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Essential role for phytol kinase and tocopherol in tolerance to combined light and temperature stress in tomato

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Abstract

In a changing environment, plants need to cope with the impact of rising temperatures together with high light intensity. Here, we used lipidomics in the tomato model system to identify lipophilic molecules that enhance tolerance to combined high-temperature and high-light stress. Among several hundred metabolites, the two most strongly up-regulated compounds were α-tocopherol and plastoquinone/plastoquinol. Both are well-known lipid antioxidants and contribute to the protection of photosystem II (PSII) against photodamage under environmental stress. To address the protective function of tocopherol, an RNAi line (vte5) with decreased expression of VTE5 and reduced levels of α-tocopherol was selected. VTE5 encodes phytol kinase, which acts in the biosynthetic pathway of tocopherols. vte5 suffered strong photoinhibition and photobleaching when exposed to combined high-light and high-temperature stress, but neither stress alone produced a visible phenotype. As vte5 had plastoquinone levels similar to those of the wild type under combined stress, the strong phenotype could be attributed to the lack of α-tocopherol. These findings suggest that VTE5 protects against combined high-light and high-temperature stress and does so by supporting α-tocopherol production.

Key words: High-light stress, high-temperature stress, lipidomics, phytol, phytol kinase, plastochromanol, plastoglobules, plastoquinone, Solanum lycopersicum, tocopherol.

Introduction

Plants are sessile organisms and are therefore constantly exposed to environmental challenges. Climate change scenarios include high temperatures (HT) that may occur in conjunction with high light (HL) intensity, which together could undermine plant survival and affect agricultural yields (Walther et al., 2002; Streb et al., 2003; Pretty et al., 2010).

Protection of the photosynthetic machinery embedded in the thylakoid membrane is paramount. Plastoquinone (mostly known as an electron transporter) and tocopherols are lipid-soluble molecules that act as antioxidants, preventing lipid peroxidation and photoinhibition by quenching reactive oxygen species (ROS) under HL intensities (Munne-Bosch and Alegre, 2002; Gruszka et al., 2008; Triantaphylidès and Havaux, 2009; Mène-Saffrané and DellaPenna, 2010; Nowicka and Kruk, 2012; Rastogi et al., 2014; Ksas et al., 2015; Miret and Munne-Bosch, 2015).

Tocopherols (collectively known as vitamin E) contribute to seed longevity, seedling development, and protection of...
the photosynthetic apparatus against oxidative stress (Sattler et al., 2004; Mène-Saffrané et al., 2010), and their synthesis is increased under these conditions (Collakova and DellaPenna, 2003; Munne-Bosch, 2005; Maeda et al., 2006; Loyola et al., 2012; Eugeni Piller et al., 2012; Quadrana et al., 2013; Spicher et al., 2016). In addition to its function as a lipid antioxidant, α-tocopherol physically stabilizes membrane structures by reducing membrane fluidity (Arora et al., 2000; Wang and Quinn, 2000). Yet, the conditions under which tocopherols are essential are currently not known. The synthesis of tocopherol requires condensation of the aromatic homogentisate ring derived from the plastidal shikimate pathway and a phytol diphosphate chain. The majority of the phytol required is salvaged from chlorophyll catabolism rather than synthesized de novo (vom Dorp et al., 2015; Almeida et al., 2016). The salvage pathway requires phytol kinase (VTE5) and phytol-phosphate kinase (VTE6), converting free phyto into phytyl diphosphate; vte5 and vte6 mutant plants accumulate less tocopherol (Valentin et al., 2006; Ischebeck et al., 2006; vom Dorp et al., 2015; Almeida et al., 2016). Apart from tocopherol, phytol may also be converted into fatty acid phytyl esters (FAPEs) (Ischebeck et al., 2006; Gaude et al., 2007; Lippold et al., 2012). This process detoxifies free phyto, which has detergent-like characteristics (Dörmann, 2007).

In this study, we demonstrate that VTE5, through its effect on tocopherol levels, plays an essential role in protection of tomato against combined HT and HL stress.

