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Abscisic Acid Accumulation and Carotenoid and Chlorophyll Content in Relation to Water Stress and Leaf Age of Different Types of Citrus

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The abscisic acid (ABA), carotenoid, and chlorophyll composition was determined before and after water stress for detached leaves of Valencia and Pineapple orange, Marsh grapefruit, Eureka lemon, Ichang lemon, and West Indian lime. ABA and base-hydrolyzable ABA conjugates were measured by ELISA using a monoclonal antibody specific for (+)-ABA. Carotenoids and chlorophylls were separated by HPLC. ABA levels increased 3–67-fold, while Chl and carotenoid levels were not affected by short-term water stress. The carotenoids were identified by comparison of HPLC retention times, by on-line spectral measurements from a diode array UV–vis detector, and from visible absorption maxima of isolated pigments in various solvents. The carotenoids in citrus leaves have not been reported previously. The different citrus species and cultivars were qualitatively similar in carotenoid content but were quantitatively different. The major xanthophylls were *trans*-lutein, *trans*-violaxanthin, 9′-*cis*-neoxanthin, and minor amounts of luteoxanthin, antheraxanthin, and zeaxanthin. The carotenes included α- and β-carotene. Cis isomers of violaxanthin and lutein were tentatively identified. The ratios of chlorophyll *a* to *b* were between 2.7 and 4.0.

INTRODUCTION

ABA, carotenoids, and the phytol side chain of chlorophylls (Chls) share mevalonate as a common precursor in the isoprenoid pathway. Endogenous levels of ABA increase considerably when plants are stressed (Davies and Mansfield, 1983, 1988; Hirai, 1986; Milborrow, 1981; Wright, 1978; Zeevaart and Creelman, 1988). There are numerous reports of the occurrence of ABA, carotenoids, and Chls in the fruit of citrus (Goldschmidt, 1984; Sinclair, 1984; Wheaton and Bausher, 1977). However, only a few reports pertain to ABA and Chl content in citrus leaves (Arguella and Guardiola, 1977; Goren et al., 1971; Mauk et al., 1987; Norman et al., 1988; Syvertsen and Smith, 1984; Wheaton and Bausher, 1977; Weill et al., 1979). The identity and quantity of carotenoids in citrus leaves have not been previously reported.

An indirect enzyme-linked immunosorbent assay (ELISA) for measurement of (+)-ABA in citrus leaves (Norman et al., 1988) makes it possible to conveniently

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analyze numerous crude extracts without cleanup procedures. In addition to measuring free ABA, we also quantitated the base-hydrolyzable ABA (hereafter referred to as ABA conjugate). In most studies, conjugated ABA refers to the ABA liberated by hydrolysis. Harris and Dugger (1986) recently identified the major ABA conjugate in citrus fruit and leaves as abscisyl β-D-glucopyranoside on the basis of immunological cross-reactivity, TLC, enzymic degradation, and mass spectroscopy. Their results do not preclude the existence of other yet unidentified ABA conjugates. Five conjugated forms of ABA have been reported, but they are not regarded as metabolites common to all plant species (Hirai, 1986). Should other less labile conjugates such as glucosides (Milborrow, 1978) prove to be present in citrus leaves, base hydrolysis may not have liberated the total amount of ABA conjugates.

HPLC allows the rapid separation of the labile carotenoids and Chls without the use of saponification and other purification procedures that produce artifacts (Braumann and Grimme, 1981; Chen and Bailey, 1987; Lichtenthaler, 1987; Roy, 1987; Schwartz and von Elbe, 1982; Khachik et al., 1986).

Since water stress causes a change in ABA concentration that is the most dramatic change in concentration of any plant hormone in response to an environmental factor, it is a useful means of inducing rapid ABA biosynthesis or metabolism for purposes of studying inhibition of these pathways. However, before inhibitor studies can be conducted, comprehensive data on the relationship between ABA, carotenoid, and Chl composition of citrus leaves were needed. This work reports the ABA and ABA conjugate accumulation and carotenoid and Chl content in relation to water stress and leaf age for a range of different citrus species and cultivars.

EXPERIMENTAL PROCEDURES

Immunoassay Equipment. Immulon II microtiter plates (96 well, flat bottom) were from Dynatech Laboratories, Alexandria, VA. The Titertek Multiskan MC plate reader and Titertek microplate incubator were from Flow Laboratories, Inc., McLean, VA.

HPLC Equipment. The liquid chromatograph consisted of two 6000 Å pumps and a Model 660 solvent programmer (Waters Associates, Milford, MA), a 7120 injector with a 100-μL injection loop for analytical and a 5-mL injection loop for preparative separations (Rheodyne Inc., Berkeley, CA), an LC-75 detector (Perkin-Elmer Corp., Norwalk, CT), and a Chromatopac CR1B integrator (Shimadzu Corp., Kyoto, Japan). Detection was at 430 nm. Pigments were quantified from calibrations obtained from the peak area ratios of the pure reference compounds to that of a constant amount of the internal standard. On-line spectra of peaks were obtained with a Hewlett-Packard 1040A rapid-scanning UV–visible photodiode array detector. The data were stored and processed by means of a Hewlett-Packard 85-B computing system and a 7470A plotter. Absorption spectra of isolated components in various solvents were recorded on a Perkin-Elmer Lambda 7 UV–visible spectrophotometer.

Chemicals and Materials. All chemicals, unless otherwise specified, were of the highest purity available from Sigma Chemical Co. Monoclonal antibody for (+)-ABA was purchased from Idetek, Inc. (San Bruno, CA). (+)-ABA was isolated and crystallized from *Cercospora rosicola* cultures according to the method of Bennett et al. (1981). Five-milliliter disposable microfilters with 0.2-μm nylon 66 membranes were Centrex DF1-1/1 tubes from Schleicher & Schuell, Inc., Keene, NH. A peristaltic pump (Cole Parmer Instrument Co.) was used to pump sample extracts through C_{18} or NH_2 Sep-Paks (Millipore Corp., Bedford, MA), which were prewashed with methanol and then water. Magnesium oxide and Celite 545 were from Fisher Scientific (Pittsburgh, PA). Reference samples of α-carotene, β-carotene, Chl *a*, and Chl *b* were from Sigma Chemical Co. β-Apo-8′-carotenal, the internal standard, was from Fluka Chemical Corp. (Ronkonkoma, NY). Other reference compounds were isolated from various plant sources. Marigold paste was obtained from Kemin Ind. (De Moines, IA). Oleander leaves were collected from bushes on our property. Yellow corn meal and fresh broccoli and kale were purchased in the local supermarket. Oleander, broccoli, and kale were lyophylized, ground to a fine powder, and stored at −16 °C. Reagent grade methanol was redistilled for HPLC. All other solvents were of HPLC grade (Burdick and Jackson). The extracting solvent mixture used for plant tissue (unless otherwise specified) was redistilled MeOH containing 100 mg/L of BHT and BHA and 0.4% Triton X-100. A Tissumizer GDT-1810 and a SDT-080EN shaft and generator (Tekmar Co., Cincinnati, OH) were used to extract samples.

