Physiological and molecular responses of the isoprenoid biosynthetic pathway in a drought-resistant Mediterranean shrub, *Cistus creticus* exposed to water deficit

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Abscisic acid; Drought stress; Gene expression; Perennials; Tocopherols

**Summary**
The goal of the present research was to obtain new insights into the mechanisms underlying drought stress resistance in plants. Specifically, we evaluated changes in the expression of genes encoding enzymes involved in isoprenoid biosynthesis, together with the levels of the corresponding metabolites (chlorophylls, carotenoids, tocopherols and abscisic acid), in a drought-resistant Mediterranean shrub, *Cistus creticus* grown under Mediterranean field conditions. Summer drought led to reductions in the relative leaf water content (RWC) by 25%, but did not alter the maximum efficiency of PSII, indicating the absence of damage to the photosynthetic apparatus. While the expression of genes encoding *C. creticus* chlorophyll \( \alpha \) oxygenase/chlorophyll \( \beta \) synthase (CAO) and phytoene synthase (PSY) were not affected by water deficit, the genes encoding homogentisate phytyl-transferase (HPT) and 9-\( \text{cis} \)-epoxycarotenoid dioxygenase (NCED) were induced in water-stressed (WS) plants. Drought-induced changes in gene expression were observed at early stages of drought and were strongly correlated with levels of the corresponding metabolites, with simultaneous increases in abscisic acid and \( \alpha \)-tocopherol levels of...
Introduction

Because most climate change scenarios suggest an increase in aridity in many areas of the globe (Houghton et al., 2001), research into plant responses to drought stress is becoming increasingly important. The Mediterranean climate is characterized by hot, dry summers, and cool, wet winters, so that plants adapted to survive under these conditions are resistant to recurrent summer droughts and therefore offer an excellent model to study plant responses to this abiotic stress. To date, a great deal of effort has been focused on physiological and biochemical processes underlying plant responses to water deficit. Research involved in drought stress resistance at the molecular level has received special attention during the last few years thanks to advances in plant molecular biology (Bartels and Sunkar, 2005; Chaves et al., 2003). Nevertheless, there is still inadequate knowledge on the molecular-to-physiological mechanisms of plant stress response within natural habitats, since complex interactions occur as an outcome of diverse stresses that occur in parallel.

Isoprenoids are a group of compounds that are extraordinarily diverse in terms of chemistry, structure and function. More than 30,000 individual isoprenoid compounds have been characterized to date, most of them of plant origin, and hundreds of new structures are reported every year. In addition to their function as pigments, phytohormones, defensive agents against pathogens and constituents of membranes, to name only a few, isoprenoids may also have a significant photoprotective role, which is of particular importance in plant stress responses (Peñuelas and Munné-Bosch, 2005). There are two biosynthetic routes to the common branched five-carbon unit, isopentenyl diphosphate [and its interconvertible isomer dimethylallyl diphosphate], from which all isoprenoids are derived (Lichtenthaler et al., 1997). Some isoprenoids (i.e. sterols and the side chain of mitochondrial ubiquinone) are synthesized from mevalonate acid-derived isopentenyl diphosphate, whereas others [i.e. isoprene (C5), monoterpenes (C10), diterpenes (C20, including gibberellins and the phytol tail of tocopherols and chlorophylls) and carotenoids (C40)] originate from isopentenyl diphosphate synthesized via the 2-C-methyl-d-erythritol 4-phosphate pathway in plastids. Most sesquiterpenes (C15) are synthesized from the mevalonic acid pathway in the cytosol, although others such as the phytohormone abscisic acid are synthesized from the 2-C-methyl-d-erythritol 4-phosphate pathway by the specific cleavage of carotenoids (Tan et al., 1997). Furthermore, plants have an enormous capacity to synthesize huge amounts of diverse isoprenoids, particularly via the combination of the isoprenoid biosynthetic route and other secondary metabolic pathways. For instance, tocopherol biosynthesis occurs as a result of a combination of the shikimate and isoprenoid pathways, which lead to homogentisic acid and phytol diphosphate, respectively, which lead to the formation of tocopherols (formed by a chromanol head group and a phytol tail) (Collakova and DellaPenna, 2003).

