognized (14, 15). ABA synthesized in the leaf is transported in the phloem along with other assimilates to sink tissues (11). The role, if any, for ABA in sink metabolism is unclear, but it may influence sink activity and phloem unloading (12, 13) and sucrose uptake (10). However, if ABA is continuously transported to sink regions, it must either accumulate to high levels or be readily metabolized. Metabolism is an important mechanism whereby plants modulate the concentration of biologically active compounds. Studies of ABA metabolism are important, therefore, because they help clarify whether an observed effect was due to ABA or to a catabolic product made by the plant when ABA was applied exogenously. Similarly, without information on ABA metabolism, a lack of any ABA effect may be an erroneous interpretation due to the possible ABA inactivation (conjugation or degradation). Knowledge about the uptake and metabolism of ABA is important to any attempt to clarify the regulation of ABA concentration and activity within plants (7, 8).

Daie and Wyse (2) have previously determined the mode of [^{14}C]ABA uptake by source and sink tissues of sugar beet. ABA diffuses across membranes as the undissociated, lipophilic species. ABA transport is the result of a pH gradient established across membranes. As a weak acid, ABA would dissociate and accumulate in the more alkaline compartment. The findings are consistent with earlier results of Kaiser and Hartung (5).

The objective of this study was to follow the fate of exogenously applied ABA in physiologically different tissues; namely, source and sink. We particularly wanted to determine if the two tissues differed in the mechanism by which endogenous ABA levels were controlled.

MATERIALS AND METHODS

Greenhouse-grown sugar beet (*Beta vulgaris* cv AH-11; Amalgamated Sugar Company) plants, 4 to 6 months of age, were utilized. Growing conditions were as described previously (2).

Preparation of Tissue Discs. Leaf discs 10 mm in diameter were obtained using a sharp corkborer. Discs from several leaves were pooled and used within 10 min with no pretreatment. Root tissue was prepared by cutting slices 1 mm thick with a hand microtome. Discs, 5 mm in diameter, were cut from these slices with a sharp corkborer and equilibrated for 90 min in a solution of 1 mM $CaCl_2$ and 200 mM mannitol buffered at pH 6.5 with 30 mM morpholinopropanesulfonic acid-BTP.[4] The equilibration medium was aerated continuously with humidified air and replaced every 15 min.

Incubation. Unless otherwise stated, the base incubation medium for leaves consisted of 10 mM Mes-BTP (pH 5.5), 1 mM $CaCl_2$, 1 μM labeled ABA (1 nCi/pmol of [^{3}H]ABA or 0.1 nCi/pmol [^{14}C]ABA). Five leaf discs (0.1 g, fresh weight) were floated (upper side up) on 3 ml of medium in a 4-cm-diameter Petri dish. The incubation medium for root tissues consisted of 30 mM Mes-BTP (pH 5.5), 1 mM $CaCl_2$, 1 μM labeled ABA (specific activity was the same as for leaves), and 200 mM mannitol as the osmoticum. Thirty discs of root tissue (0.75 g, fresh weight) were incubated in 3 ml of medium in a 10-ml vial.

Humidified air was slowly bubbled through each vial contain-

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[4] Abbreviations: BTA, bis-tris prophane; PA, phaseic acid; DPA, dihydrophaseic acid; ABA-GE, ABA glucose ester.

ing root discs to prevent anaerobiosis. All experiments, except as stated, were conducted for periods of up to 13 h at ambient room temperature and light conditions.

After incubation, the labeled medium was withdrawn by suction, and the discs were rinsed three times for 3 min each. No determination of the radioactivity in the external media was made; therefore, any metabolite that may have been excreted or exchanged is not accounted for. Each wash consisted of 3 ml of distilled H_2O for leaf tissue and 7 ml of 200 mM mannitol for root tissue. Our preliminary compartmental analysis of these tissues had shown that almost all of the radioactivity on the surface and in the free space of the tissue was removed with this washout procedure. All treatments were conducted in triplicate, and all experiments were repeated at least twice. Specific conditions for individual experiments are described in the appropriate table or graph. Results of initial experiments conducted using [^{3}H]ABA and repeated with [^{14}C]ABA were similar. Data presented here are from the first set of experiments.

