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Leaf Abscission Induced by Ethylene in Water-Stressed Intact Seedlings of Cleopatra Mandarin Requires Previous Abscisic Acid Accumulation in Roots[1]

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The involvement of abscisic acid (ABA) in the process of leaf abscission induced by 1-aminocyclopropane-1-carboxylic acid (ACC) transported from roots to shoots in Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.) seedlings grown under water stress was studied using norflurazon (NF). Water stress induced both ABA (24-fold) and ACC (16-fold) accumulation in roots and arrested xylem flow. Leaf bulk ABA also increased (8-fold), although leaf abscission did not occur. Shortly after rehydration, root ABA and ACC returned to their prestress levels, whereas sharp and transitory increases of ACC (17-fold) and ethylene (10-fold) in leaves and high percentages of abscission (up to 47%) were observed. NF suppressed the ABA and ACC accumulation induced by water stress in roots and the sharp increases of ACC and ethylene observed after rewatering in leaves. NF also reduced leaf abscission (7–10%). These results indicate that water stress induces root ABA accumulation and that this is required for the process of leaf abscission to occur. It was also shown that exogenous ABA increases ACC levels in roots but not in leaves. Collectively, the data suggest that ABA, the primary sensitive signal to water stress, modulates the levels of ethylene, which is the hormonal activator of leaf abscission. This assumption implies that root ACC levels are correlated with root ABA amounts in a dependent way, which eventually links water status to an adequate, protective response such as leaf abscission.

Citrus plants are prone to water-stress-induced leaf abscission. When trees are subjected to water stress, leaves are injured but do not abscise. They remain attached until water stress is released by rain or irrigation and, soon afterward, leaves abscise (Addicott, 1982). Similar behavior has been reported in several other plants, including cotton (Jordan and Day, 1972). In contrast, other adverse conditions such as cold temperatures (Turrell, 1972), hypoxia (Reuther, 1972), or wounding (Lewis and McCarty, 1972) do not promote leaf abscission in citrus. Thus, it appears that leaf abscission in citrus takes place during rewatering after a period of water stress. In intact plants of citrus, we have previously shown that ethylene is the pivotal hormonal factor controlling the physiological processes that promote leaf abscission after a period of water stress (Tudela and Primo-Millo, 1992). In Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.) seedlings, extreme conditions of water-stress-induced ACC accumulation in roots drastically arrested xylem flow. However, neither increases of ACC and ethylene in leaves nor leaf abscission were observed under these conditions. In contrast, rehydration of the stressed plants restored normal xylem fluid flow and promoted ACC transport to the shoots, where it was oxidized to ethylene, triggering leaf abscission (Tudela and Primo-Millo, 1992).

In addition to ethylene, ABA also has been implicated in the process of leaf abscission in citrus (Goren, 1993). It has been reported that under certain experimental conditions exogenous ABA applied to the woody base of the stem promoted leaf abscission in intact citrus plants (Plummer et al., 1991). However, many field experiments have demonstrated that ABA does not cause abscission when applied with foliar sprays to the aerial part of the plant (Goren, 1993). Furthermore, several reports indicated that ABA promoted abscission only in aged citrus explants (Altman and Goren, 1971; Rasmussen, 1974; Sagee et al., 1980; Riov et al., 1990). In these senescing materials, abscission was accompanied by a concomitant increase in ACC synthesis, ACC oxidase activity, and ethylene production (Sagee et al., 1980; Sisler et al., 1985; Riov and Hausman, 1988; Riov et al., 1990; Goren et al., 1993). Therefore, it was assumed that the abscission enhancement induced by ABA was caused by a direct ABA effect on ethylene biosynthesis. However, a similar function for ABA in nonsenescing intact citrus plants has never been reported. Therefore, it is an open question whether root ACC in intact citrus plants responds directly to water stress and promotes leaf abscission after rewatering or, alternatively, whether root ABA induced by water stress mediates this response through the modulation of ethylene synthesis. In the present work, we used NF, an indirect inhibitor of ABA biosynthesis (Sandermann and Böger, 1989), to reduce root ABA levels in intact citrus plants and to investigate the role of ABA in leaf abscission induced by water stress. The results suggest that under water-stress conditions root ABA accumulation is required for the process of leaf abscission to take place.

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Abbreviation: NF, norflurazon.

