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Structural Requirements of Abscisic Acid (ABA) and its Impact on Water Flow During Radial Transport of ABA Analogues Through Maize Roots

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ABSTRACT

The effect of (+) (ABA) and (−)-abscisic acid and nine ABA metabolites, precursors or derivatives on radial water movement through maize roots, was investigated using a suction technique (Freundl and others 1998). (+)-ABA, (+)- and (−)-abscisyl aldehyde, (+)-8′-hydroxymethyl ABA, (+)-8′-methylene, and (+)-8′-acetylene ABA stimulated radial water transport. (−)-ABA, phaseic acid, and (+)-8′-acetylene methyl ABA were ineffective. ELISA analysis for ABA detected and apparent increase of free ABA_{xyl} in xylem sap of excised root systems that were perfused with either (+)-abscisyl aldehyde, (+)-8′-methylene, (+)8′-acetylene-ABA, or ABA-glucose ester. The analogues (+)-8′-hydroxymethyl ABA and (−)-abscisyl aldehyde passed the cortex of maize roots without changing the ABA_{xyl}. The data from this study permit conclusions about the structural requirements for hormonal regulation of hydraulic conductivity.

Key words: Water conductivity; Root systems; Flows of water and abscisic acid

INTRODUCTION

Abscisic acid (ABA) has been shown to stimulate the hydraulic conductivity of root systems (Lpr) and thus influence the water relations of plants, especially when roots were subjected to mild stress as the soil starts drying (Hose and others 2002 and references cited therein). Hose and others (2000) have shown for *Zea mays* roots that plasma membranes of cortical cells are the primary site of this ABA action. Whether ABA regulates water uptake via membrane proteins such as aquaporins still remains to be investigated.

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A set of rules has been developed to establish whether a specific substance controls a given biological process (Jacobs 1959). The rules are called the PESIGS rules, **P**arallel variation, **E**xcision, **S**ubstitution, **I**solation, **G**enerality, **S**pecificities. Preliminary studies with respect to specificity have been performed by Hose and others (2000) and Sauter and Hartung (2000). Hose and others have shown that members of the other classical plant hormones did not stimulate Lpr and that (+)-ABA was much more effective than (−)-ABA. The weak activity of the latter was explained as possibly due to contamination by (+)-ABA. Sauter and Hartung (2000) have shown that the ABA conjugate ABA-glucose ester (ABA-GE) was cleaved during radial transport through maize roots by the action of cortical apoplastic glucosidases. Released free (+)-ABA then stimulated Lpr of the maize roots.

The present study further investigates the question of specificity by using ABA precursors, metabolites, and analogues with their (+)- as well (−)-enantiomers of high purity. The action of these compounds has been studied during their radial transport through maize roots using a suction technique, as described earlier by Freundl and others (1998). Using this technique, xylem sap was harvested and analyzed by ELISA for (+)-ABA during and after application of the ABA analogues.

The compounds used are listed in Table 1 and the first group of compounds tested included ABA and its immediate precursor absciscyl aldehyde. Besides (+)-ABA, the natural form of ABA, and the direct precursor of ABA, (+)-abscisyl aldehyde, their non-natural mirror image forms (−)-ABA and (−)-abscisyl aldehyde were also tested.

The second group of compounds relate to ABA degradation. (+)-8′-hydroxymethyl ABA is a stable analogue of the (+)-8′-hydroxy ABA, the first metabolite produced on oxidation of (+)-ABA. The catabolites phaseic acid and ABA glucose ester (ABA-GE) produced on conjugation of ABA were also tested.

The other analogues investigated were modified in the 8′-C-position: the 8′-methylene, the 8′-acetylene and the 8′-acetylene methyl ABA which have practical application as growth regulators, as they are persistent forms of ABA (Abrams and others 1997). Each is resistant to oxidation by ABA 8′-hydroxylase because the 8′-methyl group is less accessible to the active site of the enzyme. The 8′-acetylene ABA has been shown to be an irreversible inhibitor of ABA 8′-hydroxylase (Cutler and others 2000). Learning more about their mode of action will enable development of practical applications for crop protection from drought stress and transplant shock.