Metabolite Extraction and Analysis. Immediately after the washout procedure described above, tissues were frozen in liquid N_2, freeze-dried, and homogenized in chilled 90% methanol (20 ml/g fresh weight). After filtration through Whatman No. 1 paper, the extracts were taken to dryness under reduced pressure at 25°C and redissolved in 1.5 ml of 0.2 N acetic acid. Particulates were removed by filtration through a 1.2-μm nitrocellulose filter (RAWP, -01300; Millipore Corp., Bedford, MA).[5] The filtrate was then chromatographed by reverse-phase HPLC. The preparative HPLC column was a 150 × 10 mm C_{18} (RSIL, 10 μm particle size; Alltech Associates, Deerfield, IL). A two-step linear solvent gradient delivered at 2 ml/min was used to separate the metabolites. The initial gradient changed the solvent from 0.1 N acetic acid to 9.5% ethanol:0.1 N acetic acid (v/v) in 2 min. Immediately following this gradient, a second gradient to 47.5% ethanol:0.1 N acetic acid (v/v) in 25 min separated samples into partially resolved peaks detected by an on-line scintillation counter (model 1050; CAI Instruments, Midland, MI) fitted with Teflon flow cell packed with quartz encapsulated scintillant beads (model EGB-02; CAI Instruments).

Seven radioactive fractions (including ABA) were collected from leaf samples and eight from root samples. The sampling corresponded to the retention times of detected radioactivity (Fig. 1). Fraction collection (Ultrora 7000; LKB), sample injection, and solvent delivery (model 6000 A pumps; Waters Associates) were controlled by a SLIC 1400 (Systec Inc., Minneapolis, MN) microprocessor-based controller. Radiolabeled fractions were taken to dryness under reduced pressure at 25°C and resuspended in two rinses of 2 ml 0.1 N acetic acid. After adding 15 ml Aquasol (New England Nuclear) to each sample, radioactivity was measured as dpm in a 460 CD Packard scintillation counter.

GC Analysis. To determine endogenous ABA levels, frozen tissue samples were extracted for 48 h with two changes of 90% methanol (20 ml/g fresh weight). The extract was then partitioned against methyl chloride and water at pH 8.5 and 3.0, respectively (three partitionings with methyl chloride and water at each pH). The methyl chloride phase that contained ABA was dried on a flash evaporator, and samples were derivatized by the diazomethane methylation procedure. A Tractor 222 GC equipped with a ^{63}Ni electron capture detector was used for ABA quantification. The 2000- × 4-mm column was packed with 3% OV 17 (Gas Chrom Q, 100–120 mesh). Nitrogen delivered at a flow rate of 80 ml/min was the carrier gas. The temperatures of the injection port, column oven, and detector were isothermally maintained at 250°, 225°, and 290°C, respectively. Internal standards of [^{3}H]ABA were used to correct for ABA losses.

RESULTS

Analysis of root and leaf tissue extracts for radiolabeled metabolites from a preliminary study indicated that labeled ABA was metabolized into several different products (Fig. 1). According to their retention times, these metabolites were tentatively identified to be DPA aldopyranoside, $R_{20.5}$, DPA, PA, ABA-GE, $R_{27.8}$, and ABA. $R_{20.5}$ and $R_{27.8}$ were unidentified metabolites present in both tissues. An additional unidentified metabolite with a retention time of 25 min (R_{25}) was unique to the root extract. R_{25} was less polar than ABA but more polar than ABA-GE.

After an approximately 2-h lag, labeled ABA taken up by leaf tissue was primarily conjugated to the ABA-GE or catabolized to DPA and PA (Fig. 2). After this initial 2-h lag, the incorporation of radioactivity into these metabolites was essentially linear. At the end of the 13-h incubation period, leaf discs had converted 60% more ABA into the ABA-GE than had been accumulated in the PA fraction. However, the amount of label in the combined PA and DPA pool was about the same as that of ABA-GE.