MATERIALS AND METHODS

One-year-old Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.) seedlings were used in all experiments reported in this work. At this stage, the seedlings had approximately 52 leaves. Plants were grown in 2-L plastic pots filled with washed, inert sand. Growth chamber conditions were as previously described (Tudela and Primo-Millo, 1992). Plants were grown under constant temperature (26°C), 16 h of light, and between 60 and 95% RH. To obtain a reproducible system, a rapid and drastic treatment of water stress was developed, since preliminary experiments showed that lighter stress treatments produced basically the same tendencies, although with higher variability of the parameters studied. Thus, water-stress treatments were imposed by transplanting plants to pots with dry sand for different periods under continuous light. Water stress was relieved by placing the stressed plants in 1-L jars filled with water (Tudela and Primo-Millo, 1992). To identify the effect of mechanical damage or wounding possibly generated during transplanting, plants were also transplanted into wet sand instead of dry sand. Similarly, the effect of waterlogging was tested by transplanting plants from wet sand to either permanently oxygenated (5 L/min) water-filled jars or nonoxygenated containers. In another set of experiments, water stress was first imposed, roots were then removed inside of the water-filled jars, and plants were rehydrated as above to isolate the effect of roots on leaf abscission. During rehydration, leaves and stems gained turgor as in the experiments performed with roots. Only intermediate leaves and young roots were used for all measurements. Abscission was expressed as the percentage of leaves that shed with a gentle touch.

Chemicals

To study the effect of NF under water-stress conditions, plants were watered with 1 mM NF (Zorial, an 80% NF commercial preparation from Sandoz Agro, Basil, Switzerland) on alternate days for 3 weeks prior to the beginning of water-stress treatments. In previous experiments it was observed that longer periods of NF resulted in leaf and root injury. During rewatering, plants were similarly treated with NF.

The effect of NF and exogenous ABA (racemic mixture, Sigma) on root ACC levels was also investigated in intact, nonstressed plants. ABA (1 mM) and NF (1 mM) were applied by either irrigation (as above) or infiltration in independent experiments. Aqueous solutions of these chemicals (alone or in combination) were infiltrated via roots into intact plants under very light vacuum conditions (5–10 kPa) for 15 min; plants were then transplanted into wet sand, and ACC root levels were determined 12 h later. Control material was infiltrated with water only.

In an additional set of experiments, the effect of exogenous ABA (1 mM) on ACC levels in leaf discs (1 cm in diameter) and in detached, whole, intermediate leaves (5 cm long) was studied. ABA was infiltrated as above under light vacuum conditions (5–10 kPa) into these plant materials for 15 min, and samples were placed in Petri dishes with the same solution for different periods (0, 3, and 6 h) until ACC was measured. Control material was infiltrated with water only.

Leaf Water Potential and Stomatal Conductance Measurements

Leaf water potential was determined with a pressure chamber (model 3000, Soilmoisture Equipment, Santa Barbara, CA; Scholander et al., 1965). No pressures greater than 3.5 Mpa were applied. Stomatal conductance was measured using a diffusion porometer (AP-4, Delta-T Devices, Cambridge, UK), and extraction of xylem fluid was performed using the pressure chamber as described by Tudela and Primo-Millo (1992). No xylem fluid was obtained from plants grown under the water-stress conditions imposed in these experiments.

ABA Extraction and Analysis

ABA was extracted from intermediate leaves and young roots. Lyophilized tissue (1 g) was homogenized using a Polytron homogenizer (Kinematica, Littau-Luzerne, Switzerland) and extracted with 25 mL of 80% methanol containing 100 mg/L 2,6-di-tert-butyl-methyl phenol as an antioxidant. The samples were filtered and reextracted overnight with 25 mL of 80% methanol at 4°C. Small aliquots of tritiated ABA (2.00 Tbq/mmol, Amersham) were added at the beginning of the extraction as an internal standard. The combined filtrates were evaporated in vacuum at 35°C, and the aqueous residues were adjusted to pH 8.0 with 0.1 M K-Pi. Chlorophylls and lipids were extracted with hexane (1×, v/v), and the organic phase was discarded. The aqueous samples were then forced through a 5-cm-long column of PVPP, and the column was eluted with the same buffer, and the elutes were acidified to pH 2.8 with HCl. The acidic solutions were partitioned (3×, v/v) against diethylether. The ether phases were combined and dried in vacuum at 35°C. The samples were redissolved in methanol, a small volume of distilled water, pH 2.5, was added to the solutions, and the methanol was removed under N_2. The remaining aqueous solutions were passed through a C_{18} cartridge for further purification. The cartridges were rinsed first with 1 mM HCl, ABA was eluted with 50% methanol, and the ABA solutions were dried in vacuum and purified by C_{18} reverse-phase HPLC (Waters). To determine ABA in xylem fluid, 1-mL aliquots were acidified with HCl to pH 2.8 and partitioned against diethylether (3×, v/v). The organic phases were dried in vacuum and also processed by HPLC. The dried samples were dissolved in 20% methanol, filtered through a 0.45-μm membrane, and loaded into an injector (model U6K, Waters) with a 2.5-mL loop. A 40-min linear gradient of 20 to 100% methanol in 1% aqueous acetic acid at a flow rate of 1.5 mL/min was used. ABA was purified with an analytical column (250 × 4 mm) packed with Spherisorb C_{18} (10-μm particles, Sharlau, Barcelona, Spain) and detected by A_{254}. Fractions containing ABA were collected and dried in vacuum.