Columns. A Whatman Partisil 5/25 ODS-3 column (5 μm, 4.6 × 250 mm) protected by a Brownlee Spheri-5 ODS (5 μm) precolumn was used for quantitative measurements (eluent A and B). Two Brownlee Spheri-5 RP-18 (5 μm, 4.6 × 240 mm) columns connected in series and protected by a Brownlee Speri-5 RP 18 (5 μm) precolumn were used to separate lutein and zeaxanthin (eluent D and E). Preparative separations were carried out on a Whatman Partisil 10 ODS-3 Magnum 20 column (10 μm, 22 × 250 mm, eluent C). Open column chromatography was carried out in a glass column 18 × 300 mm packed with a mixture of activated (60 °C) magnesium oxide and Celite 545 (1:1, eluent F).

Eluent A. Separations were made on a Whatman Partisil 5/25 ODS-3 column with a 16-min gradient from acetonitrile/methanol/water (55:20:25 v/v/v) to acetonitrile/methanol/tetrahydrofuran (75:25:2 v/v/v) followed by 20 min isocratic. The flow rate was 2 mL/min (Braumann and Grimme, 1981).

Eluent B. Separations were made on the Whatman Partisil 5/25 ODS-3 column with a 30-min gradient from acetonitrile/methanol/water (55:20:25 v/v/v) to acetonitrile/methanol/tetrahydrofuran (75:25:2 v/v/v) followed by 15 min isocratic. The flow rate was 2 mL/min. On-line spectra of the peaks were collected with the diode array detector, and selected peaks were collected for further identification.

Eluent C. Preparative separations on the Whatman Magnum 20 column were with a 16-min gradient from acetonitrile/methanol/water (55:20:25 v/v/v) to acetonitrile/methanol/tetrahydrofuran (75:25:2 v/v/v) followed by 35 min isocratic. The flow rate was 9.9 mL/min. Peaks or fractions were collected to obtain absorption spectra in various solvents or for further purification and separation. The purity of the peaks collected from the preparative column was checked with the analytical columns with eluent A, B, or E.

Eluent D. Isocratic separations on two Spheri-5 RP-18 columns connected in series were made with methanol/acetonitrile/dichloromethane/hexane (20:170:5:5) at a flow rate of 1 mL/min (Khachik et al., 1986).

Eluent E. A combination of isocratic and gradient chromatography (Khachik et al., 1986) was used with the two Spheri-5 RP-18 columns to collect on-line spectra with the diode array detector. An isocratic mixture of methanol/acetonitrile/dichloromethane/hexane (20:170:5:5) at 0 time was followed by a gradient beginning after 20 min to a final composition of methanol/acetonitrile/dichloromethane/hexane (30:80:45:45), which was completed in 40 min. Solvent flow rate was 1 mL/min.

Eluent F. For open column chromatography on magnesium oxide and Celite, the sample in hexane was developed with hexane/acetone (95:5 and 90:10) followed by hexane/acetone/ethanol (89:10:1) to separate lutein and zeaxanthin. The column was extruded, and the bands were cut out and eluted with acetone. The acetone was removed on a rotary evaporator.

Citrus Seedlings. Valencia orange [*Citrus senesis* (L) Osbeck], Pineapple orange [*C. senesis* (L) Osbeck], Marsh grapefruit (*C. paradisi* Macfad.), Eureka lemon [*C. limon* (L)], Ichang lemon (*C. ichangensis* X *C. grandis*), and West Indian key lime (*C. aurantifolia* Swingle) were grown from seed in 1-gal pots in a greenhouse. The seedlings were between 18 and 24 months old. The seedlings were divided into three sections: (a) the upper leaves, which were actively growing and not fully expanded, (b) the middle leaves, which were mature, fully expanded leaves, and (c) the lower leaves, which were about 1–2 years old but did not include senescing or primary leaves. Leaves from each

section were combined from several plants (20–50 leaves per sample). The Pineapple orange seedlings were smaller than the other citrus types, and only immature and mature leaves were sampled. The leaves from each section were divided into two parts. One part was frozen immediately at -90 °C (Puffer Hubbard Ultra-low Freezer, New York, NY). The other part was placed on a screen in subdued light at room temperature until the leaves lost 12–15% of their initial weight. These stressed leaves were placed in plastic bags in the dark for 24 h at room temperature to allow ABA to accumulate and then frozen at -90 °C as above. The leaves were then lyophilized, ground to a fine powder in a small coffee mill, and stored at -16 °C until analyzed.

ABA and ABA Conjugate Analyses. Duplicate samples of leaf tissue (10–50 mg dry weight) were extracted in 1.5 mL of 80% acetone containing BHT and BHA (100 mg of each/L) by storing several days at 4 °C. A portion (750 μL) of the acetone extract was diluted to 5.0 mL with TBST buffer (6.0 g of Tris, 0.2 g of $MgCl_2$, 8.8 g of NaCl, 0.1 g of NaN_3, and 0.5 mL of Tween 20, pH 7.5) in a 5-mL Centrex tube and filtered through the 0.2-μm nylon 66 filter by centrifugation for 9 min at 1200 rpm. This extract was analyzed directly or further diluted with TBST to be in the appropriate range of ABA for the assay. Triplicate portions of 100, 75, 50, and 25 μL were placed in wells of microtiter plates. A standard curve was included on each plate. ELISA procedures have been previously described in detail (Norman et al, 1988). For alkaline hydrolysis, 1 mL of the same diluted TBST extract was adjusted to pH 11 with 0.05 N NaOH, heated 1 h at 60 °C, neutralized with 0.05 N HCl, and diluted with TBST to 2 mL for turgid leaves and 8 mL for water-stressed leaves. These hydrolyzed samples were analyzed again to measure the combined free ABA and ABA formed by base hydrolysis. The ABA conjugate concentration was calculated by the difference between free ABA from the first analysis and the total ABA from the second analysis.

Quantification of Carotenoids and Chlorophylls. Precautions to avoid exposure to light were taken as much as possible during extractions. Lyophilized leaf tissue (100 mg) was weighed directly in the top of a 5-mL Centrex tube, $MgCO_3$ (25 mg) and 5 mL of methanol extracting solvent were added, and the tissue was ground directly in the upper Centrex tube with the Tissumizer. The extract was filtered through the nylon 66 filter by centrifugation for 9 min. The tissue was extracted 3 more times with 5-mL portions of the extracting solvent as described. The combined filtrates were diluted to 60 mL with H_2O and passed through two NH_2 Sep-Paks connected in series. NH_2 Sep-Paks do not have a large capacity, and two were required to retain all of the compounds of interest for the sample size described above. The carotenoids and Chls were eluted with 4.5 mL of methanol containing BHT and BHA (100 mg of each/L) into a 5-mL volumetric flask to which 50 μg of β-apo-8'-carotenal dissolved in 0.5 mL of methanol was added as internal standard. After mixing, the samples were transferred to 3.5-mL amber vials which were filled to the brim to avoid oxidation, and the excess was discarded. Tissue samples were extracted in sets of four and analyzed as soon as possible by HPLC (eluent A).