Despite the fact that the isoprenoid biosynthetic pathway has been characterized in some detail at the molecular level over the last two decades, its regulation during plant responses to drought stress is still poorly understood and is mostly limited to the model annual plant Arabidopsis thaliana (Liu et al., 2005; Rodriguez-Concepción and Boronat, 2002). Mediterranean shrubs are excellent models to study plant responses to drought since they are generally very resistant and well adapted to decreased soil water availability during the summer. Cistus creticus L. subsp. creticus (known as rockrose) is a branched scrophyllous shrub that is naturally found in littoral brushwood growing in dry soils primarily of the Mediterranean coast, and is therefore characterized by its resistance to drought stress. Further, recent advances have been achieved in the molecular biology of this species (Pateraki and Kanellis, 2004), allowing us to study the response of this species to drought stress at the molecular level.

The aim of the present study was to gain new insights into the mechanisms of drought stress resistance in field-grown C. creticus plants, both at...
the physiological and molecular levels, with an emphasis on the response and regulation of the isoprenoid biosynthetic pathway under these conditions. To address these questions, we measured the expression of genes encoding key enzymes participating in isoprenoid biosynthesis, the levels of the corresponding isoprenoid metabolites (chlorophylls, carotenoids, tocopherols and abscisic acid), and the endogenous concentrations of other growth regulators (jasmonic acid and salicylic acid) in leaves of C. creticus exposed to water deficit under Mediterranean field conditions.

Materials and methods

Plant material, growth conditions and sampling

Plants used for the experiments were obtained as follows. C. creticus L. subsp. creticus plants were germinated by exposing the seeds at 90 °C for 10 min in an equal amount of water, in Eppendorf tubes, followed by incubation at room temperature in the dark for 3–4 days in an appropriate container. Next, seedlings were grown in a growth chamber in 0.5-L pots containing a mixture of soil:perlite (3:1, by vol.) under a photoperiod of 16:8 (light:darkness) and a temperature of 23 °C. After 1 month of growth, plants were transferred into a greenhouse with a controlled temperature (24 °C/18 °C, day/night) and were watered twice a week, once with tap water and once with Hoagland’s solution. Two months later in June of 2005, plants were transplanted to the experimental fields of the University of Barcelona (Barcelona, Spain) and 16 plants per plot were distributed homogeneously in a square, 1 m apart, such that all plants had the same orientation to sun. The experimental area consisted of two plots of 4.5 m² each of calcic Luvisol (FAO) separated by 2 m. Water deficit was imposed on a plot of plants by withholding water from 23 August to 30 September, referred to here as water-stressed (WS) plants, while the other plot of plants was watered twice a week with tap water and these plants were used as controls (IR, irrigated plants).

Environmental conditions were monitored with a weather station (Delta-T Devices, Newmarket, UK). Air temperature and relative humidity were measured with a Vaisala thermohygrometer (Vaisala, Helsinki, Finland). The vapor pressure deficit was determined from relative humidity and air temperature data following Nobel (1991). Precipitation was measured with a standard rain gauge. The environmental conditions during the experiment were typical of the Mediterranean climate, with high PPFD and VPD values during the summer (Table 1). Rainfall during the experimental period was very scarce, with rainfalls of 1.0 and 7.2 mm on 25 and 28 September only (Figure 1).

Gene expression, levels of the selected isoprenoid metabolites, endogenous concentrations of abscisic acid and other growth regulators, and stress indicators were measured from fully expanded young leaves. For gene expression and metabolite analyses, samples were collected, immediately frozen in liquid nitrogen and stored at −80 °C until analyses. All measurements were performed from leaves collected at midday (at maximum incident diurnal PPFD).

Table 1. Climatological conditions (maximum photosynthetically active photon flux density [PPFD], minimum and maximum air temperature [T min], and maximum vapor pressure deficit [VPD]), during the measurement days of the experiment.

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>Days</th>
<th>PPFD (µmol m⁻² s⁻¹)</th>
<th>T min (°C)</th>
<th>T max (°C)</th>
<th>VPD (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23 August</td>
<td>0</td>
<td>1680</td>
<td>18.1</td>
<td>29.0</td>
<td>1.76</td>
</tr>
<tr>
<td>2 September</td>
<td>10</td>
<td>1640</td>
<td>23.9</td>
<td>26.5</td>
<td>1.18</td>
</tr>
<tr>
<td>16 September</td>
<td>24</td>
<td>1500</td>
<td>20.9</td>
<td>26.5</td>
<td>1.42</td>
</tr>
<tr>
<td>30 September</td>
<td>38</td>
<td>1400</td>
<td>17.0</td>
<td>26.0</td>
<td>1.31</td>
</tr>
</tbody>
</table>

