FEBS Letters 583 (2009) 992–996

FEBS Letters

journal homepage: www.FEBSLetters.org

Ethylene signaling may be involved in the regulation of tocopherol biosynthesis in *Arabidopsis thaliana*

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ARTICLE INFO

Article history:
Received 10 February 2009
Revised 19 February 2009
Accepted 20 February 2009
Available online 1 March 2009

Edited by Ulf-Ingo Flügge

Keywords:
Ethylene
Leaf aging
Tocopherol
Vitamin E
Water-stress

ABSTRACT

Tocopherol biosynthesis was investigated in *ein3-1*, *etr1-1* and *eto1-1* mutants of *Arabidopsis thaliana*, which show a defect in ethylene signaling, perception and over-produce ethylene, respectively. A mutation in the *EIN3* gene delayed the water-stress related increase in α-tocopherol and caused a reduction in the levels of this antioxidant by *ca.* 30% compared to the wild type. In contrast to the wild type and *ein3-1* mutants, both *etr1-1* and *eto1-1* mutants showed a sharp (up to 5-fold) increase in α-tocopherol levels during leaf aging. It is concluded that ethylene perception and signaling may be involved in the regulation of tocopherol biosynthesis during water stress and leaf aging.

1. Introduction

Under water deficit, active oxygen species are produced as a consequence of stomatal closure and over-reduction of the photosynthetic electron transport chain leading to the production of superoxide anion, hydrogen peroxide and hydroxyl radicals, which in turn may cause lipid peroxidation and photo-inhibitory damage to the photosynthetic apparatus. Protection against oxidative damage in chloroplasts is provided by a number of both enzymatic and non-enzymatic antioxidants [1]. From the non-enzymatic compounds, the lipid-soluble antioxidant α-tocopherol has been proven to be especially important to maintain the integrity of thylakoids and chloroplast membranes [2]. Conditions favouring oxidative damage have been shown to induce an enhanced accumulation of endogenous α-tocopherol in order to cope with oxidative stress [3]. Moreover, tocopherol levels could be shown to increase with leaf age and during leaf senescence [4–7].

In the past decade all genes necessary for the biosynthesis of tocopherols in plants have been identified, characterized, and used for biotechnological approaches generating plants with altered tocopherol composition or levels [8,9]. However, not much is known about the regulatory mechanisms controlling the biosynthesis of tocopherols during development and under stress conditions in plants. So far, exogenous treatment with jasmonate and ethylene in barley and the use of jasmonic acid-deficient Arabidopsis plants showed that these phytohormones may induce the accumulation of transcripts of at least one gene coding for an enzyme involved in the biosynthesis of tocopherols [10,11]. Furthermore, it could be shown that endogenous levels of salicylic acid, an important phytohormone involved in oxidative stress signaling, strongly correlates with tocopherol levels in water-stressed *Phyllirea angustifolia* plants and senescing *Salvia lanigera* leaves [12,13], but that in other species, such as *Cistus creticus*, abscisic acid correlates much better than salicylic acid or jasmonic acid with α-tocopherol in water-stressed plants [14]. Ethylene has long been known as a major signal molecule in the adaptation of plants to environmental stresses [15–17]. When holm oak (*Quercus ilex*) is exposed to heat stress or drought stress a significant increase in α-tocopherol can be observed. However, when such plants are additionally fumigated with ethylene in concentrations found in polluted areas, symptoms of enhanced oxidative stress accompanied with a significant decrease in α-tocopherol levels in the leaves were observed [18].

To get further insight into the mechanisms underlining the regulation of α-tocopherol levels in plants we used in the present study the ethylene-response mutants *ein3-1* (At3g20770) [19] and *etr1-1* (At1g66340) [20] as well as the ethylene over-producer *eto1-1* (At3g51770) [21] from *Arabidopsis thaliana*. The accumulation of α-tocopherol in response to water stress and leaf aging was compared to the transcript level of selected tocopherol pathway-related genes.