Extraction of Plant Material for Preparative Separations. Corn meal was extracted according to the method of Quackenbush et al. (1961). Two to five grams of lyophilized broccoli or leaves of kale, oleander, or Pineapple orange was extracted to isolate individual carotenoids for identification. Magnesium carbonate (100 mg/g of tissue) and 50 mL of extracting solvent were added to the tissue and ground directly in a 60-mL fritted glass funnel with the Tissumizer. The mixture was filtered under suction and the plant residue reextracted until the filtrate was colorless. The filtrates were combined, diluted to 50% with water, and pumped through 8–10 NH_2 or C_{18} Sep-Paks connected in series. Pigments were eluted with methanol (NH_2) or acetone (C_{18}). The solvent was removed on a rotary evaporator at 30 °C, and the residue was dissolved in an appropriate volume of methanol and filtered through a Millex-HV 0.45-μm filter. Five-milliliter aliquots were separated on the Magnum 20 preparative column (eluent C). Fresh samples were prepared for each peak of interest and separated, the peak eluent was collected, and spectral measurements in various solvents were made on the same day. When further purification on an analytical column was required, the collected peak eluents were stored at -90 °C and completed the following day.

Saponification. The marigold paste contained esterified carotenoids and required saponification. An ethereal solution of 1 g of marigold paste was treated with 5 mL of 40% methanolic KOH for 20 min at 56 °C according to the method of Quackenbush and Miller (1972). The saponified mixture was diluted to 300 mL with 10% NaCl and partitioned three times with hexane/ether (70:30). The combined hexane/ether fractions were dried over Na_2SO_4, and solvent was removed on a rotary evaporator. The residue was taken up in methanol and a yellow-brown insoluble precipitate was removed by filtration and discarded. Aliquots of the methanol extract were evaporated onto Celite for application on the magnesium oxide/Celite column (eluent F).

Isolation of Carotenoid Reference Compounds. *Lutein and Zeaxanthin.* The extract of yellow corn meal in hexane was separated on the magnesium oxide/Celite 545 column with vacuum. Lutein separated in a band about 5 cm below the zeaxanthin band. The column was extruded and each band cut out and eluted with acetone. The acetone was removed on a rotary evaporator and the residue dissolved in methanol. Water was added dropwise until the appearance of a precipitate, which redissolved upon swirling. The compounds crystallized overnight at -16 °C. HPLC (eluent D) showed a single peak for each compound with retention times of 17.05 and 17.99 min for lutein and zeaxanthin, respectively. Visible absorption maxima (nanometers) for lutein were as follows: hexane, λ_{max} = 420, 443, 471; methanol, λ_{max} = 420, 443, 471; carbon disulfide, λ_{max} = 446, 473, 504; benzene, λ_{max} = 432, 456, 486; eluent A, λ_{max} = 424, 446, 474; eluent D, λ_{max} = 422, 446, 474. Visible absorption maxima (nm) for zeaxanthin were as follows: hexane, λ_{max} = 425, 447, 475; methanol, λ_{max} = 424, 448, 474; carbon disulfide, λ_{max} = 448, 479, 507; benzene, λ_{max} = 434, 462, 489; eluent A, λ_{max} = 425, 452, 479; eluent D, λ_{max} = 425, 452, 479. Lutein isolated from marigold paste by the same procedure had spectra and HPLC Rt identical with those from corn meal.

Neoxanthin, Violaxanthin, and Luteoxanthin. Ground kale leaves (2 g) were extracted and diluted to 50% with water as described above and pumped through five C_{18} Sep-Paks. The xanthophylls (Eskins and Dutton, 1979) were eluted with 90% methanol. HPLC (eluent A) showed that this faction contained neoxanthins, violaxanthins, and some of the lutein. The extract was separated by preparative HPLC (eluent C). Center cuts were collected from the major 9'-*cis*-neoxanthin (Rt = 25.1 min) and lutein (Rt = 37.9 min) peaks. The violaxanthin peak was collected to include three small peaks immediately following the main violaxanthin peak. Analytical HPLC (eluent A) showed neoxanthin to be a single peak. Aliquots were evaporated and dissolved in various solvents for spectral measurements. The visible absorption maximum (nanometers) for 9'-*cis*-neoxanthin were as follows: hexane, λ_{max} = 410, 434, 463; benzene, λ_{max} = 422, 447, 477; carbon disulfide, λ_{max} = 436, 462, 494; methanol, λ_{max} = 412, 434, 463; eluent A, λ_{max} = 414, 437, 466; ethanol, λ_{max} = 413, 437, 465. Addition of three drops of ethanolic HCl (0.1 M) showed a 15-nm hypsochromic shift in absorption maxima (ethanol, λ_{max} = 399, 422, 449) characteristic of neochrome. The violaxanthin fraction was further purified by HPLC (eluent B), and peaks corresponding to *all-trans*-violaxanthin and luteoxanthin were collected. Visible absorption maxima (nanometers) for *all-trans*-violaxanthin were as follows: hexane, λ_{max} = 422, 445, 468; benzene, λ_{max} = 430, 455, 481; carbon disulfide, λ_{max} = 446, 470, 498; methanol, λ_{max} = 420, 443, 467; eluent A, λ_{max} = 422, 444, 470; ethanol, λ_{max} = 422, 445, 469. Addition of three drops of ethanolic HCl showed a 44-nm hypsochromic shift (ethanol, λ_{max} = 379, 401, 426) characteristic of auroxanthin. Visible absorption spectra (nanometers) of luteoxanthin were as follows: hexane, λ_{max} = 398, 422, 448; benzene, λ_{max} = 409, 432, 460; carbon disulfide, λ_{max} = 422, 448, 476; methanol, λ_{max} = 398, 421, 447; eluent A, λ_{max} = 400, 423, 449; ethanol, λ_{max} = 400, 423, 449. Addition of three drops of ethanolic HCl showed a 22-nm hypsochromic shift (λ_{max} = 381, 401, 426) characteristic of auroxanthin. These spectra

are consistent with those for *9'-cis*-neoxanthin and *all-trans*-violaxanthin reported as constituents of kale and luteoxanthin from acid treatment of violaxanthin by Khachik et al. (1986).

The peak corresponding to lutein in the kale extract was also collected and had spectra in various solvents identical with that isolated from corn meal and marigold paste.