In the root tissue, labeled ABA was rapidly incorporated into PA and the unique metabolite (R_{25}) with no apparent lag period (Fig. 2). The ABA-GE accumulated in the root tissue at much slower rates than did PA. In contrast to the leaf tissue, after 13 h, root tissue had incorporated almost twice as much ABA into PA as into ABA-GE.

By measuring total dpm in the tissue, Daie and Wyse (2) had previously shown that an increase in the exogenous ABA concentration from 10^{-9} to 10^{-3} M resulted in a proportional increase in ABA uptake. ABA metabolism, as indicated by incorporation of label into different ABA metabolites, was also proportional to exogenous ABA concentrations (Fig. 3). Substantially larger amounts of ABA were converted into its metabolites when tissue was incubated in the presence of 10^{-5} M ABA than with 10^{-8} M ABA. In the leaf tissue, all metabolites increased in essentially a proportional fashion except for the DPA aldopyranoside, which increased exponentially when the external ABA concentration increased from 10^{-6} to 10^{-5} M ABA. In the root tissue, incorporation of label into all metabolites except DPA aldopyranoside

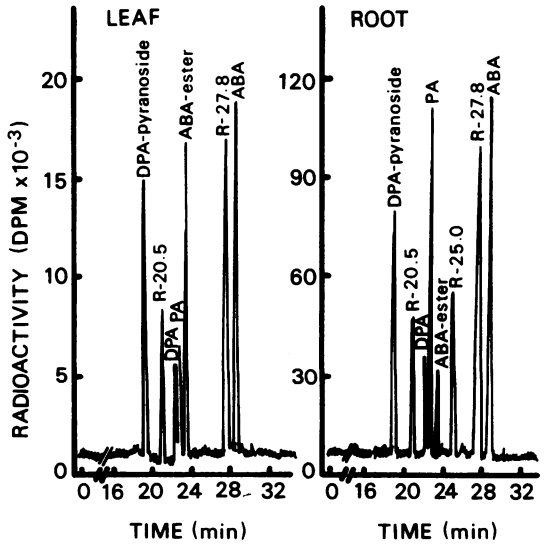

FIG. 1. On-line scintillation counter tracing from chromatogram of a sample of sugar beet leaf and root extracts. Separation was with the preparative HPLC system as described in the text.

[5] Mention of a trademark of proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

