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Accumulation of γ- Rather than α-Tocopherol Alters Ethylene Signaling Gene Expression in the *vte4* Mutant of *Arabidopsis thaliana*

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Regular Paper

Tocopherols are antioxidants found in chloroplasts of leaves, and it is a matter of current debate whether or not they can affect signaling and gene expression in plant cells. For insight into the possible effects of altered tocopherol composition in chloroplasts on gene expression in the nucleus, the expression of ethylene biosynthesis, perception and signaling genes was investigated in *vte1* and *vte4 Arabidopsis thaliana* mutants, which are impaired in tocopherol (vitamin E) biosynthesis. Changes in gene expression were measured in plants exposed to either salt or water stress, and in young and mature leaves of *vte1* and *vte4* mutants, which lack tocopherol cyclase and γ-tocopherol methyltransferase, respectively. While transcript levels of ethylene signaling genes in the *vte1* mutant and the wild type were similar in all tested conditions, major changes in gene expression occurred in the *vte4* mutant, particularly in mature leaves (compared with young leaves) and under salt stress. Accumulation of γ- instead of α-tocopherol in this mutant led to elevated transcript levels of ethylene signaling pathway genes (particularly *CTR1*, *EIN2*, *EIN3* and *ERF1*) in mature leaves of control plants. However, with salt treatment, transcript levels of most of these genes remained constant or dropped in the *vte4* mutant, while they were dramatically induced in the wild type and the *vte1* mutant. Furthermore, under salt stress, leaf age-induced jasmonic acid accumulated in both the *vte1* mutant and the wild type, but not in the *vte4* mutant. It is concluded that jasmonic acid and ethylene signaling pathways are down-regulated in mature leaves of salt-stressed *vte4* plants.

Keywords: Ethylene signaling • Salt stress • Tocochromanols • Vitamin E • Water stress.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; ACS, ACC synthase; ANOVA, analysis of variance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ROS, reactive oxygen species; RWC, relative water content; UPLC-MS/MS, ultra-high performance liquid chromatography–tandem mass spectrometry

Introduction

Tocopherols, which are part of the vitamin E group of compounds, are lipid-soluble molecules essential for human nutrition, but they are only synthesized by photosynthetic organisms, including all plants, algae and most cyanobacteria (Horvath et al. 2006, Mène-Saffrané and DellaPenna 2010). Tocopherols play an important role as antioxidants, but there is some controversy concerning whether or not they have additional functions in both animals and plants (Azzi 2007, Maeda and DellaPenna 2007, Traber and Atkinson 2007, Falk and Munné-Bosch 2010). Recent studies on vitamin E-deficient (*vte*) *Arabidopsis thaliana* mutants have revealed that one of the most important functions tocopherols play is acting as antioxidants in chloroplasts, thus protecting plants from photoinhibition and photo-oxidative stress (Havaux et al. 2005). Tocopherols, in cooperation with other antioxidants, can scavenge lipid peroxyl radicals, preventing the propagation of lipid peroxidation, and are excellent quenchers and scavengers of singlet oxygen, controlling its levels. Furthermore, studies on *vte* mutants have revealed an important role for tocopherols in low-temperature adaptation (Maeda et al. 2006, Maeda et al. 2008) and seedling germination and growth (Sattler et al. 2004, Sattler et al. 2006), in both cases influencing gene expression levels though modulation of extraplastidic polyunsaturated fatty acid metabolism. Under water deficit and salt stress, however, it is still unknown to what extent a tocopherol deficiency in these mutants can alter the plant stress response at the gene expression level.

Previous studies have shown a positive correlation between tocopherol accumulation and water stress in several plant species (Munné-Bosch et al. 1999, Munné-Bosch and Alegre 2003), including *A. thaliana* (Cela et al. 2009). However, nothing is known about how a tocopherol deficiency in *A. thaliana* affects water stress tolerance. It is noteworthy that increased tocopherol levels in transgenic tobacco plants overexpressing *VTE1* from *A. thaliana* show enhanced tolerance to water stress (Liu et al. 2008). *VTE1* encodes a tocopherol cyclase that is

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essential for the synthesis of all tocopherol forms (Porfirova et al. 2002, Sattler et al. 2003), thus this study indicates that tocopherols, which mainly accumulate in the form of α-tocopherol in leaves (Szymanska and Kruk 2008a, Szymanska and Kruk 2008b), confer resistance to water stress. Protection is thought to be exerted through their antioxidant role in chloroplasts, since reactive oxygen species (ROS) production is known to be exacerbated under drought stress (Smirnoff 1993, Smirnoff 2005), but the exact mechanisms remain unknown. Less information is available on the role of tocopherols in salt stress tolerance, another of the most important stresses limiting crop yields. Salt stress limits growth by different mechanisms, including ion toxicity, causing osmotic unbalance and leading to oxidative stress by increasing ROS production (Miller et al. 2010). However, very little is known about the role of tocopherols in protecting plants from salt stress. Interestingly, it has been shown that decreased total tocopherol contents in *HPT*:RNAi transgenic tobacco plants, which lack all tocopherol forms, increases the sensitivity to salt stress, while tolerance to osmotic stress and methyl viologen of γ-tocopherol methyltransferase transgenics, which accumulate γ- instead of α-tocopherol, is strongly increased (Abbasi et al. 2007).

Ethylene gas regulates many diverse metabolic and developmental processes in plants, ranging from seed germination to organ senescence, and is considered to play a major role as a signal molecule at low concentrations in the tolerance to environmental stresses, including water and salt stress (Bleecker and Kende 2000, Argueso et al. 2007). Ethylene production is induced by various stresses, as well as by other hormones and developmental cues (Argueso et al. 2007). The rate-limiting step in the two-step biosynthetic pathway is the production of 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase (ACS). Conversion of ACC into ethylene gas is then catalyzed by ACC oxidase (ACO). The ACS and ACO enzymes are encoded by multigene families. *A. thaliana* has nine *ACS* genes (*ACS1*, *ACS2*, *ACS4*, *ACS5*, *ACS6*, *ACS7*, *ACS8*, *ACS9* and *ACS11*), which display unique and overlapping expression patterns (Tsuchisaka and Theologis 2004, Argueso et al. 2007). *ACS* gene expression is differentially regulated by various ethylene-inducing factors. For instance, *ACS6* expression is stimulated by ozone, wounding, auxins and ethylene (Vahala et al. 1998, Tian et al. 2002). All but three of the *ACS* genes are expressed in young rosette leaves; *ACS1* is expressed in the vascular tissue, *ACS9* appears to be inactive and *ACS11* expression is restricted to the trichomes (Tsuchisaka and Theologis 2004).