Antheraxanthin. Four grams dry weight oleander leaves (Demming et al., 1988) were extracted for the isolation of antheraxanthin. The diluted extract was pumped through eight C_{18} Sep-Paks and eluted with acetone. A peak with a Rt of 27 min on the preparative column (eluent C) was collected and shown to be a single peak by analytical HPLC (Rt = 13.99 min, eluent A; Rt = 19.0 min, eluent B). Visible absorption maxima (nanometers) of antheraxanthin were as follows: hexane, λ_{max} = 422, 444, 472; benzene, λ_{max} = 432, 457, 486; carbon disulfide, λ_{max} = 448, 473, 503; methanol, λ_{max} = 420, 443, 470; eluent A, λ_{max} = 423, 446, 473; eluent D, λ_{max} = 422, 446, 473; chloroform, λ_{max} = 432, 455, 483; ethanol, λ_{max} = 423, 445, 473. The shape of the spectrum was nearly identical with that of lutein. The visible absorption maxima and Rt in eluent A are consistent with that reported by Brauman and Grimme (1981) and in hexane and benzene with that reported by Khachik et al. (1986) for semisynthetic antheraxanthin A and B which had identical spectra. Addition of three drops of ethanolic HCl showed an 18–20-nm hypsochromic shift to mutatoxanthin. Visible absorption maxima (nanometers) of mutatoxanthin were as follows: hexane, λ_{max} = 402, 425, 452; benzene, λ_{max} = 412, 436, 463; carbon disulfide, λ_{max} = 426, 452, 479; methanol, λ_{max} = 402, 424, 450; eluent A, λ_{max} = 404, 426, 452; eluent D, λ_{max} = 406, 426, 452; chloroform, λ_{max} = 411, 434, 460; ethanol, λ_{max} = 402, 424, 450.

Lutein Epoxide. Five grams dry weight broccoli (Khachik et al., 1986) was extracted for the isolation of lutein epoxide. The diluted extract was pumped through eight C_{18} Sep-Paks and eluted with acetone. A peak with Rt of 24.85 min on the preparative column (eluent C) was collected which was shown to be a single peak by analytical HPLC (Rt = 13.89 min, eluent A; Rt = 18.82 min, eluent B). Visible absorption maxima (nanometers) of lutein epoxide were as follows: hexane, λ_{max} = 415, 438, 468; benzene, λ_{max} = 426, 451, 481; carbon disulfide, λ_{max} = 440, 467, 498; methanol, λ_{max} = 415, 438, 467; eluent A, λ_{max} = 417, 440, 469; eluent D, λ_{max} = 417, 440, 469; chloroform, λ_{max} = 425, 449, 478; ethanol, λ_{max} = 417, 439, 469. The spectra and Rt in eluent A are consistent with that reported by Brauman and Grimme (1981). The visible absorption spectrum exhibited a well-defined structure consistent with that reported by Khachik et al. (1986) in contrast to that of antheraxanthin, which was not as well-defined. On treatment with ethanolic HCl the absorption maxima of lutein epoxide showed an 18–21-nm hypsochromic shift to flavoxanthin. Absorption maxima (nanometers) of flavoxanthin were as follows: hexane, λ_{max} = 397, 420, 447; benzene, λ_{max} = 407, 430, 458; carbon disulfide, λ_{max} = 422, 446, 475; methanol, λ_{max} = 397, 420, 446; eluent A, λ_{max} = 399, 421, 448; eluent D, λ_{max} = 399, 422, 448; chloroform, λ_{max} = 406, 429, 457; ethanol, λ_{max} = 399, 421, 448. Flavoxanthin also exhibited a well-defined spectrum in contrast to that of mutatoxanthin from antheraxanthin, which was not as well-defined. When lutein epoxide and antheraxanthin were cochromatographed together (eluent A and B), the two xanthophylls resulted in a double peak that was not resolved.

Identification of Carotenoids and Chlorophylls in Pineapple Orange Leaves. Five grams dry weight of immature Pineapple orange leaves was extracted as described. The residue in methanol was separated by preparative HPLC (eluent C), and peaks were collected to obtain the spectra in various solvents and also separated by analytical HPLC (eluent B), and spectral data were collected on-line for each peak from a diode array detector. HPLC retention times, on-line spectra, and spectra of the isolated components in various solvents were compared to that of reference compounds isolated from the various sources described to establish the identity of the carotenoids and Chls. Our separations with eluents D and B were also compared with literature values in eluent D (Khachik et al., 1986) and in eluent B (Braumann and Grimme, 1981). Other spectra reported in the literature were also consulted (deRitter and Purcell, 1981; Schwartz and von Elbe, 1982; Watanabe et al., 1984).

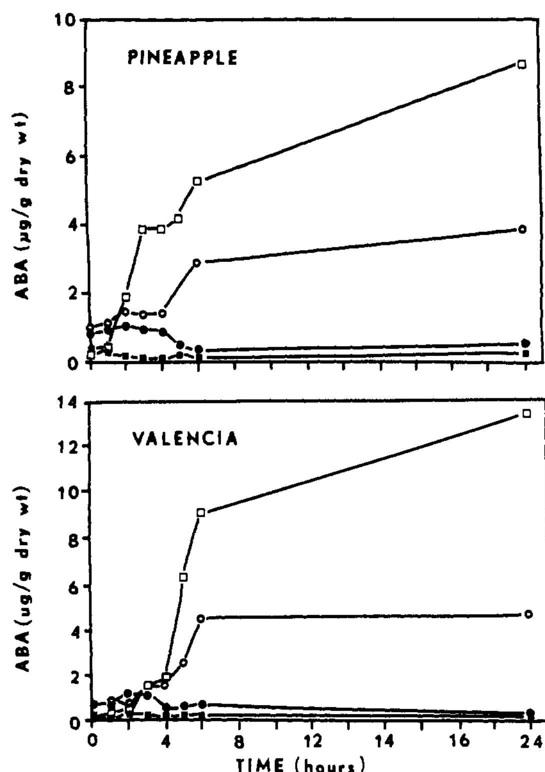

Figure 1. Accumulation of ABA and ABA conjugate in Valencia (15 leaves/sample) and Pineapple (20 leaves/sample) citrus leaves before and after fresh weight had been reduced by 12.42% and 14.46%, respectively. (□) ABA in stressed leaves; (○) ABA conjugate in stressed leaves; (■) ABA in turgid leaves; (●) ABA conjugate in turgid leaves.

RESULTS AND DISCUSSION

ABA and ABA Conjugate Content of Citrus Leaves before and after Water Stress. The monoclonal antibody used for ELISA is specific for free ABA and does not cross-react with *trans*-ABA nor with ABA conjugates. Initially, β-glucosidase was used to hydrolyze the ABA conjugate, but it was found that the enzyme interfered with the ELISA assay and could not be used. Therefore, the usual base hydrolysis was used. Solutions of (+)-ABA and (+)-abscisyl β-D-glucopyranoside were measured by HPLC before and after hydrolysis at pH 11 for 1 h at 60 °C; 100% of the ABA and 92% of the ABA released from the ester were recovered.