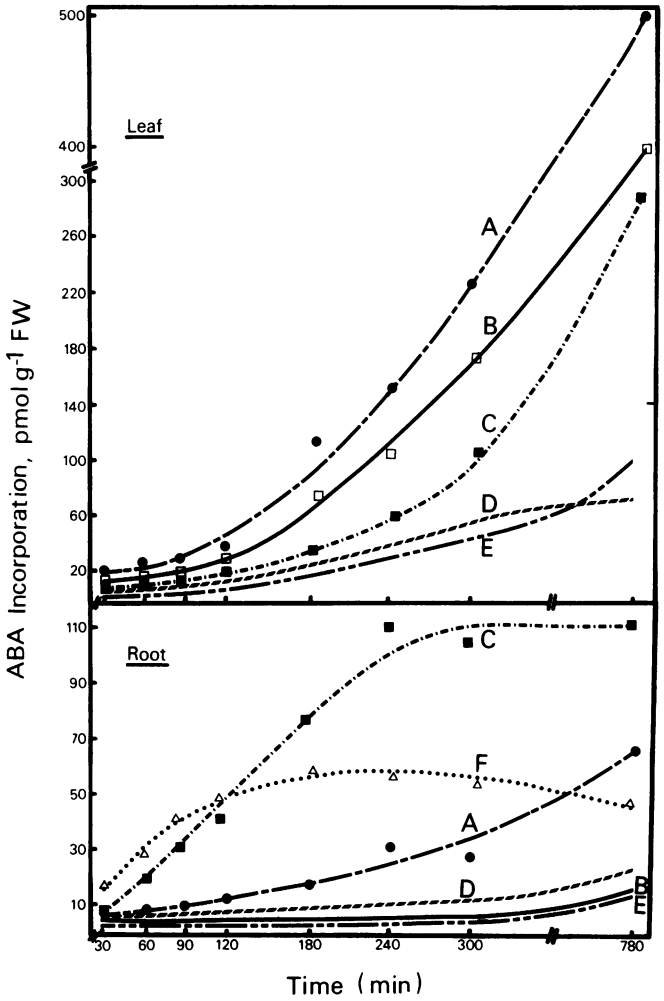

FIG. 2. Time course study of [^{3}H]ABA metabolism by sugar beet leaf and root tissue discs. Metabolites were collected as described in the text. Samples were taken at all time points shown on the horizontal axis. ABA-GE (A), DPA (B), PA (C), $R_{27.8}$ (D), DPA aldopyranoside (E), and R_{25} (F).

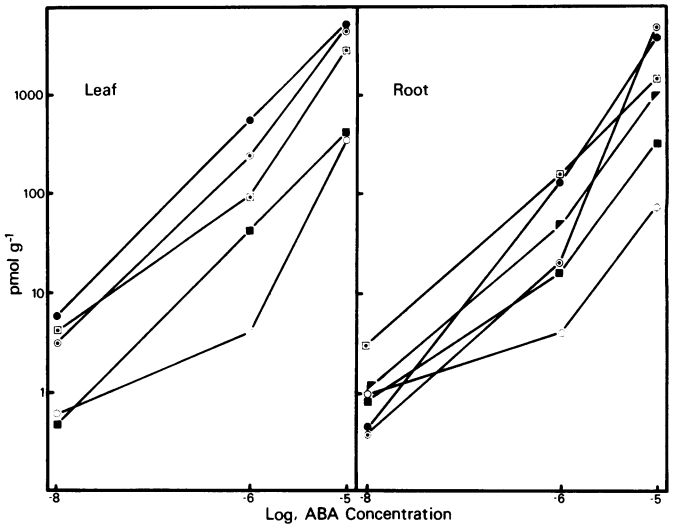

FIG. 3. Effect of exogenous ABA concentrations on [^{3}H]ABA incorporation into metabolites. Tissue was incubated for 5 h in media as described in "Materials and Methods." ABA (●), ABA glucose ester (⊙), PA (⊡), $R_{27.8}$ (■), DPA aldopyranoside (○), and the unidentified metabolite (R_{25}) of the root extract (▲).

Table I. *Endogenous ABA Levels in Leaf and Root Tissues Incubated for 5 Hours with Various Concentrations of Unlabeled ABA*

Data represent mean of four determinations ± SE.

	Time	ABA Concentration	
		Applied	Endogenous
	min	*M*	*ng g^{-1} fresh wt*
Leaf	0	0	16 ± 4
	300	0	14 ± 11
		10^{-6}	55 ± 27
		10^{-4}	640 ± 42
Root	0	0	7 ± 1
	300	0	5 ± 2
		10^{-6}	15 ± 9
		10^{-4}	1630 ± 80

and ABA-GE was proportional to the ABA concentration from 10^{-8} or 10^{-5} M.

To determine how the internal ABA pool was affected as a result of incubation with external ABA, tissues were incubated for 5 h in the presence of 0, 10^{-6}, or 10^{-4} M unlabeled ABA. Considering the previously defined rates of ABA uptake (2), and if exogenous ABA was not significantly metabolized, we expected to see increases of up to 3-fold in endogenous ABA levels during a 5-h incubation. After 5 h, tissue incubated with 10^{-4} M ABA had significantly more endogenous ABA than did the tissue incubated with 0 or 10^{-6} M ABA (Table I). This experiment indicated that no stress-induced ABA was produced by the tissue during incubation since, in the absence of any exogenous ABA, the endogenous ABA levels were the same at 0 time and after 5 h. The data suggested a relatively constant internal pool of ABA despite continued ABA uptake (2). The increase in ABA when tissues were incubated with high exogenous ABA concentrations (10^{-4} M) indicated the inability of the tissue to metabolize ABA at rates commensurate with high rates of uptake.