The ethylene signal transduction pathway, as proposed in *A. thaliana*, involves five ethylene receptors ETR1, ERS1, ETR2, EIN4 and ERS2, as well as the negative regulator CTR1, the downstream positive regulator EIN2, and the transcription factor EIN3 (among others), which up-regulates yet another transcription factor, ERF1 (Chen et al. 2005, Kendrick and Chang 2008; see also **Supplementary Fig. S1**). Recent studies have shown that alterations in ethylene signaling affect plant responses to both salt and water stress (Cao et al. 2006, Cao et al. 2008, Cela et al. 2009). For example, ethylene-insensitive mutants are hypersensitive to salt, suggesting that ethylene signaling reduces sensitivity to salt stress (Cao et al. 2008).

In a previous study, it was shown that a defect in ethylene signaling in the *ein3-1* mutant can alter tocopherol biosynthesis in *A. thaliana* (Cela et al. 2009). Since ethylene is a major player in the response of plant to water and salt stress, we aimed here to evaluate not only whether or not a deficiency in specific tocopherol forms in *vte1* and *vte4* mutants of *A. thaliana* alters plant response to salt and water stress, but also to what extent a particular differential response in these mutants is associated with a specific response at the expression level of ethylene biosynthesis, perception and/or signaling genes.

Results

Plant response to water and salt stress in *vte1* and *vte4* mutants

Salt stress (addition of 100 mM NaCl to the nutrient solution) and water stress (by withholding water) for 11 d caused dramatic effects on growth in wild-type *A. thaliana* plants (**Supplementary Fig. S2**). Rosette biomass of wild-type plants was reduced by 34 and 57% in response to salt and water stress, respectively. *vte1* and *vte4* mutants showed a response very similar to that of the wild type, in control and salt- or water-stressed conditions. It is noteworthy that water stress led to a more dramatic phenotype than salt stress; both *vte1* and *vte4* mutants and the wild type showed a similar response characterized by accelerated senescence in mature leaves, an aspect more evident than that observed under salt stress (**Supplementary Fig. S2**). Measurements of the relative water content (RWC) and the maximum efficiency of PSII photochemistry (the F_v/F_m ratio), an indicator of photoinhibition to the photosynthetic apparatus, showed that the water stress imposed on plants was much more severe than the salt stress, with RWC decreasing from values >85% in control plants to values of around 20% in water stress and 70% in salt stress (**Fig. 1**). The F_v/F_m ratio decreased from values around 0.85 in control plants to 0.30 in water-stressed plants, while salt stress did not significantly alter this parameter. Furthermore, in the wild type, the levels of α-tocopherol stayed constant throughout the study in control and salt-stressed plants, while they increased from 190 to 700 $\mu g\, g^{-1}$ DW in water-stressed plants. The levels of γ-tocopherol were much lower than those of α-tocopherol in wild-type plants, but they increased from 4 $\mu g\, g^{-1}$ DW in control to 13 and 54 $\mu g\, g^{-1}$ DW in salt- and water-stressed plants, respectively. As expected, the *vte4* mutant showed γ-tocopherol but not α-tocopherol accumulation in leaves, with γ-tocopherol levels around 150 $\mu g\, g^{-1}$ DW under control conditions, similar to α-tocopherol levels in the wild type. However, in contrast to wild-type plants, the *vte4* mutant did not increase tocopherol levels under either salt or water stress. As expected, the *vte1* mutant did not accumulate either α- or γ-tocopherol in leaves (**Fig. 1**). Despite significant

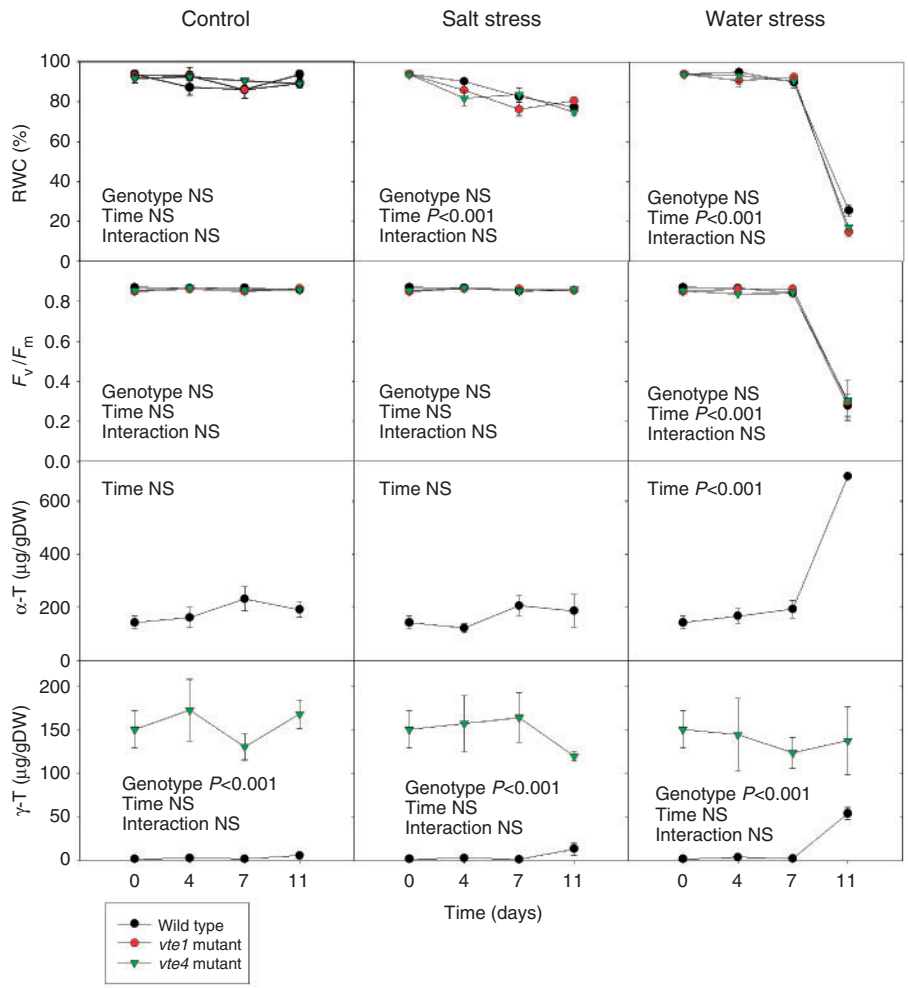

Fig. 1 Time-course evolution of relative water content (RWC), maximum efficiency of PSII photochemistry (F_v/F_m ratio) and levels of α- and γ-tocopherol (α-T and γ-T, respectively) in young leaves of the wild type and *vte1* and *vte4* mutants of *A. thaliana* exposed to control, salt stress or water stress conditions for 11 d. Data are the mean ± SE of $n = 4$ experiments. Significant differences between genotypes, time of measurements and their interaction (genotype × time) are given in the panels (ANOVA, $P \leq 0.05$). NS, not significant.

stress-induced tocopherol increases in wild-type plants, the expression levels of three of the most important genes involved in tocopherol biosynthesis (encoding homogentisate phytyltransferase, *VTE2*; tocopherol cyclase, *VTE1*; and γ-tocopherol methyltransferase, *VTE4*) were not significantly altered (**Supplementary Fig. S3**).