Materials and methods

Plant material

A tomato VTE5-knockdown transgenic line (SIVTE5-RNAi#1, here termed vte5, background in cv. Micro-Tom; MT) had been generated in a previous study (Almeida et al., 2016) by constitutively expressing an intron-spliced hairpin sequence targeting the SIVTE5 gene (Solyc03g071720). Wild-type (WT; Solanum lycopersicum, cv. MT) and vte5 plants were grown in soil under conditions referred to as control (250 μmol m⁻² s⁻¹ light, 16/8 h light/dark, at 20/18 °C, with 55% relative air humidity).

Stress treatments

Plants 5 to 6 weeks old were either kept under standard growth conditions (control, as described above) or subjected for 6 days to stress treatments: HL (800 μmol m⁻² s⁻¹ light, 16/8 h light/dark, at 20/18 °C), HT (250 μmol m⁻² s⁻¹ light, 16/8 h light/dark, at 38/30 °C), and a combination of HL and HT (800 μmol m⁻² s⁻¹ light, 16/8 h light/dark, at 38/30 °C).

Transmission electron microscopy

Transmission electron microscopy was used to analyze the chloroplast ultrastructure of WT and vte5 and to compare their ultrastructure under HL, HT, and the combination of HT+HL stress treatments. Leaf segments from tomato plants exposed for 6 continuous days to stress treatments were fixed under vacuum overnight in 0.1 M phosphate buffer (pH 6.8) containing 4% (w/v) formaldehyde and 5% (w/v) glutaraldehyde, washed three times in 0.1 M phosphate buffer (pH 6.8) for 20 min, and postfixed for 2 h with 1% (w/v) osmium tetroxide at 20 °C. Samples were washed in 0.1 M phosphate buffer and then dehydrated in a graded series of ethanol and acetone. Samples were first infiltrated overnight in Spurr resin (Polyscience). Leaf fragments were then placed in an appropriate mold in Spurr resin and heated at 60 °C for 24 h to allow solidification and embedding of the leaf section in the resin. Ultrathin sections of 90 nm were prepared using an Ultracut-E microtome (Reichert-Jung) equipped with a diamond knife (Diatome), mounted on copper grids, and contrasted with a saturated uranyl acetate solution in 50% ethanol (Watson, 1958) and with Reynolds' lead citrate (Reynolds, 1963). Ultrathin sections were observed with a Philips CM-100 electron microscope operating at 60 kV.

Determination of photosynthetic parameters

The ratio of maximum photochemical efficiency or optimum quantum yield of PSII (Fv/Fm), electron transport rate (ETR), and non-photochemical quenching (NPQ) were fluorometrically determined using a MINI-PAM Photosynthesis Yield Analyzer (Walz). Plants were dark-adapted for 15 min before Fv/Fm measurements with illumination by application of a saturation flash. Measurements were always made in the green photosynthetic tissue. At least four replicates for each treatment were performed, at day 0 as a baseline measurement before the application of stress, and at days 2, 4, and 6 after the start of continuous stress treatment. On day 7, all plants were exposed to control conditions in order to assess recovery after stress. Another measurement was taken after 5 days of recovery (day 11).

Untargeted lipid profiling

After 6 days of the HT, HL, or combined HT+HL treatment, at least four replicates of leaf material from each treatment were harvested for untargeted lipid analysis (Martinis et al., 2013). Lipids were extracted from 100 mg fresh leaf tissue using 1 ml tetrahydrofuran:methanol 50:50 (v/v) according to Spicher et al. (2016). After centrifugation, the supernatant was directly analyzed by ultra-high-pressure liquid chromatography (UPLC™, Waters) coupled to quadrupole time-of-flight mass spectrometry (QTOF-MS; Synapt G2, Waters) through an atmospheric pressure chemical ionization (APCI) interface. To evaluate variations in the WT and vte5, features were extracted from lipidomics data using Markerlynx XS (Waters), mean-centered, and Pareto-scaled before applying principal component analysis (PCA) (Martinis et al., 2011; Eugeni Piller et al., 2011). The changes in lipid content after the stress treatments were established after the PCA, reducing data complexity. Identification of the variables of interest was achieved through comparison with pure standards whenever available.

