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The role of alpha-tocopherol in the protection of tomato plants against abiotic stress

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Abstract

The ability of energy conversion by the photosynthetic machinery under stress and its capacity to adjust to an ever-changing environment is crucial for plant survival. The photosynthetic light reactions occur at the photosystems in the thylakoids of chloroplasts. The photosystems are composed of proteins in a specific lipid environment. It includes not only membrane lipids but also lipophilic pigments (chlorophylls, carotenoids) and prenylquinones (plastoquinone, phylloquinone, tocopherol). Apart from their respective roles in light harvesting and electron transport, carotenoids and prenylquinones have important antioxidant properties and protect plant cells against reactive oxygen species. The focus of this work is to understand how plants resist and adapt to environmental stress in particular high light, high temperature and the combination of the two. Lipid metabolism takes places in plastid subcompartments, at the level of envelopes, at thylakoid microdomains called plastoglobules. Plastoglobules are involved in various essential biosynthetic metabolic pathways and accumulation of prenylquinone molecules. In this thesis, we use tomato as the model system to address the role of (prenyl) lipids synthesis and remodelling to protect photosynthetic function under stress. After an introduction on the implication of photosynthetic machinery in lipid metabolism, in Chapter 2 we summarized recent advances in plastoglobule research and their findings on biosynthesis and metabolism of Vitamins E and K_1. Then in Chapter 3, we investigate the question of how the photosynthetic machinery is protected against heat stress. Amongst many hundreds of compounds that change under heat stress, we identified α-tocopherol and plastoquinone as the most significantly increased antioxidants. This finding suggests a new role for these two prenylquinones in protecting the photosynthetic apparatus against temperature stress. In Chapter 4, through a joint effort, we provided valuable information on the metabolic fluxes and biosynthesis of Vitamin E in tomato. Finally, in Chapter 5, we intended to identify molecules that contribute to the protection against combined high temperature and high light stress. To perturb α-tocopherol levels we used the tomato *vte5* knock down-line. The data indicate that VTE5 protects against combined high light and high temperature stress and does so by supporting α-tocopherol production. Overall, this

thesis contributes to a better understanding of the role of prenylquinone compounds, in the resistance of tomato plants against high light and high temperature stresses.

Keywords: lipidomics, prenylquinones, photosynthesis, temperature stress, high light stress, tocopherol, Vitamin E, plastoquinone, plastochromanol, carotenoids, *F*v/*F*m, plastoglobules, phytol, phytol kinase, *Solanum lycopersicum*, tomato.

Résumé

La capacité de conversion de l'énergie par les chloroplastes en condition de stress et son aptitude à s'adapter à un environnement en constante évolution est cruciale pour la survie des plantes. Les réactions photosynthétiques se produisent au niveau des photosystèmes dans les thylakoïdes des chloroplastes. Les photosystèmes sont composés de protéines dans un environnement lipidique spécifique. Ce dernier comprend non seulement des lipides membranaires mais aussi des pigments lipophiles (chlorophylles, caroténoïdes) et des prénylquinones (plastoquinone, phylloquinone, tocophérol). À part leurs rôles de collecteur de lumière et leurs implications dans le transport d'électrons, les caroténoïdes et les prénylquinones (respectivement) ont d'importantes propriétés antioxydantes et protègent les cellules végétales contre les espèces réactives à l'oxygène. Le but de ce travail est de comprendre comment les plantes résistent et s'adaptent aux stress environnementaux, en particulier aux fortes intensités lumineuses, à la hausse de température et à la combinaison des deux. Le métabolisme des lipides prend place dans les sous-compartiments des plastides, au niveau des enveloppes, des membranes des thylacoïdes et de ses microdomaines, appelés plastoglobules. Les plastoglobules sont impliqués dans diverses voies métaboliques biosynthétiques essentielles et dans l'accumulation des molécules de prénylquinone. Dans cette thèse, nous avons utilisé la tomate comme système modèle afin d'étudier le rôle de la synthèse des (prényl) lipides ainsi que leur remodelage dans la protection de la fonction photosynthétique en condition de stress. Après une introduction sur l'implication des chloroplastes dans le métabolisme des lipides, nous avons résumé, dans le chapitre 2, les récents progrès de la recherche sur les plastoglobules et leurs implications sur la biosynthèse et le métabolisme de la vitamine E et de la vitamine K_1. Ensuite, dans le chapitre 3, nous avons étudié comment le chloroplaste est protégé contre le stress dû à la hausse de la température. Parmi les centaines de composés qui changent sous stress thermique, nous avons identifié l'α-tocophérol et la plastoquinone comme étant les antioxydants les plus significativement en hausse. Cette découverte suggère un nouveau rôle pour ces deux prénylquinones dans la protection de l'appareil photosynthétique contre le stress thermique. Dans le chapitre 4, nous avons fourni des informations précieuses sur les flux métaboliques et de biosynthèse impliqués dans l'accumulation de la vitamine E chez la tomate. Enfin, au chapitre 5, nous avons cherché à identifier les molécules qui contribuent à la protection contre le stress dû à la hausse des températures combiné au stress de haute

intensité lumineuse. Pour perturber les niveaux d'α-tocophérols, nous avons utilisé un mutant chez la tomate ayant perdu la fonction du VTE5 (*vte5*). Les données indiquent que le VTE5 protège la plante contre la hausse des températures combinée au stress de forte intensité lumineuse en soutenant la production d'α-tocophérol. D'une manière générale, cette thèse contribue à une meilleure compréhension du rôle des prénylquinones impliqués dans la résistance chez la tomate au stress de forte intensité lumineuse combiné à la hausse de température.

Mots-clés: lipidomique, prénylquinones, photosynthèse, stress de température, lumière de forte intensité, tocophérol, Vitamin E, plastoquinone, plastochromanol, caroténoïdes, *F*v/*F*m, plastoglobules, phytol, phytol kinase, *Solanum lycopersicum*, tomate.

Contents

1 General introduction

1.1 Introduction

Plants have to adapt continually to environmental changes. With the changes in climate, including increased temperatures in combination with high light intensity, plants incur losses and shifts of their original habitat (Pretty et al., 2010; Streb et al., 2003; Walther et al., 2002). The inability of plants to adapt to environmental changes leads to significant physiological perturbations that affect photosynthesis (Mishra and Singhal, 1992). As a consequence climate changes increase pressure on agriculture, especially crop plant productivity.

Plant growth and development depends on photosynthesis. Photosynthesis is an essential process that enables life on Earth (Jajoo, 2014) and the major bioenergetic activity that takes place at the thylakoid membranes in the chloroplasts (Eberhard et al., 2008). The photosynthetic light reactions generate ATP, NADPH, and molecular oxygen. Using the ATP and NADPH, the "dark" reactions assimilate carbon dioxide into organic compounds, initially in the form of starch (Waters and Langdale, 2009). Starch degradation products will then either be exported to the cytosol or directly used in the chloroplast stroma as a primary carbon source for plant biomass production.

Light is essential for plant growth and productivity. However excessive light can cause severe stress in plants by damaging the photosynthetic reaction centers (Lichtenthaler, 1999; Takahashi and Badger, 2011). In combination with increased temperature, excessive light leads to dramatic changes in the structure of photosynthetic machinery, consequently altering the photosynthetic capacity of plants, and disrupting cellular homeostasis (Bita and Gerats, 2013; Wahid et al., 2007).

1.1.1 Plastids adapt to changes in environmental conditions

Plastids are essential organelles of endosymbiotic origin. Plastid occur in higher plants in various sizes, shapes, and functions (Thomson and Whatley, 1980). Depending on environmental stimuli and developmental signals, plastids develop into distinct, tissue-specific types, including chloroplasts, chromoplasts, amyloplasts, elaioplasts, gerontoplasts (Figure 1.1) (Jarvis and López-Juez, 2013; Lopez-Juez and Pyke, 2005; Wise, 2006).