Expression of ethylene biosynthesis, perception and signaling genes in response to water and salt stress in *vte1* and *vte4* mutants

Expression levels were analyzed for the nine genes of the ACS family (*ACS1*, *ACS2*, *ACS4*, *ACS5*, *ACS6*, *ACS7*, *ACS8*, *ACS9* and *ACS11*), and *ACS2*, *ACS6*, *ACS8* and *ACS11* were shown to have high expression levels in leaves by quantitative PCR (data not shown). Later, it was confirmed that the *ACS2* gene showed the highest expression levels by quantitative PCR (**Supplementary Fig. S4**). Transcripts of both *ACS2* and *ACS6* increased significantly in response to water deprivation in both mutants and the wild type, while *ACS8* also increased in response to water stress but differences were not significant. In contrast, transcript levels of *ACS6* increased in the *vte4* mutant exposed to salt stress, while levels remained unaltered in the wild type and the *vte1* mutant (**Supplementary Fig. S4**). Transcript levels of *ACS11* stayed at values <0.00012 throughout the study (data not shown). Increases in *ACS2* and *ACS6* transcripts in water-stressed plants were accompanied by significant increases in *ACO4* and *ACO1* expression levels (**Fig. 2**). Furthermore, *ACO4* expression levels increased more in the *vte4* mutant than in the *vte1* mutant or the wild type exposed to water stress, a result that was also observed, although to a lesser extent, in plants exposed to salt stress. Expression levels of ethylene perception genes also revealed a differential response in tocopherol mutants (**Fig. 3**). The *vte4* mutant showed lower transcript levels of *ERS1* than the *vte1* mutant in all tested conditions, although differences were significant under salt stress only. Analysis of the ethylene signaling genes *EIN2*,

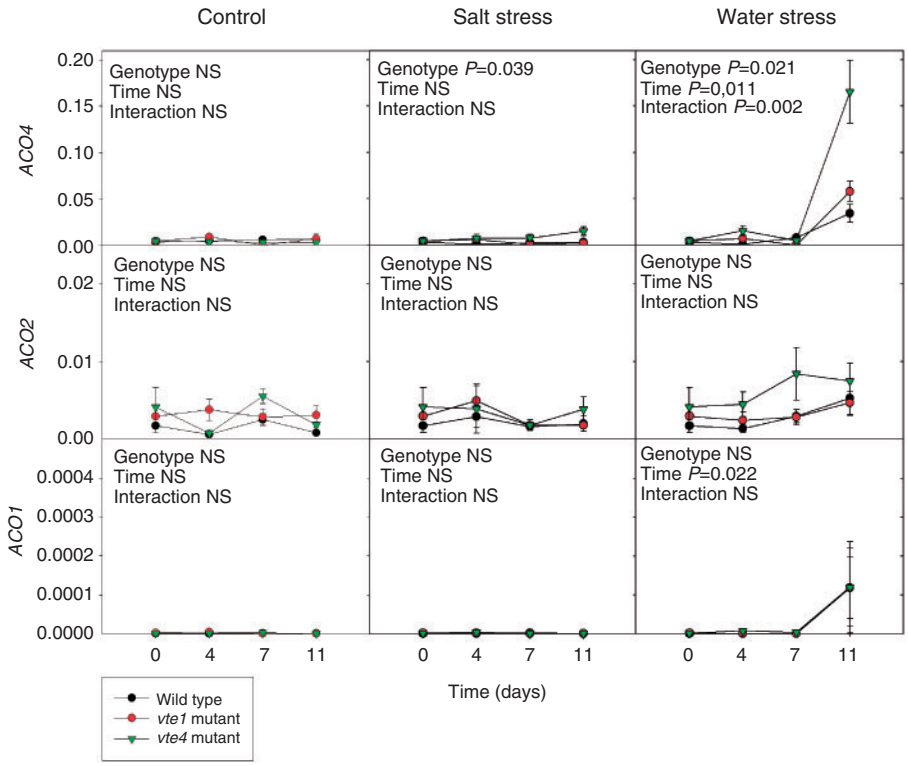

Fig. 2 Time-course evolution of relative expression of the ethylene biosynthesis genes, *ACO4*, *ACO2* and *ACO1* in young leaves of the wild type and *vte1* and *vte4* mutants of *A. thaliana* plants exposed to control, salt stress or water stress conditions for 11 d. Data are the mean ± SE of $n = 4$ experiments. Significant differences between genotypes, time of measurements and their interaction (genotype × time) are given in the panels (ANOVA, $P \leq 0.05$). NS, not significant. *GAPDH* was used as the reference gene to calculate the relative expression of the ethylene biosynthesis genes.

EIN3, *CTR1* and *ERF1* did not reveal any effect of tocopherol deficiency or stress treatments, except for an increase in *ERF1* transcript levels under 11 d of water stress in all genotypes (**Supplementary Fig. S5**).

Influence of leaf age on expression of ethylene biosynthesis, perception and signaling genes and their interaction with salt and water stress

The influence of leaf age on gene expression was evaluated in control conditions and in plants exposed to 7 d of salt or water stress, before stress treatments caused severe cell death in mature leaves. Values of RWC and the F_v/F_m ratio in young and mature leaves did not reveal differences in the degree of stress suffered by both leaf types in all treatments and mutants (**Fig. 4**). α-Tocopherol increased significantly from 190 to 400 $\mu g\, g^{-1}$ DW in mature leaves compared with young leaves under water stress, but leaf age did not cause any effect under control or salt stress conditions, although levels increased slightly under salt stress. γ-Tocopherol levels changed in parallel to those of α-tocopherol, with a significant increase in wild-type plants under salt stress. Mature leaves of the *vte4* mutant also showed significant increases in γ-tocopherol levels in response to water stress (**Fig. 4**). Again, transcript levels of tocopherol biosynthetic genes did not always correlate with tocopherol levels in leaves. Transcript levels of *VTE2* increased in parallel with tocopherol levels in mature leaves of wild-type plants exposed to water stress, but transcript levels of *VTE1*, *VTE2* and *VTE4* increased sharply in mature leaves of wild-type plants in response to salt stress with no concomitant increases in tocopherol (**Supplementary Fig. S6**; and **Fig. 4**).

