Molecular and Metabolic Analyses in Developing Olive Fruit in Relation to Different Water Regimes

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Abstract

Despite the global economic importance of olive (*Olea europaea* L.), little is known about the molecular and metabolic changes during fruit development and the modulation of quality-related metabolic pathways during drought stress. In this work, we report the expression pattern of genes involved in important pathways of secondary metabolism (polyphenols, terpenoids) during fruit development in rainfed or fully irrigated olive plants.

Phenolic compounds represent a complex mixture in both olive fruits and oil. The interest on these compounds is due to their demonstrated anti-atherogenic and anti-cancerogenic effects providing to the olive oil important nutraceutical properties. Several parameters affect olive fruit phenolic content and these include genotype, pedo-climatic conditions, agronomic techniques (e.g. irrigation) and the developmental stage of the drupe. To gain insight into the transcriptional regulation of phenolic pathways we analysed the pattern of expression of important genes during olive fruit development. Transcript levels of phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), and dihydroflavonol reductase (DFR) genes showed different expression patterns during fruit development. CHS and DFR showed stronger expression in the skin than in the pulp. PAL expression was strong before pit hardening and decreased thereafter. CHS expression peaked at pit hardening and at veraison stage, whereas DFR was only strongly expressed at fruit ripening, suggesting a crucial role for these genes in the regulation of flavonoid biosynthesis in the last developmental stages of olive fruit. Water stress enhanced the expressions of PAL and CHS at pit hardening and of DFR in the skin at ripening. Of the terpenoids, amyrin synthase (AS) showed a decreasing expression trend throughout development, whereas lupeol synthase (LUS) was highly expressed in the skin of ripe fruit. Induction of LUS gene expression was observed at the early developmental stages in fruit of water-stressed plants.

To gain insight into the metabolic changes in the fruit under different water regimes in the field, we initiated, in a high-throughput approach, analysis of the mesocarp of ripe fruits by GC-TOF mass spectrometry, and differences in metabolite composition were determined.

INTRODUCTION

Olive is a drought-tolerant species that can survive under prolonged periods of drought by developing resistance mechanisms to water stress (Chartzoulakis et al., 1999; Xiloyiannis et al., 1999). In Mediterranean agro-ecosystems, water shortage is one of the main growth-limiting factors and olive plants must finely regulate water balance to optimize their adaptation to the environment while reducing the intensity of the water stress. In olive orchards, knowledge of these characteristics is a relevant prerequisite for planning effective irrigation-scheduling protocols (Fernández et al., 2008).

Several studies have shown that irrigation enhances yield through increases in the size and number of fruits, and in the production of oil per unit surface. Less information is

available on the composition and molecular and metabolic processes in olives grown under different water regimes. Phenolic compounds are among the qualitative parameters most affected by irrigation, generally showing a negative trend with increasing water supply (d'Andria et al., 2009).

Fruit development is a genetically programmed process that is markedly influenced by environmental factors, such as the plant-water relationship. In this context, and to better understand basic mechanisms regulating fruit development, molecular, genomic and postgenomic approaches are extremely useful and relatively novel for *O. europaea*. Recently, a large set of differentially expressed genes in developing olive fruits of cultivar 'Leccino' have been identified (Galla et al., 2009), thus equipping the olive sequence database for further analyses.

In this work, we report the expression pattern of some genes involved in important pathways of secondary metabolism (polyphenols, terpenoids) and the results of a preliminary metabolomics approach in fruit from rainfed (control) and fully irrigated 'Leccino' olive trees.

MATERIALS AND METHODS

The experimental site is located near Benevento (41°06'N, 14°43'E; 250 m.a.s.l.), in a hilly olive-growing area of southern Italy. The soil is sandy loam (1.76% organic matter, 1% $CaCO_3$, 0.15% N, pH 7.2), characterized by a volumetric water content (m^3/m^3) of 35.6% at field capacity (soil matric potential of -0.03 MPa) and 21.2% at wilting point (soil matric potential -1.5 MPa), and an apparent bulk density of 1.25 t/m^3. Trees are planted 6 m apart at a density of 555 plants/ha. The olive (cultivar 'Leccino') trees used for the experiment were 15 years old. Plants (n=3) were selected for uniformity in size and randomly assigned to two treatments: non-irrigated (water-stressed) and irrigated (control) with a seasonal water amount equivalent to 100% of maximum crop evapotranspiration (ETc). Irrigation water was delivered by a drip irrigation system from the beginning of pit hardening (end of July) to early fruit ripening (beginning of October). ETc was estimated from Class 'A' pan evaporation and data were corrected with a pan coefficient (kp) of 0.8 (to obtain reference crop evapotranspiration, ETo, mm), a crop coefficient (kc) equal to 0.65 and a tree ground cover coefficient (kr) of 0.85. Irrigation volume of controls was 181 mm while ETc during the irrigation period was 191 mm. A Scholander type pressure chamber was used to assess predawn and midday leaf water potential.