detected autoradiographically. Membranes were stripped with boiled 0.1% SDS and re-used. For hybridizations, homologous probe was used for 9-cis-epoxycarotenoid synthase gene (CcNCED) (accession no. EU244637). For chlorophyll a/oxygenase/chlorophyll b synthase (CAO), homogentisate phytyl-transferase (HPT) and phytoene synthase (PSY) genes, appropriate clones were obtained from Solanaceae Genomic Network database (Clone’s name cLEM-25-A20, cTOS-1-J4 and TUS 41-K22, respectively) and were used as heterologous probes.

Determination of isoprenoid metabolites

For the determination of chlorophylls and carotenoids, leaves were ground in liquid nitrogen and extracted repeatedly with 80% (w/w) acetone using ultrasonication (Vibra-Cell Ultrasonic Processor). The resulting extracts were immediately assayed spectrophotometrically. Specific absorption coefficients of chlorophylls and total carotenoids reported by Lichtenthaler and Wellburn (1983) were used. Extraction and HPLC analysis of α-tocopherol were carried out as described by Munne-Bosch and Alegre (2000). Extraction and HPLC-MS/MS analysis of abscisic acid were carried out as described by López-Carbonell and Jáuregui (2005).

Determination of jasmonic acid and salicylic acid

Extraction and HPLC-MS/MS analysis of jasmonic acid and salicylic acid were carried out as described by Abreu and Munne-Bosch (2008).

Statistical analyses

Statistical differences between measurements on different days and treatments were analyzed using ANOVA with Tukey’s post-hoc analyses. Differences were considered significant at a probability level of $P < 0.05$. Differences between treatments at each sampling point are indicated by an asterisk in the figures. These analyses, as well as additional regression analyses were conducted using SPSS (Chicago, IL, USA).

Results

Resistance of Cistus creticus plants to water deficit

Water deficit over 24 days (23 August–16 September) led to a 25% reduction in the RWC, with RWC values at midday around 60% in IR plants (controls) and 48% in WS plants. Light rainfall at days 33 (1 mm) and 36 (7.2 mm) led to a partial recovery of the RWC in WS plants, although values were slightly smaller than those measured in IR plants (Figure 1). Despite the 25% decrease of the RWC in WS plants at day 24 (maximum stress), the maximum efficiency of PSII photochemistry ($F_v/F_m$) was not altered during water deficit relative to controls. The $F_v/F_m$ ratio stayed around 0.80 throughout the experiment in IR and WS plants, indicating the absence of damage to the photosynthetic apparatus (Figure 1). WS plants were therefore exposed to mild water deficit in the field, which did not cause any damage to the photosynthetic apparatus in C. creticus plants.

Biosynthesis of chlorophylls and tocopherol in WS plants

The transcript accumulation of the CAO gene was slightly higher in WS than in IR plants at the beginning of the experiment, and increased in both plant groups at the end of the experiment (Figure 2). However, CAO mRNA levels were not affected by water deficit. In contrast, HPT mRNA

![Figure 1](image_url)

Figure 1. Rainfall, and changes in the relative water content (RWC) and the maximum efficiency of PSII ($F_v/F_m$) during the experiment in leaves of irrigated (black symbols) and water-stressed (white symbols) field-grown C. creticus plants. Data correspond to the mean ± SEM of 5 measurements. IR plants were watered throughout the experiment, while WS plants were exposed to summer drought.
levels increased in WS plants relative to controls, as shown in the northern blots (Figure 2). The increases in HPT mRNA abundance paralleled the increases in \(\alpha\)-tocopherol levels. While chlorophyll (Chl) \(a+b\) levels and the Chl \(a/b\) ratio were not affected by water deficit, \(\alpha\)-tocopherol levels increased sharply in response to summer drought reaching levels, to 62% higher than controls. Exposure to prolonged water deficit (day 24) did not lead to further increases in HPT mRNA abundance or \(\alpha\)-tocopherol levels in leaves.

**Biosynthesis of carotenoids and abscisic acid in WS plants**

Water deficit did not lead to any effect on PSY mRNA steady-state levels. However, the same conditions induced the NCED transcript accumulation in WS plants (Figure 3). In particular, the expression of the NCED gene increased after 10 days of water deficit, which correlated with enhanced accumulation of abscisic acid levels in leaves. While carotenoid levels were similar.