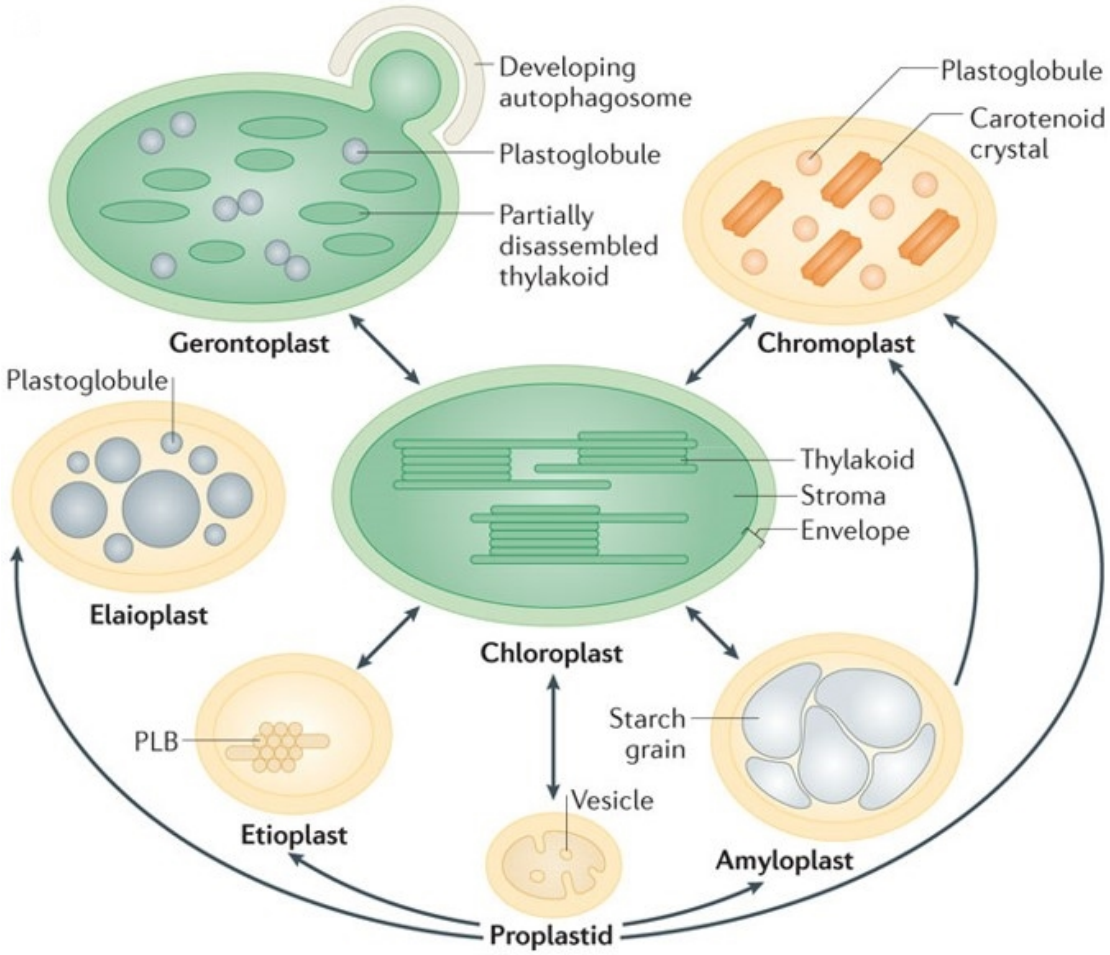


Fig. 1.1: Plastid diversity and interconversions. Plastids differentiate in various functional organelles depending on tissue, developmental stage, hormonal and environmental cues. Proplastids are totipotent plastids present in the meristematic tissue. Etioplasts are chloroplast precursors that develop in the absence of light and accumulate the chlorophyll precursor protochlorophyllide in the prolamellar body (PLB, paracrystalline membranous structure). Upon illumination etioplasts, rapidly differentiate into chloroplasts containing the vast thylakoid membrane system. Leucoplasts are non-pigmented, storage plastids. This group comprises amyloplasts and elaioplasts. Amyloplasts store starch and are present in storage tissues such as cotyledons, endosperm, and tubers. Elaioplasts store lipids and exist, for example, in the functional layer of nutritive cells within the anther during pollen development or epidermal cells of some monocotyledonous families. Chromoplasts accumulate colored carotenoids and are associated with reproductive tissues in flowers and fruits, having roles in attracting pollinators and seed disseminators. Gerontoplasts differentiate from chloroplasts during senescence and have highly regulated catabolic activities including the disassembly of the photosynthetic machinery, autophagic recycling and reallocation of resources to seed and perennial tissues (Jarvis and López-Juez, 2013; Neuhaus and Emes, 2000; Rottet et al., 2015; Waters and Langdale, 2009)

Moreover, in its photosynthetic role, the chloroplast adapts to varying environmental conditions, remodeling its membrane system to increase or decrease light harvesting surface (Figure 1.2) (Lichtenthaler and Burkart, 1999).

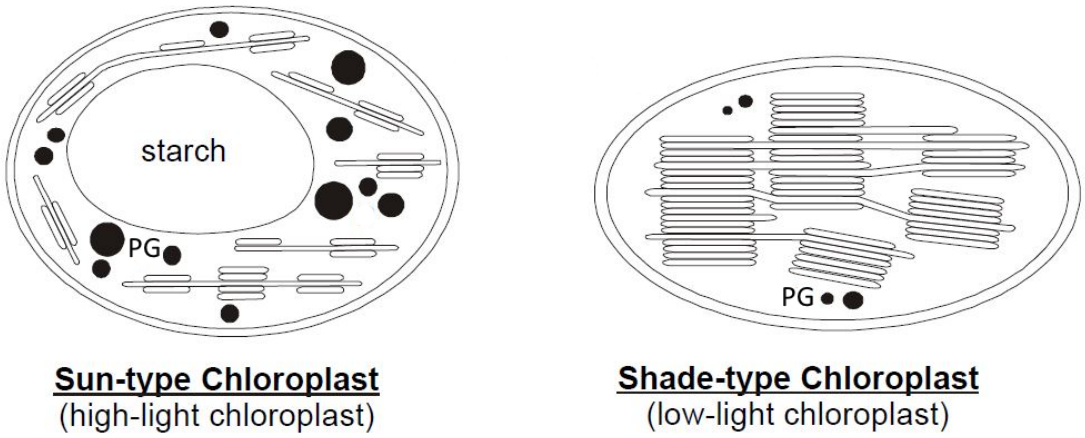


Fig. 1.2: Chloroplast ultrastructural adaptation in response to high-light and low-light. Sun-type chloroplasts exhibit a reduction of the grana stacks and abundance of light-harvesting complex-LHCII, large starch grains, and enlargement of plastoglobules when compared to chloroplasts in shade-grown plants (Lichtenthaler, 2010). Adapted from Lichtenthaler and Burkart (1999)

1.1.2 Chloroplasts - a primary site of diverse metabolic pathways

Besides supplying energy through photosynthesis, chloroplasts, fulfill other important metabolic roles (Lopez-Juez and Pyke, 2005; Neuhaus and Emes, 2000; Rolland et al., 2012). Chloroplasts synthesize an immense variety and quantity of lipophilic compounds that serve different purposes within the cell. The implication of these chloroplast lipids range from structural roles, such as membrane biogenesis (Andersson et al., 2001; Benning, 2008, 2009; Kobayashi et al., 2007, 2013) and remodeling (Kelly and Dörmann, 2004; Kirchhoff, 2014; Shimojima and Ohta, 2011) to protection against oxidative stress under high light (Nowicka et al., 2016; Szymańska and Kruk, 2010) and to lipid-derived signaling in response to biotic stress, e.g. wounding, herbivory and pathogens (Howe and Schilmiller, 2002; Upchurch, 2008; Wasternack, 2007).

Lipid metabolism takes places in plastid subcompartments, at the level of envelopes, thylakoid membranes and at thylakoid microdomains called plastoglobules. The processes involved in chloroplast lipid metabolism are tightly regulated depending on developmental stage and environmental factors (Zhang et al., 2010).

The chloroplast structural core is constituted by a complex membrane system, the thylakoids, that are formed mainly by galactolipids, approximated 50% monogalactosyl-

diacylglycerol, 26% digalactosyldiacylglycerol, sulfoquinovosyldiacylglycerol and phosphatidylglycerol comprise most of the remaining lipids (Boudière et al., 2014; Dörmann, 2013; Kobayashi, 2016). Chloroplast photosynthetic membranes also contain embedded proteins and lipophilic compounds including chlorophylls, carotenoids (β-carotene, lutein, neoxanthin and the three xanthophyll cycle carotenoids zeaxanthin, violaxanthin and antheraxanthin) and prenylquinones (plastoquinone, phylloquinone, tocopherol and plastochromanol) (Dekker and Boekema, 2005; Lichtenthaler, 2007; Lichtenthaler and Calvin, 1964; Lichtenthaler and Park, 1963; Matringe et al., 2008). While carotenoids play structural and light harvesting roles and have a protective function against excessive light Frank and Cogdell, 1996; Gruszecki and Strzałka, 2005; Niyogi et al., 2000, prenylquinones have essential roles as antioxidants and in electron transport (Havaux et al., 2005). Together, the carotenoids and prenylquinones unfold protective functions under environmental stress and shield plant cells against ROS (reactive oxygen species) (Havaux and Kloppstech, 2001; Horvath et al., 2006).

1.1.3 Regulatory roles of plastids in isoprenoid compounds biosynthesis

The prenylquinones, as well as the carotenoids, belong to the plastid isoprenoid family. Due to their diverse roles in a wide range of biological processes, they are essential for plant growth and development. Also known as terpenoids, isoprenoid compounds are the most abundant class of metabolites and more than 50 000 different molecules have been identified and reported to date (Banerjee and Sharkey, 2014; Thulasiram et al., 2007). In higher plants, two independent biosynthetic pathways supply the universal C5 precursor, isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP) (Eisenreich et al., 1998), the cytosolic mevalonate (MVA) and the plastidial methylerythritol-4-phosphate (MEP) pathways (Lange and Croteau, 1999; Lange et al., 1998; Lichtenthaler, 1999, 2010; Paseshnichenko, 1998; Rodríguez-Concepción and Boronat, 2015; Vranová et al., 2013; Wanke et al., 2001). Whilst the MVA pathway supplies precursors for the synthesis of sesquiterpenes, triterpenes and sterols (e.g. brassinosteroid hormones) (Mendoza-Poudereux et al., 2015; Rodríguez-Concepción, 2006), the MEP pathway is involved in the production of monoterpenes, plastoquinones, carotenoids, the phytol tail of chlorophylls, and phytohormones (e.g. cytokinins, gibberellins, abscisic acid) (Boronat, 2010; Rodríguez-Concepción, 2006; Rodríguez-Concepción and Boronat, 2002). The separation of the two pathways in different compartments and its advantages are not yet fully understood (Hemmerlin et al., 2012). The plastid pathway is probably conserved from the endosymbiotic event, whereas the cytosolic one is eukaryotic. Moreover, many isoprenoid compounds from the MEP pathway are required in the plastid for their roles in photosynthesis (Figure 1.3). Thus the physical separation is likely to facilitate the optimal supply of precursors for each pathway (Rodríguez-Concepción and Boronat,

2015). In this chapter, I will focus on plastidial derived pathway, with particular emphasis into prenyllipids and tocopherol metabolism.