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0014-5793/$36.00
doi:10.1016/j.febslet.2009.02.036

2. Materials and methods

2.1. Plant material, growth conditions and treatments

Seedlings of the *A. thaliana* Columbia ecotype (Col-0) and the *ein3-1* mutant, which shows reduced responsiveness to ethylene (At3g20770, N8052 [19]) were used in the present study. Plants were grown in pots containing a mixture of peat/perlite/vermiculite (1:1:1, v/v/v) in a constant environment chamber (8-h photoperiod, 90–110 μmol quanta m^{-2} s^{-1}, air temperature 21–23 °C) and were watered with Hoagland's solution every 3 days during 8 weeks until the experiment started. Then, plants were either exposed to water deficit (water-stressed plants) by withholding water, or were watered with Hoagland's solution every 3 days (irrigated plants) during 10 days. To confirm the role of ethylene in the regulation of tocopherol biosynthesis, the ethylene-response mutant *etr1-1* (At1g66340, N237 [20]) as well as the ethylene overproducer mutant *eto1-1* (At3g51770, N3072 [21]) were compared to the wild type in terms of tocopherol accumulation. Upper (young, not fully expanded) and lower (older, fully expanded but not senescing) leaves of the rosette were collected and used for the experiments.

Leaf water status and PSII efficiency (as stress indicators), as well as α-tocopherol contents, together with expression of major tocopherol biosynthetic genes were measured in leaves collected at the middle of the photoperiod. For measurements of α-tocopherol and relative transcript levels leaves were collected, frozen in liquid nitrogen, and stored at −80 °C until analysis. Experiments were fully replicated twice giving very similar results.

2.2. Stress indicators

Leaves were weighed, re-hydrated for 24 h at 4 °C in darkness and subsequently oven-dried for 24 h at 80 °C. The relative leaf water content (RWC) was determined as $100 \times (FW - DW)/(TW - DW)$, where FW is the fresh weight, TW is the turgid weight after re-hydrating the leaves at 4 °C in darkness, and DW is the dry weight after oven-drying the leaves at 80 °C to constant weight. The relative and maximum efficiencies of photosystem II photochemistry (φ_{PSII} and F_v/F_m, respectively) were determined by using a pulse-modulated fluorimeter mini-PAM (Walz, Effeltrich, Germany) in the light and after 1 h of dark adaptation, respectively, as described [22].

2.3. Analyses of α-tocopherol

To measure α-tocopherol, leaf samples (100 mg) were extracted two times with ice-cold methanol using sonication and determined by HPLC as described [23]. In short, tocopherols were separated on a Partisil 10 ODS-3 column (250 × 4.6 mm, Scharlau, Barcelona, Spain) at a flow rate of 1 mL min^{-1}. The solvents consisted of (A) methanol/water (95:5, v/v) and (B) methanol. The gradient used was: 0–10 min 100% A, 10–20 min decreasing to 0% A, 20–25 min 0% A, 25–28 min increasing to 100% A, and 28–33 min 100% A. α-Tocopherol was quantified by its absorbance at 295 nm (Diode array detector 1000S, Applied Biosystems) and identified by its characteristic spectrum and by co-elution with an authentic standard provided by Sigma (Steinheim, Germany).

2.4. Gene expression analyses

RNA was isolated from leaf material by a modified hot borate method [24]. Leaf samples (100 mg) were ground to a fine power in liquid nitrogen with a modified Retsch mill (Haan, Germany), the powder transferred into a 2 ml reaction cap containing 750 μl of pre-heated borate extraction buffer (0.2 M sodium borate, 1% (w/v) SDS, 30 mM EGTA) and 750 μl phenol, and then mixed and incubated for 30 min at 30 °C. The samples were centrifuged for 20 min at 25 000×*g*, the upper phase mixed with 1 ml phenol/chloroform and centrifuged again. This procedure was repeated twice, before the RNA was precipitated with LiCl at a final concentration of 3 M on ice for 4 h and centrifuged for 15 min at 4 °C. The pellet was washed with 70% (v/v) ethanol, air-dried, resuspended in 20 μl water, and the RNA concentrations determined at 260 nm.