Materials and Methods

Plant Material and Culture

Seeds of maize (*Zea mays* L. cv. Helix, Kleinwanzlebener Saatzucht AG, Einbeck, Germany) were germinated on filter paper soaked with 0.5 mM $CaSO_4$ for 4 days at 25°C in the dark. The seedlings developed primary roots up to 10 cm long and coleoptiles of up to 3 cm long. They were transferred to 1.8 l aerated hydroponic containers with nutrient solution (1.5 mM KH_2PO_4, 2.0 mM KNO_3, 1.0 mM $CaCl_2$, 1.0 mM $MgS0_4$ and 18 μM FeNaEDTA, 8.1 μM H_3BO_3, 1.5 μM $MnCl_2$ at a pH of 5.8). The plants were kept in a greenhouse with an additional light source (mercury vapor lamp; 200 μmol m^{-2} s^{-1}; day/night 16/8 h; 25/17°C). Plants were cultivated an additional 8 days in this culture. A more detailed description of root and shoot anatomy for this culture has been published by Freundl and others (1998, 2000). After 12 days of cultivation plants developed roots with an average surface of 64.3 ± 11.2 cm^2 and a fresh weight of 1.3 ± 0.2 g.

Root Surface Area

An image analyzing system based on a video camera and software (BIAS from Delta-T Device, Cambridge, UK) was used to determine root surface areas. Roots were stained for 60 sec with 0.25 mM methyl violet to obtain a better contrast. The measurements were performed as described earlier by Freundl and others (1998).

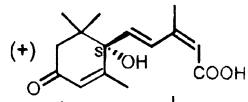
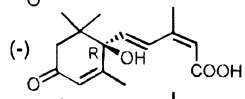
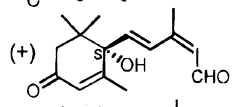
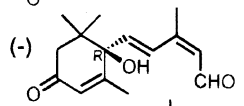
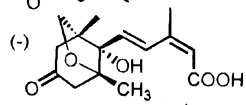
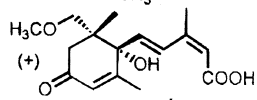
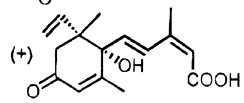
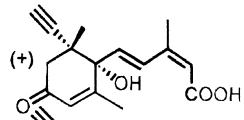
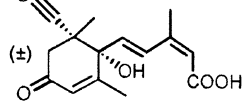
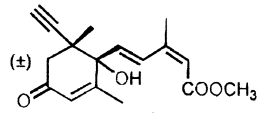
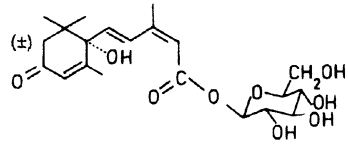
ABA Analogues

All compounds used are listed in Table 1. Stock solutions were stored in the dark at −25°C. The crystalline powder of each ABA analogue was first dissolved in 20–40 μl of methanol (100%), then diluted with water to a concentration of 10^{-3} M. The same amount of methanol was given to the controls. To avoid light-induced isomerization from cis to trans in the side chain of ABA and related compounds, small portions were kept in black eppendorf cups, immediately frozen in liquid nitrogen, and stored at −25°C.

Collection of Xylem Sap of Excised Maize Root Systems

An experimental setup was chosen in which water flow can be forced across an excised root system by suction pressure imitating the situation

Table 1. Compounds Used in this Study

Analogue	Chemical structure	Mr [g mol^{-1}]	Literature
(+)-ABA		264.3	Freundl and others 1998 Dunstan and others 1992
(−)-ABA		264.3	Hose and others 2000 Dunstan and others 1992
(+)-abscisyl aldehyde		248.3	Hays and others 1996
(−)-abscisyl aldehyde		248.3	Hays and others 1996
(−)-phaseic acid		280.3	Balsevich and others 1994
(+)-8′-hydroxy methyl ABA		294.3	Rose and others 1997
(+)-8′-methylene ABA		276.3	Abrams and others 1997
(+)-8′-acetylene ABA		274.3	Cutler and others 2000 Rose and others 1997
(±)-8′-acetylene ABA		274.3	Rose and others 1997
(±)-8′-acetylene ABA methyl ester		288.3	Rose and others 1997
(±)-ABA glucose ester		426.5	Sauter and Hartung 2000 Hogge and other 1993

in a transpiring plant (Freundl and others 1998). Hydroponically grown maize seedlings were decapitated directly above the mesocotyl and attached to a capillary with a pressure-tight silicone seal. A vacuum pump was connected to the capillary and the suction pressure was adjusted with a pressure valve and a manometer. The roots were left in the same nutrient medium in which they were grown, and carefully aerated. A suction pressure of −0.06 MPa was applied causing xylem sap flow into the capillary where it could be collected with a syringe. The water flow was determined by weighing the harvested xylem sap fractions. It is important to note that sub-atmospheric pressure was used as reference (zero pressure) throughout this paper. After 30–40 min, the flow across the root system was steady, then xylem sap was collected in 10 min intervals. After 60 min, ABA or an ABA analogue was added to the nutrient solution to give a concentration of 100 nM. The experimental setup was wrapped with aluminium foil to avoid light-induced changes of the analogues. A detailed critical evaluation of the suction technique was published recently by Freundl and others (1998). Discussing the flow equation with its diffusional, solvent drag and active component, it was demonstrated that the diffusional and active components can be

neglected. This is also supported by the fact that blocking the xylem vessels by mild squeezing reduces water flow to zero.