The rise in the amount of ABA during water stress generally starts about the time plant leaves begin to wilt or reach a certain water potential. This rise in ABA occurs during the first hour of wilting in many plants and then reaches an equilibrium. This sudden increase in ABA can be up to 40 times the prestress levels in many plants (Milborrow, 1981). When citrus leaves lose 12–15% of their initial weight, the leaves do not become flaccid as annual plants do but tend to curl slightly. We determined the time course of ABA accumulation in a mixture of immature and mature Valencia and Pineapple orange leaves after water stress (Figure 1). The ABA content increased rapidly between 1 and 6 h in Pineapple orange and between 4 and 6 h in Valencia orange leaves. Over 1 h was required for the leaves to lose 12–14% of the initial weight, and samples were not measured between 6 and 24 h to determine the time that an equilibrium was reached; however, the ABA content was highest at 24 h. The ABA conjugate did not increase much

Table I. ABA and Base-Hydrolyzable ABA Conjugate Content of Turgid and Water-Stressed Detached Leaves of Different Maturity from Different Types of Citrus Seedlings

			μg/g dry wt ± SD (no. of assays)			
			ABA		ABA conjugate	
type of citrus	leaf type	% dry wt	turgid	stressed	turgid	stressed
Valencia orange	immature	25.82	0.19 ± 0.02 (8)	11.39 ± 0.87 (8)	0.63	2.99
	mature	35.28	0.05 ± 0.01 (7)	1.78 ± 0.45 (8)	0.64	1.81
	old	35.43	0.13 ± 0.05 (7)	1.63 ± 0.57 (7)	0.76	3.89
Pineapple orange	immature	24.08	0.49 ± 0.05 (6)	22.49 ± 5.20 (6)	0.30	2.35
	mature	29.91	0.20 ± 0.01 (7)	13.31 ± 4.50 (8)	0.49	1.68
Marsh grapefruit	immature	25.19	0.34 ± 0.03 (8)	10.99 ± 4.63 (7)	1.29	6.58
	mature	29.13	1.01 ± 0.14 (8)	5.52 ± 0.64 (8)	1.25	3.03
	old	30.44	1.13 ± 0.16 (8)	3.62 ± 0.31 (8)	1.07	3.48
Eureka lemon	immature	21.53	0.84 ± 0.10 (8)	12.19 ± 0.73 (8)	0.49	5.63
	mature	26.70	0.72 ± 0.06 (8)	6.86 ± 0.55 (6)	0.95	3.58
	old	29.87	0.50 ± 0.03 (8)	4.09 ± 0.88 (8)	1.10	1.53
Ichang lemon	immature	21.80	0.81 ± 0.10 (8)	10.44 ± 1.08 (8)	0.00	0.00
	mature	34.26	0.15 ± 0.02 (7)	3.67 ± 0.39 (7)	0.30	2.30
	old	35.40	0.07 ± 0.02 (8)	1.40 ± 0.07 (6)	0.20	1.02
West Indian lime	immature	25.16	0.61 ± 0.06 (8)	7.30 ± 1.01 (6)	0.85	3.04
	mature	32.44	0.37 ± 0.06 (8)	1.68 ± 0.19 (7)	1.13	2.62
	old	31.52	0.31 ± 0.04 (8)	1.90 ± 0.10 (4)	2.28	3.69

after 6 h. The ABA content did not increase in turgid leaves held overnight under the same conditions. Therefore, as a standard procedure, in subsequent work, we stressed detached leaves to 12–15% weight loss and then enclosed the leaves in plastic bags and stored the leaves in the dark 24 h.

The ABA and the ABA conjugate content in detached leaves of six different citrus types before and after water stress were measured by ELISA (Table I). The ABA and ABA conjugate level in turgid leaves from different species and cultivars and maturities fell within the range 0.05–1.13 and 0.20–2.28 μg/g dry weight, respectively. The amount of ABA was generally highest in young, rapidly growing leaves, and the ABA conjugate was usually highest in old leaves. Without exception, when the leaves were water stressed, there was a substantial increase in ABA levels. The increase was most pronounced with immature leaves, ranging from 12- to 60-fold (average, 30). The stress-induced increase declined with the age of the leaf. For mature leaves it ranged from 5- to 67-fold (average, 25) and for old leaves 3- to 20-fold (average, 10). The increase in ABA conjugate content of leaves when stressed was less dramatic. It ranged between 3- and 11-fold (average, 7) for immature leaves, between 2- and 8-fold (average, 4) for mature leaves, between and 1- and 5-fold (average, 3) for old leaves. In all cases, the increase in the amount of free ABA with stress was substantially greater than the amount of ABA conjugate in unstressed leaves. Therefore, the stress-induced ABA most likely comes from new synthesis rather than conversion of the ABA conjugate. The fact that the conjugate level increases rather than decreases also supports this interpretation. Our data for citrus leaves are in agreement with reports on other plants in which the stress-induced ABA arises from biosynthesis rather than a release from a pool of conjugated ABA and in which the formation of the glucose ester is irreversible and leads to biological inactivity (Milborrow, 1978).

Little information is available concerning the ABA content of citrus leaves. The ABA content was reported to range in concentration from 0.09 to 0.25 μg/g fresh weight in stems and expanding leaves of Valencia oranges, from 0.14 to 0.59 μg/g fresh weight in Wilking mandarin tree leaves, and from 0.14 to 0.59 μg/g fresh weight in navel orange leaves (Wheaton and Bausher, 1977; Goldschmidt, 1984; Harris and Dugger, 1986; Norman et al., 1988). ABA-like inhibitors increased in water-stressed detached leaves of *C. sinensis* cv. Shamouti and *C. aurantium* L. from about 0.1 to 1.1 μg/g dry weight (Weill et al., 1979). The only report of ABA conjugate content for mature and newly expanding leaves of navel orange was 11.1 and 0.75 μg/g fresh weight, respectively (Harris and Dugger, 1986). Our results with citrus leaves are in agreement with reports for other plants in which the ABA content per unit weight is highest in young leaves and declines as the leaves expand (Zeevaart, 1979).

Table II. HPLC Retention Times (Rt) and Peak Identification for the Chlorophylls and Carotenoids from Leaf Tissue of Pineapple Orange Seedlings

	HPLC Rt, min		
Peak	eluent B	eluent E	pigment
1	13.02		neoxanthin *a*
2	13.69	9.41	9′-*cis*-neoxanthin
3	16.99	10.61	*all-trans*-violaxanthin
4	17.85	10.98	luteoxanthin
5	18.76		violeoxanthin
6	19.47	12.81	13- or 13′-*cis*-violaxanthin
7	20.86	13.50	antheraxanthin
8	24.14	16.73	*all-trans*-lutein
9	24.14	17.68	zeaxanthin
10	25.59	20.03	13-*cis*-lutein
11	33.76	40.33	chlorophyll *b*
12	34.94	43.19	chlorophyll *b′*
13	36.72	45.00	chlorophyll *a*
14	38.93	46.33	chlorophyll *a′*
15	42.18	51.35	α-carotene
16	43.32	52.02	β-carotene
17	43.92	52.56	15,15′-*cis*-β-carotene

Identification of Carotenoids and Chlorophylls. The HPLC chromatograms of the different citrus types were qualitatively similar but quantitatively different. Immature Pineapple orange leaves contained the highest amount of total carotenoids and were therefore used for the identification of the carotenoids and Chls. Peak assignments are given in Table II for the HPLC chromatogram (eluent B) of a concentrated extract of Pineapple orange leaves (Figure 2). Visible absorption maxima of the carotenoids collected on-line with a diode array detector and/or spectrophotometrically in various solvents are given in Table III. Carotenoids were isolated by preparative chromatography and further purified by analytical HPLC for spectrophotometric measurements.