For semi-quantitative RT-PCR, equal amounts of RNA (1 μg) from each sample were used for reverse transcription and 1/80 of the cDNA for the following PCR reaction. The amounts of cDNAs of all samples were calibrated using the Quantum RNA™ 18S Internal Standard (Ambion). The ratio of 18S to 18S competimer primer pair was 1:9 or 2:8. For the PCR amplification gene specific primers listed in Supplementary Table 1 were used. Conditions were established such that the PCR reactions would remain in the linear range for all primer pairs used as follows: 94 °C (1 min), 30–38 cycles of 94 °C (45 s), 60 °C (45 s), 72 °C (1–2 min). PCR products were electrophoretically separated on a 1% (w/v) agarose gel containing ethidium bromide and the intensity of the PCR products measured with an AlphaImager gel documentation system using AlphaEaseFC Software (Alpha Innotech Corporation, San Leandro, USA). First the relative transcript level of each transcript was calculated as the ratio of the band intensities of gene specific to 18S product. To compare changes in transcript levels over time the relative transcript level observed for each gene in experiment 1 of irrigated wild type leaves (control) was set as 1. Each time point is based on at least three quantifications ($n \geqslant 3$).

2.5. Statistical analyses

Statistical variations between measurements of different treatments at different times were analyzed with an analysis of variance ANOVA or the Student's *t*-test using SPSS software (Chicago, IL, USA). Differences were considered significant at a probability level of $P < 0.05$.

3. Results

Wild type and *ein3-1* mutant plants were morphologically indistinguishable after 8 weeks of growth and showed similar symptoms of wilting after 10 days of water deficit. The relative water content (RWC) showed a similar trend in both plant groups in response to water deficit. The RWC kept nearly constant during the first 7 days of water deficit treatment but then decreased sharply from *ca.* 80% at day 7 to values below 40% at day 10 in both plant groups (Fig. 1). The relative efficiency of PSII photochemistry (ϕ_{PSII}) followed a similar trend in response to water deficit in both plant groups, showing a strong depletion in this parameter to values around 0.3 at day 10. In contrast, the maximum efficiency of PSII photochemistry (F_v/F_m ratio), which is an indicator of damage to the photosynthetic apparatus, decreased more in wild type than in *ein3-1* mutants. In other words, while PSII efficiency partly recovered by exposure of plants to darkness in the mutants, damage to photosynthetic electron transport appeared to be irreversible in wild type plants (Fig. 2).

α-Tocopherol levels were similar in leaves of wild type and *ein3-1* mutants under control conditions, but they increased progressively, and particularly after 7 days of water deficit, in wild type plants (Fig. 3). By contrast, increases in this antioxidant were not observed until day 10 of water deficit in the *ein3-1* mutant. The defect in ethylene signaling also caused a reduction in the extent of α-tocopherol accumulation in leaves, with levels of this antioxi-

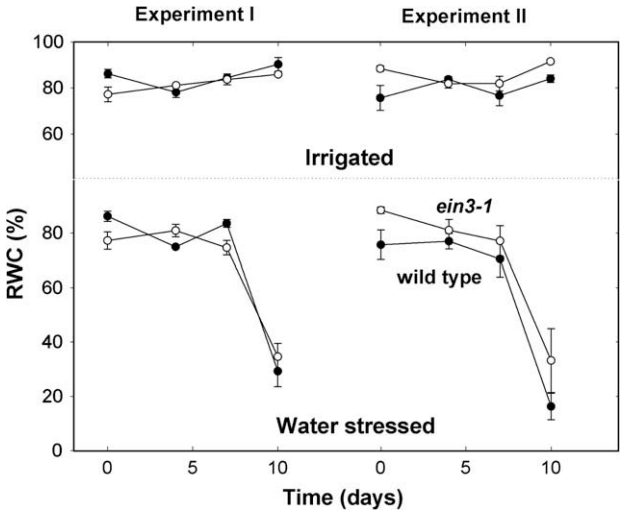

Fig. 1. Changes in relative water contents (RWC) in leaves of irrigated and water-stressed wild type and *ein3-1* mutants of *Arabidopsis thaliana*. Results of two independent experiments are shown. Data represent the mean ± SE of five individuals. No significant differences were observed between plant groups in any of the days of measurements (Student's *t*-test, $P \leqslant 0.05$).