ELISA Analysis of (+)-ABA

Xylem sap samples were analyzed without further purification. They were taken up in TBS-buffer (Tris buffered saline: 50 mM Tris, 150 NaCl, 1 mM MgG_2, adjusted to pH 7.8 with HCl) and analyzed immunologically by ELISA, as described by Weiler (1986).

All analogues that influenced hydraulic conductivity of the root were tested by ELISA for cross-reactivity. Because the optimum concentration for detection is 1 nM, the analogues were tested at 1 nM. Only (+)-8′-hydroxymethyl ABA cross-reacted with the antibody by 25%. Contrary to the observation of Walker-Simmons (personal communication to S. Abrams) no cross-reaction with 8′-acetylene ABA occurred under the conditions of our assay.

Results

ABA Effect

When natural (+)-ABA was added to the medium surrounding the root, the radial water flow induced by a vacuum of −0.06 MPa reached a steady rate 130 min after the addition and increased 2.2-fold (Figure 1, Table 2). Simultaneously the flow of ABA into the xylem was immediately stimulated, indicating that ABA was pulled apoplastically into the xylem vessels, as shown earlier by Freundl and others (1998). The unnatural enantiomer (−)-ABA was ineffective in increasing radial water flow through roots.

Effect of (+)- and (−)-Abscisyl Aldehydes

Both enantiomers of abscisyl aldehyde stimulated the radial water flow similarly by a factor of 2.5 ± 0.5 and 3.3 ± 0.7, respectively (Figure 2, Table 2). The (+)-form of abscisyl aldehyde led to an increase in water transport within the same time scale as (+)-ABA, whereas (−)-abscisyl aldehyde took 250 min after its addition to the surrounding medium until the water flow reached steady state conditions. Upon addition of the (+)-abscisyl aldehyde, (+)-ABA was detectable in the xylem sap.

Effect of ABA Catabolites and Related Analogues

After addition of racemic ABA glucose ester to the medium, water flow was again stimulated 2.2- fold,