Quantification of ABA was performed by competition ELISA using Ephyscience CEP kits from Mayoly Spindler Laboratoires (Chatou, France). The polystyrene microtiter plates in these kits are coated with conjugated ABA-4-BSA. Samples were redissolved in 1 mL of methanol, and the volume was adjusted to 10 mL with water. The organic solvent was removed under N_2, and several dilutions of each sample were made. Aliquots of 0.1 mL were placed in individual wells and incubated for 30 min at 4°C. Then, 0.05 mL of diluted anti-ABA monoclonal antibodies was added to the wells, and the plates were further incubated for 2 h at 4°C. The solutions were discarded, the plates were washed, and 200 μL of diluted (1:5000), conjugated anti-mouse peroxidase was added to each well. After 1 h at 37°C, the solutions were poured off and the plates were rinsed again. The diluted substrate for the peroxidase (200 μL of 2,2-azino-di-[3-ethylbenzo-thiazoline-6-sulfonique] in H_2O_2, v/v) was then added, and the plates were placed at room temperature in the dark until the A_{405} of controls containing no ABA was approximately 1.0. Each plate contained a duplicate standard curve of ABA (3–300 pmol/mL) and three replicates per dilution. The ABA content of the extracts was calculated by interpolation of the log transformation of the ABA standard curve, taking into account the recoveries of the procedure determined with the tritiated ABA internal standard. The accuracy of the ELISA for the ABA determination in the samples was previously evaluated with internal standardization experiments. The data obtained in these experiments showed the absence of noncompetitive interferences when exogenous ABA was added to the diluted extracts (data not shown), as reported previously in other citrus species and tissues (Zacarias et al., 1995). The values for the endogenous ABA obtained with the ELISA were also comparable to those obtained by GC with an electron capture detector (data not shown).

Ethylene and ACC Measurements

Ethylene was quantified using a gas chromatograph (model 3500, Perkin-Elmer) equipped with an activated alumina column and a flame ionization detector. The procedures for ethylene handling and determination were reported previously (Tudela and Primo-Millo, 1992). Quantitation of ACC was performed by the method reported by Lizada and Yang (1979), and GC and operating parameters were as described by Tudela and Primo-Millo (1992). Xylem fluid (150 μL) without further purification was used for ACC measurements in xylem sap. Recovery of ACC in the assay was always greater than than 70%.

All ABA, ACC, and ethylene determinations were repeated at least twice in independent experiments. The data presented in the figures are results from a typical experiment with at least three replicates per treatment.

RESULTS

Water Status and Stomatal Conductance

We have recently shown that Cleopatra mandarin possesses a high capacity for osmotic adjustment under rapidly imposed, short-term water deficit (Tudela and Primo-Millo, 1992). In the present work, well-watered Cleopatra mandarin seedlings were transplanted to new pots containing dry sand. Plants were severely stressed for 24 h under these conditions and subsequently rehydrated to release water stress. In stressed plants, the rates of decline in leaf water potential were high during the first 6 h of water deficit (−0.3 MPa/h) and moderate thereafter (Fig. 1A). During the period of water stress, xylem flow was interrupted (data not shown). After release of drought, leaf water potential again reached control values (−0.9 MPa) in 12 h. Figure 1A also shows that the pretreatment with 1 mM NF for 3 weeks had no detectable effects on leaf water potential. The changes in stomatal conductance paralleled those observed for leaf water potential in water-stressed plants (Fig. 1B). After 6 h of water stress, stomatal conductance decreased from 121 to 34 mmol m^{-2} s^{-1} and then decreased slowly to reach its lowest value (18 mmol m^{-2} s^{-1}) 18 h later. Thus, as water potential decreased from −0.9 to −3.5 MPa, conductance was reduced by about 85%.

