Effect of Paclobutrazol on Water Stress-Induced Abscisic Acid in Apple Seedling Leaves

SHIOW Y. WANG*, TUNG SUN, ZUO L. JI1, AND MIKLOS FAUST

Fruit Laboratory, Agricultural Research Center, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland 20703 (S.Y.W., Z.L.J., M.F.); and Food Safety Inspection Service, United States Department of Agriculture, P. O. Box 6085, Athens, Georgia 30604 (T.S.)

Received for publication July 29, 1986 and in revised form March 16, 1987

ABSTRACT

Abscisic acid (ABA) was quantitated by enzyme-linked immunosorbent assay (ELISA) in water-stressed leaves from control apple seedlings, and also from apple seedlings treated for 28 days with paclobutrazol (2RS, 3RS)-1-[4-chlorophenyl]-4,4-dimethyl-2-[1,2,4-triazol-1-yl] pentan-3-ol. The ELISA quantitative estimates were also validated by gas chromatography-electron capture detector and lettuce seed germination inhibition bioassay. Paclobutrazol treatment reduced endogenous ABA levels by about one-third, and prevented the marked accumulation of water-stress-induced ABA that occurred in untreated seedlings. The presence of ABA in the apple leaf extracts was confirmed by gas chromatography-mass spectrometry.

The accumulation of ABA in the leaves of water-stressed plants has been shown by numerous investigators (12, 18, 22, 23). Our previous reports showed that the accumulation of water-stress-induced 1-aminocyclopropane-1-carboxylic acid, 1-(malonylamino)cyclopropene-1-carboxylic acid, ethylene production, and polyamines also occurred in water-stressed apple leaves, but these increases were prevented by the treatment with the plant bio-regulant (2RS, 3RS)-1-[4-chlorophenyl]-4,4-dimethyl-2-[1,2,4-triazol-1-yl] pentan-3-ol (paclobutrazol; PP333)2 (19).

Paclobutrazol is a triazol derivative (4, 10) and has been shown to inhibit shoot growth of apple trees (4, 16, 21). Coobbaugh et al. (2, 3) showed that ancymidol, another growth retardant, blocks with high specificity the oxidative steps leading from ent-kaurene to ent-kaurenoic acid in the pathway of GA biosynthesis. The same oxidative steps are thought to be inhibited by the active triazol derivatives (10). Paclobutrazol has also been reported to inhibit GA biosynthesis in plants by inhibiting kaurene oxidase, a Cyt P-450 oxidase, thus blocking the oxidation of kaurene to kaurenoic acid (6, 9). The growth inhibitory activity of paclobutrazol can be reversed by GA3 (16, 21). Recently, Norman et al. (13) reported that paclobutrazol inhibited ABA biosynthesis in Cercospora rosicola. Since the levels of ABA increase markedly following water stress, the present study was undertaken to determine if paclobutrazol could reduce either normal or water stress-induced ABA levels in apple leaves.

MATERIALS AND METHODS

Plant Material and Treatments. The growing conditions and treatments of the apple seedlings ('York Imperial' Malus domestica Borkh) were as described previously (19). Apple seeds were germinated in the greenhouse and grown in sand until they were approximately 7.0 cm in height. After the sand was washed from the roots, the seedlings were grown for 1 week in a continuously aerated nutrient solution. The treatments were initiated by adding 0.68 μm paclobutrazol (95% active; ICI Americas, Inc.)3 in the nutrient solution. Radiation sources in the greenhouse consisted of natural daylight and 400 W high pressure sodium lamps which provided a PAR level of about 400 to 500 μmol m−2 s−1 for 12 h/d (0700–1900 h). Temperatures were approximately 25°C during the day and 20°C at night. Solutions were changed weekly. Seedlings were harvested 28 d after paclobutrazol treatment and subjected to different degrees of water stress. The technique used for inducing water stress was described by Wright (23), and Apelbaum and Yang (1). Batches of excised apple seedling leaves (3rd expanded leaf from the tip) were spread out on sheets of filter paper and allowed to wilt at room temperature (22°C) and 60% RH. When the fresh weight loss in the leaves reached 10, 20, and 30%, respectively, of their original weight, the leaves were collected and used for ABA analysis.