dant being *ca.* 30% lower in the mutant compared to the wild type after 10 days of stress. It is noteworthy that the increase in α-tocopherol levels in the wild type was observed before the RWC decreased sharply. In order to better understand the regulatory mechanisms underlying the water-stress-induced accumulation of α-tocopherol, the transcript levels of tocopherol biosynthesis genes were analyzed by semi-quantitative RT-PCR. The relative transcript levels of the genes encoding for the 4-hydroxyphenylpyruvate dioxygenase *(HPD)*, tocopherol cyclase (*VTE1*), homogentisate prenyltransferase (*VTE2*) and γ-tocopherol methyltransferase (*VTE4*) were not significantly different between both plant groups. Moreover, water deficit did not induce an increase in transcript levels of these genes (Supplementary Fig. 1). Therefore, the increase in α-tocopherol observed under water stress conditions was not accompanied by an increase of transcript levels of central genes encoding for enzymes of the tocopherol biosynthetic pathway.

In order to confirm the influence of ethylene on the accumulation of α-tocopherol in leaves, young and older leaves of two other mutants were analyzed. A second ethylene-response mutant, *etr1-1*, showed 2-fold higher α-tocopherol levels in young leaves compared to wild type and *ein3-1* mutants (Table 1). In contrast to the *ein3-1* mutant, tocopherol levels were about 10-fold higher in the older leaves of the *etr1-1* mutant compared to those of the wild type. On the other hand, when young and old leaves of the ethylene over-producer *eto1* were analyzed a dramatic higher tocopherol level was also observed in older leaves (Table 1), while transcript levels were not increased (Supplementary Table 2).

4. Discussion

Tocopherol contents are known to change during leaf development and appear to be strongly influenced by different stress conditions. Drought stress is known to increase α-tocopherol levels in several species, including *A. thaliana* [25,26], and it has been recently shown that enhanced production of α-tocopherol may improve drought tolerance in tobacco [27]. On the other hand, ethylene, the simplest unsaturated hydrocarbon, regulates many diverse metabolic and developmental processes in plants, ranging from seed germination to organ senescence, and it is considered to play a major role as a signal molecule at low concentrations in the tolerance of several species to environmental stresses (for review, see [15,28,29]). In previous studies, it was shown that exogenous treatment with ethylene can induce transcription of the *hpd* gene, which encodes for 4-hydroxyphenylpyruvate dioxygenase, a key enzyme in tocopherol biosynthesis [10]. Also, it was shown that sustained accumulation of ethylene, at concentrations similar to those found in polluted areas, can give rise to enhanced oxidative stress and lowered tocopherol levels in holm oak plants exposed to heat stress or a combination of heat and drought stress [18]. We were therefore interested in unraveling the possible involvement of ethylene in the regulation of tocopherol biosynthesis in plants, and with this purpose we compared the water-stress response of the *ein3-1* mutant, which shows a defect in ethylene signaling, to that of wild type plants. Tocopherol levels increased progressively during water stress in wild type plants, but this water-stress-induced increase was delayed and reduced in *ein3-1* mutants, thus suggesting that ethylene signaling may be involved in tocopherol biosynthesis. EIN3 is a transcription factor involved in ethylene signaling and loss-of-function mutations in *ein3* gene cause partial ethylene insensitivity. This insensitivity can be rescued by expression of *EIL1* or *EIL2* indicating that, along with EIN3, at least these two EIN3-like (EIL) proteins can mediate an ethylene-response [19]. It is therefore likely that EIL proteins may account for the partial increase in α-tocopherol levels observed in the *ein3-1* mutants. Further insight into the signaling components involved in the regulation of tocopherol biosynthesis was obtained by comparing the water-stress response of *ein2-1* and *eil1-1* mutants to that of *ein3-1* mutants (Table 2). EIN2, which has similarity to the Nramp family of metal ion transporters [30], acts downstream of ETR1 and upstream of EIN3 and EIL proteins [31]. When exposed to water deficit for 7 days, *ein3-1* and *ein2-1* mutants showed 33% and 43% reductions, respectively, in α-tocopherol accumulation in leaves compared to wild type, while tocopherol levels in *eil1-1* mutants were reduced by 15% compared to wild type. This suggests that the regulation of tocopherol biosynthesis is specifically regulated by EIN2, EIN3 and EIL1 proteins. It appears that EIN2 proteins have a major role in the control of tocopherol biosynthesis, since *ein2-1* mutants could only increase tocopherol levels by 33% under water deficit (relative to irrigated plants), while wild type plants increased the levels of this antioxidant 2.5 fold under the same conditions (water-stressed plants relative to irrigated ones, Table 2). Loss-of-function mutations in *EIN3*