The fact that endogenous ABA levels reach steady state conditions after about 4 h of incubation is illustrated in Figure 4. The total amount of radioactivity present in the tissue continued to rise in both root and leaf tissues, but after 3 to 4 h, the amount of radioactivity found in the ABA fraction stabilized. During the last 9 to 10 h of incubation, the ABA pool was in a steady state condition, and the specific activity remained constant.

DISCUSSION

Our data confirmed the existence of the two metabolic pathways reported for ABA inactivation (14), namely, conjugation and catabolism to PA and DPA (Fig. 5) in both source and sink tissues of sugar beet. Although ABA was conjugated in both root and leaf tissue, ABA and DPA were conjugated (ABA-GE and DPA aldopyranoside) more rapidly in the leaf tissue, possibly because of more efficient compartmentalization mechanism or the presence of more active enzymes in the leaf tissue. Another speculation as to why sink tissue would preferentially catabolize ABA as opposed to conjugating it relates to the importance of sugars in sink *versus* source tissue. While glucose may be readily available in source tissue for different processes, in sink tissue its use in structure or storage carbohydrates may result in its limited availability. In leaves of *Xanthium*, ABA is efficiently and rapidly conjugated to its glucose ester and is presumably stored without further metabolism (17). This observation on the conversion of ABA to its glucosyl ester in *Xanthium* plants even after they are released from wilt conditions is especially interesting because the

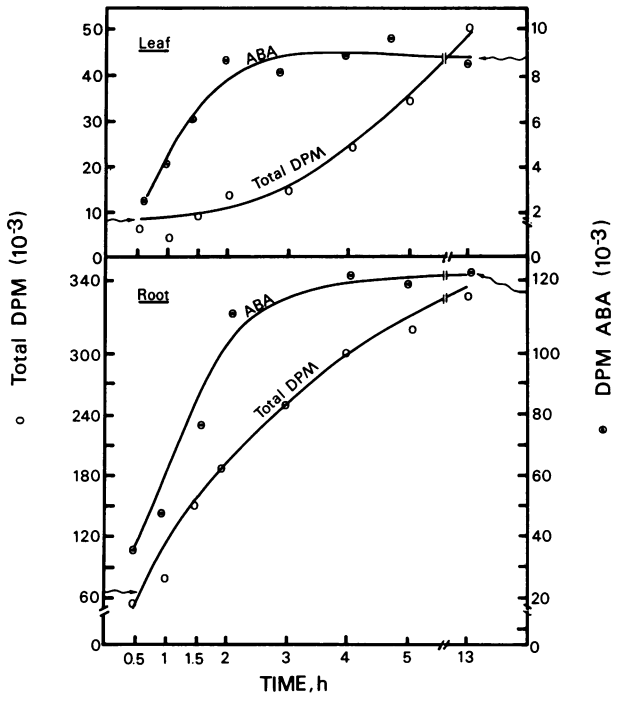

FIG. 4. Relationship between total radioactivity present in the tissue and the radioactivity in the ABA fraction.

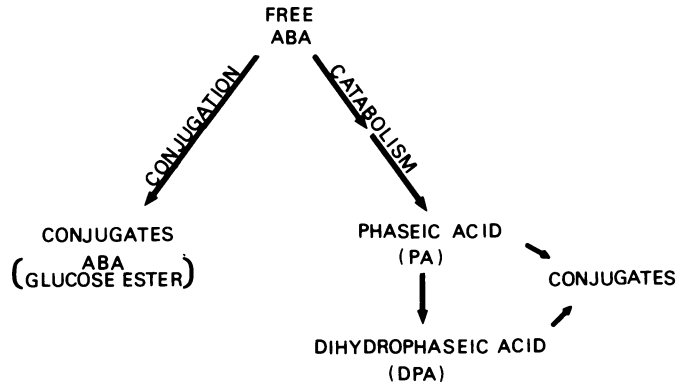

FIG. 5. The two proposed metabolic pathways for ABA (Walton, 1980).

plant can thus dispose of large quantities of ABA by converting it into a compound with little or no biological activity. The compartmentalization of the ABA-GE with no further metabolism implies *de novo* synthesis of ABA (rather than release from the bound form) under most conditions (1, 8, 14, 16). Although Zeevaart recently (18) reported that the ABA-GE levels of *Xanthium* remain unchanged 34 d after release from stress, there is no conclusive evidence that further metabolism of the ABA-GE does not occur over the long term. These data collectively suggest the central role of metabolism and synthesis in the regulation of endogenous ABA levels.