While leaf age did not induce changes in *ACS2*, *ACS6* and *ACS8* transcript levels (**Supplementary Fig. S7**), the *ACO1* and *ACO2* transcript levels were drastically affected by leaf age, with particularly interesting interactions with salt and water stress (**Fig. 5**). In control plants, leaf age reduced *ACO2* transcript levels, particularly in the wild type and the *vte1* mutant, an aspect that was also observed in *ACO4* and *ACO1* expression, though to a lower extent. These effects were not so evident in the *vte4* mutant, to the extreme that *ACO1* transcript levels increased in mature leaves of this mutant under control conditions. A similar response was observed under water stress, especially exacerbated in *ACO1* transcript levels of the *vte4* mutant, which increased particularly in mature leaves of water-stressed plants. In contrast, this response was not observed under salt stress (**Fig. 5**). Furthermore, transcript levels of ethylene perception genes, namely *ERS1*, *ERS2* and *ETR2*, increased in mature leaves of control plants of the *vte4*

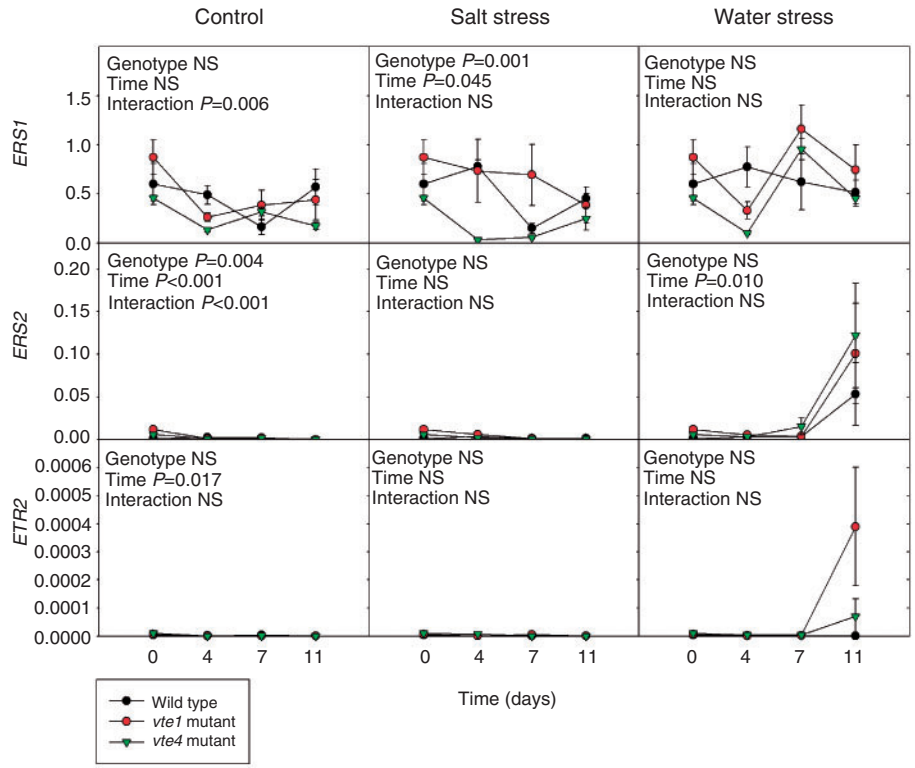

Fig. 3 Time-course evolution of relative expression of the ethylene perception genes, *ERS1*, *ERS2* and *ETR2* in young leaves of the wild type and *vte1* and *vte4* mutants of *A. thaliana* plants exposed to control, salt stress or water stress conditions for 11 d. Data are the mean ± SE of $n = 4$ experiments. Significant differences between genotypes, time of measurements and their interaction (genotype × time) are given in the panels (ANOVA, $P \leq 0.05$). NS, not significant. *GAPDH* was used as the reference gene to calculate the relative expression of the ethylene perception genes.

mutant only, a response that was also observed only for this mutant for the *ERS1* gene under water stress (**Fig. 6**). Major changes in gene expression of ethylene signaling genes also occurred in mature leaves of the *vte4* mutant under both control and water-stressed conditions (**Fig. 7**). Elevated levels of all the ethylene signaling transcripts, particularly the downstream components *CTR1*, *EIN2*, *EIN3* and *ERF1*, were observed in mature leaves of the control and water-stressed *vte4*. However, with salt treatment these transcript levels remained constant or dropped in the *vte4* mutant, while they were dramatically induced in the wild type (particularly *CTR1*, *EIN2* and *ERF1*). Furthermore, the *vte1* mutant and wild-type plants behaved similarly in all tested conditions, particularly for *CTR1*, *EIN3* and *ERF1* expression (**Fig. 7**).

Changes in jasmonic acid levels

To unravel whether or not those changes in gene expression could be associated with a modulation of oxylipin levels, a time course of the evolution of endogenous jasmonic acid levels was evaluated under control, salt and water stress conditions (**Supplementary Fig. S8**), and in young and mature leaves at 7 d of stress (**Fig. 8**). Jasmonic acid levels did not significantly differ between *vte1* or *vte4* mutants and the wild type in young leaves during the 11 d of treatment (**Supplementary Fig. S8**). However, jasmonic acid levels increased in mature leaves of the *vte1* mutant under control conditions, but not in those of the *vte4* mutant or the wild type. Under salt stress, leaf age-induced jasmonic acid accumulated in both the *vte1* mutant and the wild type, but not in the *vte4* mutants (**Fig. 8**). No differences between mutants and the wild type were observed in jasmonic acid levels under water stress (**Supplementary Fig. S8**).