Table 1
Relative leaf water content (RWC), relative and maximum PSII efficiency (ϕ_{PSII} and F_v/F_m, respectively), and α-tocopherol levels in young and old leaves of wild type and *eto1–1*, *etr1–1*, and *ein3–1* mutants of *Arabidopsis thaliana*. Data represent the mean ± SE of five randomly chosen plants. Significant differences between young and old leaves are indicated by an asterisk (Student's *t*-test, $P \leqslant 0.05$).

	Wild type		*ein3-1*		*etr1-1*		*eto1-1*	
	Young	Old	Young	Old	Young	Old	Young	Old
RWC (%)	78.10 ± 2.24	83.71 ± 1.20	81.11 ± 0.90	81.86 ± 1.92	80.18 ± 5.61	77.63 ± 1.75	81.34 ± 3.61	84.39 ± 2.47
ϕ_{PSII}	0.71 ± 0.03	0.70 ± 0.03	0.75 ± 0.01	0.75 ± 0.01	0.73 ± 0.02	0.75 ± 0.01	0..73 ± 0.03	0.72 ± 0.01
F_v/F_m	0.82 ± 0.01	0.83 ± 0.02	0.80 ± 0.02	0.85 ± 0.02	0.77 ± 0.02	0.81 ± 0.01	0.78 ± 0.01	0.77 ± 0.02
α-Tocopherol (μmol [g DW]$^{-1}$)	0.81 ± 0.16	1.05 ± 0.02	0.80 ± 0.10	1.16 ± 0.20	2.19 ± 0.66	10.97 ± 0.64*	1.01 ± 0.12	6.60 ± 0.36*

Table 2
Relative leaf water content (RWC), relative and maximum PSII efficiencies (ϕ_{PSII} and F_v/F_m, respectively), and α-tocopherol levels in leaves of wild type, and *ein3-1*, *ein2-1* and *eil1-1* mutants of *Arabidopsis thaliana* either grown under irrigated conditions (irrigated plants) or exposed to water deficit for 7 days (water-stressed plants). Data represent the mean ± SE of five randomly chosen plants. Significant differences between irrigated and water-stressed plants are indicated by an asterisk.

	Wild type		*ein3-1*		*ein2-1*		*eil1-1*	
	Irrigated	Water-stressed	Irrigated	Water-stressed	Irrigated	Water-stressed	Irrigated	Water-stressed
RWC (%)	89.47 ± 0.75	85.54 ± 1.48*	90.40 ± 2,44	84.07 ± 1.36*	90.46 ± 0.22	85.08 ± 2.02*	89.88 ± 0.73	84.48 ± 1.66*
ϕ_{PSII}	0.78 ± 0.04	0.74 ± 0.02	0.78 ± 0.02	0.76 ± 0.02	0.77 ± 0.03	0.75 ± 0.02	0.81 ± 0.03	0.82 ± 0.01[a]
F_v/F_m	0.84 ± 0.04	0.78 ± 0.01	0.85 ± 0.02	0.82 ± 0.01[a]	0.84 ± 0.02	0.81 ± 0.01[a]	0.84 ± 0.03	0.85 ± 0.01[a]
α-Tocopherol (μmol [g DW]$^{-1}$)	0.82 ± 0.09	2.12 ± 0.08*	0.81 ± 0.05	1.44 ± 0.14*,[a]	0.90 ± 0.10	1.20 ± 0.02*,[a]	0.78 ± 0.06	1.81 ± 0.10*,[a]

[a] Indicates significant differences between mutants and the wild type for each of these treatments (Student's *t*-test, $P \leqslant 0.05$).