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throughout the experiment in both plant groups, abscisic acid values sharply increased after 10 days of water deficit, reaching levels 4 times higher in WS than in IR plants. Thereafter, NCED mRNA abundance and abscisic acid levels decreased (day 24). Small rainfalls led to a further decrease in abscisic acid levels in WS plants (day 38). It is worth noting that abscisic acid levels peaked at very early stages of drought (day 10), to decrease later (day 24, Figure 3) although water deficit progressed further (Figure 1).

**Regulation of the isoprenoid biosynthetic pathway in WS plants**

In an attempt to better understand the regulation of these isoprenoid biosynthetic pathways in WS plants, we measured the levels of jasmonic acid and salicylic acid in addition to those of abscisic acid. Salicylic acid levels were similar in WS and IR plants throughout the experiment, whereas the contents of jasmonic acid slightly decreased under water deficit. Jasmonic acid levels were between 11% and 21% lower in WS than in IR plants (Figure 4). Interestingly, \( \alpha \)-tocopherol levels strongly positively correlated with abscisic acid contents (\( r^2 = 0.90 \), Figure 5), but not with those of jasmonic acid and salicylic acid. Indeed, \( \alpha \)-tocopherol levels negatively correlated with those of jasmonic acid (\( r^2 = 0.59 \), order 1 regression, and \( r^2 = 0.88 \), order 2 regression) (Figure 5).

**Discussion**

Despite the fact that the isoprenoid biosynthetic pathway has been characterized in some detail at the molecular level during the last two decades, its regulation during plant responses to drought stress...
is still poorly understood (Liu et al., 2005; Rodríguez-Concepción and Boronat, 2002). In the present study, we analyzed the response of the isoprenoid biosynthetic pathway in *C. creticus* plants exposed to summer drought under Mediterranean field conditions by using combined molecular and biochemical approaches. This study is of particular relevance since experiments were performed in a drought-resistant species growing in the field. It is noteworthy that WS plants were exposed to mild water deficit in the field and that this stress did not cause any damage to the photosynthetic apparatus in *C. creticus* plants, thus reflecting the capacity of this species to withstand drought stress. It is also worth noting that the water deficit response observed in this study suggests a well-adapted species that will likely withstand the predicted increase in climate change-associated aridity in the Mediterranean region.

In the present study, we identified two key points of the isoprenoid biosynthetic pathway in drought-stressed *C. creticus* plants. The first was the induction of the expression of the *NCED* gene in WS plants, in agreement with results reported in other species (Agustí et al., 2007; Iuchi et al., 2001; Yang and Guo, 2007). The first dedicated step in abscisic acid biosynthesis in plants is the oxidative cleavage of 9-*cis*-epoxycarotenoids, which is indeed catalyzed by NCED (Schwartz et al., 2003; Taylor et al., 2005). This enzyme therefore catalyzes a key rate-limiting step in abscisic acid biosynthesis, and it is considered to play a major role in controlling the levels of this phytohormone, which modulates key aspects of plant responses to water deficit such as transpiration efficiency, hydraulic conductivity and leaf expansion (Thompson et al., 2007). Indeed, endogenous contents of

Figure 4. Changes in the levels of jasmonic acid (JA) and salicylic acid (SA) during the experiment in leaves of irrigated (black symbols) and water-stressed (white symbols) field-grown *C. creticus* plants. Data correspond to the mean ± SEM of at least 3 measurements.

Figure 5. Correlations between endogenous abscisic acid (ABA), jasmonic acid (JA) or salicylic acid (SA) levels and α-tocopherol contents during the experiment in leaves of field-grown *C. creticus* plants.
abscisic acid increased up to 4 times at day 10 in WS C. creticus plants, which may contribute to preventing water loss, as shown by constant RWC contents between 10 and 24 d of water deficit.