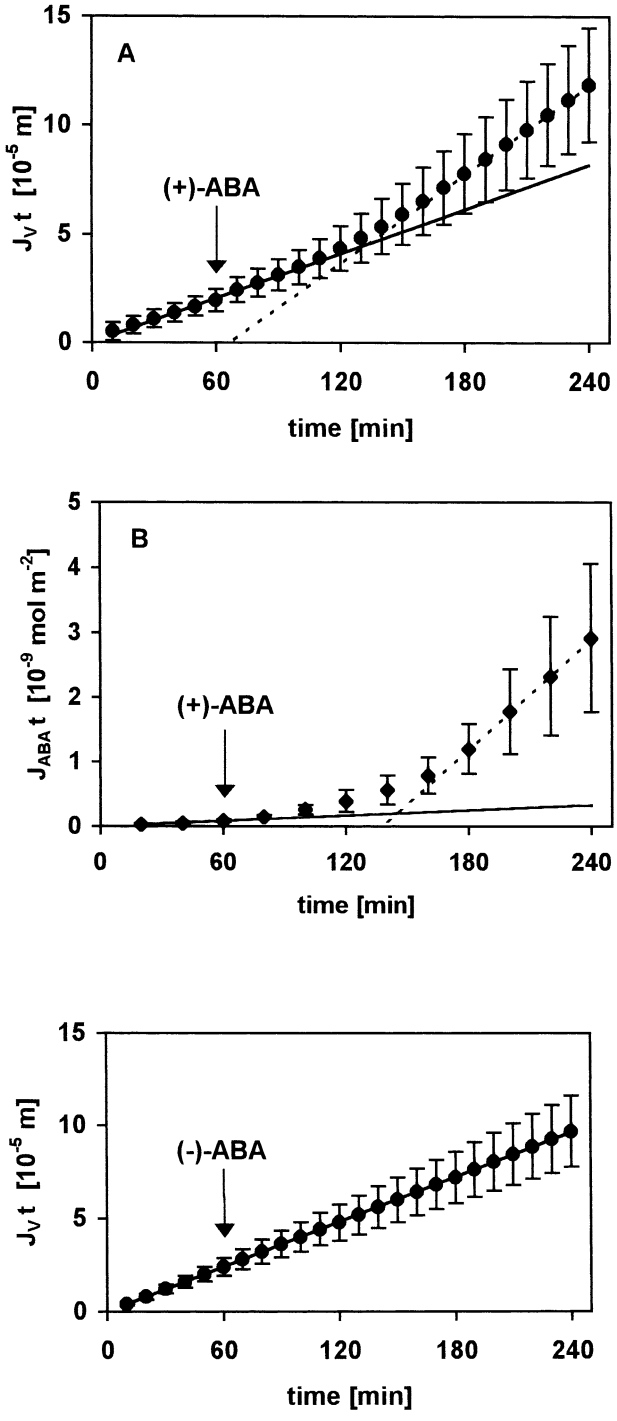


Figure 1. The effect of 100 nM (+)- and (−)-ABA on radial movement of water ($J_v t$) and ABA ($J_{ABA} t$) through maize roots. Using a suction pump a hydrostatic pressure gradient of −0.06 MPa was applied to the excised root system and xylem sap was collected in intervals, as shown in the Figure ($n = 4 \pm$ SD). The arrow denotes the time when (+)- or (−)-ABA were added. pH of the medium: 5.8.

Table 2. The Ratio of Water and ABA Flows Before and After Addition of ABA, its Analogues or Metabolites and the Time Period Until Steady State Water Flow was Reached (MW ± SD, $n = 4$)

	Ratio $J_{v\ after}/J_{v\ before}$ addition of the analogue	Time [min] required to reach steady state water flow after addition of the analogue	Ratio $J_{ABA\ after}/J_{ABA\ before}$ addition of the analogue
(+)-ABA	2.2 ± 0.6	130	19.0 ± 7.4
(+)-abscisyl aldehyde	2.5 ± 0.5	130	52.2 ± 19.3
(−)-abscisyl aldehyde	3.3 ± 0.7	250	0.6 ± 0.4
(+)-8′-hydroxymethyl ABA	2.5 ± 0.7	130	1.2 ± 0.4
(+)-8′-methyleneABA	2.2 ± 0.3	130	12.4 ± 6.5
(+)-8′-acetyleneABA	2.8 ± 0.6	250	10.8 ± 3.8
(±)-8′-acetyleneABA	2.9 ± 0.8	310	12.9 ± 6.6
(+)-8′-acetylene ABA methyl ester	0.9 ± 0.1	–	–
(−)-phaseic acid	1.0 ± 0.0	–	–
(−)-ABA	1.0 ± 0.0	–	–
(±)-ABA glucose ester	2.2 ± 0.6	250	6.8 ± 2.5