Neoxanthin a. Identification of peak 1 (Figure 2) as neoxanthin *a* is tentative since the amount was too small to isolate and obtain spectra. The spectrum from the diode array detector exhibited a well-defined fine structure similar to that of 9′-*cis*-neoxanthin but showed a

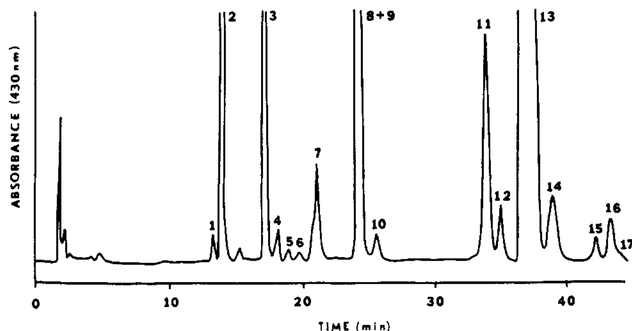

Figure 2. HPLC chromatogram of concentrated Pineapple orange leaf extract. Chromatographic conditions were a 30-min linear gradient from $CH_3CN/MeOH/H_2O$ (55:20:25) to $CH_3CN/MeOH/THF$ (75:25:2) at a flow rate of 2 mL/min and detection at 430 nm. Peak identification is described in Table II.

4–7-nm hypsochromic shift. The spectrum of peak 1 had cis peaks at λ_{max} = 314 and 327. The shape of the spectrum did not correspond to that of *trans*-neoxanthin reported by Khachik et al. (1986) in which the fine structure of the 9′-*cis*-neoxanthin spectrum was lost when isomerized to *trans*-neoxanthin and the λ_{max} remained the same. *trans*-Neoxanthin was not detected in the Pineapple orange leaf extract.

9′-cis-Neoxanthin. Peak 2 had the same Rt as 9′-*cis*-neoxanthin isolated and identified from kale and was collected from the preparative column separation of the Pineapple orange leaf extract. The HPLC Rt (eluents A, B, and D) and visible absorption maxima in various solvents were identical with those of 9′-*cis*-neoxanthin isolated from kale. The cis peaks at 314 and 328 nm were minor peaks. The retention times were also consistent with similar peaks in extracts of oleander and broccoli. On treatment with dilute acid the absorption maxima showed a 14–17-nm hypsochromic shift (hexane, λ_{max} = 399, 421, 448; benzene, λ_{max} = 407, 431, 459; carbon disulfide, λ_{max} = 421, 446, 475; methanol, λ_{max} = 397, 420, 447; eluent A, λ_{max} = 399, 422, 449) typical of neochrome.

trans-Violaxanthin. Peak 3 with the same Rt as *trans*-violaxanthin isolated from kale was collected from preparative chromatography of the Pineapple orange leaf extract. The HPLC retention times (eluents A and B) and visible absorption maxima in various solvents were identical with those of *all-trans*-violaxanthin isolated from kale. On treatment with dilute acid the absorption maxima showed a 39–40-nm hypsochromic shift (hexane, λ_{max} = 379, 400, 425; benzene, λ_{max} = 387, 410, 436; carbon disulfide, λ_{max} = 402, 425, 452; methanol, λ_{max} = 379, 400, 425; eluent A, λ_{max} = 380, 401, 426) typical of auroxanthin.

Luteoxanthin and Cis Isomers of Violaxanthin. Identification of peaks 4–6 (Figure 2) is tentative since we did not isolate the pure compounds. The spectra from the diode array detector of peak 4 exhibited a 19-nm hypsochromic shift compared with *trans*-violaxanthin, a well-defined fine structure, and the absorption maxima at 422 and 449 nm were the same height. The spectra were similar to that of luteoxanthin isolated from kale. The visible absorption maxima from the diode array detector of peak 5 were similar to those of *trans*-violaxanthin but exhibited a 5–6-nm hypsochromic shift similar to 9-*cis*-violaxanthin (violeoxanthin) with minor cis peaks at 312 and 326 nm. The spectrum from the diode array detector of peak 6 was similar to the spectrum of *trans*-violaxanthin but exhibited a 7-nm hypsochromic shift and intense cis peaks at 314 and 327 nm which are similar to those of 13- or 13′-*cis*-violaxanthin. The spectra of peaks 5 and 6 are similar to those reported for stereoisomers

Table III. Visible Absorption Maxima Obtained Spectrophotometrically for Isolated Carotenoids from Leaf Tissue of Pineapple Orange Seedlings (Except Where Noted Otherwise)

		visible absorption maxima (nm)							
peak	carotenoids	eluent A	eluent D	hexane	benzene	carbon disulfide	ethanol	ethanol + HCl	reference source
1	neoxanthin *a*	313,[a] 327, 411, 433, 461							
2	9′-*cis*-neoxanthin	314, 328, 414, 437, 466	414, 437, 466	328, 411, 435, 464	315, 328, 422, 447, 477	434, 463, 494	314, 328, 413, 437, 467	399, 422, 449	kale
3	*all-trans*-violaxanthin	418, 441, 470	417, 441, 470	417, 439, 469	427, 452, 482	442, 468, 499	418, 441, 470	380, 401, 426	kale
4	luteoxanthin	399,[a] 422, 449	400,[a] 423, 448						kale
5	violeoxanthin	312,[a] 328, 413, 437, 465							kale
6	13- or 13′-*cis*-violaxanthin	314,[a] 330, 412, 435, 463							kale
7	antheraxanthin	425, 447, 474	424, 447, 474	422, 444, 472	432, 457, 486	448, 474, 503	424, 446, 474	403, 426, 452	oleander
8	*all-trans*-lutein	423, 447, 475	423, 446, 474	420, 444, 473	431, 456, 486	446, 473, 504	422, 445, 474		corn meal, marigold
9	zeaxanthin		424,[a] 451, 480						corn meal
10	13-*cis*-lutein	331,[a] 416, 441, 468	330,[a] 417, 444, 469						corn meal, kale
15	α-carotene	423, 447, 475	423, 447, 474	420, 444, 473	432, 457, 487	448, 474, 504	423, 445, 474		Sigma
16	β-carotene	423, 452, 477	424, 452, 478	423, 449, 475	435, 463, 491	450, 481, 509	423, 450, 475		Sigma, kale
17	15,15′-*cis*-β-carotene	339, 422, 447, 472	341, 423, 449, 473	336, 417, 444, 469	345, 430, 458, 494	446, 475, 504	335, 422, 445, 472		Sigma, kale

[a] Spectra collected only on-line with diode array detector, and identification is therefore tentative.

of *trans*-violaxanthin by Khachik et al. (1986, 1988). Peaks 5 and 6 were too small to obtain suitable spectra in eluent D on-line.