When standards were not available, tentative identification was performed by combining determination of elemental compositions with accurate mass and isotopic ratios provided by QTOF-MS, fragmentation by collision-induced dissociation to obtain characteristic fragments, and searches in online databases such as LIPID MAPS (http://www.lipidmaps.org/data/structure/LMSDSearch.php?Mode=SetupTextOntologySearch) and PUBCHEM (https://pubchem.ncbi.nlm.nih.gov/search/search.cgi#).

Targeted lipid profiling: prenylquinone, carotenoid, and glycolipid profile

Absolute concentrations of δ-tocopherol (δ-T), γ-tocopherol (γ-T), α-tocopherol (α-T), α-tocopherolquinone (α-TQ), plastoquinone (PQ-9), plastochoanol (PC-8), and phylloquinone (vitamin K) were measured based on calibration curves obtained from standards as described by Martinis et al. (2013). Plastoquinol (PQH-9), hydroxy-plastoquinone (PQ-OH), and hydroxy-plastochoanol (PC-OH) were quantified as PQ-9 and PC-8 equivalents, respectively. Tocopherol and phylloquinone standards were purchased from Sigma-Aldrich. Pure standards of PQ-9 and PC-8 were obtained by Sigma-Aldrich. Preparative liquid chromatography (UPLC) coupled to quadrupole time-of-flight mass spectrometry (QTOF-MS; Synapt G2, Waters) through an atmospheric pressure chemical ionization (APCI) interface. To evaluate variations in the WT and vte5, features were extracted from lipidomics data using Markerlynx XS (Waters), mean-centered, and Pareto-scaled before applying principal component analysis (PCA) (Martinis et al., 2011; Eugeni Piller et al., 2011). The changes in lipid content after the stress treatments were established after the PCA, reducing data complexity. Identification of the variables of interest was achieved through comparison with pure standards whenever available.

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was obtained by saponification of the oil followed by partition with a hexanecetyl acetate mixture (Siger et al., 2014). The resulting extract was then separated by open-column chromatography and semi-preparative HPLC as for PQ-9 isolation. A detailed description of the purification process is given in Supplementary Protocol S1 at JXB online. The two carotenoids violaxanthin and neoxanthin were measured as a sum, since they could not be resolved in either the chromatographic or the mass dimensions under the conditions employed.

The other molecules identified for which pure standards were unavailable were quantified relatively based on peak intensity measurements in the chromatograms. The data obtained from the measurements were subjected to one-way ANOVA, followed by Holm-Sidak test comparisons versus control in WT, to determine any significant differences between different temperatures and light conditions over the time course of the experiment.

**Free phytol and fatty acid phytol ester quantification**

Total lipids were extracted from ~20 mg lyophilized leaf tissue with diethyl ether and 300 mM ammonium acetate. Next, FAPes and free phytol were purified via solid-phase extraction on silica columns (Strata SI-1, 100 mg, Phenomenex) using a step gradient of n-hexane and diethyl ether (www.cyberlipid.org): FAPes were eluted with n-hexane-diethyl ether 99:1 (v/v), while free phytol was eluted with n-hexane-diethyl ether 92:8 (v/v). FAPes and free phytol were analyzed as previously described (von Dorp et al., 2015).

**Quantitative real-time PCR analysis**

RNA extraction, cDNA synthesis, and quantitative real-time PCR (qPCR) assays were performed as described by Quadrana et al. (2013). Specific primers to genes in the tocopherol biosynthetic and related pathways were designed. Primer sequences are listed in Supplementary Table S1. qPCRs were performed in a 7500 real-time PCR system (Applied Biosystems) using 2x SYBR Green Master Mix reagent (Applied Biosystems). Expression values were normalized against the geometric mean of two reference genes, CAC and EXPRESSED (Quadrana et al., 2013). RNA samples with absorbance ratios at 260/280 nm between 1.95 and 2.1, indicating high purity, and showing sharp and clear rRNA 28S/18S bands on an agarose gel, as an indication of structural integrity, were used for subsequent cDNA synthesis. A permutation test lacking sample distribution assumptions (Pfaffl et al., 2002) was applied to detect statistically significant differences (P<0.05) in expression ratios using the algorithms in the fgStatistics software package (Di Rienzo et al., 2009).