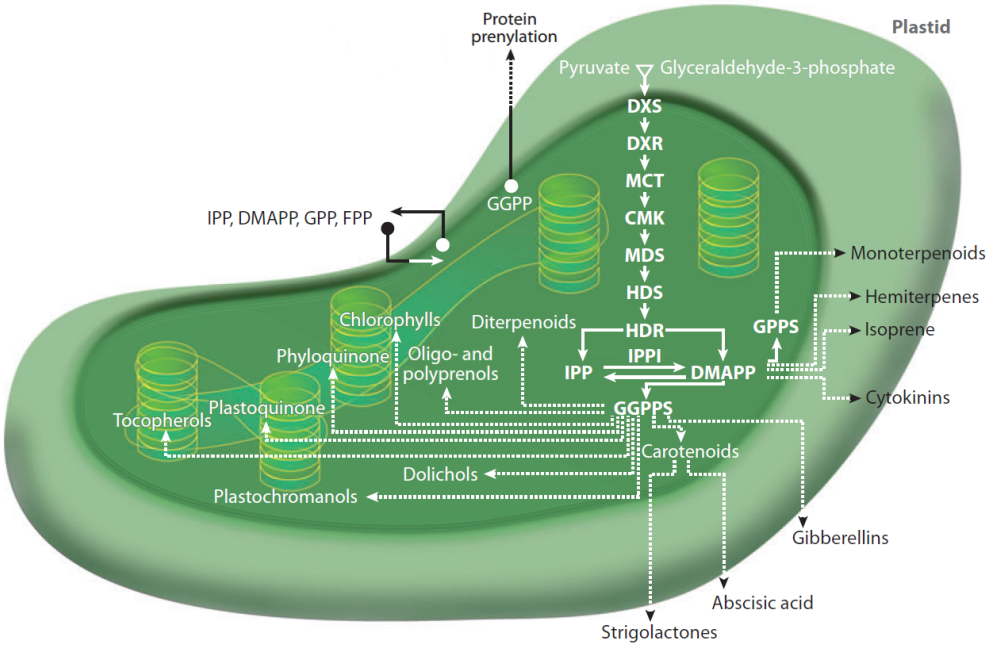


Fig. 1.3: Plastid MEP pathway in plant cells. Solid-line arrows indicate a single enzymatic step, dashed-line arrows indicate more than one enzymatic step, and circle-ended arrows indicate cross-membrane transport. White arrows indicate enzymatic reactions in plastids. Short-chain prenyl diphosphates such as isopentenyl diphosphate (IPP), dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP), and farnesyl diphosphate (FPP) translocate across the plastid membrane (Bick and Lange, 2003). Geranylgeranyl diphosphate (GGPP) can be used for protein prenylation in the cytosol of tobacco cells (Gerber et al., 2009). Specific transporters for prenyl diphosphates have not yet been identified. Other abbreviations: 1-Deoxy-D-xylulose 5-phosphate synthase (DXS); 1-Deoxy-D-xylulose 5-phosphate reductoisomerase (DXR); 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (MCT); 4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase (CMK); 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS); 4-Hydroxy-3-methylbut-2-enyl-diphosphate synthase (HDS); 4-Hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR); Isopentenyl diphosphate-isomerase (IPPI); Geranyl diphosphate synthase (GPPS); Geranylgeranyl diphosphate synthase (GGPPS). Adapted from Vranová et al. (2013).

The MEP pathway (also known as 1-deoxy-D-xylulose 5-phosphate (DXP) pathway or pyruvate/glyceraldehyde-3-phosphate pathway) consists of seven enzymatic steps (Rodríguez-Concepción and Boronat, 2002; Rohdich et al., 2001; Rohmer, 1999; Rohmer et al., 1996; Sharkey et al., 2007). DXP is biosynthesized from pyruvate and D-glyceraldehyde 3-phosphate (GAP) that are catalyzed by the enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXS) (Rodríguez-Concepción and Boronat, 2002; Rohmer et al., 1993). Next, the enzyme 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) converts DXP into MEP. Then, through three consecutive enzymatic steps involving cytidylation (CTP-dependent), phosphorylation (ATP-dependent), and cyclization, MEP is converted to the cyclic intermediate methylerythritol 2,4-cyclodiphosphate (MEcDP). In the following step, catalyzed

by HMBDP synthase (HDS) MEcDP is converted into hydroxymethylbutenyl diphosphate (HMBDP). Finally, HMDBP is reduced to IDP and DMADP by HMBDP reductase (HDR). IDP and DMADP are also isomerized by isopentenyl diphosphate isomerase (IDI) (Banerjee and Sharkey, 2014)).

1.1.4 Prenylquinone biosynthesis also derives from the MEP pathway

Prenylquinones, like all isoprenoids, are assembled from the condensation of monomers of isopentenyl diphosphate (IPP) and its double bond isomer dimethylallyl diphosphate (DMAPP). A series of subsequent condensation reactions yields geranylgeranyl diphosphate (GGDP) from IPP. GGDP is an essential intermediate for isoprenoid-derived metabolism shared in the biosynthesis of carotenoids, tocochromanols, a group that includes tocopherols, tocotrienols and plastochromanol (PC-8), and other isoprenoid metabolic pathways isoprenoids (DellaPenna and Pogson, 2006; Kruk et al., 2014). Prenylquinone biosynthesis also depends on aromatic precursors from the shikimate pathway: *p*-hydroxyphenylpyruvate for plastoquinone, plastochromanol, and tocopherols, or chorismate, for phylloquinone, ubiquinone, and menaquinones (Figure 1.4).

1.1.5 Tocochromanols essential plastid antioxidant metabolites

Tocochromanols make up a small family of amphipathic molecules that is composed of four tocopherols and four tocotrienols. This group of molecules does not only have potent lipid-soluble antioxidant activity, but its members are also essential as nutrients for human health (Kamal-Eldin and Appelqvist, 1996; Munné-Bosch and Alegre, 2002; Schneider, 2005; Wolf, 2005). Discovered as a reproductive factor in 1922 (Evans and Bishop, 1922), the tocopherol biosynthetic pathway in plants and algae was decoded at the beginning of the 1970ies from precursor and products studies using radiolabeled intermediates (Grusak and DellaPenna, 1999; Whistance and Threlfall, 1970). Tocopherol biosynthetic reactions were unraveled in the following decade (Soll et al., 1980; Soll and Schultz, 1980; Soll et al., 1983, 1985), whereas the genes involved were only recently identified (Cheng et al., 2003; Collakova and Dellapenna, 2001; Porfirova et al., 2002; Rohmer, 2003; Savidge et al., 2002; Shintani and Dellapenna, 1991; van Eenennaam et al., 2003). Almost all of the enzymes responsible for tocopherol biosynthesis were localized to the inner envelope membrane (Bouvier et al., 2005; DellaPenna and Pogson, 2006) with the exception of VTE1 that is present predominantly in plastoglobules (Austin II et al., 2006; Lundquist et al., 2012; Vidi et al., 2006; Ytterberg et al., 2006).

Tocochromanols occur naturally as α, β, γ, and δ-tocopherol and -tocotrienols that differ in the number and position of methyl groups at the aromatic ring (Grusak and DellaPenna,

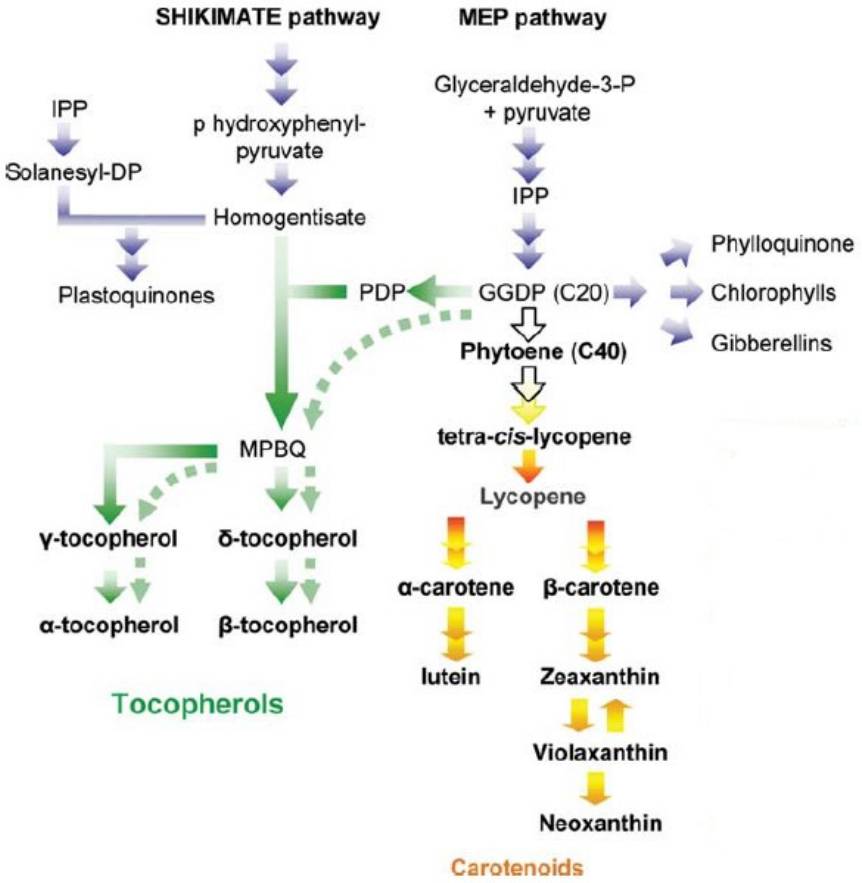
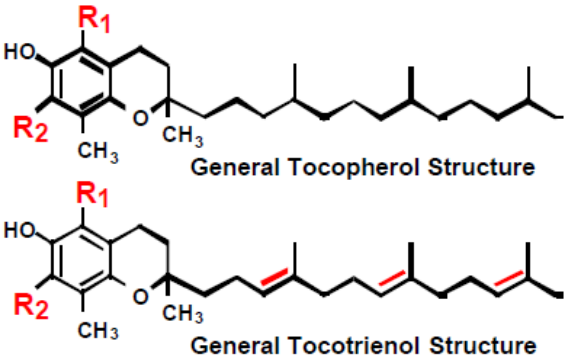


Fig. 1.4: Overview of plastidic isoprenoid, tocopherol, and carotenoid biosynthesis in plants. The MEP pathway provides IPP for the synthesis of GGDP. Orange arrows relate to abundant carotenoids. Minor products of the carotenoid pathway are not shown. Green arrows regard tocopherol synthesis starting with homogentisate, a product of the shikimate pathway. The tocotrienol product of the condensation of GGDP with homogentisate produced by the same pathway is not shown, adapted from DellaPenna and Pogson (2006).