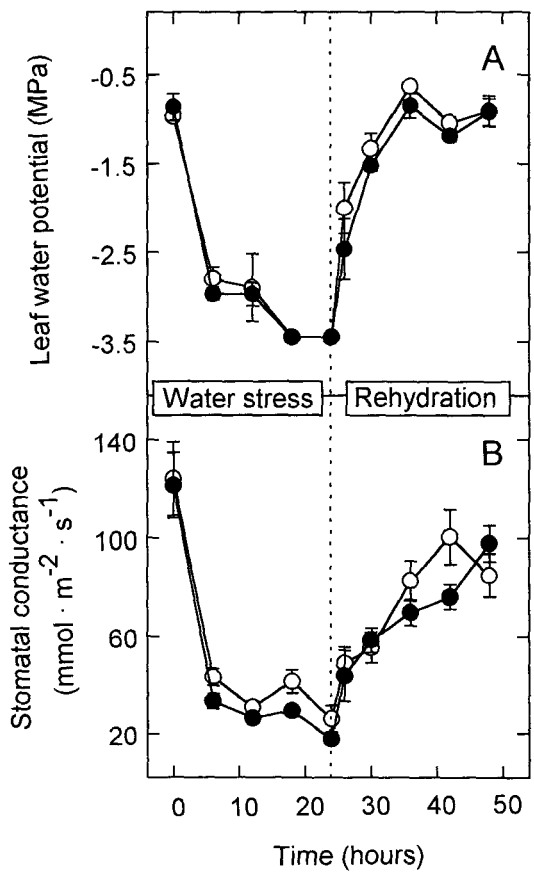

Figure 1. Effect of NF on leaf water potential (A) and stomatal conductance (B) in Cleopatra mandarin seedlings during water stress and after rewatering. Plants were either nontreated (●) or treated (○) with 1 mM NF for 3 weeks before water stress was imposed. Plants pretreated with NF were also supplemented with 1 mM NF during rewatering. Data are means ± SE of a typical experiment. Each value was determined in at least three different plants with three replicates per plant. The whole experiment was repeated twice and the trends were similar.

After rehydration, conductance increased during the following 24 h, but it did not fully recover in comparison with the control values of the nonstressed seedlings. Pretreatment with NF had little influence on stomatal conductance (Fig. 1B).

Leaf Abscission

Leaf abscission in seedlings of Cleopatra mandarin occurred between the petiole and the leaf blade, in the abscission zone known as LA-AZ, according to the description reported in Goren (1993). In well-watered seedlings, as well as in 24 h water-stressed plants, leaf abscission did not take place (Tables I and II; for further details concerning leaf abscission, see Tudela and Primo-Millo, 1992). However, high percentages of defoliation (between 27 and 47%) were observed after the plants previously stressed for 24 h were rewatered. It is interesting that the pretreatment with NF partially reversed the effect of rehydration on leaf abscission (Table I), since NF-treated plants had lower percentages of leaf abscission (between 7 and 10%). The data also indicated that mechanical damage (wounding), hypoxia (in presence or absence of O_2), and water stress generated during the experiment had no influence on leaf abscission (Table II). Leaf abscission took place only during rehydration after a period of water stress; however, leaf abscission was completely inhibited (0%) in intact water-stressed plants that were rehydrated in the absence of roots, which suggests that the causal agent of leaf abscission is generated in the roots.

Changes in ACC Levels Induced by Wounding, Hypoxia, and Water Stress

In a previous paper, we suggested that ACC is the water-stress-induced root signal that promotes leaf abscission after translocation to the leaves, where it is oxidized to ethylene (Tudela and Primo-Millo, 1992). Table II shows that neither wounding nor waterlogging (with or without O_2) increased ACC levels in roots and leaves of Cleopatra mandarin plants. Again, ACC accumulation in roots was found only in water-stressed plants, although in these conditions leaf abscission was not observed. When water-stressed plants were rehydrated, ACC levels decreased in roots (65 to 6 nmol/g dry weight after 24 h) and increased in leaves (0.7 to 12.2 nmol/g dry weight after 6 h), and leaf abscission was triggered (from 0 to 38% after 24 h). Accordingly, no ACC increases were detected in leaves of water-stressed plants that were rehydrated in the absence of roots (Table II).

Table I. *Effect of NF on leaf abscission of Cleopatra mandarin seedlings during water stress and rehydration*

Plants were either nontreated or treated with 1 mM NF for 3 weeks before water stress was imposed. Plants pretreated with NF also were fed 1 mM NF during rewatering. Abscission is expressed as the percentage of leaves that shed with a gentle touch at the end of either a 48-h period of water stress (WS) or a 24-h period of drought followed with an additional 24-h period of rehydration (WS+R). Data are means ± SE of four plants per treatment (about 205 leaves).

Treatment	Experiment 1		Experiment 2	
	WS	WS+R	WS	WS+R
	%		%	
−NF	0	47 ± 6	0	27 ± 4
+NF	0	10 ± 2	0	07 ± 2

Pattern of Change in ABA, ACC, and Ethylene Levels in Water-Stressed Plants

The above results suggested that root ACC is the leaf abscission signal generated during water stress. Since ABA also has been implicated in leaf abscission, we studied the relationship of endogenous ABA and ACC on this process. In young roots of well-watered plants, ABA levels were lower than 0.2 nmol/g dry weight; however, the ABA concentration increased continuously up to 4.9 nmol/g dry weight (24-fold) during the 24-h period of water stress (Fig. 2A). The relatively high levels of ABA detected in stressed roots also were reduced progressively after the release of drought stress. After 12 h of rewatering, ABA levels reached basal values of nonstressed plants and remained constant thereafter. The ABA accumulation observed in roots from stressed plants was effectively suppressed by the NF pretreatment. Because of this, roots from plants treated with NF and grown subsequently under water-stress conditions contained low and constant levels of ABA (between 0.4 and 0.7 nmol/g dry weight) during the whole period of water stress. Rehydrated roots pretreated with NF had even lower ABA amounts. Levels of ACC also accumulated considerably (from 4 to 65 nmol/g dry weight; 16-fold) in roots from water-stressed Cleopatra mandarin seedlings (Fig. 2B) and decreased rapidly after rehydration, confirming previous observations (Tudela and Primo-Millo, 1992). Although in drought-stressed roots the ABA accumulation started sooner (6 h after the beginning of the water stress) than the ACC increase (12 h), when water stress was released, the ACC decline was faster (2 h) than the ABA reduction (6 h). Pretreatment with NF also inhibited ACC accumulation in water-stressed roots. Levels of ACC were high (65 nmol/g dry weight) in roots stressed for 24 h and much lower (17 nmol/g dry weight) in NF-treated and water-stressed roots. Twenty-four hours after rehydration, basal ACC levels similar to those detected in nonstressed plants were found in both NF-treated and nontreated roots.