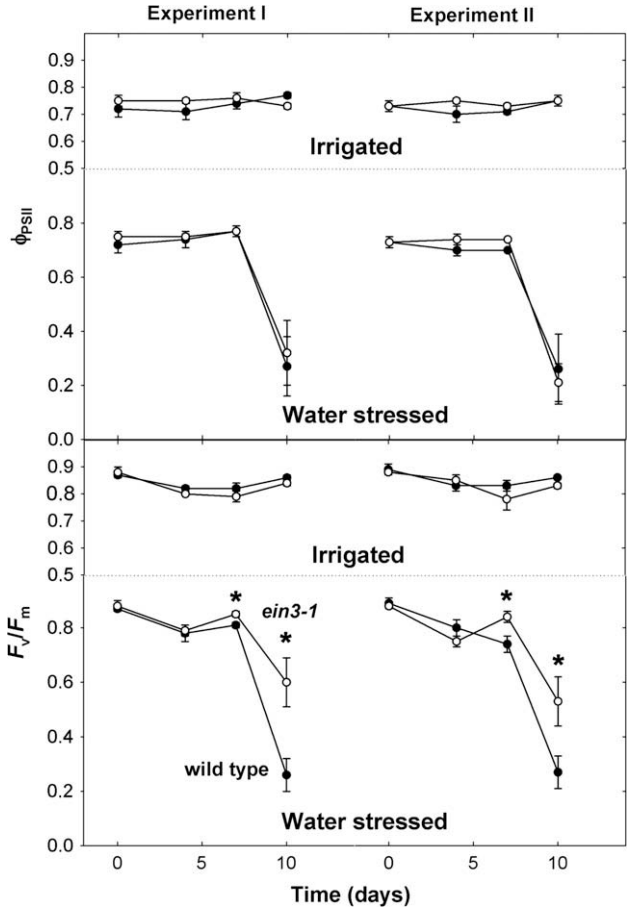

Fig. 2. Changes in relative and maximum efficiency of PSII photochemistry (ϕ_{PSII} and F_v/F_m, respectively) in leaves of irrigated and water-stressed wild type and *ein3-1* mutants of *Arabidopsis thaliana*. Results of two independent experiments are shown. Data represent the mean ± SE of five individuals. Significant differences between plant groups are indicated by an asterisk (Student's *t*-test, $P \leqslant 0.05$).

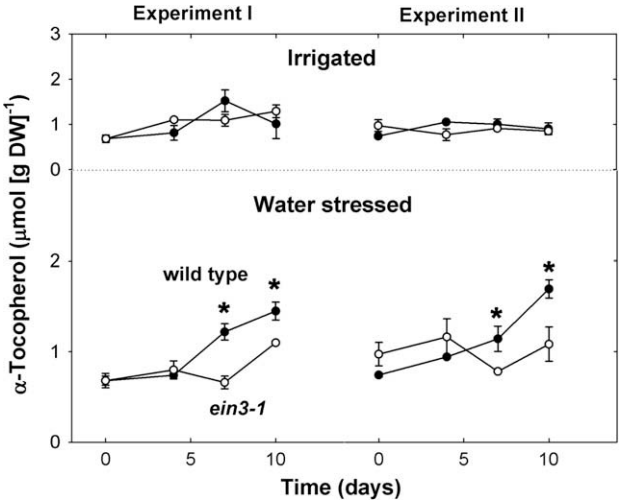

Fig. 3. Changes in α-tocopherol levels in leaves of irrigated and water-stressed wild type and *ein3-1* mutants of *Arabidopsis thaliana*. Results of two independent experiments are shown. Data represent the mean ± SE of four individuals. Significant differences between plant groups are indicated by an asterisk (Student's *t*-test, $P \leqslant 0.05$).

and *EIL1* genes also reduced tocopherol biosynthesis, although to a lower extent, thus suggesting that EIN3 and EIL can compensate each other to some extent. It is also noteworthy that, while ethylene-response mutants altered tocopherol accumulation under water stress, clearly other factors additionally regulate tocopherol accumulation since these mutants still show accumulation of this compound under water stress.