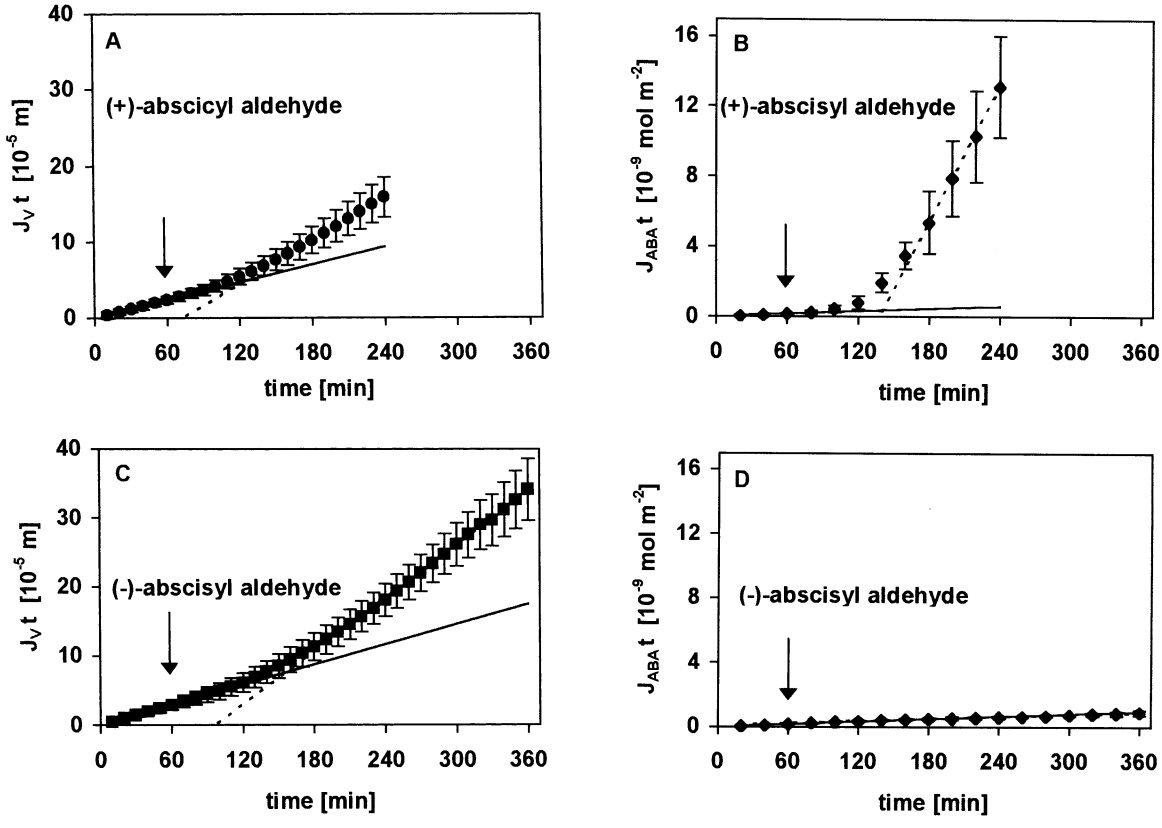


Figure 2. The effect of 100 nM (+)- and (−)-abscisic aldehyde on radial movement of water ($J_v t$) and ABA ($J_{ABA} t$) through maize roots. Experimental details are as given in Figure 1. MW ± SD, $n = 4$.

accompanied by a simultaneous increase of (+)-ABA in the xylem (Figure 3, Table 2). Phaseic acid showed no effect on water uptake into maize root systems. The (+)-8′-hydroxymethyl ABA stimulated radial water flow through maize roots 2.5-fold. Although (+)-8′-hydroxymethyl ABA showed a 25% cross-reactivity with the antibody, no significant increase in immunoreactive material in the xylem sap was observed after its addition to the medium. (+)-ABA and (+)-8′-hydroxymethyl ABA affected