Extraction and Purification of ABA. All manipulations were carried out under dim green light at 4°C. Tissue was extracted with 80% methanol (containing 10 mg/L of 2, 6-di-t-buty1-4-methyl phenol [BHT]) (pH 4.8) for 72 h at 4°C with intermittent stirring. The extract solution was replaced with fresh solvent every 24 h. The combined extract was evaporated under vacuum (40°C) to the aqueous phase. The aqueous phase was then centrifuged at 5000 rpm for 15 min at 0°C. An equal volume of 0.1 M phosphate buffer (pH 8.0) was added to the supernatant. The pH of the solution was adjusted to 8.0 with 1 N NaOH. The solution was then partitioned with an equal volume of petroleum ether (3 times). The pH of the aqueous solution was then acidified to pH 2.8 with 6 N HCl, and the solution was extracted with ethyl acetate (3 times). Hexane was added to the buffer saturated ethyl acetate fraction to make a hexane/ethyl acetate ratio of 7:3. After centrifugation at 1000 rpm for 5 min, the water layer at the bottom of the tube was discarded. The supernatant was passed through a 'Baker'-10 SPE 3ml column prepacked with:

1 On leave from the Department of Horticulture, South China Agricultural University, Guangzhou, People's Republic of China.

2 Abbreviations: paclobutrazol, (2RS, 3RS)-1-[4-chlorophenyl]-4,4-dimethyl-2-[1,2,4-triazol-1-yl]-pentan-3-ol; CI, chemical ionization; ELISA, enzyme-linked immunosorbent assay; EC, electron capture detector; EI, electron impact; GA, gibberellin; Me-ABA, methyl ester of abscisic acid; SIM, selected ion monitoring.

3 Mention of a trademark, proprietary product, or vendor 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 or vendors that may be suitable.
Table 1. Relative Abundances of Ions of the Methyl Derivative of ABA using GC-MS in Multiple Ion Monitoring Mode

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative Abundances to m/z 125</th>
<th>Relative Abundances to m/z 260</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>125</td>
<td>134</td>
</tr>
<tr>
<td>Authentic ABA</td>
<td>100</td>
<td>42</td>
</tr>
<tr>
<td>Isolated from leaves</td>
<td>100</td>
<td>40</td>
</tr>
</tbody>
</table>

Fig. 1. Methane chemical ionization mass spectrum of the methyl ester of ABA from apple leaves extract.

Fig. 2. ABA content in apple seedling leaves in response to paclobutrazol treatment following different degrees of water stress. Paclobutrazol at 0.68 μM was supplied continuously via the nutrient solution to seedlings. Samples were taken after 28 d and subjected to water stress. Free ABA was determined by ELISA after silica column purification. ABA content was expressed as nmol/g of initial fresh weight.

500 mg of silica gel (15) which retain the ABA. The ABA was then eluted with 2 ml methanol/acetonitrile (1:3) which was evaporated to dryness under N2. The residue, which contains the ABA, was taken up with phosphate-buffer saline for ELISA quantitation (14, 20), lettuce seed germination inhibition bioassay (24), or methylation with diazomethane (8) for GC-MS analysis. An external standard of unlabeled authentic ABA (1.5 μg/g fresh weight) was added to apple leaves from all treatments and carried through the same procedures. Quantification of the external standard was by GC-SIM. Recovery rates in all external standard leaf samples were comparable, with an average of 51.7 ± 6.2%. All of the experiments were repeated three times, each with three replications for each treatment.

Confirmation of ABA by GC-MS. After elution from the silica gel column, all the samples containing ABA were taken to dryness and methylated with diazomethane (8). A Pye Unicam model 104 GC equipped with a 0.46 m × 2.0 mm (i.d.) 3% OV 1 packed glass column and Kratos AEI MS 30 double beam mass spectrometer was used to confirm the presence of Me-ABA. Column temperature was 160°C. Ultrapure He was used as a carrier gas at a flow rate of 40 ml/min. Injector was 240°C. Jet separator was 165°C. The typical retention time for Me-ABA was 3 min 20 s. Data acquisition was accomplished with DS-50 S software and a Data General NOVA 2 computer. The resolution at 10% valley definition was 1400. The full EIMS was carried out by magnetic scanning. Selected ions were analyzed by voltage jumping. Two sets of ions were selected and analyzed with the SIM mode. One set was m/z 125, m/z 134, m/z 162, m/z 190 with dwell time in seconds as 0.5, 0.75, 1.25, and 1.25, respectively. The other set was m/z 247, m/z 260, m/z 278 with dwell time in seconds as 0.75, 1, and 1, respectively. The second set of ions was analyzed at 30 times higher electron multiplier gain than the first set of ions. The ion peak areas were integrated with baseline substractions. The mass chromatograms of lower and higher masses were normalized to the strongest ion in each set of ions. In addition to using GC-EIMS-SIM, Me-ABA was further confirmed by methane positive ion CI (12) with a 15 m SE 30 fused silica glass capillary column interfaced to an Extrel ELTQ-400-3 mass spectrometer. Initial column temperature was 150°C for 1 min and then programmed at 30°C/min to 250°C. Temperature of GC injector was 275°C. Ultrapure He was used as a carrier gas at a flow rate of 1 ml/min. Me-ABA was quantitatively estimated with a Hewlett-Packard 5880 GC equipped with a 63Ni EC detector (7, 11, 17) and a fused silica capillary column (methyl silicone fluid, 12.5 m × 0.2 mm). The GC-MS quantitative validation was performed on the same sample which was used for ELISA, lettuce seed bioassay, and GC-EC.