The involvement of ethylene in the regulation of tocopherol accumulation in Arabidopsis plants was confirmed by using *etr1-1* and *eto1-1* mutants. Ethylene is perceived by a family of five membrane-bound receptors (ETR1, ETR2, ERS1, ERS2, EIN4) that have similarity to two-component regulators from bacteria [32]. Interestingly, mutation of the etr1 gene led to drastic changes in α-tocopherol levels during leaf development, the oldest leaves showing up to 5-fold higher tocopherol levels than young leaves in the *etr1-1* mutant. By contrast, tocopherol levels were lower in wild type plants, and increased only slightly during leaf development. Although mutations in any single gene encoding an ethylene receptor have little or no effect upon seedling growth, consistent with functional overlap within the receptor family, it appears that ETR1 may play a central, specific role in the regulation of tocopherol biosynthesis. Since ethylene receptors are negative regulators of ethylene signaling [33], the tocopherol increase observed in *etr1-1* mutants confirms the involvement of ethylene signaling in the regulation of tocopherol biosynthesis. Furthermore, tocopherol biosynthesis was also enhanced in the ethylene over-producer *eto1−1* mutant, thus indicating that ethylene promotes tocopherol biosynthesis in *A. thaliana*. An experiment conducted to unravel the effects of the interaction between water deficit and leaf aging on tocopherol biosynthesis showed that old leaves accumulate α-tocopherol to a similar extent during the first 4 days of water deficit, despite relative leaf water contents decreased to *ca.* 60% in *eto1−1* mutants (Supplementary Fig. 2). Even, tocopherol levels decreased significantly in old leaves of water-stressed *etr1-1* mutants, while transcript levels kept similar (Supplementary Fig. 3), thus suggesting degradation of tocopherol under stress. Indeed, *etr1-1* and *eto1-1* mutants showed symptoms of photo-oxidative damage in old leaves after one week of stress, which were accompanied by visible death of photosynthetic tissues in the ethylene over-producer *eto1−1* mutant (data not shown).

Tocopherol biosynthesis was most likely not regulated at the transcriptional level in the present study, while it has been previ-

ously shown that some tocopherol biosynthetic genes, and particularly *VTE2*, which encodes for homogentisate prenyltransferase and plays a rate limiting step in tocopherol biosynthesis, increase in response to water deficit in field-grown *C. creticus* plants [14] and in response to a combination of high light and nutrient stress in *A. thaliana* [34]. In the present study, however, stress-induced increases in tocopherol were moderate (up to 2-fold), much lower than in the those previous studies, which might explain why we failed to detect by semi-quantitative RT-PCR any significant increase in transcript levels of tocopherol biosynthetic genes in wild type plants. Up to 5-fold increases in α-tocopherol levels were however observed in the leaf aging experiment, while transcript levels were not increased, thus confirming that the ethylene-mediated effects on tocopherol biosynthesis appear not to be regulated at the transcriptional level. Since most components of ethylene signaling, including transcription factors, influence tocopherol accumulation and this is not reflected at the transcript level of the tocopherol biosynthetic genes examined, further research is needed to unravel ethylene-regulated genes involved either directly or indirectly in the regulation of tocopherol biosynthesis.

Acknowledgments

We are very grateful to Joseph Ecker and the the Arabidopsis Biological Resource Center for kindly providing Arabidopsis seeds, and the Serveis de Camps Experimentals and Serveis Científico-Tècnics (University of Barcelona) for technical assistance. This research was supported by the BFU2006-01127 grant from the Spanish Government. Support for the research of S.M. was also received through the prize ICREA Academia for excellence in research, funded by the Generalitat de Catalunya.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.02.036.

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