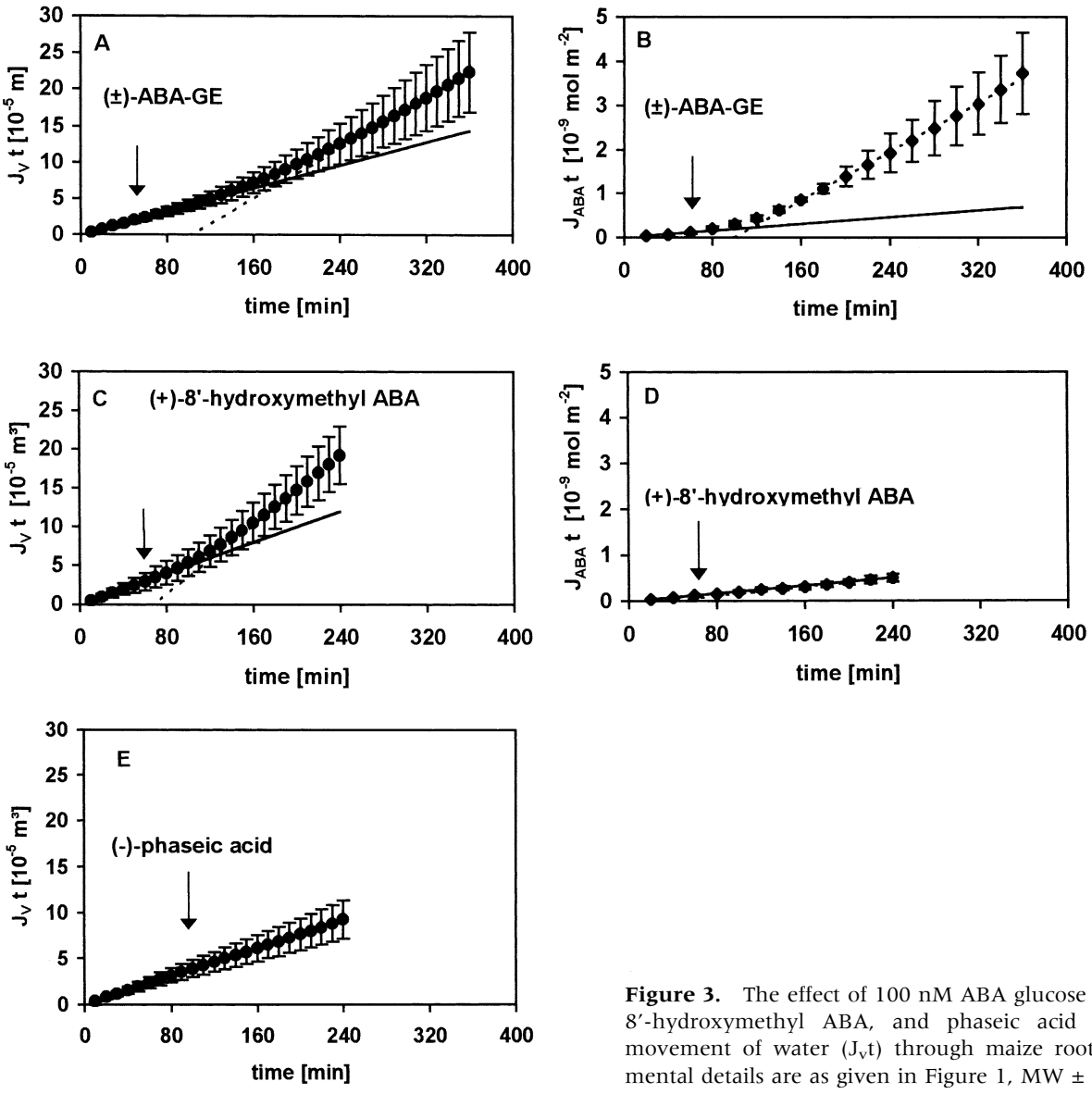


Figure 3. The effect of 100 nM ABA glucose ester, (+)-8′-hydroxymethyl ABA, and phaseic acid on radial movement of water (J_vt) through maize roots. Experimental details are as given in Figure 1, MW ± SD, n = 4.

radial water flow over similar time periods. Steady state water flow was reached 130 min after the addition of (+)-8′-hydroxymethyl ABA.

Effects of ABA Analogues Modified at the 8′-C-Atom of the ABA Molecule

(+)-8′-methylene ABA and (+)-8′-acetylene ABA increased water movement through root systems to a similar extent (Figure 4, Table 2). (+)-8′-acetylene ABA influenced water flow over a longer period of time (250 min) than (+)-8′-methylene ABA or (+) ABA (130 min). In both cases, ABA_{xyl} increased simultaneously. The racemate (±)-8′-acetylene ABA raised the water movement in the same range as its (+) enantiomer (Table 2) but required more than 310 min, to reach steady state conditions. The apparent ABA concentration in the xylem was also enriched during that period. As with ABA methyl ester (Hose and others 2000), the methyl ester of 8′-acetylene ABA proved to be inactive.

Discussion

The stress hormone abscisic acid influences the water relations of plants on the leaf (stomatal closure) and root level (hydraulic conductivity, Lpr). The effects on roots have been recently investigated in detail by Hose and others (2000) on the organ, tissue, and cellular level. They found the (−) enantiomer of ABA to be much less active on Lpr of

