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Changes in carotenoids and ABA content in *Citrus* leaves in response to girdling

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ABSTRACT

The effect of girdling on abscisic acid, carotenoids content, xanthophylls cycle and non-photoquemical quenching was studied in leaves from different shoot types of containerized two-year-old 'Loretina' mandarin during spring flush period. Girdling increased abscisic acid in young leaves and decreased it in mature leaves. These changes were accompanied by a significant increase in leaf carotenoids, carotenoids:chlorophylls ratio, xanthophylls and xanthophylls cycle pool size and its de-epoxidation state in vegetative and multiflowered young leafy shoots. However, changes in de-epoxidation state did not correlate with the energy-dependent non-photochemical quenching. The role of carotenoids and abscisic acid as photoprotecting agents during the oxidative stress caused by girdling is discussed. In conclusion, this study provides evidence linking carotenoids-based photoprotecting mechanisms in the response to the stress induced by girdling in *Citrus* trees.

1. Introduction

Girdling is widely used in Citriculture to influence growth and reproductive behaviour of most varieties of *Citrus* genus. In fact, girdling is used to increase flowering, improve fruit set and increase fruit size (Goren et al., 2003). The effects of girdling have been linked to the interruption of the downward phloem transport, thereby increasing carbohydrate availability (Wallerstein et al., 1974; Rivas et al., 2006, 2007) and modifying the hormonal balance in the canopy (Goren et al., 1971). However in some cases girdling may induce excessive accumulation of carbohydrates originating a feedback inhibition of photosynthesis by reducing photosynthetic capacity (Rivas et al., 2007) and CO_2 assimilation rate (Iglesias et al., 2003). Other works reported that the build up of carbohydrate reserves increase the size and number of starch granules in chloroplasts causing physical damage to the thylakoid ultrastructure (Schaffer et al., 1986; Bondada and Syvertsen, 2005), which may explain the symptoms of leaf chlorosis often appearing after the treatment (Noel, 1970).

Impairment of the photochemical apparatus and photoinhibition frequently induce oxidative damage since a significant fraction of the absorbed light energy cannot be diverted to the electron transport chain; then reactive oxygen species (ROS) can be establish in the chloroplast. To prevent cell collapse, ROS must be rapidly processed if oxidative damage is to be averted (Noctor and Foyer, 1998). For this purpose, plants usually maintain the levels of ROS under tight control by activating an integrated system of enzymatic and non-enzymatic antioxidants that are located in the chloroplast (Asada, 1999). The activity of the ROS scavenging enzymes, including those of the water–water cycle, has been shown to be induced in *Citrus* after girdling as a way to prevent further cellular damage (Rivas et al., 2008). Additionally to this enzymatic ROS-scavenging mechanism, many plants may also accumulate carotenoids (Car) and other metabolites as a way to prevail over-stress conditions, dissipating the excess of absorbed energy as heat and balancing cellular redox state (Demmig-Adams, 1990; Telfer et al., 1994; Hare and Cress, 1997).

Car are recognized to be essential for the survival of illuminated plants, not only because they can act as accessory light-harvesting pigments, but also by the ability of their conjugated double-bound to quench Chl triplet state (^{3}Chl*) and scavenge singlet oxygen ($^1O_2$) and other highly reactive ROS (e.g. O_2^-, OH^-) which can photooxidize Chl (Demmig-Adams et al., 1996; Miller et al., 1996; Davison et al., 2002). Thermal dissipation by direct quenching of singlet excited Chl (^{1}Chl*) is also possible and is manifested as non-photochemical quenching (NPQ; Horton et al., 1996). This process involves Car pigments that comprise the so-called 'xanthophylls cycle' (XC) in which three X participate in a cyclic reaction involving two de-epoxidations of violaxanthin (V) to zeaxanthin (Z), with antheraxanthin (A) as intermediate (Demmig-Adams, 1990). This reaction is driven by ascorbic acid oxidation (Bratt et al., 1995). However, some reports indicated a poor correlation between NPQ and the de-epoxidation state (DPS) of the XC (Davison et al., 2002; Lu et al., 2003; Bailey et al., 2004; Chen et al., 2005).