**Isolation of thylakoid membranes and Hill reaction**

Thylakoid membranes were prepared from 50 g of fresh leaves from 18-day-old pea (Pisum sativum) plants, which were grown under control conditions with minor modifications (Fitzpatrick and Keegstra, 2001; Smith et al., 2003; Kummerova et al., 2006, 2008). The Hill reaction was adapted and carried out to measure the direct effect of phytol on the photosynthetic primary reaction at the level of PSII. The total reaction mixture (4 ml) contained 0.03 mM sodium salt solution of 2,6-dichlorophenolindophenol (DCPIP; redox system), 20% dimethyl sulfoxide (DMSO, Sigma-Aldrich) and 0.5 and 1 mg thylakoid membranes in suspension buffer. Phytol (97%), mixture of isomers) was purchased from Sigma-Aldrich and suspended in DMSO at a final concentration of 10 mM. Phytol was used to obtain final concentrations of 500 μM and 50 μM. The reactions were incubated for 5 minutes and then exposed for 6 minutes to an irradiance of 200–250 μmol m−2 s−1 at room temperature. A 1 ml aliquot was removed at 3 and 6 minutes, centrifuged for 1 min at 16 000 g, and measured spectrophotometrically at 600 nm. Photosynthetic activity was determined by measuring DCPIP reduction using an UltraViolet-Visible Spectroscopy (UV/VIS) Ultraspec 3100 pro (Amersham Biosciences) in 1 cm UV-visible cuvettes. Measurements were repeated at least three times at each time point.

**Results**

**Untargeted lipidomics demonstrate changes in lipid composition upon combined high-light and high-temperature stress**

To determine variations in the lipid composition of WT tomato plants after exposure to HL, HT, and the combination of HT+HL, we carried out untargeted lipidomics analysis (Fig. 1). Lipid extracts were isolated from fresh leaf tissue and analyzed by ultrahigh-pressure liquid chromatography coupled with atmospheric pressure chemical ionization-quadrupole time-of-flight mass spectrometry (UHPLC-APCI-QTOF-MS). PCA identified four distinct clusters when comparing the control condition with the different stress conditions (Fig. 1A). In the loadings plot (Fig. 1B), the most contributive features of the first principal component (PC1) were selected and characterized by a combination of tandem mass spectrometry analysis and consultation of databases such as LIPID MAPS. This revealed that prenylquinones, α-tocopherol, and plastoquinol (PQH2-9) contributed most to the separation of the HT+HL cluster. The HT cluster was characterized by the accumulation of the saturated galactolipid MGDG-18:3/16:0, and the control and HL clusters were specifically separated from the other clusters, represented by MGDG-18:3/16:3, MGDG-18:3/18:3, and DGDG-18:3/18:3 (Fig. 1B). Loadings located near the center of the plot, (i.e. the vast majority) made a negligible contribution to metabolic variation.

**vte5 develops a chlorotic phenotype under combined high-light and high-temperature stress**

To investigate the role of tocopherol in resistance to HT+HL stress, a VTE5-deficient tomato line (vte5) was used (Almeida et al., 2016). The vte5 line contains diminished levels of phytol kinase and therefore accumulates reduced levels of tocopherol in leaves and fruits (Almeida et al., 2016). The line was subjected to the HL, HT, and HT+HL conditions. HL treatment alone had little effect on WT and vte5 plants. Under HT treatment, no differences were apparent between WT and vte5 or when compared with control conditions. While WT plants developed a mild bleaching phenotype only in the oldest leaves after 6 days under the HL+HT condition, vte5 plants were extensively photobleached and developed chlorotic leaves (Fig. 2); the chlorosis became visible after 2 days under HL+HT. The visible phenotype after 4 days is shown in Supplementary Fig. S1.