Enzyme-linked Immunosorbent Assay (ELISA). Monoclonal antibody (IgG1) was used for the detection and quantitation of endogenous ABA in apple leaves during water stress (20). The procedure used was described by Idetek (14). Each well in a polystyrene plate (96-well) was coated with monoclonal antibody IgG. One hundred μl of the diluted, purified silica column samples or ABA standard solutions (0.0 to 2.0 pmol) and along with a 100 pmol of ABA for nonspecific binding) were added to each well. This was followed by 100 μl of diluted enzyme (alkaline phosphatase) conjugated ABA. The mixtures were incubated for 3 h at 4°C. The plates were then decanted and rinsed with water (3 times). The activity of the enzyme-ABA conjugate bound to the polystyrene-adsorbed antibodies was then determined using p-nitrophenyl-P as substrate. The enzyme reaction was allowed to proceed at 37°C for 1 h and was stopped with 50 μl of 1 M KOH per well. After waiting for 5 min, the readings were taken at 405 nm. Calculations were done as described (14, 20).

Lettuce Seed Germination Inhibition Bioassay. The solutions
were separately streaked on Whatman No. 1 paper and chromatographed with a solvent of: 1-butanol:ethanol:water: ammonia (58:18:14:5). Rf bands of the chromatogram were assayed for germination inhibition of lettuce seed (Black Seeded Simpson) (24).

RESULTS AND DISCUSSION

Confirmation of ABA from Apple Leaf Tissue Extract. The significant ions and relative intensities (in parentheses) of the full EIMS of Me-ABA were as follows: m/z 190 (100), m/z 125 (40), m/z 134 (20), m/z 162 (20), m/z 91 (18), m/z 260 (2), m/z 278 (1), m/z 247 (0.5), and m/z 219 (0.5). This was similar to that reported by Rivier et al. (17). The ions with higher masses (m/z 247, m/z 260, and m/z 278) were about 100 times less intense as compared to the intense peak of m/z 190. However, they were very important for confirmation, because they contained molecular ion m/z 278, (M-H2O)+ at m/z 260 and (M-OCH3)+ at m/z 247. The SIM (m/z 125, m/z 134, m/z 162, m/z 190, m/z 247, m/z 260, and m/z 278) mass chromatograms of Me-ABA from apple leaf extracts and authentic ABA had the same retention time (data not shown) and their relative abundance also fell in ±15% of the standard (Table I). The methane positive ion of CIMS of Me-ABA from apple leaves extract was plotted in Figure 1 and was similar to that reported by Netting et al. (12). The mol wt was proven by showing the characteristic adduct ions m/z 279 (M + 1), m/z 307 (M + 29), and m/z 319 (m + 41). The base peak was m/z 261 (M + H2O) indicating that the molecule still had one underivatized -OH functional group. The second intense peak was m/z 247 (M+CH2OH) indicating that the molecule had one hydroxy group methylated. With the presence of m/z 219 (M + H-COOC2H5) and m/z 247, the methylated hydroxy group was determined to be on the carboxylic acid site. Ions not present in the EIMS were m/z 229 (M + CH2O-H2O) and m/z 275 (M + CH2OH2O).

The Rf value (Rf = 0.8) which showed the most inhibitory activity in lettuce seed bioassay from the purified silica column tissue extract separated by paper chromatography coincided closely with that of synthetic ABA (data not shown). Based on above information, our results indicate that the inhibitory compound from apple leaf extracts was indeed ABA.