Discussion

A number of environmental stress factors have been associated with an increase in photosynthesis-derived ROS, and the levels of tocopherols and other antioxidants have been related to stress tolerance. Whereas stress-tolerant plants normally display increased tocopherol levels, the most sensitive ones show net tocopherol loss under stress, which is thought to lead to oxidative damage and cell destruction (reviewed in Munné-Bosch and Alegre 2002). However, the function of tocopherols might be more complex, especially considering their role in cellular signaling. Tocopherols are part of an intricate signaling network controlled by ROS, antioxidants and hormones,

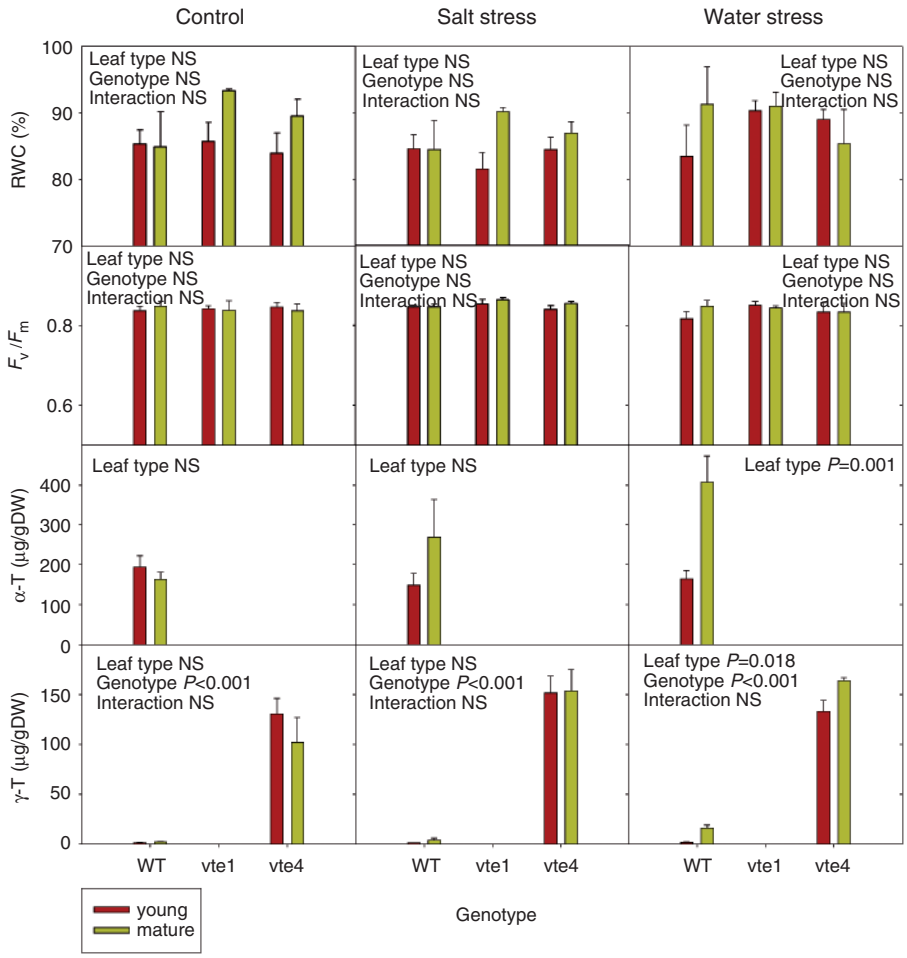

Fig. 4 Relative water content (RWC), maximum efficiency of PSII photochemistry (F_v/F_m ratio) and levels of α- and γ-tocopherol (α-T and γ-T, respectively) in young and mature leaves of the wild type, and *vte1* and *vte4* mutants of *A. thaliana* exposed to control, salt stress or water stress conditions for 7 d. Data are the mean ± SE of $n = 4$ experiements. Significant differences between genotypes, leaf type and their interaction (genotype × leaf type) are given in the panels (ANOVA, $P \leq 0.05$). NS, not significant.

and are therefore good candidates to influence signaling in plant cells. Tocopherols, ascorbate and glutathione are interdependent in the control of ROS levels (Foyer and Noctor 2005, Kanwischer et al. 2005, Munné-Bosch 2005, Asada 2006), and ROS are crucial modulators of gene expression in chloroplasts (Apel and Hirt 2004) and the nucleus (Vandenabeele et al. 2003). Furthermore, ROS and hormones interact in the regulation of signal transduction and gene expression in plants (Pastori and Foyer 2002). By controlling ROS levels and the extent of lipid peroxidation in chloroplasts, tocopherols may indirectly regulate the amounts of extraplastidic lipid peroxidation products and affect gene expression in the nucleus. Hofius et al. (2004) showed that tocopherols may affect source–sink transitions and may alter gene expression in plants, thus providing the first steps towards understanding the role of tocopherols in signaling in plant cells. Later, it was found that tocopherols play an essential role in low-temperature adaptation (Maeda et al. 2006, Maeda et al. 2008, Song et al. 2010) and seedling germination and growth (Sattler et al. 2004, Sattler et al. 2006), in both cases influencing gene expression levels though modulation of extraplastidic polyunsaturated fatty acid metabolism. Under water deficit and salt stress, however, it was still unknown to what extent a tocopherol deficiency can alter the plant stress response at the gene expression level.

The results clearly indicate that major changes in transcript levels of ethylene biosynthesis, perception and signaling genes occur in the *vte4* mutant, which lacks α-tocopherol, but accumulates γ-tocopherol in leaves. Transcript levels of *ACS6* increased in the *vte4* mutant exposed to 11 d of salt stress, while levels were unaltered in the wild type and the *vte1* mutant. *ACO4* expression levels also increased more in the *vte4* mutant than in the *vte1* mutant or the wild type exposed to severe water stress, a result that was also observed, although to a lesser extent, in plants exposed to salt stress, probably partly because the salt stress was not as severe as the water stress. Another important factor controlling the extent of tocopherol effects on the expression of ethylene biosynthesis,