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Recent works have also related lutein (L) content with enhanced photoprotection since L is located at sites L2 and V1 in light-harvesting chlorophyll protein complexes of photosystem II, facilitating enhanced ^{1}Chl* and ^{3}Chl* quenching capacity (Matsubara et al., 2005). In apple leaves L have been suggested to play a role in thermal dissipation (Cheng, 2003), but the exact contribution of L to thermal dissipation remains to be determined.

Furthermore, mechanical damage elicits several signalling pathway including the increase of endogenous abscisic acid (ABA; Herde et al., 1999) being its synthesis dependent on the presence of epoxycarotenoids (Rock and Zeevaart, 1991). ABA plays a crucial role in the adaptation to several environmental stresses (Zeevaart and Creelman, 1988). In fact a photoprotective function has been conferred to ABA since it has been shown to induce the rise of Car content, XC activity (Ivanov et al., 1995; Herde et al., 1999) and the activity of the ROS scavenge enzymes (Hung and Kao, 2003).

Based on these previous evidences, we hypothesized that the impairment of photosynthetic apparatus induced by girdling (see Rivas et al., 2008) may also trigger Car and ABA synthesis as a complementary mechanism to provide photoprotection to the photochemical apparatus. However, information about carotenoid-based photoprotecting mechanisms acting under these stress conditions has not been reported. Thus, the aims of this work was to evidence carotenoids-based photoprotecting mechanisms and verify hormonal-related changes operating in young and mature *Citrus* leaves in response to girdling.

2. Materials and methods

2.1. Plant material, growth conditions and treatment

Experimental conditions were as described previously by Rivas et al. (2008). Briefly, ten containerized two-year-old trees of 'Loretina' mandarin (*Citrus reticulata* Blanco), grafted onto Carrizo citrange (*Citrus sinensis* [L.] Osbeck × *Poncirus trifoliata* Raf.) were used in the experiment. Trees were grown outdoors, in 10-L plastic containers with a sandy-loamy soil and fertilized with nitrogen at 20 g tree^{-1} year^{-1}. The plants were daily drip irrigated and pests were controlled when necessary.

At anthesis (when 60% of the flowers were opened), five trees were girdled on the trunk, 10 cm above the rootstock, taking care to avoid injuring the xylem or removing bark. The other five trees were used as ungirdled controls. Each tree was considered as a replicate in the design of the experiment.

Thirty days after girdling (DAG), three types of leaves were sampled: (1) mature leaves (ML), approximately 14-month old from the spring flush of the year before; (2) growing leaves from vegetative shoots (leafy shoots; VG) of the current spring flush (two-month old), and (3) growing leaves from mixed shoots (multiple-flowered leafy shoots; MLY) also from the current spring flush (two-month old). For each sample, 25 leaves of each type were picked from every tree. Leaves were immediately freeze-ground with liquid N_2 and stored at −70 °C until analysis. For ABA analysis, tissue was lyophilized. For Chl and Car measurement only fresh leaf tissue was used. All samples were analyzed at least three times.

2.2. Chl a fluorescence measurements

Chl *a* fluorescence was assessed using a pulse amplitude modulated system (Junior-PAM, Walz, Gademann Instrument, Germany) under the same conditions and cares previously described by Rivas et al. (2007). Measurements were performed before mid-day (08.30–10.30 h), and prior to sampling leaves for analysis. For all shoots, measurements were carried out on 15 leaves per tree, performing six readings per leaf. Prior to the measurements leaves were dark-adapted for 1 h and then maximal Chl fluorescence (F_m) was obtained giving a 1-s saturation pulse (10,800 μmol m^{-2} s^{-1}). Afterward, leaves where light-acclimated for 40 min and maximal fluorescence of light-adapted leaves (F'_m) was achieved by applying an actinic white-light pulse (270 μmol m^{-2} s^{-1}). Non-photochemical quenching of Chl fluorescence (NPQ) was calculated as $F_m/F'_m - 1$ (Bilger and Björkman, 1994).