The ABA and ACC reductions detected in rehydrated roots may suggest that both compounds were translocated via the xylem to the shoots. In addition, evidence has been previously presented (Tudela and Primo-Millo, 1992) indicating that ACC was transported from roots to shoots under the experimental conditions described in this work. It is clear from the data shown in Figure 3 that 2 h after rehydration the xylem fluid from the previously stressed plants had a higher ABA (4-fold) and ACC (17-fold) content than the xylem sap of the nonstressed seedlings. As time of rehydration increased, ABA and ACC amounts in xylem fluid decreased. Eighteen hours after rehydration, these amounts were similar to those observed in non-

Table II. *Effect of wounding (mechanical damage during transplanting), hypoxia, and water stress on leaf abscission and ACC levels in roots and leaves of 1-year-old Cleopatra mandarin seedlings*

Leaf abscission and ACC levels were determined 24 h after the beginning of the stress, except where indicated. Abscission is expressed as the percentage of leaves that shed with a gentle touch. Data are means ± SE of five plants. Controls were not stressed.

Parameters Measured	Control	Wounding	Waterlogging		Water Stress			
						(+) Rehydration		
			(−) Oxygenation	(+) Oxygenation	(−) Rehydration	With roots		Without roots
						6 h	24 h	24 h
Leaf abscission (%)	0	0	0	0	0	4 ± 1	38 ± 5	0
ACC (nmol g^{-1} dry wt)								
Roots	3.7 ± 0.2	4.3 ± 0.3	5.2 ± 0.4	4.8 ± 0.2	65.6 ± 3.3	17.2 ± 0.7	6.0 ± 0.9	–[a]
Leaves	0.9 ± 0.1	1.2 ± 0.1	0.8 ± 0.1	1.2 ± 0.2	0.7 ± 0.1	12.2 ± 0.9	1.8 ± 0.1	1.0 ± 0.2

[a] Plants without roots.

stressed seedlings. NF also reduced the increases in ABA and ACC observed 2 h after rehydration in the xylem content of the untreated plants (Fig. 3). ABA and ACC levels in xylem sap of NF-treated seedlings also declined thereafter, and 12 h after rewatering, these levels were the same as those found in nonstressed plants.

Water stress induced a marked ABA accumulation in leaves of Cleopatra mandarin seedlings (Fig. 4A). In well-watered plants, ABA was relatively low (8.8 nmol/g dry weight). In water-stressed plants, the ABA concentration increased constantly and rapidly as soon as water stress started. Elevated ABA values (about 70 nmol/g dry weight) were reached after 24 h of water stress (8-fold increase). Rehydration had the opposite effect and decreased gradually from the high amounts of ABA detected in stressed leaves. Values of ABA similar to those of nonstressed plants were observed 18 h after rewatering. The pattern of ABA change in leaves from plants treated with NF, kept under drought conditions and finally rehydrated, was the same as that described for the plants that did not receive the NF treatment. In contrast to ABA, water stress did not induce ACC accumulation in leaves (Fig. 4B). However, a sharp and transitory ACC increase (from 0.7 to 12 nmol/g dry weight, a 17-fold increase) occurred in leaves 6 h after rewatering.

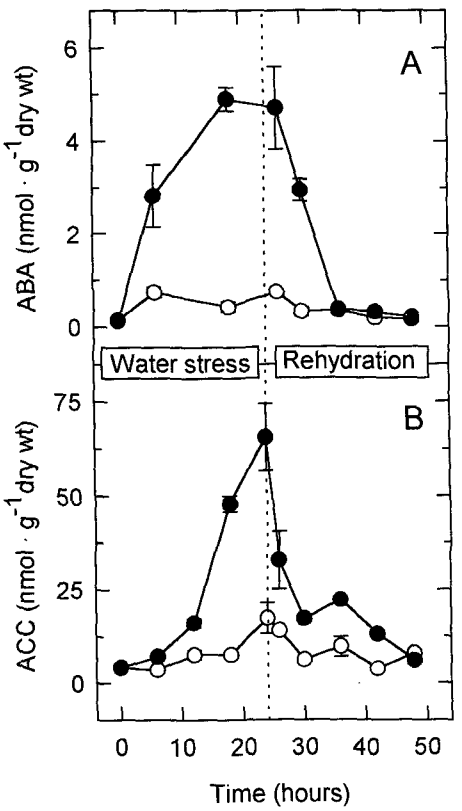

Figure 2. Effect of NF on ABA (A) and ACC (B) levels in young roots from Cleopatra mandarin seedlings during water stress and rehydration. Plants were either nontreated (●) or treated (○) with 1 mM NF for 3 weeks before water stress was imposed. Plants pretreated with NF were also supplemented with 1 mM NF during rewatering. Data are means ± SE of three measurements in a typical experiment. The whole experiment was repeated twice and the trends were similar. dry wt, Dry weight.