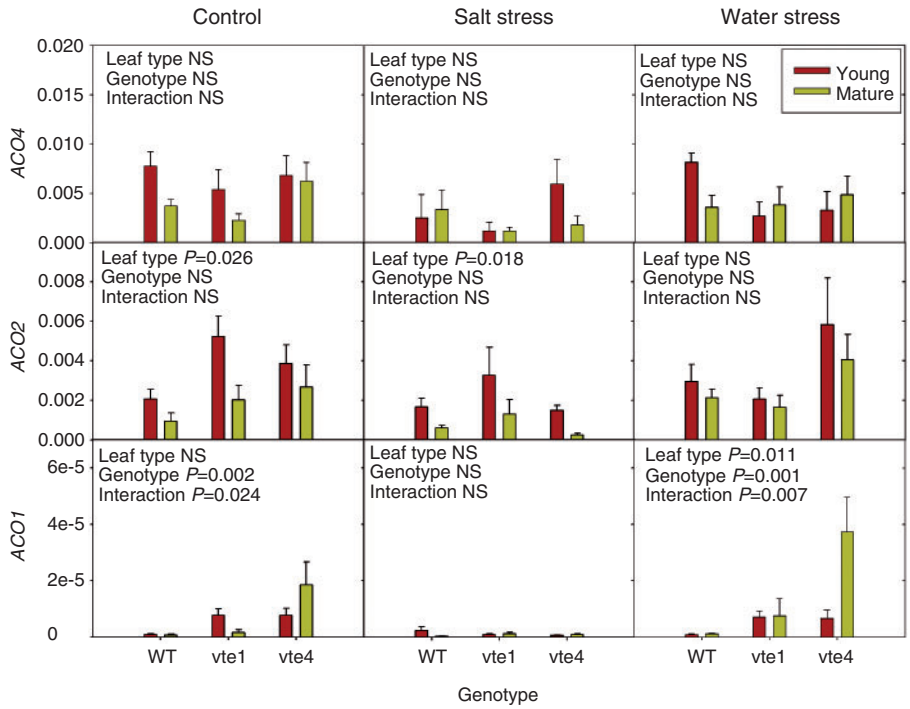

Fig. 5 Relative expression of the ethylene biosynthesis genes, *ACO4*, *ACO2* and *ACO1* in young and mature leaves of the wild type and *vte1* and *vte4* mutants of *A. thaliana* plants exposed to control, salt stress or water stress conditions for 7 d. Data are the mean ± SE of $n = 4$ experiments. Significant differences between genotypes, leaf type and their interaction (genotype × leaf type) are given in the panels (ANOVA, $P \leq 0.05$). NS, not significant. *GAPDH* was used as the reference gene to calculate relative expression of the ethylene biosynthesis genes.

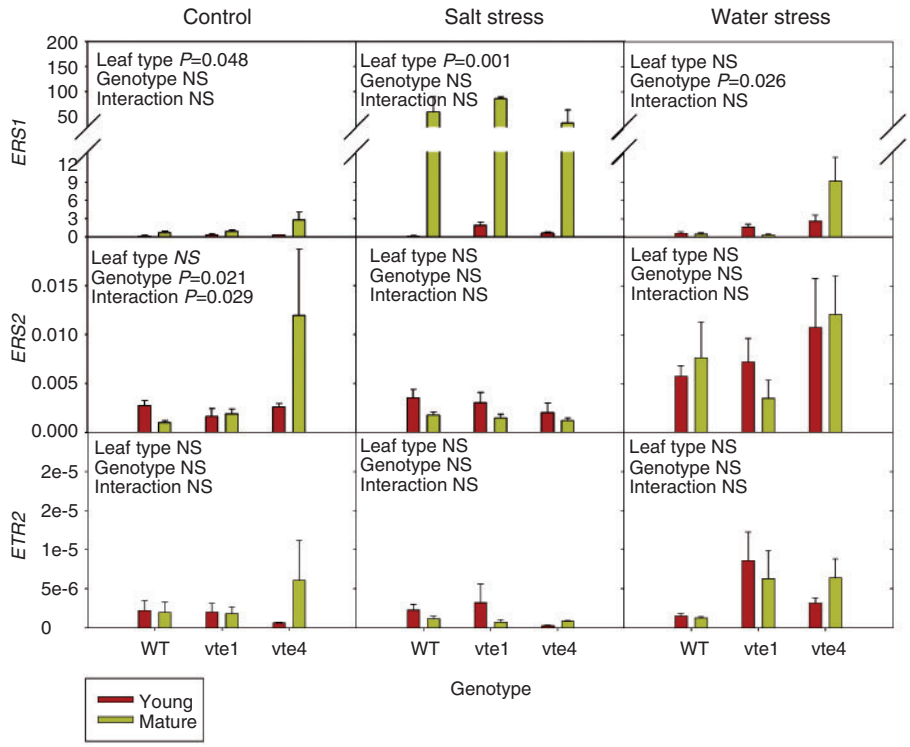

Fig. 6 Relative expression of the ethylene perception genes, *ERS1*, *ERS2* and *ETR2* in young and mature leaves of the wild type and *vte1* and *vte4* mutants of *A. thaliana* plants exposed to control, salt stress or water stress conditions for 7 d. Data are the mean ± SE of $n = 4$ experiments. Significant differences between genotypes, leaf type and their interaction (genotype × leaf type) are given in the panels (ANOVA, $P \leq 0.05$). NS, not significant. *GAPDH* was used as the reference gene to calculate relative expression of the ethylene perception genes.