ABA Content in Apple Leaves. In apple leaves, water stress substantially increased ABA accumulation as quantitatively estimated by the ELISA and qualitatively validated by GC-MS (Fig. 2). ABA content increased progressively with increasing water stress. A rise in the level of ABA in plant tissues subjected to water stress has also been demonstrated in a number of other species (7, 12, 18, 22, 23). However, both control (nonstress) and water stress-induced ABA could be suppressed by paclobutrazol treatment (Fig. 2). In untreated apple leaves, when water loss reached 30% of the initial fresh weight, ABA accumulated in excess of 2 times that produced by nonstressed untreated leaves, whereas in the paclobutrazol-treated leaves, ABA was reduced to circa two-thirds of nonstress leaves, and showed only a slight increase during water stress. A comparison of quantitative estimates of ABA in the plant extracts by ELISA, GC-EC, and the lettuce seed bioassay is shown in Table II. A good correlation was found between results obtained from ELISA and GC. A similar correlation for these methods was reported by Leroux et al. (11). The lettuce seed bioassay had the greatest variation. When water loss reached 20% of the initial leaf fresh weight, the amounts of ABA in the untreated apple leaves were calculated to be 1.82, 1.77, and 1.65 nmol/g of original fresh weight by ELISA, GC-EC, and lettuce seed bioassay, respectively. Since an external standard of authentic ABA was used for calculations of recovery, the absolute ABA values from experimental tissue may differ from calculations based on the use of an internal standard. Nonetheless, we have demonstrated that a relative difference in ABA levels occurred between paclobutrazol-treated and non-treated water-stressed tissues.

Creeelman and Zeevaart (5) found that one of the oxygens in the carbonyl group of the rapidly formed ABA in water-stressed leaves of Xanthium strumarium was derived from molecular oxygen, implying that either the oxygen atoms present in the ABA arise from water or the oxygen atoms already present in a stored precursor which is converted to abscisic acid under water stress condition. Recently, it was reported (13) that paclobutrazol blocked the ABA biosynthetic pathway in Cercospora rosicola at a point after farnesyl pyrophosphate. However, the oxidation at the 4'-position of (2Z,4E)-a-ionylideneacetic acid was not prevented in the presence of the paclobutrazol (13). Dalziel and Lawrence (6) also reported that paclobutrazol was a potent inhibitor of kaurene oxidase in the GA biosynthetic pathway in pepper. The decrease of ABA content in paclobutrazol-treated, apple seedling leaves could thus also be due to the blocking of one or more oxidation steps in the ABA biosynthetic pathway by paclobutrazol. Since paclobutrazol had presumably been inhibiting ABA biosynthesis for at least several weeks, it is also not clear whether the marked reduction in water stress-induced accumulation of ABA is due to a block of newly synthesized ABA, or to an appreciably lowered pool of an immediate precursor, or conjugate (e.g. ABA glucosyl ester). Further research is warranted to clarify this relationship.

LITERATURE CITED


5. CREEELMANN RA, JAD ZIEVAART 1984 Incorporation of oxygen into abscisic acid and phasic acid from molecular oxygen. Plant Physiol 75: 166-169


7. DAVIES FS, AN LARSO 1978 Water relations in apple seedlings: changes in water potential components, abscisic acid levels and stomatal conductances under irrigated and non-irrigated conditions. J Am Soc Hortic Sci 103: 310-

Table II. ABA Content in Water-Stressed Leaves from Apple Seedlings Treated and Untreated

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ABA Content</th>
<th>Lettuce seed bioassay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ELISA</td>
<td>GC-EC</td>
</tr>
<tr>
<td>Control</td>
<td>1.82 ± 0.13</td>
<td>1.77 ± 0.12</td>
</tr>
<tr>
<td>Paclobutrazol</td>
<td>1.01 ± 0.07</td>
<td>1.00 ± 0.09</td>
</tr>
</tbody>
</table>

nmol/g of original fresh wt ± se


22. Wright STC 1969 An increase in the "inhibitor-β" content of detached wheat leaves following a period of wilting. Planta 86: 9-20

23. Wright STC 1977 The relationship between leaf water potential (ψ leaf) and the levels of abscisic acid and ethylene in excised wheat leaves. Planta 134: 183-189