2.3. ABA analysis

Quantification of ABA in leaf tissue was performed by indirect enzyme-linked immunosorbent assay as reported previously (Zacarías et al., 1995; Lafuente et al., 1997). The samples were extracted overnight at 4 °C with acetone 80% containing citric acid (0.5 g L^{-1}) and butylated hydroxytoluene (BHT; 100 mL L^{-1}). The extracts were centrifuged and 5 μL of the supernatant were used for ELISA assay following the procedure proposed by Walker-Simmons (1987). Four replicates per sample were incubated with 500 μL of monoclonal antibody (MAb) and 480 μL of Tris-buffered saline (TBS, pH 7.8) (1 tablet and 0.2 g $Cl_2Mg{\cdot}6H_2O$ dissolved in 15 mL of double distilled water) at 4 °C overnight. Plate wells were incubated at 4 °C overnight with 200 μL of ABA-4′ Bovine albumin (BSA) conjugate, prepared according to Weiler (1979). Wells were washed three times with 200 μL TBS-tween (1 L TBS with 0.5 mL Tween-20) and 0.2 g BSA. Two-hundred μL aliquots of sample incubated with MAb was pipetted into the wells and then plates were kept 2 h at room temperature. After washing three times with 200 μL of TBS-tween, each well was filled with 200 μL of the rabbit anti-mouse alkaline phosphatase conjugate (20 mL TBS containing 20 μL IgG). Plates were incubated at room temperature for 2 h. Wells were washed three times with TBS-tween and then 200 μL of nitrophenyl phosphate solution (20 mL 0.05 M $NaHCO_3$ containing 20 mg 4-nitrophenyl phosphate disodium salt hexahydrate) were added to each well. Plates were incubated for around 30 min in a forced-draft oven (35 °C) until the absorbance at 405 nm of control sample containing no ABA was approximately 1.0. Replicate ABA standards (ranging from 15 to 250 pg 100 μL^{-1} TBS) were assayed and a linear regression analysis was computed. The amount of ABA in leaf extract samples was calculated based on the coefficient of the ABA standard curve for each plate. (+)-ABA standards, TBS, BSA, anti-mouse IgG, 4′-nitrophenyl phosphate disodium salt hexahydrate were purchase in Sigma. MAb was obtained from Idetek (Inc.). BHT, citric acid, acetone, Tween-20, $NaHCO_3$ were purchase in Scharlau. Immuno plates (F96 Maxisorp) were obtained from NUNC™ (Roskilde, Denmark) which showed a better ABA-4′BSA conjugate binding than other plates tested. Results are expressed as μg g^{-1} DW.

2.4. Chlorophyll and carotenoids (carotenes and xanthophylls) extraction and quantification

Leaf pigments were extracted as described previously by Rodrigo et al. (2003). All the extraction and quantification process was carried out at 4 °C under dim light condition to avoid photodegradation, isomerizations and structural changes of Car. Each sample was extracted at least twice.

Fresh freeze ground tissue (0.25 g) was extracted with a solution containing 2 mL MeOH and 1.5 mL of Tris–HCL (50 mM, pH 7.5 containing NaCl 1 M) during 30 min. Chloroform (4 mL) was added to the mixture, stirred during 30 s and centrifuged. The aqueous phase was re-extracted with chloroform until it was colourless. The combined chloroform extracts were dried on a rotary evaporator at 40 °C and re-dissolved with a mix of acetone:petroleum ether:diethyl ether (0.7:9:1, v/v/v). The Chl ($a+b$) content was determined by measuring the absorbance at 644 and 662 nm and