The relative activity of the two pathways in the two tissues studied can be estimated by comparing the relative amounts of [^{3}H]ABA converted into the end products of either the PA or ABA conjugation pathways. In leaves [^{3}H]ABA was converted to its glucosyl ester at much faster rates and with a shorter lag time than it was incorporated into PA. However, assuming that all the labeled DPA was the result of PA breakdown, the activity of each pathway would be about equal in leaves. The lag in the formation of PA and DPA indicated slow initial rates, possibly due to limited substrate. In contrast, the root tissue clearly showed much more activity in the PA pathway than in the conjugation pathway. Also, the 1 to 2 h lag in the leaf tissue was not observed in the root tissue. Another difference between the tissues seemed to be the reduction of PA to DPA—rapid in the leaves but very slow in the roots (Fig. 2).

Since synthetic (±)-ABA was used in these studies, we do not know how the data would differ if only the naturally occurring (+)-ABA enantiomer had been utilized. Although (−)-ABA is reported to be inactive in many tests involving 'fast' responses such as stomatal closure (6), it has been described as affecting 'slow' growth responses such as germination and growth of bean and barley shoot and root axes (8). Milborrow (8) suggested that the (−)-ABA enantiomer is mainly derivatized to the (−)-ABA-GE.

In the present study, the observed high incorporation of radioactivity into the ABA-GE by leaf tissue may have been a contribution of the (−)-ABA enantiomer. Root tissue, however, received the same ratio of (−)-ABA and yet contained much smaller quantities of ABA-GE than did leaf tissue. We were unable to calculate the differences in rates of conversion of ABA to different metabolites caused by the presence of (−)-ABA. If we assume a 50:50 mixture of the two enantiomers, however, the *in vivo* rates for conversion of ABA into its glucosyl ester may be half of the observed *in vitro* rates in either tissue.

The rise in labeled metabolite concentrations that was concurrent with increases in exogenous ABA concentrations and ABA uptake rates suggested that the plant has the capacity to rapidly metabolize ABA to modify endogenous ABA levels. This conclusion seems logical since unusually large quantities of ABA are known to be synthesized in a relatively short period of time to trigger a specific response in the plant, *i.e.* stomatal closure (4). Such high concentrations do not seem to be necessary during the post-response period and, therefore, must be disposed of by endogenous mechanisms.

Our data are consistent with the observation that plants have a substantial capacity to degrade ABA (3, 16). This capacity was observed in experiments where plants were subjected to excessive, stress-induced, internal concentrations of ABA (16–18). Our data indicated that the degradative capacity was also operative when ABA was applied exogenously. Since the ABA status of the tissue depends on its rates of uptake and metaboism of exogenous ABA, and because activity and export of metabolites as a result of ABA application may be involved in an observed physiological response, we believe that the responses of plants to applied ABA should be interpreted with these points in mind.

Based on the results presented here and those by Daie and Wyse (2) on ABA membrane transport and uptake studies using these same tissues, we conclude that two control mechanisms may be operating at two levels to modify ABA concentrations within the plant. pH may be the major factor for ABA distribution at the subcellular level. The pH differential across membranes regulates the localized ABA concentration changes, such as between compartments within a cell or between adjacent cells. However, overall ABA levels among different parts of the whole plant seem to be regulated by metabolism. ABA concentrations and effects manifest themselves throughout the integrated plant system by controlling rates of synthesis and rates and direction of the metabolism (conjugation *versus* catabolism).

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