1999) (Figure 1.5). α-tocopherol is the most active form as vitamin E due to its retention by a hepatic α-tocopherol binding protein (α-TTP) (Hosomi et al., 1997; Terasawa et al., 2000).



Form	R_1	R_2	*Relative Activity (toco vs trienol)
α-tocopherol / trienol	$-CH_3$	$-CH_3$	100% vs 30%
β-tocopherol / trienol	$-CH_3$	-H	50% vs 5%
γ-tocopherol / trienol	-H	$-CH_3$	10% vs 0%
δ-tocopherol / trienol	-H	-H	3% vs 0%

Fig. 1.5: Tocopherol and tocotrienol structures. Differences are indicated in red. The table shows the number/position of methyl substituents in α, β, γ, and δ-tocopherol and tocotrienols. *Vitamin E activity of each tocopherol and tocotrienol with α-tocopherol being 100% (DellaPenna, 2005).

Tocopherols and tocotrienols are differentiated by their isoprenyl side chains, derived from phytyl-PP or GGDP, respectively. The polar head group (homogentisate) of tocochromanols is derived from the Shikimate pathway of aromatic amino-acid metabolism (DellaPenna and Pogson, 2006; Kamal-Eldin and Appelqvist, 1996; Munné-Bosch and Alegre, 2002). The condensation of the prenyl moiety phytyl diphosphate and homogentisate by homogentisate phytyl transferase, (HPT, VTE2) initiates tocopherol synthesis. The 2-methyl-6-phytylbenzoquinol (MPBQ) derived from the previous step can be methylated on the C-3 position on the aromatic ring by the dimethyl-phytylquinol methyl transferase (VTE3) to give 2,3-dimethyl-6-phytyl-1,4-benzoquinone (DMPBQ). The cyclization of either MPBQ or DMPBQ by the tocopherol cyclase (VTE1) results in δ- and γ-tocopherol, respectively. The further methylation at the C-5 position of these two molecules, mediated by the tocopherol γ-methyl transferase (VTE4), leads to the formation of β- and α-tocopherol (DellaPenna and Pogson, 2006; Zbierzak et al., 2010). Phytyl diphosphate, besides de novo synthesis, may originate from an alternative salvage pathway dependent on the phytol kinase (VTE5) and phytyl-phosphate kinase (VTE6), which by phosphorylation recycle the phytol moiety released from the chlorophyll tetrapyrrole ring into tocopherol biosynthesis (Almeida et al., 2016; Ischebeck et al., 2006; Valentin et al., 2006; vom Dorp et al., 2015).

Tocochromanols react with polyunsaturated acyl groups and protect membrane lipids from oxidative damage by scavenging lipid peroxyl radicals and quenching highly reactive singlet oxygen ($^1O_2$) as well as other reactive oxygen species (DellaPenna and Pogson, 2006; Mène-Saffrané and DellaPenna, 2010; Wolf, 2005). Their chromanol ring is responsible for their spectral and antioxidant properties (Kruk et al., 2014). In the first mechanism, the tocochromanol ring hydroxyl will donate a hydrogen atom to a highly reactive PUFA peroxyl radical, which will convert into its much less reactive hydroperoxide form, preventing the propagation of PUFAs peroxidation in the cell membranes (Serbinova et al., 1991; Traber and Atkinson, 2007). In the second mechanism, $^1O_2$ will be either quenched physically or chemically (Krieger-Liszkay and Trebst, 2006; Triantaphylidès and Havaux, 2009). The physical quench of $^1O_2$ relies on charge transfer and thermal dissipation mechanisms to return the oxygen to its ground state ($^3O_2$) (Dellapenna and Mène-Saffrané, 2011). The $^1O_2$ chemical quenching occurs by the opening of the chromanol ring, resulting in the tocopherol oxidation product, α-tocopherolquinone. The latter can still fulfil roles in electron transfer, and it can be reconverted and recycled into α-tocopherol, by the re-introduction of the chromanol ring by the activity of tocopherol cyclase (VTE1) (Gruszka et al., 2008; Kobayashi and DellaPenna, 2008; Kruk et al., 2005; Munné-Bosch, 2005; Triantaphylidès and Havaux, 2009).

Even though relative tocopherol composition can vary among species (Grusak and DellaPenna, 1999; Horvath et al., 2006). Tocopherols are found in most plant organs, while tocotrienols are mostly present in monocotyledon seeds. The levels of α-tocopherol in most seeds are low and limited by the expression of VTE4. Arabidopsis and soybean plants overexpressing VTE4 have strikingly increased α-tocopherol levels (Shintani and Dellapenna, 1991; van Eenennaam et al., 2003). Besides its photoprotective roles (Falk and Munné-Bosch, 2010), tocopherol participates in seed longevity and germination (Chen et al., 2016; Mène-Saffrané and DellaPenna, 2010; Sattler et al., 2004). The loss of function mutant *vte2* exhibited reduced seed longevity, and during germination, seedlings showed severe growth defects, presumably caused by increased levels of lipid hydroperoxides and hydroxyl fatty acids. VTE5 was initially characterized in Arabidopsis. The *vte5* mutant had only 20% of the tocopherol normally found in wild-type seeds, and even less so in leaves. Surprisingly, however, no effect on seed germination has been reported (Valentin et al., 2006).

1.1.6 Plastoglobules are more than lipid droplets

Chloroplast lipid droplets, also known as plastoglobules are microdomains of the thylakoid membranes (Austin II et al., 2006). They were first believed to be just a storage site for lipid metabolites (Bailey and Whyborn, 1963; Greenwood et al., 1963). Plastoglobules are physically connected to thylakoids through the outer membrane leaflet (Austin II et al., 2006) and their lipid monolayer boundary hosts a small number of proteins (Kessler et al.,

1999), that are mostly involved in lipid metabolism (Eugeni Piller et al., 2012; Grennan, 2008). Since the analysis of plastoglobule proteomes of Arabidopsis chloroplasts and red pepper chromoplasts, an active metabolic role of plastoglobules in the biosynthesis of neutral lipids, has been established (Lundquist et al., 2012; Vidi et al., 2006; Ytterberg et al., 2006).

It has been demonstrated that plastoglobules are involved in various essential biosynthetic metabolic pathways. In the context of this thesis, metabolism and/or accumulation of prenyl quinone molecules, e.g. tocopherol, plastoquinone and plastochromanol in plastoglobules (Vidi et al., 2006; Zbierzak et al., 2010). Beyond lipid metabolism and storage, plastoglobule protein composition changes in response to stress or developmental transitions. During chloroplast senescence, which entails catabolic processes such as the dismantling of the thylakoid membrane, plastoglobules play roles in metabolic detoxification. The degradation of chlorophyll (liberating phytol) and monogalactosyldiacylglycerol (MGDG) was correlated with the accumulation of triacylglycerol (TAG) and fatty acid phytyl ester (FAPE). Both TAG and FAPE are products of phytyl ester synthases 1 (PES1) and 2 (PES2) that localize to plastoglobule (Besagni and Kessler, 2013; Gaude et al., 2007; Ischebeck et al., 2006; Lippold et al., 2012; Rottet et al., 2015; Tevini and Steinmüller, 1985). Likewise, in reproductive tissues and fruit maturation, plastoglobules have roles in disassembly of thylakoids, and concomitant accumulation of carotenoids in plastoglobules and plastoglobule-derived carotenoid fibrils (Deruère et al., 1994; Steinmüller and Tevini, 1985).

Not only plastoglobules fascinate by their dynamic ultrastructural changes, in constant adaptation to plant development stage, stresses or changes in the environment; but also their direct involvement in essential lipid metabolism highlights their importance in plant development and survival. This present thesis explores and confirms the role of prenylquinone lipids, which metabolism takes place in the plastoglobules, in the resistance of tomato plants against high light and high temperature stresses.

1.2 General thesis outline

The focus of this work is to understand how plants resist and adapt to environmental stress in particular high light, high temperature and the combination of the two. I took strong interest in lipid synthesis and re-modeling as the two are key factors in photosynthetic function under environmental stress conditions I addressed the role of (prenyl) lipids in these processes taking a broad metabolomics approach to lipophilic compounds. I chose tomato as the model system. As a crop plant this prominent member of the Solanaceae family is not only of importance in agriculture, but it has also become an important laboratory model system for fleshy fruit plants. In addition to its nutritional value, tomato

is also a suitable model to study prenylquinone metabolism. The use of a crop systems may allow a more rapid translation of the findings into agriculture. Our knowledge of the role of prenylquinones in stress resistance was expanded by the use of new lipidomic-based methods, which allowed the simultaneous and rapid profiling of prenylquinones and carotenoids in plant extract based-methods.