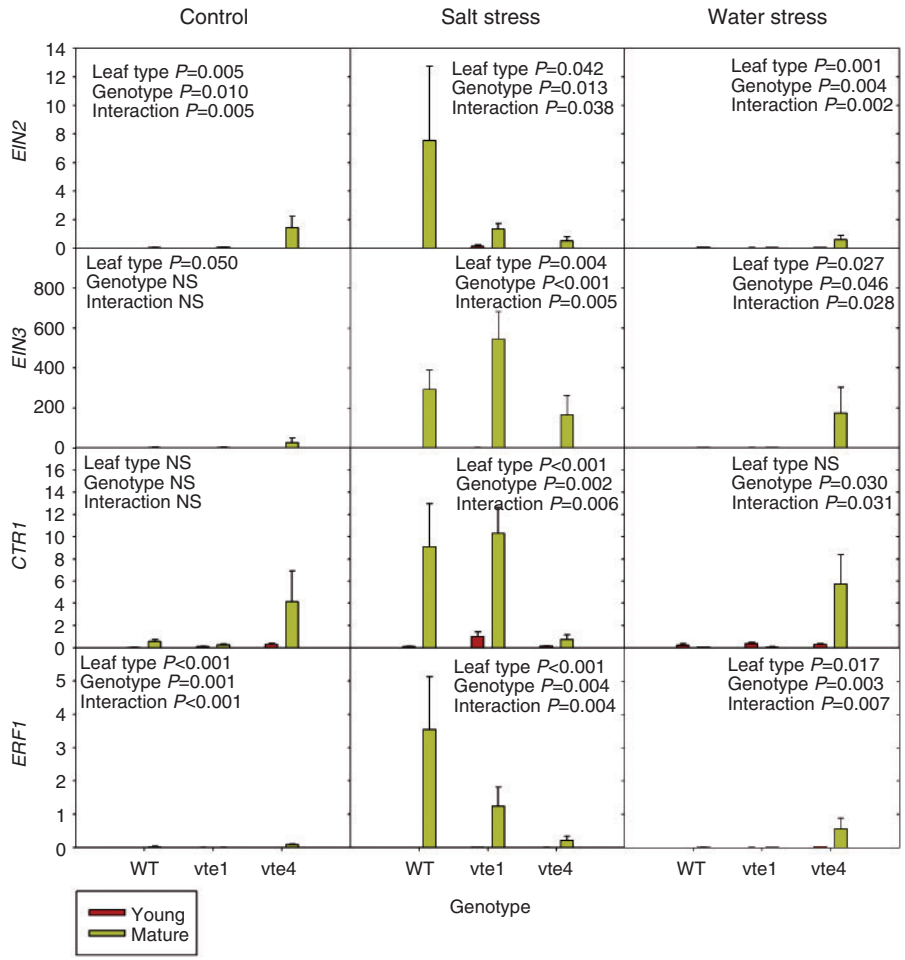

Fig. 7 Relative expression of the ethylene signaling genes, *EIN2*, *EIN3*, *CTR1* and *ERF1* in young and mature leaves of the wild type and *vte1* and *vte4* mutants of *A. thaliana* plants exposed to control, salt stress or water stress conditions for 7 d. Data are the mean ± SE of $n = 4$ experiments. Significant differences between genotypes, leaf type and their interaction (genotype × leaf type) are given in the panels (ANOVA, $P \leq 0.05$). NS, not significant. *GAPDH* was used as the reference gene to calculate relative expression of the ethylene signaling genes.

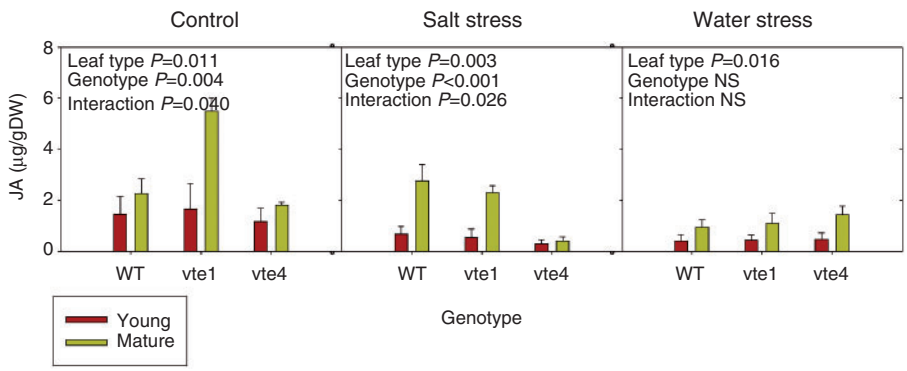

Fig. 8 Jasmonic acid (JA) levels in young and mature leaves of the wild type and *vte1* and *vte4* mutants of *A. thaliana* plants exposed to control, salt stress or water stress conditions for 7 d. Data are the mean ± SE of $n = 4$ experiments. Significant differences between genotypes, leaf type and their interaction (genotype × leaf type) are given in the panels (ANOVA, $P \leq 0.05$). NS, not significant.

perception and signaling genes is leaf age. In control plants, reduced *ACO2* transcript levels were observed in mature leaves of the wild type and the *vte1* mutant, while *ACO1* transcript levels increased in those of the *vte4* mutant. Furthermore, transcript levels of *ERS1*, *ERS2* and *ETR2* increased in mature leaves of the *vte4* mutant only, and elevated levels of *CTR1*, *EIN2*, *EIN3* and *ERF1* were observed in mature leaves of the control and water-stressed *vte4* mutant. However, with salt treatment, these transcript levels remained constant or dramatically dropped in the *vte4* mutant. In addition, leaf age-induced jasmonic acid accumulated in both the *vte1* mutant and the wild type, but not in the *vte4* mutant under salt stress. It has been shown that the transcription factor ERF1 acts downstream of the interaction between the ethylene and jasmonic acid pathways and is a key element in the integration of both signals for the regulation of defense response genes (Lorenzo et al. 2003), thereby suggesting that jasmonic acid and ethylene signaling transcript levels are down-regulated in mature leaves of the salt-stressed *vte4* mutant.

Interestingly, this differential response at the gene expression level in the *vte4* mutant results in a similar resistance to salt and water stress, as indicated by the F_v/F_m ratio and plant biomass, which were similar in the *vte4* mutant and the wild type. Similar stress sensitivity in the *vte4* mutant and the wild type was also observed when plants were subjected to light, heat and cold stress (Bergmüller et al. 2003). Indeed, neither the *vte1* nor the *vte4* mutants were more sensitive than the wild type to any of the stress conditions applied in the present study, thus suggesting that γ-tocopherol is at least as efficient as its α homolog in the protection of plants against salt and water stress. It appears, however, that the mechanisms to achieve this similar response differ between the mutants, as indicated by changes in transcript levels of ethylene biosynthesis genes under both salt and water stress. Obviously, this does not exclude many other mechanisms that may operate in these mutants, and their response to salt and water stress may not necessarily be the same as their response to other types or magnitudes of stresses. Indeed, transgenic tobacco accumulating γ- instead of α-tocopherol was more sensitive to high doses of salt stress (≥200 mM NaCl) than the wild-type strain, which accumulates α-tocopherol (Abbasi et al. 2007). Taken together, this suggests that plants constitutively accumulating γ- instead of α-tocopherol show an altered response to salt stress, since sustained accumulation of γ-tocopherol represses jasmonic acid and ethylene signaling response pathways, which leads to reduced expression of transcription factor ERF1. One may speculate that this will predispose plants to suffer the negative effects of salt stress when the duration and/or magnitude of stress is increased (Abbasi et al. 2007), effects that were not observed in our study due to exposure of plants to low doses of salinity. It is interesting to note that such effects were specific to the plant response to salinity and were not observed under water deficit.

Furthermore, the apparent lack of differential response in the *vte1* mutant might be associated with the antioxidant potential of the tocopherol precursor, 2,3-dimethyl-5-phytylhydroquinol, which accumulates in this mutant (Liebler and Burr 2000). Although the *vte1* mutant lacks both tocopherols and plastochromanol, which account for 5–10% of total tocochromanols in *A. thaliana* grown under low light conditions (Zbierzak et al. 2010), this mutant accumulates plastoquinol, an important lipid-soluble antioxidant which in addition to tocopherols protects chloroplasts from photooxidative stress (Szymanska and Kruk 2010). It is therefore very likely that the accumulation of plastoquinol and 2,3-dimethyl-5-phytylhydroquinol can substitute for the lack of tocochromanols in the *vte1* mutant and therefore no apparent phenotype in plant response to water deficit and salt stress in the low light conditions used in the present study was observed.