**Combination of high light and high temperature triggers photoinhibition in vte5 plants**

To determine the effects of HL, HT, and HT+HL stress on photosynthetic activity, photosynthetic parameters were determined by measuring chlorophyll fluorescence
(Supplementary Fig. S2). After 6 days of stress, WT and vte5 plants were allowed to recover for 5 days under control conditions. The control plants were permanently grown under control conditions. Significant differences in the photosynthetic parameters were observed under HT+HL when comparing the two genotypes (Fig. 3). In vte5 a strong and increasing reduction of the photochemical efficiency of PSII ($F_{v}/F_{m}$) was observed, resulting in almost complete photoinhibition after 6 days. The vte5 plants reached close to normal values after 5 days of recovery under control conditions (Fig. 3, top panel). In WT, $F_{v}/F_{m}$ decreased slightly under HT+HL but recovered while still under the combined stress. Both WT and vte5 exhibited a significant reduction of NPQ under HL+HT. However, NPQ in WT began to recover at day 6 of HT+HL, whereas in vte5 this parameter improved only during the 5-day recovery period under control conditions. ETR in WT under the HT+HL treatment remained almost constant throughout the stress treatment and recovery period. In
contrast, HT+HL provoked a rapid and severe drop in ETR in vte5, with a recovery to control values after a 5-day recovery period.

**Plastoglobules accumulate under HT+HL in vte5**

Electron microscopy was carried out on leaf sections of plants exposed to control, HL, HT, and HT+HL conditions to determine the impact of the different conditions on chloroplast ultrastructure (Fig. 4 shows representative images). Under control conditions, WT (Fig. 4A) and vte5 (Fig. 4B) chloroplasts were very similar. Under HL conditions, chloroplasts in both WT and vte5 contained large starch granules (Fig. 4C and D) and larger plastoglobules also appeared, particularly in vte5. The vte5 plastoglobules were surrounded by a non-osmiophilic ring appearing to contain additional, smaller globular structures (Fig. 4D). Under HT treatment, highly stacked thylakoids were observed and large plastoglobules surrounded by non-osmiophilic rings appeared in both WT and vte5 (Fig. 4E and F). Under HL+HT, WT chloroplasts contained large plastoglobules with non-osmiophilic rings, and thylakoids appeared disorganized and swollen (Fig. 4G). vte5 chloroplasts under HT+HL contained diminished and scattered thylakoids, increased numbers of plastoglobules (mostly without non-osmiophilic rings), as well as areas that contained amorphous material (Fig. 4H).

**Stress treatments change prenylquinone and carotenoid metabolism**

Knockdown of vte5 results in tocopherol deficiency (Almeida et al., 2016). Under control conditions, the α-tocopherol content in vte5 was one-third of that in WT, and other tocopherols displayed similar ratios (Fig. 5). Under both HL and HT stress, a 1.6-fold increase in α-tocopherol content was observed in WT. When exposed to the combination of HT+HL stress, WT plants showed a striking 2.8-fold increase in α-tocopherol. This indicates that HL and HT have additive effects on α-tocopherol accumulation in WT. In vte5, α-tocopherol also increased in an additive manner under the HT+HL condition, but only to around half the value observed in WT (Fig. 5). In addition, vte5 showed a 3-fold increase in α-tocopherolquinone (α-TQ) when compared with WT under the HT+HL stress condition (Fig. 5).

Plastoquinone [consisting of oxidized PQ-9 (plastoquinone) and reduced PQH$_2$-9 (plastoquinol)] and its derivatives PQ-OH (hydroxy-plastoquinone), plastochromanol-8 (PC-8), and hydroxy-plastochromanol-8 (POH) were also measured (Fig. 6). PQH$_2$-9 and PQ-OH concentrations were similar in WT and vte5 under all conditions. PQ-9 was similar in WT and vte5 under control conditions and the single-stress conditions, but was increased 1.5-fold in vte5 compared with WT under the combined HT+HL stress (Fig. 6). PC-8 was reduced by around half in vte5 compared with WT under HT and HT+HL, while PC-OH was similar in WT and vte5. Phylloquinone concentrations were not strongly affected in vte5 and WT under any of the conditions (Fig. 6).

No significant changes in β-carotene and violaxanthin+neoxanthin concentrations were observed under any conditions when comparing WT with vte5 (Supplementary Fig. S3). However, lutein increased 1.5- and 1.3-fold under HT in WT and vte5 with respect to control conditions.