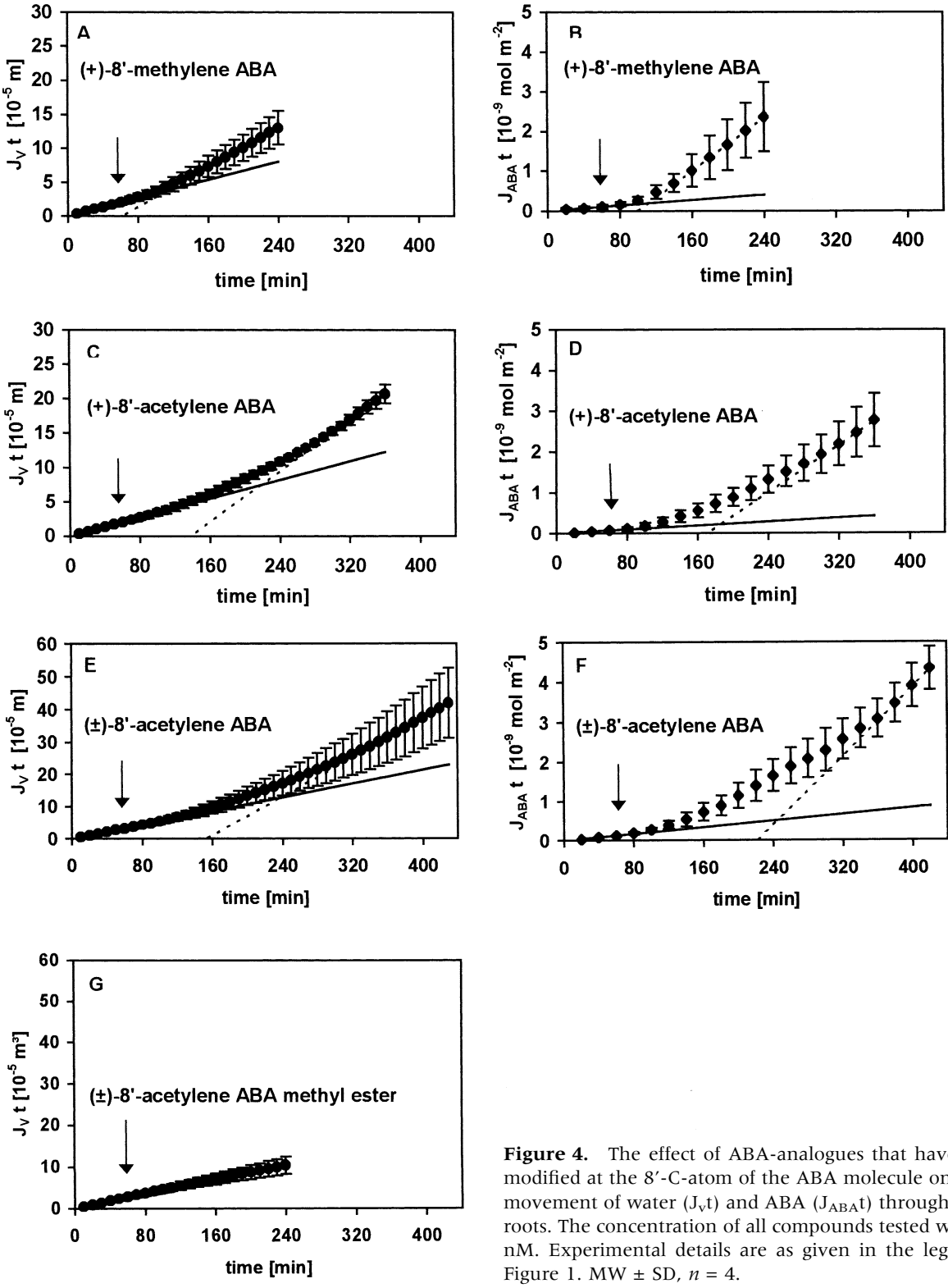


Figure 4. The effect of ABA-analogues that have been modified at the 8′-C-atom of the ABA molecule on radial movement of water (J_vt) and ABA (J_{ABA}t) through maize roots. The concentration of all compounds tested was 100 nM. Experimental details are as given in the legend of Figure 1. MW ± SD, n = 4.

maize roots than the naturally occurring (+)-ABA. The authors explained the weak activity of the (−) ABA by the contamination of the commercially available (−) enantiomer with some (+) ABA.

In the experiments of this study (−)-ABA of established optical purity was used, which indeed proved to be ineffective. The data showed that it is the (+)-ABA exclusively that increases radial water

movement through maize roots. The specificity for the natural isomer is similar to that observed for germination of cress seeds (Gusta and others 1992) and some gene expression assays (Walker-Simmons and others 1992; Wilen and others 1993).

A strong stimulating effect also developed when the root systems were treated with the analogue (+)-8′-hydroxymethyl ABA. This compound is a stable derivative of the (+)-8′-hydroxy ABA, the first degradation product of (+)-ABA and the precursor of phaseic acid (PA). These findings agree with those of Walker-Simmons and coworkers (1997) who reported that the (+)-8′-hydroxy ABA was a more effective inducer of LEA-proteins in wheat than (+)-ABA. Zou and colleagues (1995) studied the formation of very long chain monosaturated fatty acids, an oleosin protein and the Δ15 desaturase during embryogenesis in *Brassica napus* embryos. They found similar stimulatory effects of the (+)-8′-hydroxy ABA and (+)-ABA on lipid and oleosin biosynthesis. One could argue that a metabolite formed from ABA, and not ABA itself, could be responsible for the physiological effects that occur after treatment with ABA. In the maize root system this seems to be unlikely because Hose (2000) observed stimulating ABA effects on Lpr when roots were treated with tetcyclacis–a plant growth retardant that inhibits the hydroxylation of the 8′-methyl group (Daeter and Hartung 1990). Consequently, both compounds, (+)-ABA and its metabolite the 8′-hydroxy ABA, must be physiologically active. In these experiments, no apparent ABA increase was observed on treatment with (+)-8′-hydroxymethyl ABA. This supports the idea that the analogue itself is active, and does not work through stimulation of ABA in the plant.