Longer rehydration periods did not further modify the basal ACC levels, as previously reported (Tudela and Primo-Millo, 1992). During water stress, ACC levels in leaves of NF-treated plants were low and similar to those detected in plants not treated with NF. Moreover, the sharp ACC increase induced by rehydration was reduced dramatically in NF-treated plants. Leaves from these plants showed only a minor ACC increase (1.0 to 4 nmol/g dry weight) 2 h after rewatering (Fig. 4B). The basal levels of ethylene produced in leaves under normal conditions (hardly detectable) were unmodified by water stress during the whole period of drought (Fig. 4C). However, 2 to 6 h after rewatering, ethylene increased (about 10-fold) and subsequently decreased to reach control values 24 h later. NF had no effect on ethylene production in leaves from plants under water stress; however, the ethylene increase observed after rewatering was dramatically reduced. Leaves from rehydrated plants previously treated with NF showed a small increase in ethylene over a shortened interval of time in comparison with the high ethylene production of the leaves from plants not treated with NF.

Effect of Exogenous ABA and NF on Root ACC Levels

The data in Table III show that 1 mM exogenous ABA increased ACC levels in roots of intact nonstressed plants. Root ABA infiltration for 15 min enhanced (3-fold) ACC levels 12 h later, and the ACC increase observed in roots of

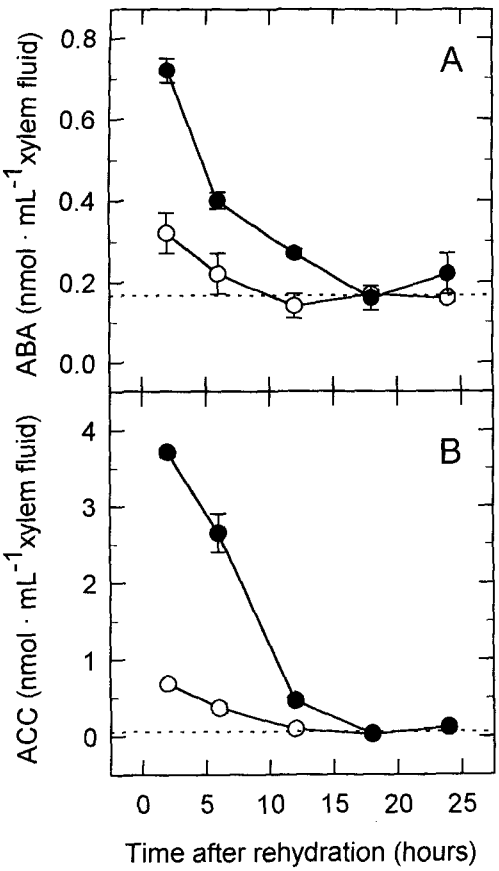

Figure 3. Effect of NF on ABA (A) and ACC (B) content in xylem fluid of rehydrated Cleopatra mandarin seedlings previously maintained under water-stress conditions for 24 h. Plants were either nontreated (●) or treated (○) with 1 mM NF for 3 weeks before water stress was imposed. Plants pretreated with NF were also supplemented with 1 mM NF during rewatering. Dotted lines indicate content in xylem fluid of nonstressed plants. Data are means ± SE of three measurements in a typical experiment. The whole experiment was repeated twice and the trends were similar.

water-stressed plants also occurred 12 h after the onset of the ABA increase (Fig. 2). Similarly, irrigation for 16 d with this solution also increased root ACC levels (2-fold). On the other hand, NF (1 mM) did not significantly reduce ACC amounts increased by a simultaneous ABA application. Moreover, NF alone had no effect on ACC levels.

Effect of Exogenous ABA on ACC Levels of Green Tissues

Exogenous application of ABA had no effect on ACC levels from intact green tissues (data not shown). Furthermore, data presented in Table IV show that 1 mM ABA infiltrated into nonaged leaf discs and detached whole leaves did not modify the ACC levels 6 h later, the period observed between the increases of ABA and ACC in leaves of water-stressed plants (Fig. 4). The fact that exogenous ABA does not influence ACC levels in nonaged green tissues also has been reported several times in other citrus species (Riov et al., 1990).