In Chapter 2, I present a review published in Current Opinions in Plant Biology entitled "Unexpected roles of plastoglobules (plastid lipid droplets) in vitamin K_1 and E metabolism." In this review, I summarized recent advances on plastoglobule research and their findings on biosynthesis and metabolism of Vitamins E and K_1.

Climate change and rising temperatures are amongst the most serious issues on this planet. In Chapter 3, I present a manuscript that was published in Frontiers in Plant Science entitled "Lipid antioxidant and galactolipid remodeling under temperature stress in tomato plants." To investigate the question of how the photosynthetic machinery is protected against heat stress, I employed a non-targeted lipidomics approach. Amongst many hundreds of compounds that change under heat stress, I identified α-tocopherol and plastoquinone as the most significantly increased antioxidants. This suggest a new role for these two prenyl quinones in protecting the photosynthetic apparatus against temperature stress.

In Chapter 4, I present an article published in the Journal of Experimental Botany entitled "Down-regulation of tomato PHYTOL KINASE strongly impairs tocopherol biosynthesis and affects prenyllipid metabolism in an organ-specific manner." This article was the result of a collaboration with the University of São Paulo. My contribution to this study, together with Neuchâtel Platform of Analytical Chemistry (NPAC), was the non-targeted lipidomic analysis of prenyllipids and galactolipids in leaves and fruits of a tomato vte5 knock down-line (SIVTE5). I designed the extraction method for mature-green and ripe fruit analysis. The results obtained through this joint effort provided valuable information on the metabolic fluxes implicated in Vitamin E accumulation as well as biosynthetically related pathways which impact tomato physiology.

In Chapter 5, I intended to identify molecules that contribute to the protection against combined high temperature and high light stress. The ability of energy conversion by the photosynthetic machinery under stress and its capacity to adapt to an ever-changing environment is crucial for plant survival. Here, we use the non-targeted lipidomics approach in the tomato model system to identify lipophilic molecules that may contribute to protection against this combined stress. Among several hundred compounds, the two most strongly upregulated compounds were α-tocopherol and plastoquinone/-ol. To perturb α-tocopherol levels we used the tomato vte5 knock down-line (SIVTE5). The data indicate that VTE5 protects against combined high light and high temperature stress and do so by supporting α-tocopherol production. The manuscript entitled "VTE5 phytol

kinase is essential for resistance to combined light and temperature stress in tomato" will be submitted shortly.

The use of plants perturbed in biosynthetic pathways has deepened our understanding of the importance of prenyllipid metabolism in plants (Mène-Saffrané and DellaPenna, 2010). Currently, climate change scenarios include rising temperatures in conjunction with high light (HL) intensity that may undermine plant survival and affect agricultural yields (Streb et al., 2003; Walther et al., 2002). This thesis provides valuable insights into how prenyllipids, notably α-tocopherol, contributes to plant resistance to high light and high temperature stress.

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2

Unexpected roles of plastoglobules (plastid lipid droplets) in vitamin K_1 and E metabolism

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Abstract

Tocopherol (Vitamin E) and phylloquinone (Vitamin K_1) are lipid-soluble antioxidants that can only be synthesized by photosynthetic organisms. These compounds function primarily at the thylakoid membrane but are also present in chloroplast lipid droplets, also known as plastoglobules (PG). Depending on environmental conditions and stage of plant development, changes in the content, number and size of PG occur. PG are directly connected to the thylakoid membrane via the outer lipid leaflet. Apart from storage, PG are active in metabolism and likely trafficking of diverse lipid species. This review presents recent advances on how plastoglobules are implicated in the biosynthesis and metabolism of Vitamin E and K.

2.1 Introduction

Tocopherols (Vitamin E) and phylloquinone (Vitamin K_1) are essential nutrients for humans and can be only synthesized by photosynthetic organisms such as cyanobacteria and plants (Nowicka and Kruk, 2010). Tocopherols and phylloquinone belong to the family of prenylquinone compounds that also includes plastoquinone (PQ) and its chromane derivative plastochromanol (PC8). The family consists of lipid-soluble molecules, which act as antioxidants preventing lipid peroxidation in the thylakoid membrane and quenching harmful reactive oxygen species (ROS) (Havaux et al., 2005; Kobayashi and DellaPenna, 2008; Krieger-Liszkay and Trebst, 2006). Phylloquinone and PQ also act as electron carriers in photosystem I (PSI) and II (PSII), respectively. Phylloquinone deficiency causes a reduction of PSI and increase of PQ leading to the unbalanced functioning of PSII (Lohmann et al., 2006; Shimada et al., 2005). Phylloquinone is present in the PsaA and PsaB subunits of the PSI complex. Phylloquinone, located at A_1 acceptor locus, receives an electron from the chlorophyll a acceptor molecule (A_0) and then donates an electron to the membrane-associated iron-sulfur protein acceptor cluster (F_x, F_A /F_B) (Brettel and Leibl, 2001; Itoh and Iwaki, 1989; Shimada et al., 2005; van Oostende et al., 2008, 2011).

Tocopherols belong to the eight-member tocochromanol family, consisting of four tocopherols and four tocotrienols. Tocopherols have a phytyl side chain whereas tocotrienols have an unsaturated geranylgeranyl side chain with three double-bonds. α-, β-, γ- and δ-tocopherols and -tocotrienols are all active and collectively referred to as vitamin E. α-, β-, γ- and δ only differ in the number and position of the methyl groups in the hydrophilic chromane group. α-, β-, γ- and δ-tocopherols and tocotrienols have many activities in plants as detailed in recent reviews (DellaPenna, 2005; Dörmann, 2007) and highlighted in subsequent sections. These compounds also act as important lipid soluble antioxidants in the diet of mammals (including humans) and in this context are collectively referred to as vitamin E. The vitamin E activity of the different compounds ranges from zero for δ- and γ-tocotrienol to 100% for α-tocopherol, the most active form.

The majority of the chloroplast enzymes implicated in the biosynthesis of prenylquinones have been localized at the inner membrane of the chloroplast envelope (Schultz et al., 1981; Soll et al., 1980; Soll et al., 1985), with the exception of part of the phylloquinone biosynthetic pathway that is compartmentalized in peroxisomes (Babujee et al., 2010; Reumann, 2013) (Figure 2.1).

However, recent studies have demonstrated that several important steps in the metabolic pathways localize at plastoglobules (PG) (Vidi et al., 2006; Ytterberg et al., 2006).

Vitamin K_1 is synthesized *de novo* from a naphthoquinone ring derived from chorismate in the shikimate pathway and a prenyl side chain derived from phytyldiphosphate (Eugeni Piller et al., 2011; van Oostende et al., 2011) (Figure 2.1). Similarly, the tocopherol benzoquinone group is a product of the shikimate pathway and prenyl side chains originate from plastidic isoprenoid pathway (Kobayashi and DellaPenna, 2008; Munné-Bosch and Alegre, 2002; Sattler et al., 2003; Soll et al., 1985). The benzoquinone head group is derived from the homogentisic acid (HGA) that is formed from p-hydroxyphenylpyruvic acid by p-hydroxyphenylpyruvic acid dioxygenase (HPPD) (Lohmann et al., 2006). The isoprenoid phytyl or geranylgeranyl (in the case of tocotrienols) tail derives from the 1-deoxy-D-xylulose-5-phosphate pathway located in the plastid. All the subsequent steps in the tocopherol synthesis have been reported to occur in the inner envelope of the chloroplast (Eisenreich et al., 1998; Lichtenthaler, 1998; Sattler et al., 2003) (Figure 2.1).

2.2 Plastoglobules change with plant developmental stages and function as microdomains for (prenyl-) lipid metabolism

The photosynthetic light reactions take place at the chloroplast thylakoid membranes. Thylakoid membranes are composed mainly of galactolipids but also contain prenylquinones as well as chlorophylls and carotenoids that are mostly attached to thylakoid membrane proteins (Lichtenthaler and Calvin, 1964; Lichtenthaler and Park, 1963). At the curved stromal margins of thylakoid membranes, PG may emerge and constitute thylakoid membrane microdomains (Eugeni Piller et al., 2012; Kessler et al., 1999; Lichtenthaler, 1968; Smith et al., 2000).

PG were long thought to be just storage droplets for excess neutral lipids. However, recent studies show that PG also play important metabolic roles in the metabolism of prenyl lipids (Austin II et al., 2006; Bréhélin et al., 2007; Eugeni Piller et al., 2011, 2012; Ytterberg et al., 2006). PG consist of an outer, polar lipid monolayer, which is contiguous with the thylakoid outer lipid leaflet (Austin II et al., 2006; Bréhélin et al., 2007). The PG core contains neutral lipids such as prenylquinones (tocopherol, DMPBQ, phylloquinone, PQ, carotenoids, fatty acid phythyl esters, and triacylglycerols). The PG surface is studded with proteins. Among these are the fibrillins (FBN), previously known as plastoglobulins (PGL). FBNs are considered structural proteins (Bréhélin et al., 2007), that are also known or predicted to be involved in various lipid metabolic pathways (Ytterberg et al., 2006).