Fruits were sampled from water-stressed and control plants at the four phenological stages of fruit development (from pit-hardening to ripening). RNA was extracted from the entire fruit according to Galla et al. (2009), and semi-quantitative RT-PCR was performed using specific primers for phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), dihydroflavonol reductase (DFR), amyrin synthase (AS) and lupeol synthase (LUS) genes according to the manufacturer's instructions (Reverse Transcription System, Promega). 18S RNA was used as the housekeeping gene, with cycle conditions adapted for each gene following the instructions of the Quantum 18S RNA Universal kit (Ambion Inc.). The PCR products were separated by electrophoresis on 1.5% agarose gels. Amplification mix was prepared using the goTaq protocol (Promega). The amplification products were scanned and quantified using Quantity One software (BioRad).

Metabolomics analysis was performed by GC-TOF mass spectrometry. Three replicates composed by a pool of 5-6 fruits for each tree of control and water-stressed treatment were frozen in liquid nitrogen, and stored at -80°C until analysis. For each sample, 20 mg pulp was ground in 2 ml pre-chilled extraction solvent ($MetOH:CHCl_3$, 1:1, v/v). After vortexing and centrifugation (5000 rpm for 2 minutes at 4°C), the supernatant was analyzed with a Pegasus III TOF GC mass spectrometer with a resolution power of 400 compound profiles. The relative concentrations were determined by peak area (mm^2). All peak detections were manually checked for false-positive and false-negative assignments.

RESULTS AND DISCUSSION

Water stress induced a slight decrease in PAL gene expression at the first sampling date (early pit hardening), but its upregulation was observed at the second sampling date (late pit hardening) when the highest level of transcript accumulation throughout fruit development was detected (Fig. 2) and when the highest rate of polyphenol accumulation occurs (Martinelli and Tonutti, unpublished). Similarly, CHS showed increased expression in control fruit at late pit hardening, and water-stress conditions further enhanced its transcription. However, in contrast to PAL, the highest CHS expression level was observed at the ripening stage in skin tissues (Fig. 2). DFR transcripts were detected only in the last stages of fruit development in both pulp and skin under control conditions, whereas under water stress, its increased expression was only induced in the skin (Fig. 2). The increased expression of PAL and CHS might account for the higher accumulation of phenols observed in olives from water-stressed trees (Tovar et al., 2002). The increased expression of both CHS and DFR observed in the last developmental stage suggests that the flavonoid biosynthetic pathway, leading to the production of compounds such as flavonols, flavones and anthocyanins (Fig. 2, simplified scheme), is regulated by these two genes. Both genes showed high expression levels in the skin of ripening fruit indicating that, as observed in other fruit species, peel is highly active in terms of flavonoid metabolism in olives.

Expression analysis of two oxidosqualene cyclase genes, AS and LUS, which are responsible for specific steps of the triterpenoid biosynthetic pathway (Fig. 3, simplified scheme) indicated that AS is actively transcribed during the early growth stages and that water stress induces higher expression throughout development (Fig. 3). Marked upregulation of LUS expression was detected in fruit from water-stressed trees on two sampling dates (early development and before the onset of ripening). In contrast, LUS appeared to be strongly expressed in the skin of both control and irrigated ripe fruit.

To gain insight into overall changes in fruit metabolism under water stress, we also initiated an analysis of the pulp of mature fruits from water-stressed and control plants using a metabolomics approach by means of GC-TOF mass spectrometry and data analysis is in progress.

Further studies will need to be performed using different water regimes and changing timing of water treatment to identify if this induces differences in phenol compounds in olive fruits. The use of metabolomics will allow to determine changes in the entire metabolic profile identifying which important quality and nutraceutical metabolites are affected by irrigation regimes.