ABA catabolites were also tested. Phaseic acid, which is formed from the 8′-hydroxy ABA, proved to be inactive. Phaseic acid was also shown to be inactive in induction of long chain fatty acid synthesis (Zou and others 1995), and induction of freeze tolerance in brome grass cells (Robertson and others 1994). Findings of Sauter and Hartung (2000) obtained after treatment of maize roots with the ABA conjugate ABA-GE were confirmed in the present study.

The direct precursor of (+)-ABA, the (+)-abscisyl aldehyde also increased radial water movement. This stimulation was accompanied by a simultaneous increase of ABA in the xylem sap. During its radial passage through the maize roots the (+)-abscisyl aldehyde may be oxidized to (+)-ABA. The aldehyde oxidase (AO) must be very active and easily accessible for the abscisyl aldehyde during its transport. However, AO has been shown to be cytosolic (Koshiba and others 1996; Zimmer and Mendel 1999). It is therefore unlikely that the AO could be located in the plasma membrane facing the apoplast. Abscisyl aldehyde must then be taken up rapidly by the cortical cells and converted to free ABA. From the particular distinct increase of ABA_{xyl} up to 120 nM (not shown) during radial transport of (+)-abscisyl aldehyde, it may be concluded that endogenous abscisyl aldehyde of cortical cells is also oxidized to ABA. This may contribute to the high ABA_{xyl}. Again ABA has to be partly redistributed to the apoplast where it is dragged directly across the endodermis to the xylem, as shown earlier by Freundl and coworkers (1998). These results do not provide definitive evidence about the activity of the aldehyde itself.

Surprisingly, the (−)-abscisyl aldehyde also increases radial water movement to about the same extent as the (+)-enantiomer without a simultaneous increase of (+)-ABA_{xyl}. The (+)- and (−)-enantiomers of abscisyl alcohol and aldehyde have also shown biological activity in *B. napus* microspore embryos, in inhibiting germination and induction of the napin and oleosin mRNA accumulation (Hays and others 1996). Generally, abscisyl aldehyde has been tested as the racemic mixture, and the biological activity observed ascribed to conversion of the (+)- form to natural ABA (Gusta and others 1992). Oxidation of the (−)-abscisyl aldehyde to (−)-ABA was not measured, so no inferences can be made about the specificity of the AO. However, the lack of activity of (−)-ABA and the strong activity observed with (−)-abscisyl aldehyde suggest that the aldehyde possesses activity.

Other compounds that have been modified at the 8′-C atom, the 8′-methylene, the (+) 8′-acetylene ABA and its racemic mixture have also been tested. After addition of these substances, water transport was stimulated 2-3 fold and an increase of ABA_{xyl} was observed. The analogue (+) 8′-methylene ABA showed a similar activity as the natural enantiomer (+)-ABA on both, J_v and J_{ABA}. The stimulation of water flow by (+) 8′- and (±) 8′-acetylene ABA was clearly weaker compared with (+)-ABA. It is unlikely that the increased ABA_{xyl} is due to enzymatic conversion of these compounds to ABA during passage through root tissue because these analogues are resistent to oxidation by ABA 8′-hydroxylase, and have an additional carbon at the 8′-carbon atom. The increase of ABA_{xyl} may be a result of stimulated ABA efflux into the xylem, or production of some other cross-reacting substance. This possibility will be investigated in future studies. It can be concluded that the analogues altered at the 8′-carbon atom of ABA possess biological activity in

this assay on stimulating radial movement of water, and have effects similar to ABA.

No effect was observed when roots were treated with the methyl ester of 8′-acetylene-ABA. This is consistent with earlier results. The methyl ester of ABA has already been investigated by Hose and others (2000) and proved to be physiologically inactive in the maize root system.

Conclusions

In this study, the specificity of the ABA effect on water flow during radial transport through maize roots was examined by using precursors, catabolites, and analogues of the plant hormone. The natural hormone (+)-ABA and its precursor (+)- abscisyl aldehyde induced the response, whereas the catabolite in the ABA pathway, phaseic acid did not.

The positive effect observed for the 8′-hydroxmethyl ABA is consistent with the immediate catabolite also controlling the process. As seen in other ABA-regulated responses, small alterations to the structure of ABA are tolerated, and these unnatural analogues modified at the 8′-carbon atom can be used to stimulate longer-lasting ABA responses in plants. The (+)abscisyl aldehyde and the ABA glucose ester are very likely converted to ABA during their radial transport in the roots. The 8′-altered compounds may stimulate ABA synthesis in the plants. We will be investigating this question using deuterated compounds so that we can observe conversion to ABA in the plants. Further work with labelled ABA metabolites and analogues will be undertaken to probe the conversion and action of compounds in the ABA pathway.

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