PG numbers, size and lipid content change, depending on environmental conditions and plant developmental stage (Besagni and Kessler, 2013). These changes at the ultrastructural level (visible by electron microscopy) correlate with the remodeling of lipid content

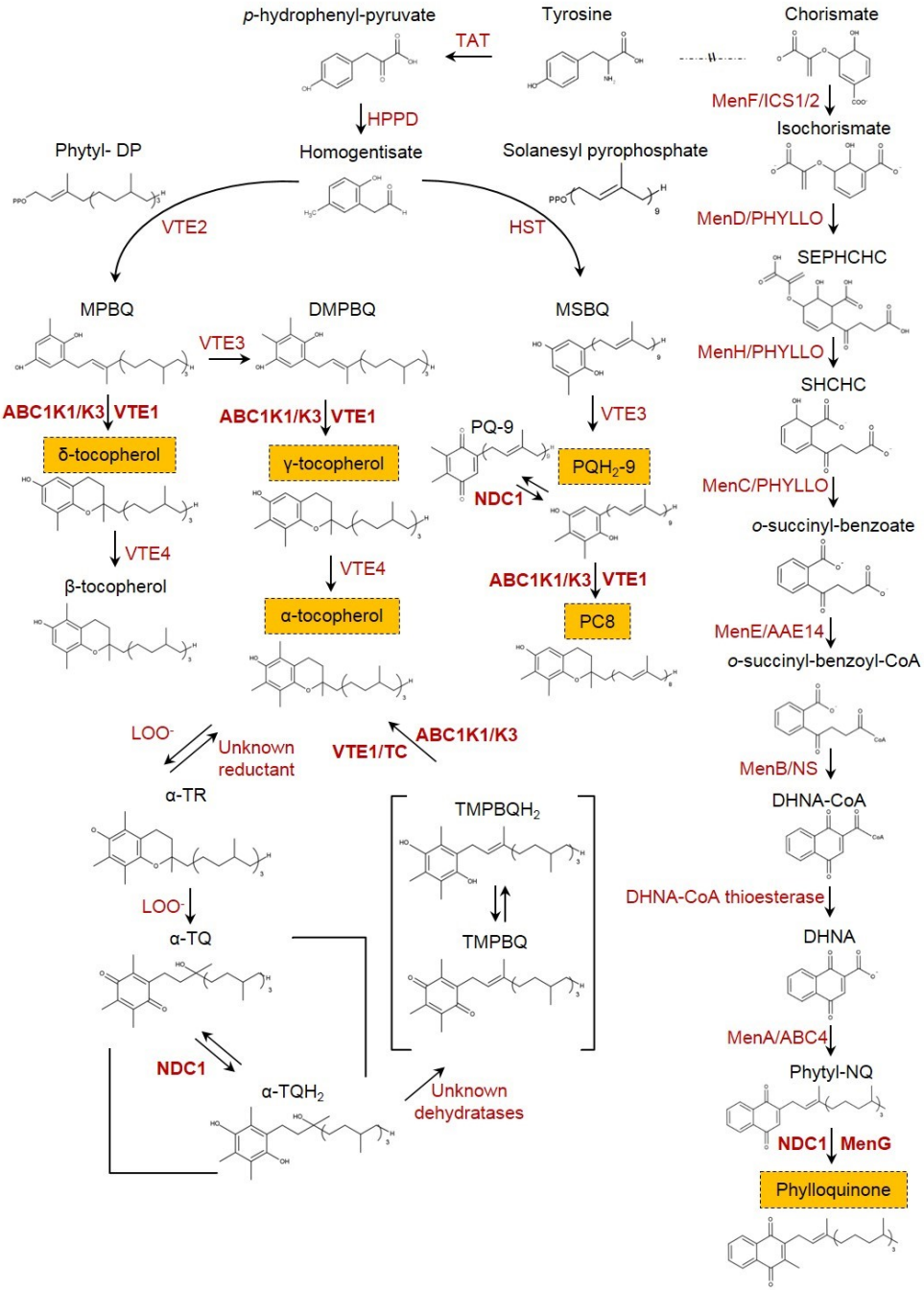

Fig. 2.1: Overview of the prenylquinone biosynthetic pathways in Arabidopsis. Phylloquinone *de novo* synthesis. Four Men enzymes are responsible for the conversion of chorismate to o-succinyl-benzoate. They are referred to as MenF, D, H and C, respectively (Babujee et al., 2010; Gross et al., 2006; Reumann, 2013).The three next steps consist of the activation of *o*-succinyl-benzoate into *o*-succinyl-benzonyl-CoA followed by the formation of the naphtoate ring. Then, production of the DHNA-CoA is compartmentalized in peroxisomes where MenE, B and H act sequentially (Babujee et al., 2010; Reumann, 2013). The pathway then returns to the plastid where the CoA moiety of DHNA-CoA is removed, and conversion to DHNA is catalyzed by a thioesterase hotdog-fold enzyme (Furt et al., 2013). DHNA phytyltransferase (MenA) converts DHNA to Phytyl-NQ. Phytyl-NQ is methylated at the C3-position of the naphthoquinone moiety by the MenG gene yielding phylloquinone (Lohmann et al., 2006). *Continued on next page.*

Fig. 2.1: *continued:* Tocopherol *de novo* synthesis: the benzoquinone head group is derived from the shikimate pathway and the phytyl tail from the plastidic 1-deoxy-D-xylulose-5-phosphate isoprenoid pathway. Phytyl-DP prenylation by homogentisate phytyltransferase (HPT/VTE2) converts HGA into MPBQ (Besagni and Kessler, 2013; Eugeni Piller et al., 2014). TC/VTE1 catalyzes chromanol ring formation in MPBQ leading to the formation of δ-tocopherol. Alternatively, MPBQ methyltransferase (encoded by the Arabidopsis VTE3 locus) may methylate MPBQ to give DMPBQ. Cyclization DMPBQ by VTE1 leads to the formation of γ-tocopherol. The addition of a methyl group to the sixth position of the chromanol ring by VTE4 is responsible for conversion of δ- and γ-tocopherols into β- and α-T, respectively (Cheng et al., 2003; DellaPenna, 2005; Eugeni Piller et al., 2014; Kobayashi and DellaPenna, 2008; Porfirova et al., 2002; Sattler et al., 2003; Shintani et al., 2002). Enzymes are shown in red and enzymes which are discussed in more detail are shown in bold letters. The prenylquinone metabolites highlighted in yellow are probably synthesized at the plastoglobule. Abbreviations: TAT, tyrosine aminotransferase; HPPD, p-hydroxyphenyl-pyruvate dioxygenase; HST, homogentisic acid solanesyl transferase; VTE, enzymes of vitamin E synthesis; LOO-, lipid peroxy radical; TC, tocopherol cyclase; Men, menaquinone synthesis; ICS 1/2, isochorismate synthase 1 and 2; AAE14, acyl-CoA activating enzyme isoform 14; NS, naphtoate synthase; DHNA-CoA, 1,4-dihydroxy-2-naphthoyl-CoA; DHNA-CoA thioesterase, 1,4-dihidroxy-2naphthoyl-CoA thioesterase; DHNA, 1,4-dihydroxy-2-naphthoate; Phytyl-NQ, 2-phytyl-1,4-naphthoquinone ; ECHld, enoyl-CoA hydratase/isomerase; DP, diphosphate; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinone; DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinone; MSBQ, 2-methyl-6-solanesyl-1,4-benzoquinol; PQH_2-9, plastoquinol; PQ-9, plastoquinone; PC8, plastochromanol; SEPHCHC, 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate; SHCHC, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate; NDC1, NAD(P)H-dependent:quinone oxidoreductase; ABC1K1/K3, activity of bc1 kinases 1 and 3 complex; $TMPBQH_2$, Trimethylbenzoquinol; TMPBQ, Trimethylbenzoquinone; α-TQH_2, α-tocopherol quinol; α-TQ, α-tocopherol quinone; α-TR, α-tocopheroxyl radical. Adapted from Eugeni Piller et al. (2014) and Ytterberg et al. (2006).

of the thylakoid membrane. Moreover, PG participate in remodeling by metabolizing and storing lipid molecules. Their role in trafficking from and to the thylakoid is inferred from the existence of PG-thylakoid contact sites that may serve as conduits for lipid molecules.

2.3 Vitamin K_1 in the thylakoids and plastoglobules

Phylloquinone is present mainly in thylakoid membrane and, two molecules localize to each PSI complex. However, it has been demonstrated through chloroplast isolation and subplastidial fractionation that phylloquinone is not restricted to PSI. Around a third of the total phylloquinone was found to be located in PG, suggesting that PG function as a reservoir for excess phylloquinone (Lohmann et al., 2006).

In the final step of phylloquinone biosynthesis, AtMENG functions as the methylase for 2-phytyl-1,4-naphthoquinone (Phytyl-NQ) (Lohmann et al., 2006) (Figure 2.2). The AtmenG knock-out mutant is devoid of phylloquinone but accumulates Phytyl-NQ. Unexpectedly, the lack of phylloquinone does not provoke a drastic phenotype as reported previously for the *abc4* mutant, in which the absence of phylloquinone led to a lethal albino phenotype (Shimada et al., 2005). AtmenG only exhibited reduced growth and anthocyanin accumulation under normal light condition, besides there was a slight decrease in photosynthetic efficiency upon high light (HL) treatment due to accelerated degradation of PSI (Lohmann et al., 2006). It therefore appears that the Phytyl-NQ precursor of phylloquinone is able to replace phylloquinone under non-stress conditions.

Strangely, the mutant of the PG protein NDC1 (NAD(P)H-dependent:quinone oxidoreductase) has a molecular phenotype that is similar to that of *atmenG*. Lipidomics analysis in Arabidopsis showed that the most significant difference between the *ndc1* mutant and the wild type (wt) consisted in the absence of phylloquinone in wt and the presence of the Phytyl-NQ precursor in *ndc1* (Eugeni Piller et al., 2011). An ortholog of NDC1 exists in the green alga *Chlamydomonas reinhardtii*. In this organism the NDA2 NAD(P)H:quinone oxidoreductase, takes over the function of the NDH complex in cyclic electron flow and chlororespiration (Eugeni Piller et al., 2011; van Oostende et al., 2011). NDC1 in Arabidopsis is a member of a family of seven homologs. Apart from NDC1 that is present in chloroplasts the homologs have been localized in mitochondria. However, NDC1 may be dually localized and also be present in mitochondria (Eugeni Piller et al., 2011). NDC1 does not appear to play a role in a major electron pathway. Yet it keeps the PQ in PG reduced resulting in an overall reduced state of total chloroplast PQ. The consequences of this for chloroplast Redox signaling have not yet been studied. How NDC1 is implicated in the AtmenG-dependent methylation step of phylloquinone synthesis remains a mystery. It will be interesting to see whether NDC1 is uniquely required for phylloquinone in

Arabidopsis or whether this is also the case in other species (Babujee et al., 2010; Furt et al., 2013; Reumann, 2013).