DISCUSSION

We have shown that ABA (Fig. 2A) and ACC (Fig. 2B) accumulated in water-stressed roots and that these high amounts decreased to basal levels soon after rewatering. Rehydration also restored xylem movement and increased the ABA (Fig. 3A) and ACC (Fig. 3B) content of the xylem fluid. These observations may suggest that ABA, in addi-

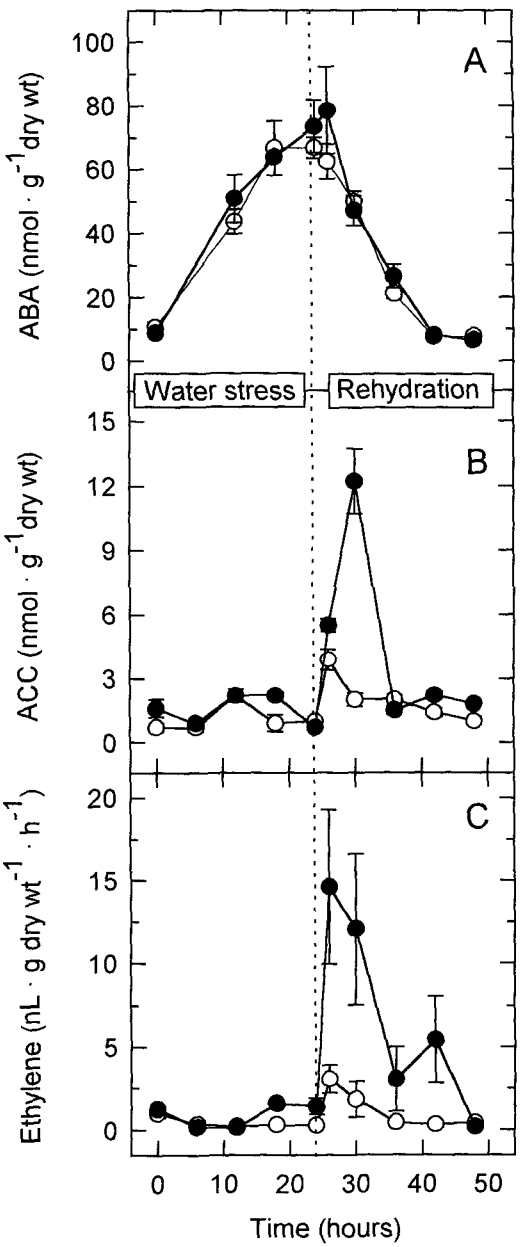

Figure 4. Effect of NF on ABA (A), ACC (B), and ethylene (C) in leaves from Cleopatra mandarin seedlings during water stress and rehydration. Plants were either nontreated (●) or treated (○) with 1 mM NF for 3 weeks before water stress was imposed. Plants pretreated with NF were also supplemented with 1 mM NF during rewatering. Data are means ± SE of three measurements in a typical experiment. The whole experiment was repeated twice and the trends were similar. dry wt, Dry weight.

Table III. *Effect of ABA (1 mM) and NF (1 mM) on ACC levels extracted from roots of intact, nonstressed 1-year-old Cleopatra mandarin seedlings*

Roots were either infiltrated for 15 min under very light vacuum conditions or watered for 16 d with these chemicals. In the infiltration experiment, ACC levels were measured 12 h after the treatment. Five different plants per treatment in each experiment were used, and controls were treated similarly with water. Data are means ± SE.

Treatment	Infiltration	Irrigation
	ACC nmol g^{-1} dry wt	
Control	3.7 ± 0.1	4.9 ± 0.3
ABA	12.3 ± 2.1	10.7 ± 0.4
ABA + NF	9.6 ± 0.6	9.0 ± 0.2
NF	N.D.[a]	4.1 ± 0.3

[a] N.D., Not determined.

tion to ACC (Tudela and Primo-Millo, 1992), was actively transported through the xylem after rehydration. In roots, ABA synthesis induced by water stress (Cammue et al., 1989; Tardieu et al., 1992) and/or subsequent ABA transport from roots to shoots (Zeevaart and Boyer, 1984; Zhang and Davies, 1989; Neales and McLeod, 1991; Tardieu et al., 1991; Trejo and Davies, 1991) previously have been reported in a number of species. Water stress also induced ABA accumulation in leaves (Fig. 4A), although ACC (Fig. 4B) and ethylene (Fig. 4C) were unmodified in these organs. In contrast, rewatering of the stressed plants reduced leaf ABA, increased both ACC and ethylene in leaves, and, in addition, promoted leaf abscission (Table I). Thus, it is clear that leaf abscission was associated with the presence of high levels of ACC and ethylene in leaves. Taken together, these observations reinforce the conclusion that ethylene, and not ABA, is the ultimate hormonal activator controlling leaf abscission in water-stressed citrus plants.