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Figures

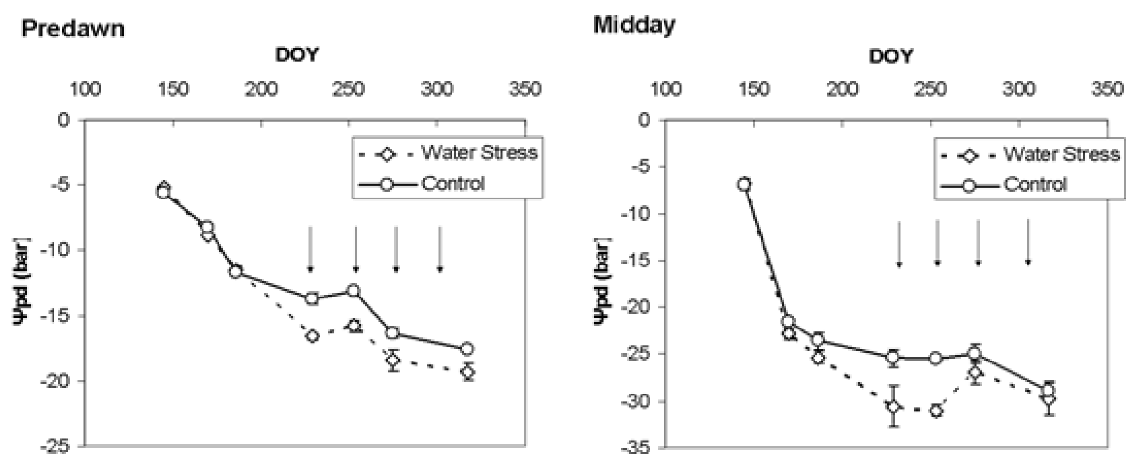

Fig. 1. Predawn and midday (covered leaves) leaf water potential dynamics in water-stress and control treatments. Arrows = olive sampling dates. DOY = day of the year.