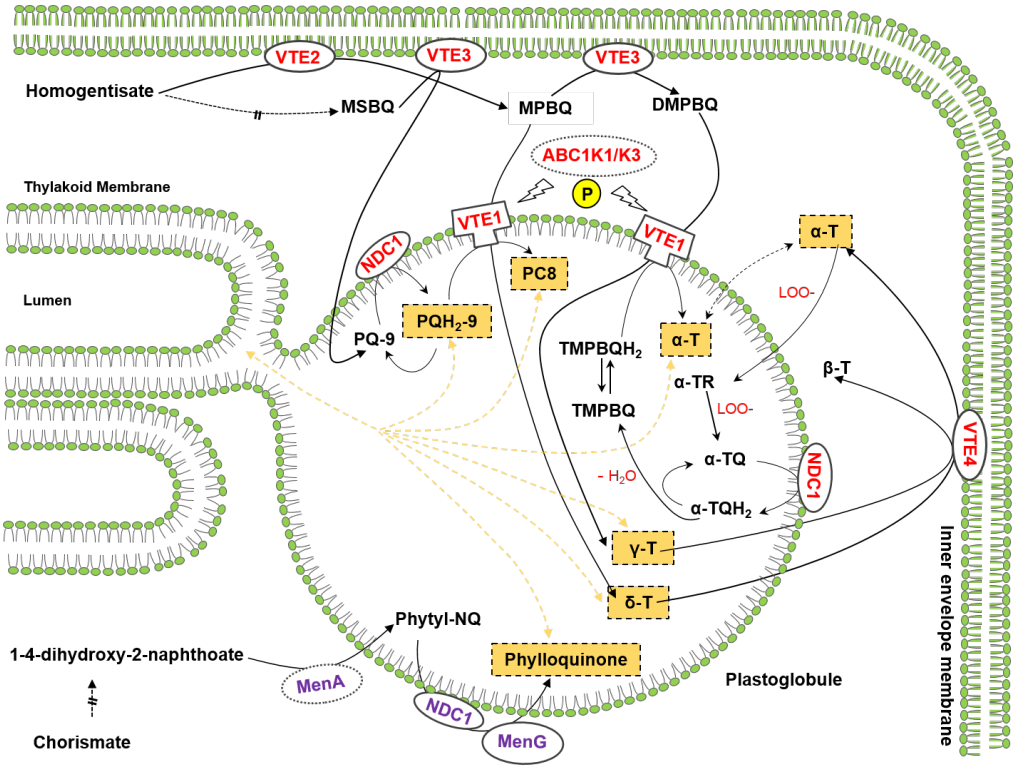

Fig. 2.2: Biosynthetic pathways and enzymes of Vitamin E and K metabolism in plastoglobules. Enzymes in red participate in α-tocopherol metabolism, including its redox cycle, as well as the conversion of plastoquinone to plastochromanol. Enzymes in purple participate in phylloquinone metabolism. In light orange connectors represent bidirectional trafficking of metabolites between plastoglobules and thylakoids. Final products are highlighted in yellow. MPBQ, 2-methyl-6-phytyl-1,4-benzoquinol; DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinol; MSBQ, 2-methyl-6-solanesyl-1,4-benzoquinol; PQ-9, plastoquinone; PQH2-9, plastoquinol; PC8, plastochromanol-8; α-T, alpha-tocopherol; α-TR, alpha-tocopheroxyl radical; α-TQ, alpha-tocopherolquinone; TMPBQ, trimethylbenzoquinone; $TMPBQH_2$, trimethylbenzoquinol; γ-T, gamma-tocopherol; δ-T, delta-tocopherol; β-T, beta-tocopherol; Phytyl-NQ, 2-phytyl-1,4-naphthoquinone.

2.4 Tocopherol cyclase (VTE1) located at plastoglobules fulfills diverse roles in the tocopherol metabolism

A large proportion of VTE1 (tocopherol cyclase - TC) is localized at PG (Lundquist et al., 2012). This was one of the first indications that PG function not only in the storage but also participate in the metabolism of tocopherol (Besagni and Kessler, 2013; Schultz et al., 1981) (Figure 2.2). The *vte1* mutant lacking TC and *vte2* mutant lacking the homogentisate phytyl transferase are both devoid of α-tocopherol. Instead of α-tocopherol,

vte1 accumulates its precursor DMPBQ and also lacks PC8 (Eugeni Piller et al., 2014; Mène-Saffrané and DellaPenna, 2010). It has been suggested that in *vte1* DMPBQ can substitute for α-tocopherol, for example, in seed germination (Sattler et al., 2004). However, under HL *vte1* displays a drop in PSII efficiency. Under HL, α-tocopherol concentrations increase in wt but in *vte1* the DMPBQ concentrations remain constant (Kobayashi and DellaPenna, 2008). This supports the idea that the accumulation of DMPBQ under HL is not sufficient to completely replace α-tocopherol and assume its membrane protective role in the thylakoid membranes (Havaux et al., 2005; Martinis et al., 2013; Zbierzak et al., 2010). *vte2* displayed a similar phenotype under short-term HL stress as *vte1* (Havaux et al., 2005). In *vte2*, both tocopherols and DMPBQ are entirely absent. However, in *vte2* PC8 may partially assume the antioxidant protection role of a-tocopherol attenuating or suppressing perturbed phenotypes under stress. In support of this notion, the absence of both tocopherol and PC8 in the *vte1/vte2* double mutant leads to a dramatic reduction in seed longevity and seedling viability (Mène-Saffrané and DellaPenna, 2010). The role of VTE1 in the production of PC8 is clearly apparent in overexpressing lines that show a massive increase of PC8 (Mène-Saffrané and DellaPenna, 2010). Moreover, much of the PC8 accumulates in PG.

2.5 VTE1 and NDC1 enzymes are directly implicated in the redox cycle of α-tocopherol

The oxidation of α-tocopherol involves donation of two electrons. First, a lipid peroxy radical (LOO^-) is reduced to a more stable hydroperoxide (LOOH) yielding α-tocopheroxyl radical (α-TR). Subsequently, α-TR is oxidized to α-tocopherol quinone (α-TQ) by LOO^-. α-TQ accumulates in PG. A PG-based repair system enables the reconversion of the oxidation products to tocopherol (DellaPenna and Pogson, 2006; Eugeni Piller et al., 2012, 2014; Kobayashi and DellaPenna, 2008; Mène-Saffrané and DellaPenna, 2010) (Figure 2.2).

Initially, α-TQ is reduced to yield α-tocopherol quinol (α-TQH_2), a reaction that is facilitated by NDC1. This is not surprising because NDC1 has a wide substrate specificity (Eugeni Piller et al., 2011). The following step consists of the conversion of α-TQH_2 to trimethylbenzoquinol ($TMPBQH_2$) by an unknown dehydratase. Finally, the conversion of $TMPBQH_2$ to a-tocopherol is carried out by VTE1 (Eugeni Piller et al., 2014; Kobayashi and DellaPenna, 2008; Mène-Saffrané and DellaPenna, 2010; Schultz et al., 1981) (Figure 2.2). The presence of a large proportion of VTE1 suggests that it may be committed to tocopherol recycling in PG rather than to *de novo* synthesis in this compartment.

2.6 ABC1-like kinases affect prenyl lipid composition of the chloroplast

BC1 (activity of bc1 complex)-like kinases are essential regulators of ubiquinone synthesis in bacteria and mitochondria of all lineages (Ytterberg et al., 2006). In plants, surprisingly, a group of 8 ABC1-like kinases family are present in chloroplasts, six of which are associated with PG. By analogy to bacterial and mitochondrial ABC1-like kinases implication in prenylquinone metabolism was proposed and demonstrated using reverse genetic experimentation in conjunction with lipidomics (Lundquist et al., 2012; Martinis et al., 2013, 2014). The ABC1K1 homolog was identified earlier as PGR6 (proton gradient regulation 6). The *pgr6* mutant has a high fluorescence phenotype indicative of a defect in the formation of the proton gradient across the thylakoid membrane. Lipidomics analysis revealed that the *abc1k1/pgr6* mutant is defective in prenyl lipid metabolism. Under HL conditions, *abc1/pgr6* failed to accumulate α-, γ- and δ- tocopherols to the high levels observed in wt. At the same time, *abc1k1/pgr6* showed a striking 6-fold increase of α-TQ, the oxidation product of α-tocopherol over wt under HL. These findings indicated a perturbation in tocopherol metabolism at the level of VTE1. The kinase nature of ABC1K1/PGR6 and the common localization in PG suggested that VTE1 might be a substrate of ABC1/PGR6. This was confirmed by *in vitro* experimentation. Moreover, VTE1 is a known phosphoprotein containing a phosphorylation hotspot near its N-terminus (Martinis et al., 2013). However, the range of other metabolic effects (reduced levels of β-carotene and lutein, altered PQ/PQH_2 ratio) observed in the mutant suggests that ABC1K1/PGR6 has many more targets. This molecular phenotype resulted in reduced photosynthetic electron transport, strong but reversible photodamage under HL and decrease of carbon fixation. Thus, the mutant had lower photosynthetic rates which led to reduced starch production and perturbation in the starch and sugar ratios after recovery from initial photodamage (Bayer et al., 2012; Martinis et al., 2014).