Antheraxanthin. Antheraxanthin and lutein epoxide have similar HPLC retention times in eluents A, B, and D and are not resolved but result in double peaks. Peak 7 collected from preparative chromatography of the Pineapple orange leaf extract was compared with antheraxanthin isolated from oleander leaves and lutein epoxide isolated from broccoli. The light absorption maxima of the component we isolated from Pineapple orange leaves are more consistent with that of antheraxanthin than with that of lutein epoxide. The visible absorption maxima in various solvents were consistently at 5–7 nm longer wavelengths than lutein epoxide and did not exhibit a well-defined fine spectra characteristic of lutein epoxide. The shape of the spectrum was nearly identical with that of lutein. The absorption maxima of the rearrangement product from acid treatment of antheraxanthin isolated from the citrus leaves were also more consistent with those of mutatoxanthin from acid treatment of antheraxanthin from oleander. Visible absorption maxima (nanometers) of the rearrangement product were as follows: hexane, λ_{max} = 402, 425, 450; benzene, λ_{max} = 422, 435, 461; carbon disulfide, λ_{max} = 426, 452, 479; methanol, λ_{max} = 402, 424, 449; eluent A, λ_{max} = 405, 426, 452; eluent D, λ_{max} = 403, 426, 450. The visible absorption spectra of mutatoxanthin from acid treatment of antheraxanthin did not exhibit a well-defined fine spectra characteristic of flavoxanthin from lutein epoxide. The occurrence of antheraxanthin is not surprising since small amounts of zeaxanthin were also detected in citrus leaves.

Lutein. Peak 8, corresponding to *all-trans*-lutein reference compound, was isolated by preparative chromatography of the Pineapple orange leaf extract. The compound was further purified by analytical HPLC (eluent D) to separate lutein from minor amounts of zeaxanthin. Lutein was the most abundant xanthophyll in the citrus leaves and exhibited spectra in the various solvents identical with those of lutein isolated from corn meal, marigold paste, and kale.

Zeaxanthin. Zeaxanthin and lutein were not separated on the ODS-3 column but were separated on the two Brownlee columns in series (eluent E) and by open column chromatography on magnesium oxide/Celite (eluent F). Zeaxanthin, peak 9, from the Pineapple orange leaf extract had identical Rt and spectra from the diode array detector as that isolated from corn meal. The amount of zeaxanthin was too small to isolate.

13-cis-Lutein. Peak 10 was not isolated as pure compounds. The spectra from the diode array detector were similar to those of lutein but exhibited a 6-nm hypsochromic shift and exhibited an intense cis peak at 331 nm. The Rt (eluents A and D) and spectra are consistent with those of 13- or 13′-cis isomers of lutein reported by Khachik et al. (1986) and had the same Rt and spectra as similar peaks that we found in kale extracts.

α-Carotene. Peak 15 isolated by preparative chromatography (eluent C) had the same Rt and visible absorption maxima in various solvents as the authentic reference compound of α-carotene from Sigma. α-Carotene was also detected in the oleander leaves.

β-Carotene. Peak 16 isolated by preparative chromatography (eluent C) had the same Rt and visible absorption maxima in various solvents as the authentic reference compound of β-carotene from Sigma. β-Carotene was also found in broccoli, kale, and oleander extracts, and the spectra and Rt were consistent with those reported by Khachik et al. (1986).

15,15′-cis-β-Carotene. Peak 17 isolated by preparative chromatography (eluent C) had the same Rt and visible absorption maxima in various solvents as those found in kale and are consistent with that reported by Khachik et al. (1986) for 15,15′-*cis*-β-carotene. This compound appeared as a tailing shoulder of β-carotene on the analytical columns but was a distinct peak separate from β-carotene on the preparative column. This compound was also present in trace amounts in the authentic β-carotene from Sigma.

Chlorophylls a and b. The major Chls *a* and *b* were identified by their Rt and absorption spectra as well as by comparison with those of authentic samples. Chls *a′* and *b′* were also identified by their Rt, absorption spectra, and comparison with trace amounts present in the authentic samples of Chls, *a* and *b*. Rt and spectra were the same as those in broccoli, kale, and oleander.

Citrus leaves contain the same major carotenoids reported for other photosynthetic tissue (Goodwin, 1980; Lichtenthaler, 1987; Khachik et al., 1986).

Quantification of Carotenoids and Chlorophylls. Carotenoids and Chls were more readily extracted from kale, oleander, and broccoli than from citrus tissue. We examined several solvents for the extraction of the carotenoids and Chls from the lyophilized citrus tissue. Generally, the addition of water to dry tissue aids in extraction, but we found that water in the extracting solvent caused rapid conversion of the Chls to chlorophyllides. Addition of 0.4% Triton X-100 to MeOH gave the highest recovery and the least amount of degradation of Chls and carotenoids from the dry citrus tissue. Citrus tissue is very high in chlorophyllase activity which rapidly converts Chls to chlorophyllides (Amir-Shapira et al., 1986; Hirschfeld and Goldschmidt, 1983). Previous values reported for Chl *a* and *b* content of citrus leaves (Arguella and Guardiola, 1977; Mauk et al., 1987; Syvertsen and Smith, 1984) involved extraction with DMF or aqueous acetone and determination spectrophotometrically. These determinations relied on the absorbance measurements of the crude extracts at two different wavelengths, which does not distinguish between Chls *a* and *b* and their isomerization and degradation products. Our results establish individual values for Chls *a* and *b* which are clearly separated from each other and from their isomerization and degradation products by HPLC.

We established carotenoid and Chl values for a range of citrus species and cultivars. The total amount of the major carotenoids, obtained by adding the amounts of neoxanthin, violaxanthin, lutein, and α- and β-carotene, and the amounts of the Chls for turgid and water-stressed leaves at different stages of maturity for the six different citrus types are given in Table IV. Total carotenoid and Chl content varied among the different citrus types, but there was no consistent pattern among leaves of different maturity before and after water stress. Chl *a* and Chl *b* content ranged between 1.61 and 4.59 and between 0.58 and 1.73 mg/g dry weight, respectively. The values do not include Chl *a′* or *b′*. The ratio of Chl *a*/*b* for turgid leaves averaged 3.2, 2.8, 4.0, 3.1, 3.8, and 3.1 for Valencia orange, Pineapple orange, Marsh grapefruit, Eureka lemon, Ichang lemon, and West Indian lime, respectively. Small amounts of Chl *a′* and *b′* were always present but were less than 0.1 mg/g dry weight.