It is concluded that jasmonic acid and ethylene signaling pathways are down-regulated in mature leaves of the salt-stressed *vte4* mutant, thus indicating that an altered tocopherol composition in chloroplasts of this mutant alters the physiological response of mature leaves to salt stress. It is still to be determined, however, how enhanced γ-tocopherol levels lead to reduced jasmonic acid and ethylene signaling gene expression levels in mature leaves, and why this occurs under salt stress conditions only.

Materials and Methods

Plant material, growth conditions and treatments

Seeds of *A. thaliana* Columbia ecotype (Col 0) and *vte1* and *vte4* mutants, which were provided by Kathleen Brückner (University of Kiel, Germany), were used in this study. *vte1* and *vte4* mutants have T-DNA insertions in the *VTE1* and *VTE4* genes, which encode tocopherol cyclase and γ-tocopherol methyltransferase, respectively, so that the *vte1* mutant lacks both α- and γ-tocopherol, and the *vte4* mutant lacks α-tocopherol but accumulates γ-tocopherol (Porfirova et al. 2002, Bergmüller et al. 2003). 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 d during 8 weeks until the experiment started. Then, plants were exposed for 11 d to either (i) water deficit (water-stressed plants) by withholding water; (ii) salt stress by watering with Hoagland's solution but with an additional 100 mM of NaCl; or (iii) watering with Hoagland's solution (controls).

For analysis of tocopherols, jasmonic acid and the genes involved in the biosynthesis, perception and signaling of ethylene, young leaves were collected in the middle of the photoperiod at 0, 4, 7 and 11 d of treatment, immediately frozen in liquid nitrogen and stored at −80°C until analyses. Young and mature leaves were additionally collected at day 7 of

the experiment (before severe necrosis occurred in mature leaves of water-stressed plants) to examine the influence of leaf age.

Biomass, leaf water content and the F_v/F_m ratio

Biomass was determined after 11 d of treatment by measuring the rosette weight after drying the leaves in an oven at 80°C for 24 h. RWC was determined as 100 (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 for 24 h, and DW is the dry weight after oven-drying the leaves at 80°C to constant weight. The F_v/F_m was determined by using a pulse-modulated fluorimeter mini-PAM (Walz) after 1 h of dark adaptation, as described (Genty et al. 1989).

Analyses of tocopherols

For the extraction of α- and γ-tocopherol, leaf samples were ground in liquid nitrogen and extracted with ice-cold methanol using a Branson 2510 ultrasonic cleaner (Bransonic) for 45 min. The samples were then centrifuged for 15 min at 4°C and transferred to vials for analysis. The HPLC analysis was carried out as described (Amaral et al. 2005). In brief, the HPLC equipment consisted of an integrated system with a Waters 600 controller pump, a Waters 714 plus auto-sampler and an FP-1520 fluorescence detector (Jasco). Tocopherols were separated on an Inertsil 100A (5 μm, 30 × 250 mm, GL Sciences Inc.) normal-phase column operating at room temperature. The mobile phase used was a mixture of *n*-hexane and 1,4-dioxane (95.5 : 4.5, v/v) at a flow rate of 0.7 ml min^{-1}, and the injection volume was 10 μl. Detection was carried out for excitation at 295 nm and emission at 330 nm. Quantification was based on the fluorescence signal response compared with authentic standards of each compound (Sigma-Aldrich).

Gene expression analyses

RNA was isolated from leaf material using the RNEasy Plant Mini Kit (Qiagen) following the kit instructions, but incubated with DNase [RNase-Free DNase Set (Qiagen)] to eliminate the genomic DNA. The RNA was used for cDNA synthesis using GeneAmp RNA PCR (Applied Biosystems).

The most important genes related to ethylene biosynthesis, perception and signaling were analyzed by real-time PCR for each sample. At the same time, genes of tocopherol biosynthesis were analyzed only in wild-type plants. cDNAs were amplified using LightCycler 480 SYBR Green 1 Master (Roche), and the specific primers for each gene (**Supplementary Table S1**) were purchased from Eurofins MWG Operon. For the great majority of primers, the conditions were: 45 cycles of 95°C (10 s); 63°C (20 s); 72°C (20 s). For quantification, the ΔC_T method was used with the equation: ratio (reference gene, target gene) = $2^{CT\ (reference\ gene) - CT\ (target\ gene)}$, where CT is the cycle number at which enough amplified product accumulates to yield a detectable fluorescent signal, the reference gene was *GAPDH* (which encodes glyceraldehyde-3-phosphate dehydrogenase) and the target gene was the gene of interest.

Jasmonic acid analyses

Levels of jasmonic acid were analyzed by UPLC-MS/MS (ultra-performance liquid chromatography–tandem mass spectrometry) with a modification of the method described by Abreu and Munné-Bosch (2009). In brief, leaf samples were ground in liquid nitrogen and, after addition of deuterated jasmonic acid as an internal standard, extracted with methanol using sonication. The supernatant was dried completely under a nitrogen stream and the extract reconstituted in methanol. The extracts were then filtered through a 0.22 μm PTFE filter (Waters), and injected into the UPLC-MS/MS system. The UPLC system consisted of an Acquity Waters binary pump equipped with an autosampler and a UV detector. For analysis of the extracts, a HALO C18 column (2.1 × 75 mm, 2.7 μm) was used with a binary solvent system comprising acetonitrile (A) and deionized water (B), both containing 0.005% (v/v) glacial acetic acid. Separations were performed using a gradient of increasing acetonitrile content, at a constant flow rate of 0.4 ml min^{-1}. The gradient used was: 0 min, 1% A; 0–4.5 min, increasing to 45% A; 4.5–5 min, increasing to 99% A; 5–6 min, 99% A; 6–6.2 min, decreasing to 1% A; 6.2–7 min, 1% A. MS/MS analyses were performed on an API 3000 triple quadrupole mass spectrometer (PE Sciex). All the analyses were performed using the Turbo Ion spray source in negative ion mode. MRM acquisition was carried out by monitoring the following transition: JA, 209/59; and for the deuterated internal standard: JA* (d5), 214/62. Quantification by MS/MS using the MRM method was performed as described (Abreu and Munné-Bosch 2009). Results were corrected taking into account the specific recovery rates using the internal deuterated standard.

Statistical analyses

Statistical differences between measurements of the different treatments and ages were analyzed with analysis of variance (ANOVA) using SPSS software. Differences were considered significant at a probability level of $P < 0.05$.

Supplementary data

Supplementary data are available at PCP online.

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