**Fatty acid phytyl esters accumulate massively in the vte5 mutant**

Quantification of FAPES indicated a striking accumulation under combined HT+HL stress in WT, but at far lower levels than in vte5. The most notable molecular species were 16:0-, 18:3-, 18:2-, 18:1-, and 18:0-phytol (Fig. 7). 18:0-phytol was the most abundant phytyl ester. In vte5 its accumulation was 36-fold greater than in the WT exposed to HT+HL treatment.

Free phytol levels increased under combined high-light and high-temperature stress

vte5 plants accumulate free phytol in the leaf (Fig. 8) (Almeida et al., 2016). When exposed to combined HT+HL, vte5 plants had 7.6-fold higher concentrations than under control
conditions. Even more strikingly, free phytol concentrations under HT+HL were 26-fold greater than in WT.

Phytol toxicity assessed by electron transport activity

High free phytol concentrations were detected in vte5 exposed to combined HT+HL. Phytol is presumed to be toxic due to its detergent-like structure and may therefore contribute to the photobleaching phenotype observed in vte5 under HT+HL. To determine whether phytol may exert toxicity via perturbation of the thylakoid membrane and of photosynthetic electron transport, we carried out the Hill reaction in the presence of phytol. The Hill reaction is a method to measure photosynthetic electron transport in isolated thylakoid membranes (P. sativum) at the level of PSII (Fig. 9). In the Hill reaction, the synthetic electron acceptor DCPIP is reduced; this reaction can be measured spectrophotometrically. In the absence of phytol, DCPIP reduction was arbitrarily set to 100%; in a negative control experiment in the absence of thylakoids, DCPIP reduction did not occur and was set to 0%. The effects of the presence of two concentrations of phytol (50 µM and 500 µM) on the Hill reaction were measured. The phytol concentrations were calculated to be 50-fold and 500-fold those measured in vivo in vte5 leaves under HT+HL. Two separate experiments were carried out using isolated thylakoids corresponding to either 0.5 mg or 1 mg chlorophyll per reaction. The Hill reaction was significantly inhibited only at 500 µM phytol.

Gene expression profiles

To determine whether the observed biochemical changes could be due to altered gene expression under the HL, HT, and HT+HL conditions, transcript levels of genes encoding proteins involved in methyl-D-erythritol 4-phosphate, carotenoid, prenylquinone, chlorophyll, and phytol metabolism (Almeida et al., 2016; Lira et al., 2016) were measured by qPCR (Supplementary Fig. S4).

VTE5 showed a down-regulation of 76% in the vte5 line under control conditions, close to the value previously published in Almeida et al. (2016). Under both the HL and HT conditions the expression patterns of genes encoding enzymes involved in tocopheran, prenylquinone, chlorophyll degradation, carotenoid, and FAPE metabolic pathways (see Supplementary Fig. S4 for genes) were remarkably similar in both WT and vte5. Even under combined HT+HL stress, differences between WT and vte5 were rather limited. However, some differences were observed: First, in WT as well as vte5, the abundance of the 1-deoxy-D-xylulose-5-P synthase (DXS1) transcript was diminished under HL and HT+HL. This potentially limits carbon supply to the plastidial isoprenoid pathway. However, this did not appear to be the case: with the exception of α-tocopherol in vte5, prenyllipids were unchanged or increased, particularly under the combined HT+HL stress.

NDC1 expression was remarkably up-regulated in vte5 under HT+HL. In contrast, VTE1 expression was lower in vte5 than in WT under the same stress conditions. The low VTE1 transcription level correlates with (i) with the accumulation of α-TQ, indicating insufficient activity in the redox recycling of α-TQ by VTE1, and (ii) the reduced levels of PC-8 that were observed in vte5 (Fig. 6). In both WT and vte5, geranylgeranyl reductase (GGDR) mRNA abundance was diminished under all stress conditions except HL in WT, potentially provoking a reduction of chlorophyll synthesis. Higher expression of the plastoglobule-associated enzyme PES (phytol ester synthase) was observed to similar degrees under the HL and HT+HL conditions in both WT and vte5. However, only in vte5 was a dramatic increase of FAPEs actually observed. This suggests that the transcriptional
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regulation had only a limited role in diverting the metabolic flux into the FAPE biosynthetic pathway.