The NF experiments showed that this chemical inhibited ABA (Fig. 2A) and ACC (Fig. 2B) accumulation in stressed roots, reduced xylem transport of both (Fig. 3), and limited ACC (Fig. 4B) and ethylene (Fig. 4C) increases in leaves from the rehydrated plants. NF also considerably reduced leaf abscission in these seedlings (Table I). The inhibitory effect of NF on abscission has also been described in cotton (Suttle and Hultstrand, 1993). Current data (Tudela and Primo-Millo, 1992) suggest that the inhibition of ACC accumulation in stressed roots by NF (Fig. 2B) is responsible for the reduction in leaf abscission. The NF effect on the ACC levels is a striking observation, since NF is known to inhibit phytoene desaturase activity, which is an early step in carotenoid biosynthesis (Sandermann and Böger, 1989). This explains the ABA reduction observed in NF-treated roots (Fig. 2A). Since we have demonstrated that NF has no direct effect on ACC root levels (Table III), we suggest that the reduction of root ABA with NF inhibited the increase in root ACC. Therefore, we propose that the ACC accumulation that occurs in roots in response to water stress requires previous ABA accumulation. We have demonstrated also that exogenous ABA increases ACC levels in roots (Table III), whereas NF is not able to reduce ACC levels in the absence or presence of ABA. The timing of the ABA and ACC accumulation in roots of water-stressed plants and the timing of the ACC increase in the ABA infiltration experiments also appear to be compatible with the above suggestion.

In contrast to the effect of exogenous ABA on root ACC levels, ABA does not modify ACC production in citrus leaves (Table IV), as it does in wheat (McKeon et al., 1982) or *Radermachera* explants (Dunlap et al., 1994). In addition, there have been several reports that foliar ABA applications do not promote leaf abscission in citrus (Goren, 1993). Actually, ABA has been found to increase ethylene production and abscission only in aged citrus organs (Altman and Goren, 1971; Rasmussen, 1974; Sagee et al., 1980; Riov and Hausman, 1988; Riov et al., 1990; Goren, 1993; Goren et al., 1993). In contrast, there is one report showing that the ABA application to the woody tissues of the basal part of the stem was able to induce leaf abscission (Plummer et al., 1991). Thus, evidence for a role of leaf ABA on ethylene production and abscission of intact, nonsenescing plants has not been obtained. Moreover, ABA accumulation took place in water-stressed leaves, and no leaf abscission was observed. This occurred only during rehydration after a period of water stress, and even in these conditions leaf abscission was suppressed if roots were removed before rewatering, which suggests that leaf ABA accumulation has no role in leaf abscission.

In our system, NF inhibited the root ABA accumulation induced by water stress, which may well act as an activator of ACC synthesis and limit the amounts of substrate for the hormonal inductor of leaf abscission. As a result, defoliation is reduced, even under conditions of extreme water deficit (Table I). These observations suggest that ABA levels, the primary "sensitive" hormonal signal to water stress, modulate the synthesis of root ACC and the increase in ethylene that triggers the events leading to leaf abscission. Therefore, we suggest that the intensity of the water stress is transduced through the modulation of the ABA synthesis to ethylene synthesis in leaves for a proportional protective response. Moreover, it has been demonstrated that the rate of ABA synthesis in roots is related to the soil or root water status (Tardieu et al., 1992).

Whereas NF efficiently inhibited ABA accumulation in young roots (Fig. 2A), this compound was not effective in

Table IV. *Effect of ABA (1 mM) and NF (1 mM) on ACC levels extracted from leaf discs and whole, detached leaves of intact, nonstressed 1-year-old Cleopatra mandarin seedlings*

Plant materials were infiltrated for 15 min under very light vacuum, and ACC levels were measured 3 and 6 h after the treatment. Five different plants were used, and controls were infiltrated with water. Data are means ± SE.

Treatment	ACC	
	3 h	6 h
	nmol g^{-1} dry wt	
Leaf discs		
Control	1.6 ± 0.3	0.9 ± 0.1
ABA	2.1 ± 0.3	1.1 ± 0.2
Detached leaves		
Control	0.9 ± 0.1	0.9 ± 0.2
ABA	0.9 ± 0.1	1.2 ± 0.1

reducing ABA concentration in leaves (Fig. 4A). This is not surprising, since NF has a limited effect on green tissues, which have high amounts of carotenoids, whereas in roots the carotenoid pool is very small (Li and Walton, 1990; Parry and Horgan, 1992). Moreover, NF may also be a relatively immobile compound. Furthermore, the ABA levels found in stressed leaves were much higher than those detected in roots. It has been demonstrated in citrus, as in many other species, that detached, intact leaves respond rapidly to water deficit, dramatically increasing the ABA concentration (Norman et al., 1990).

In conclusion, we suggest that leaf abscission induced by ethylene in water-stressed citrus plants requires previous root ABA accumulation. Accordingly, the reported data suggest that the sequence of events induced under water stress leading to leaf abscission in citrus is as follows: ABA accumulation in roots → ACC accumulation in roots → ACC transport from roots to shoots → ACC oxidation to ethylene in leaves → leaf abscission.

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