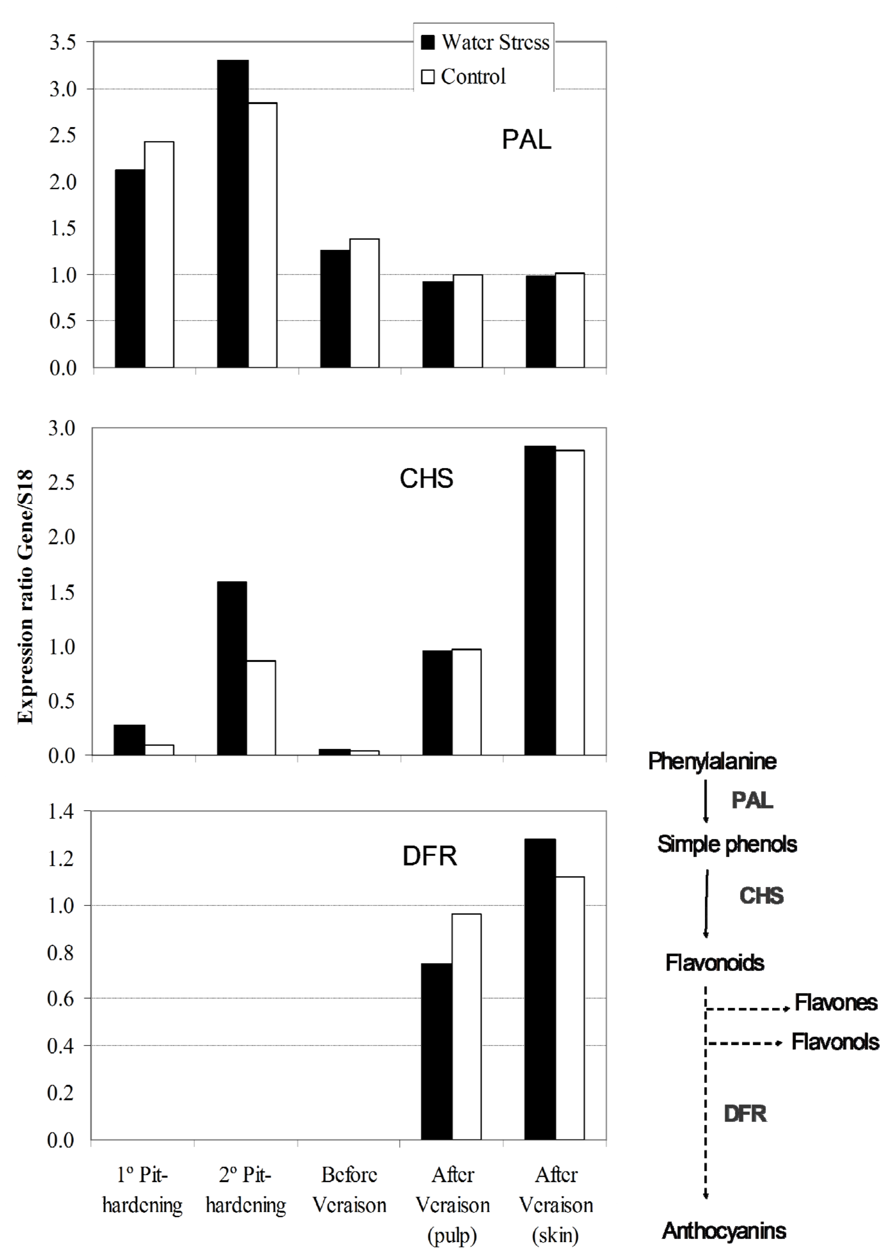

Fig. 2. Transcript accumulation of PAL, CHS and DFR in olives sampled at different developmental stages from water-stressed and control plants (left). Simplified scheme of flavonoid biosynthetic pathway (right).

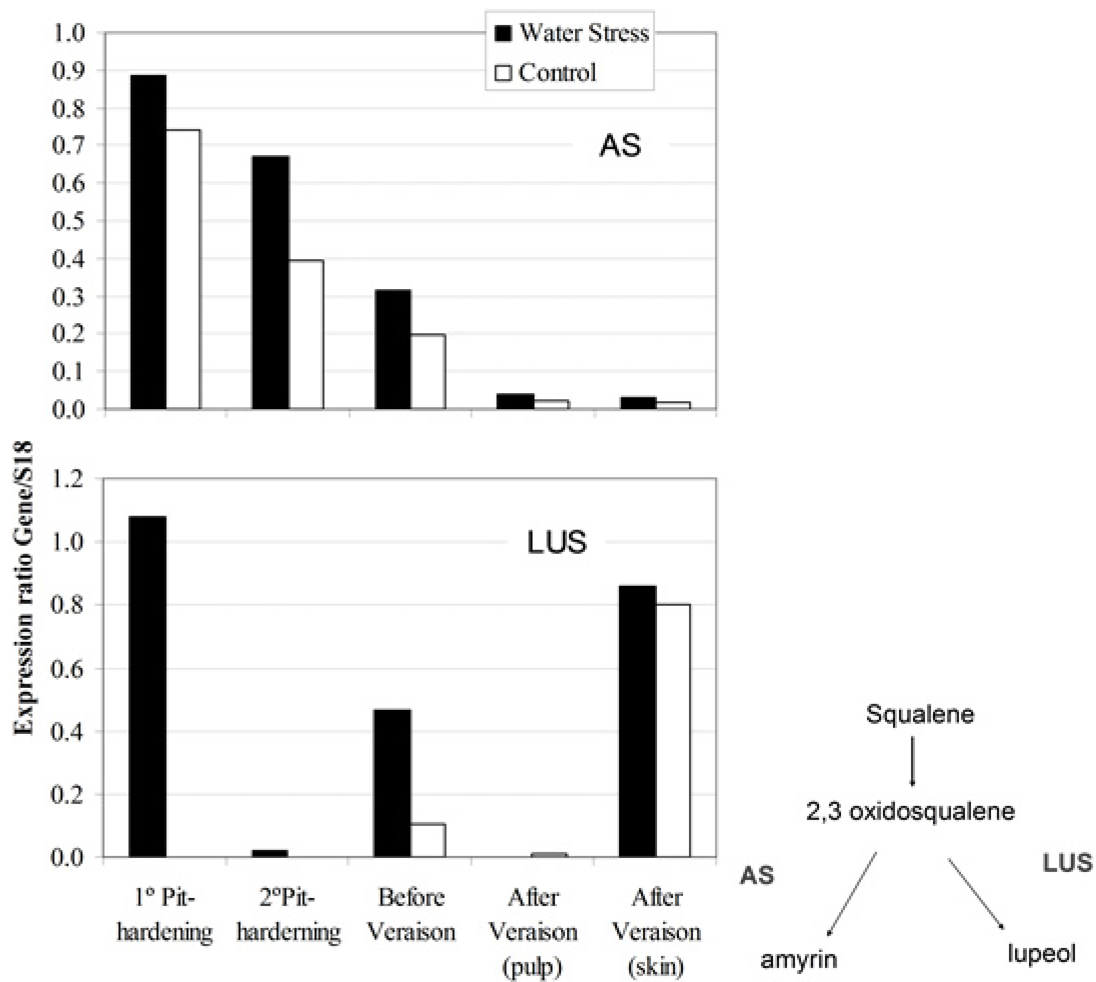

Fig. 3. Transcript accumulation of AS and LUS in olives sampled at different developmental stages from water-stressed and control plants (left). Simplified scheme of triterpenoid biosynthetic pathway (right).