The second key point of the isoprenoid biosynthetic pathway revealed in the present study was the induction of the gene encoding HPT in WS plants. To our knowledge, the induction of this gene in response to water deficit is reported here for the first time, and is similar to what occurs in response to high light stress in Arabidopsis (Collakova and DellaPenna, 2003). HPT is a membrane-bound enzyme found in chloroplasts that catalyzes a key committed step of tocopherol biosynthesis, that is the condensation of homogentisate and phytol diphosphate to form the tocopherol precursor 2-methyl-6-phytyl-1,4-benzoquinol. Elevated levels of HPT mRNA abundance were correlated with increased \( \alpha \)-tocopherol values (up to 62% in WS C. creticus plants). Increasing tocopherol levels are a common response in other drought-resistant Mediterranean shrubs, including species of the genus Cistus (Hernández et al., 2004; Munné-Bosch and Alegre, 2000), and it is shown here for the first time that this is regulated at the transcript level. \( \alpha \)-Tocopherol is thought to have a number of functions in plants, including the prevention of lipid peroxidation during germination and seedling growth (Sattler et al., 2006), protection against photo-oxidative stress (Havaux et al., 2005) and regulation of carbohydrate export from leaves (Hofius et al., 2004; Maeda et al., 2006). Among other possible functions, enhanced \( \alpha \)-tocopherol levels in leaves of WS C. creticus plants may therefore help preventing, in cooperation with other mechanisms, photo-oxidative damage in chloroplasts.

It is also shown here that the abscisic acid and tocopherol biosynthetic pathways were simultaneously activated in WS plants. Indeed, a strong positive correlation between abscisic acid and \( \alpha \)-tocopherol levels was observed \((r^2 = 0.90)\). It may be therefore hypothesized that the tocopherol biosynthetic pathway may be regulated by abscisic acid levels in WS plants. Recently, El Kayal et al. (2006) reported that applications of this phytohormone in suspension cultures of Eucalyptus gunnii increased expression of the gene encoding tocopherol cyclase, which catalyzes the penultimate step of \( \alpha \)-tocopherol biosynthesis. Moreover, an abscisic acid-specific motif has been identified in the promoter region of the gene encoding \( p \)-hydroxyphenylpyruvate dioxygenase (J. Falk, University of Kiel, pers. comm.), therefore supporting the contention that tocopherol biosynthesis may be stimulated by abscisic acid in leaves of WS plants. To our knowledge, this is the first study demonstrating a positive correlation between abscisic acid and tocopherol levels in WS plants.

Although a positive correlation between the levels of these isoprenoids was also observed in aging Arabidopsis plants \((r^2 = 0.61\), calculated from Munné-Bosch et al., 2007\), plant age-induced increases in abscisic acid levels did not lead to enhanced tocopherol accumulation in C. clusii plants (Munné-Bosch and Lalauze, 2007), suggesting that abscisic acid levels do not always regulate tocopherol contents in plants and that the levels of this antioxidant are additionally modulated by other factors.

It has been shown that the expression of the genes encoding for tyrosine aminotransferase and \( p \)-hydroxyphenylpyruvate dioxygenase, which catalyze the transamination from tyrosine to phydroxypheisolpyruvate and its conversion to homogentisate in tocopherol biosynthesis, respectively, is regulated by jasmonic acid (Falk et al., 2002; Sandorf and Holländler-Czytko, 2002). In the present study, however, \( \alpha \)-tocopherol levels increased, while jasmonic acid levels decreased in WS plants. It appears therefore that, under the tested conditions, jasmonic acid did not promote tocopherol biosynthesis in WS C. creticus plants. In fact, a negative correlation was observed between these parameters. Thus, it appears that increased tocopherol levels leads to decreases in jasmonic acid content in WS C. creticus plants, supporting the contention that tocopherol levels may exert, at least in some cases, an influence on the accumulation of jasmonic acid levels in plants, as it has been recently suggested in Arabidopsis plants (Munné-Bosch et al., 2007). In addition, salicylic acid levels did not increase in C. creticus plants exposed to drought, which contrasts with increases of this compound in other species exposed to drought such as Phillyrea angustifolia, in which a strong correlation between salicylic acid and tocopherol levels was reported (Munné-Bosch and Peníuelas, 2003). It appears, therefore, that under the tested conditions (mild drought), abscisic acid may be more important than salicylic acid or jasmonic acid in the activation of drought stress resistance mechanisms in C. creticus.

We conclude that the isoprenoid biosynthetic pathway might be regulated at the transcript level in leaves of field-grown C. creticus plants exposed to water deficit, and that the genes encoding HPT and NCED could play a key role in the drought stress resistance of this perennial plant by modulating abscisic acid and \( \alpha \)-tocopherol biosynthesis. Further research is needed to confirm the regulation of tocopherol biosynthesis by abscisic acid levels in WS plants of other species.
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