The average content of each of the major carotenoids and Chls *a* and *b* for turgid and stressed leaves of each citrus type are given in Table V. Lutein comprised from 35% to 73% and violaxanthin and neoxanthin from 8%

Table IV. Total Carotenoid and Chlorophyll Content of Turgid and Water-Stressed Detached Citrus Leaves of Different Maturity

		mg/g dry wt[a]					
		total carotenoids		chlorophyll *a*		chlorophyll *b*	
citrus	leaf maturity	turgid	stressed	turgid	stressed	turgid	stressed
Valencia orange	immature	0.77	0.84	2.54	3.07	0.86	1.09
	mature	0.85	1.01	2.79	3.63	0.81	1.05
	old	0.96	1.10	3.28	4.07	1.01	1.26
Pineapple orange	immature	1.80	0.79	4.59	2.03	1.67	1.00
	mature	1.14	0.53	3.90	2.00	1.07	0.74
Marsh grapefruit	immature	0.76	1.00	2.67	4.12	0.77	1.32
	mature	0.98	0.87	3.61	3.43	1.04	1.02
	old	0.63	0.87	2.32	3.12	0.64	0.90
Eureka lemon	immature	1.23	0.69	4.72	2.80	1.47	0.92
	mature	1.71	1.30	5.33	4.36	1.73	1.35
	old	1.27	0.87	4.59	3.03	1.52	1.01
Ichang lemon	immature	0.39	0.54	1.61	2.09	0.58	0.68
	mature	1.45	1.45	5.81	6.05	1.66	1.83
	old	0.96	1.11	3.29	3.99	1.10	1.22
West Indian lime	immature	1.06	0.99	4.25	3.82	1.47	1.38
	mature	0.89	0.83	3.25	2.14	1.00	0.68
	old	1.28	0.85	4.88	3.08	1.59	1.08

[a] Percent dry weight is given in Table I.

Table V. Average Carotenoid and Chlorophyll Content of Turgid and Water-Stressed Detached Leaves of Citrus

		mg/g dry wt ± SD[a]						
citrus leaf tissue	treatment	9′-*cis*-neoxanthin	*all-trans*-violaxanthin	lutein + zeaxanthin	α-carotene	β-carotene	Chl *a*	Chl *b*
Valencia orange	turgid	0.11 ± 0.02	0.13 ± 0.03	0.33 ± 0.06	0.18 ± 0.18	0.20 ± 0.04	3.25 ± 0.67	1.01 ± 0.18
	stressed	0.10 ± 0.02	0.14 ± 0.03	0.39 ± 0.04	0.20 ± 0.08	0.20 ± 0.03	3.89 ± 0.50	1.20 ± 0.13
Pineapple orange	turgid	0.24 ± 0.09	0.21 ± 0.06	0.69 ± 0.05	0.15 ± 0.08	0.26 ± 0.15	4.03 ± 1.63	1.46 ± 0.49
	stressed	0.09 ± 0.03	0.12 ± 0.02	0.56 ± 0.04	trace	trace	2.50 ± 0.66	0.95 ± 0.16
Marsh grapefruit	turgid	0.10 ± 0.03	0.12 ± 0.03	0.32 ± 0.06	0.14 ± 0.05	0.21 ± 0.03	3.78 ± 1.67	0.95 ± 0.23
	stressed	0.08 ± 0.02	0.13 ± 0.05	0.39 ± 0.07	0.16 ± 0.05	0.22 ± 0.04	3.83 ± 0.73	1.14 ± 0.22
Eureka lemon	turgid	0.16 ± 0.01	0.14 ± 0.02	0.48 ± 0.03	0.24 ± 0.04	0.33 ± 0.17	4.88 ± 0.32	1.56 ± 0.12
	stressed	0.11 ± 0.04	0.12 ± 0.05	0.42 ± 0.08	0.20 ± 0.14	0.24 ± 0.14	4.11 ± 1.58	1.25 ± 0.36
Ichang lemon	turgid	0.11 ± 0.05	0.08 ± 0.05	0.33 ± 0.14	0.17 ± 0.13	0.19 ± 0.09	3.88 ± 1.71	1.03 ± 0.47
	stressed	0.12 ± 0.05	0.13 ± 0.04	0.38 ± 0.10	0.20 ± 0.14	0.18 ± 0.08	4.00 ± 1.62	1.25 ± 0.45
West Indian lime	turgid	0.14 ± 0.02	0.11 ± 0.02	0.41 ± 0.08	0.15 ± 0.04	0.27 ± 0.03	4.15 ± 0.67	1.35 ± 0.25
	stressed	0.10 ± 0.02	0.13 ± 0.05	0.33 ± 0.08	0.11 ± 0.02	0.20 ± 0.05	3.30 ± 0.88	1.14 ± 0.33

[a] Mean of immature, mature, and old leaves (n = 4 for oranges and grapefruit; n = 6 for lemons and lime).

to 16% of the total carotenoids. α- and β-carotene were present in amounts between 1% and 24% of the total carotenoids. The other xanthophylls were present in amounts of less than 0.1 mg/g dry weight and were not quantified. Our results show that Chl and carotenoid levels are not affected by short-term water stress of detached leaves as are ABA levels.

Citrus leaves are very high in carotenoids and Chls. When our values are expressed on a fresh basis, citrus leaves range between 0.09 and 0.51 mg/g in total carotenoids, between 0.98 and 1.99 mg/g in Chl *a*, and between 0.29 and 0.57 mg/g in Chl *b*. Depending on growth conditions and stress factors, the weight percentage of carotenoids in other plants varies within the following ranges: β-carotene, 25–40%; lutein, 40–57%; violaxanthin, 9–20%; and neoxanthin, 5–15; (Lichtenthaler, 1987). The α- and β-carotene content of 24 different trees ranged between 0.02 and 0.19 and between 0.11 and 0.40 mg/g dry weight, respectively (Goodwin, 1980). Kale, which we used as a source of some of the reference compounds, contains 0.63 mg/g fresh weight of total carotenoids and 1.37 and 0.46 mg/g fresh weight of Chls *a* and *b*, respectively (Khachik et al., 1986). The average carotenoid value is lower and the Chl content similar in citrus leaves compared to that reported for kale leaves.

We are interested in studying the effect of various plant growth regulators and/or inhibitors to find ways to regulate the ABA biosynthetic pathway in citrus. We have established comprehensive information on the content of carotenoids, Chls, and ABA of various species and cultivars of citrus to facilitate future studies. Citrus is very high in ABA content, and young developing leaves have substantial capacity for synthesis of stress-induced ABA. Such synthesis is easily manipulated in the laboratory and produces a dramatic change in concentration of ABA. This system should prove to be very effective for studies of the regulation of ABA biosynthesis in citrus.

ABBREVIATIONS USED

ELISA, enzyme-linked immunosorbent assay; Chl(s), chlorophyll(s); HPLC, high-pressure liquid chromatography; Rt, retention time; TBST, Tris-buffered saline with Tween 20; BHT, 2,6-di-*tert*-butyl-4-methylphenol; BHA, 2(3)-*tert*-butyl-4-hydroxyanisole.

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