Discussion

Photosynthetic metabolism is among the primary processes that are strongly affected by environmental perturbations (Lichtenthaler and Burkart, 1999; Tezara et al., 1999; Chaves et al., 2009; Mathur et al., 2014). Changes in temperature, in combination with other parameters such as high incident light intensity, constitute powerful challenges to plants. There is a compelling need to understand the mechanisms underlying plant adaptation to such multiple stresses connected to climate change. Only a few studies have investigated the impact of simultaneous HL and HT on the photosynthetic apparatus of plants (Qiu and Lu, 2003; Streb et al., 2003; Quiles, 2006; Buchner et al., 2015; Krause et al., 2015; Gerganova et al., 2016; Faik et al., 2016).

Under combined HT+HL, adaptation of the photosynthetic machinery is mediated by changes in protein levels and remodeling of the thylakoid membrane (Burgos et al., 2011; Szymanski et al., 2014; Zhao et al., 2016). This response includes not only membrane lipids but also lipophilic antioxidants, including tocopherols, plastoquinone, and plastochochromanol. Both tocopherols and plastoquinone have been implicated in resistance to HL. A recent study that carefully

Fig. 4. Changes in chloroplast ultrastructure under the control condition and HL, HT, and combined HT+HL stress. Transmission electron micrographs of WT (A, C, E, G) and vte5 (B, D, F, H) leaves after 6 days of control (A, B), HL (C, D), HT (E, F), and combined HL+HT (G, H) treatments. Bar=500 nm.
dissected the distinct roles of tocopherols and plastoquinone indicated a predominant role for plastoquinone under HL (Kas et al., 2015). This was somewhat surprising, as plastoquinone is normally associated with electron transport and the role of a lipid antioxidant is usually attributed to tocopherols. Our experiments aimed to determine what makes plants (using tomato as the model system) tolerant to prolonged HL, HT, and combined HL+HT stress.

WT tomato plants exposed to HL, HT, or HT+HL stresses exhibited a striking increase in prenylquinone contents. In particular, α-tocopherol and plastoquinol were elevated. Compared with HT or HL alone, a near doubling of both compounds under combined HT+HL stress was observed, indicating an additive effect of the two stress conditions. The production of tocopherols has previously been correlated with oxidative stress (Havaux et al., 2005; Kobayashi and DellaPenna, 2008). Our own recent work has suggested a correlation of the increase in α-tocopherol and plastoquinone with the normal functioning of the photosynthetic apparatus under HT stress (Spicher et al., 2016). That study was, however, unable to resolve whether tocopherol or plastoquinone was the main contributor.

As a tool to investigate the role of tocopherol in protecting the photosystem, we used a line (vte5) in which phytol kinase (VTE5) is silenced. VTE5, which salvages phytol from chlorophyll degradation, contributes to ~70% of tocopherol biosynthesis. vte5 plants were highly sensitive to combined HT+HL but not the single stresses; HT+HL resulted in extensive photobleaching (Fig. 2). This phenotype was likely brought about by photoinhibition, as was apparent from the severe reduction of Fv/Fm in vte5 (Fig. 3). The tolerance of WT but not vte5 plants to HT+HL suggested that tocopherol metabolism holds the key to HT+HL resistance in tomato.

When comparing the prenyllipid profiles of WT and vte5 under HT+HL, tocopherols were diminished in vte5, in agreement with the defect in the tocopherol salvage pathway. In contrast, PQH2-9 (the reduced form of plastoquinone) was unchanged, and PQ-9 (the oxidized form of plastoquinone) was increased in vte5. This increase in PQ-9 is probably due to photoinhibition affecting PSII and its ability to reduce PQ-9. These findings provide strong evidence for the role of tocopherol rather than plastoquinone in tolerance to HT+HL stress. The amount of PC-8, the VTE1-dependent chromanol derivative of plastoquinone, was reduced under HT+HL. However, PC-8 is present at a far lower concentration than the oxidized and reduced forms of plastoquinone and α-tocopherol, and is therefore unlikely to contribute in a major way to HT+HL resistance. A possible explanation for the reduced concentration of PC-8 is the observed down-regulation of VTE1 in the vte5 background. This down-regulation under HT+HL may also be responsible for the accumulation of α-TQ, an α-tocopherol oxidation product that depends on VTE1 for recycling to α-tocopherol (Eugeni Piller et al., 2014). Moreover, this is additional evidence for the profound defect in tocopherol metabolism in vte5.

Why would tocopherols be particularly important for tolerance to combined HT+HL stress? Besides its antioxidant functions, α-tocopherol acts as a membrane component. It partitions into the hydrophobic phase of a membrane lipid bilayer and reduces membrane fluidity by physical stabilization of the membrane structure (Arora et al., 2000; Wang and Quinn, 2000). Reduction of membrane fluidity may therefore contribute to the adaptation of the thylakoid
membrane to HT. Although the membrane-stabilizing effect has not been shown in artificial membranes with thylakoid lipid composition, it is tempting to propose that the protective function under HT+HL lies in the combination of α-tocopherol’s antioxidant and membrane-stabilizing properties.

The inability of vte5 to salvage phytol resulting from chlorophyll degradation leads to the accumulation of free phytol and FAPE biosynthesis (Lippold et al., 2012; Almeida et al., 2016). The FAPE content rose significantly in response to HL, HT, and HT+HL in both vte5 and WT, but in a far more dramatic fashion in vte5 (Fig. 7). FAPE synthesis is boosted by the high levels of free phytol that arise due to the increase in chlorophyll catabolism and simultaneous lack of VTE5 activity. However, not only FAPEs increased; free phytol also reached much higher levels in vte5 than in WT. Under HT+HL the levels were particularly high, and this can probably be attributed to increased chlorophyll catabolism under these photobleaching conditions.

Phytol is a long-chain alcohol which, similar to free fatty acids, may exert detergent-like effects that may be detrimental to membrane function (Sikkema et al., 1995; Löbbecke and Cevc, 1995). In contrast, FAPEs (which lack a detergent-like structure) are not considered to be toxic and, in addition, they are sequestered in plastoglobules (Fig. 4) (Gaude et al., 2007; Lippold et al., 2012). Indeed, plastoglobule numbers visibly increased in vte5 under HT+HL (Fig. 4). Free phytol may affect photosynthetic function and lead to photoinhibition and photobleaching in its own right. To determine the integrity of the photosynthetic electron transport chain, we carried out the Hill reaction in the presence of two concentrations of free phytol. Concentrations that were 500 times those measured in vte5 under HT+HL only slightly reduced electron transport. These results strongly suggested that photoinhibition and photobleaching in vte5 were not due to free phytol accumulation. Taken together, the evidence presented here indicates that tocopherols, more than any other of the metabolites

Fig. 6. Plastoquinone, plastoquinone-derived quinones, and phyloquinone content in WT and vte5 after exposure to control, HL, HT, and combined HT+HL stresses. Lipids were extracted from WT and vte5 plants subjected to control or stress conditions for 6 days; lipids were analyzed by UHPLC-APCI-QTOF-MS. Values are the means±SE of at least four biological replicates. Asterisks indicate significant differences between the WT (control) and vte5 under the various stress treatments (one-way ANOVA, followed by Holm-Sidak post hoc test): *P<0.05, **P<0.01, ***P<0.001.
analyzed, are responsible for tolerance to combined HT and HL stress in tomato.

Supplementary data

Supplementary data are available at JXB online.
Protocol S1. PQ and PC purification.
Table S1. Primers used for each experiment.
Fig S4. Expression of genes encoding isoprenoid metabolism-related enzymes.

Author contributions

FK and LS designed the research. LS carried out the experimental work and statistical analysis. MR provided tee5 line. LS, MR, and JA conducted gene expression analysis. KD and PD measured phytol and fatty acid phytyl esters. RP helped to prepare TEM cuts and observations. GG developed the gene expression analysis. KD and PD measured phytol and fatty acid phytyl esters. Nitrogen deficiency in Arabidopsis affects galactolipid composition and affects prenyllipid metabolism in an organ-specific manner. Journal of Experimental Botany 67, 919–934.

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