Another ABC1 homolog in PG, ABC1K3 has a mutant phenotype related to that of ABC1K1/PGR6, but photosynthetic efficiency was as in wt. *abc1k3* was affected in prenylquinone composition but, unlike *abc1k1*, α-tocopherol accumulation was as in wt. In *abc1k3*, α-TQ over accumulated under HL and PC8 was sharply reduced under all conditions. These results suggest that, ABC1K1 specifically regulates tocopherol recycling and plastochromanol production, functions that have been attributed to PG-localized VTE1. Like ABC1K1, ABC1K3 kinase phosphorylates VTE1 *in vitro*, which may provide a basis for regulation of VTE1 activity *in vivo*.

Under HL stress conditions, gene expression of ABC1K3 and VTE1 were highly correlated. At the protein level, however, Martinis et al. (2013, 2014) showed significantly lower contents of VTE1 in the *abc1k1/pgr6* and *abc1k3* mutants than in wt. These data suggest

that ABC1K1/PGR6 and -K3 stabilize VTE1 levels. However, these data differ from the data by Lundquist et al. (2013) that show no difference in VTE1 levels between the *abc1k1/abc1k3* double mutant and wt. Currently, the reasons for this discrepancy are not known. The ABC1K1 and -K3 kinases have been shown to interact with each other forming a protein complex (ABC1K1/3 complex). Analysis of the *abc1k1/abc1k3* double mutant revealed additional defects suggesting a premature senescence phenotype by activation of jasmonate pathways, including chlorophyll and PSII degradation (Lundquist et al., 2013).

2.7 Conclusion

PG functions have been reported throughout all stages of plant development, from plastid biogenesis to senescence and fruit maturation (Shimada et al., 2005). This review focuses on the role of PG in phylloquinone synthesis and tocopherol metabolism including the Redox cycle and accumulation under HL (Bréhélin et al., 2007; Eugeni Piller et al., 2011, 2012; Kobayashi and DellaPenna, 2008; Mène-Saffrané and DellaPenna, 2010; Ytterberg et al., 2006). Astonishingly, NDC1 a PG enzyme with no apparent link to phylloquinone synthesis is required for its accumulation in Arabidopsis. The mechanism of NDC1 is currently unknown.

It is important to note that not only metabolic enzymes reside at the PG but also regulatory ABC1-like kinases that affect tocopherol accumulation and Redox recycling under HL. VTE1 is the likely target of the ABC1K1 and -K3 kinases. However, these kinases are likely to have far more targets judged by the multitude of processes that are perturbed in the single and double *abc1k1* and *-k3* mutants.

How can PG exert their role in remodeling chloroplast prenyl lipid composition? It is hard to imagine that remodeling could take place if the thylakoid and PG did not form a continuum, in which the PG serve as a functional membrane microdomain bearing an arsenal of specific enzymes (Bréhélin and Kessler, 2008; Bréhélin et al., 2007; Lundquist et al., 2012; Ytterberg et al., 2006). Implicitly, trafficking of metabolites between PG and the thylakoid membrane is required. The conduit formed by the common outer membrane leaflet is likely the "bottle-neck" in metabolite trafficking between the thylakoid and the PG. A lot remains to be discovered as PG are clearly tied into a larger metabolic network that includes pathways beyond prenylquinone metabolism, affecting the metabolisms of galactolipids (Lippold et al., 2012), carotenoids (Gonzalez-Jorge et al., 2013), chlorophyll, sugars and more.

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3

Lipid antioxidant and galactolipid remodeling under temperature stress in tomato plants

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Abstract

Increased temperatures are a major scenario in climate change and present a threat to plant growth and agriculture. Plant growth depends on photosynthesis. To function optimally the photosynthetic machinery at the thylakoid membrane in chloroplasts continuously adapts to changing conditions. Here, we set out to discover the most important changes arising at the lipid level under high temperature ($38\,^{\circ}\mathrm{C}$) in comparison to mild ($20\,^{\circ}\mathrm{C}$) and moderately cold temperature ($10\,^{\circ}\mathrm{C}$) using a non-targeted lipidomics approach. To our knowledge, no comparable experiment at the level of the whole membrane system has been documented. Here, 791 molecular species were detected by mass spectrometry and ranged from membrane lipids, prenylquinones (tocopherols, phylloquinone, plastoquinone, plastochromanol), carotenoids (β-carotene, xanthophylls) to numerous unidentified compounds. At high temperatures, the most striking changes were observed for the prenylquinones (α-tocopherol and plastoquinone/-ol) and the degree of saturation of fatty acids in galactolipids and phosphatidyl ethanolamine. Photosynthetic efficiency at high temperature was not affected but at moderately cold temperature mild photoinhibition occurred. The results indicate that the thylakoid membrane is remodeled with regard to fatty acid saturation in galactolipids and lipid antioxidant concentrations under high temperature stress. The data strongly suggest that massively increased concentrations of α-tocopherol and plastoquinone are important for protection against high temperature stress and proper function of the photosynthetic apparatus.

3.1 Introduction

Plants have the ability to acclimate to changing environmental conditions. However, long-term climate change driven by rising temperatures may have a deleterious impact on plant physiology and in turn negative effects on crop yields. Plant growth is directly dependent on photosynthesis that takes place in the chloroplast. The chloroplast contains an extensive membrane system, the thylakoids that harbor the photosynthetic machinery responsible for the light reactions.

The thylakoid membrane consists mostly of galactolipids, composed of 50% of monogalactosyldiacylglycerol (MGDG) and 26% digalactosyldiacylglycerol (DGDG), phosphatidylglycerol and sulfoquinovosyldiacylglycerol making up most of the remainder. The abundance of galactolipids in the photosynthetic membranes suggests they have not only typical bilayer functions but also specific roles, such as the stabilization of photosynthetic complexes, membrane architecture (curvature), thylakoid stack (grana) formation (Dörmann, 2013).

In addition to membrane lipids, the thylakoids contain embedded lipid antioxidants such as tocopherols, plastochromanol as well as plastoquinone (that is better known as an electron transporting redox molecule) (Gruszka et al., 2008; Ksas et al., 2015; Mène-Saffrané and DellaPenna, 2010; Munné-Bosch and Alegre, 2002; Nowicka and Kruk, 2012; Rastogi et al., 2014).

Chloroplasts and photosynthetic membranes respond to environmental changes. They acclimate to intensity and quality of light, with changes in the structure of thylakoid membranes, the size of grana stacks, chlorophyll content, amount, and localization of light harvesting complexes (LHCII), to mention some (Kanervo et al., 1997; Lichtenthaler, 2010; Rochaix, 2007; Rochaix et al., 2012). The modification of the saturation level of membrane lipids is an important acclimation strategy of plants in response to temperature changes, allowing to maintain parameters such as membrane fluidity and permeability in response to varying temperature (Zheng et al., 2011). Recent findings also reported that reversible increase in the size of chloroplasts and number of plastoglobules occurs under moderate temperature stress (Zhang et al., 2010).

Upon abiotic stress, such as high or low temperature and high-light, the homeostasis of reactive oxygen species (ROS) metabolism in the plant cell is challenged (Apel and Hirt, 2004; Lichtenthaler, 1999; Zhang et al., 2010). A protective system in part based on lipid soluble molecules is in place to protect plant cells and the photosynthetic membranes against the action of ROS. Carotenoids - such as β-carotene, lutein, neoxanthin and three xanthophyll cycle carotenoids, zeaxanthin, violaxanthin, and antheraxanthin, - besides structural stabilization and light harvesting roles, play important photoprotective roles

in scavenging of singlet oxygen species and quenching of chlorophyll triplet states, and in excess energy dissipation by non-photochemical quenching (NPQ) that implicates xanthophylls and lutein (Choudhury and Behera, 2001; Frank and Cogdell, 1996; Gruszecki and Strzałka, 2005; Mimuro and Katoh, 1991; Shumskaya and Wurtzel, 2013). Another category of molecules, the prenylquinones (including tocopherols, plastoquinone, plastochromanol) are lipid soluble compounds that function as membrane-protective antioxidant molecules. Tocopherols have widely been described as antioxidants against high-light triggered oxidative stress (Havaux et al., 2005; Kobayashi and DellaPenna, 2008; Krieger-Liszkay and Trebst, 2006). Plastoglobule-localized enzymes are actively implicated in the lipid metabolic pathways during high-light stress: NAD(P)H-dependent quinone oxidoreductase (NDC1), and tocopherol cyclase (VTE1) participate in the synthesis and the recycling of tocopherols and plastochromanol (PC-8). In addition, NDC1 is essential for phylloquinone production (Besagni and Kessler, 2013; Eugeni Piller et al., 2011; Fatihi et al., 2015).

Therefore, the content of carotenoids and prenylquinones changes in response to stress as well as throughout the development stages of the plant. These changes can be seen as an integral part of the lipid remodeling taking place at the thylakoid membrane system. This paper analyses changes in lipid composition during temperature stress using liquid chromatography-mass spectrometry based lipidomics methods. Lipidomics are a powerful tool set enabling identification and fingerprinting of different classes of lipids, ranging from galactolipids and their degree of fatty acid saturation, to antioxidant prenylquinones and carotenoids.

The study was carried out in tomato (*Solanum lycopersicum* L.). Even though it is a species of tropical origin, it is cultivated worldwide and has become an important agricultural crop. Therefore tomato is a relevant model system to study how variation in temperature affects the lipid composition in leaves.

3.2 Material and Methods

3.2.1 Plant material and stress treatments

Tomato plants (*S. lycopersicum* L.) variety M82, grown on soil under conditions referred to as control ($250\,\mu\mathrm{mol\,m^{-2}\,s^{-1}}$ of light, 16-h light/8h dark, at 20/$18\,^{\circ}\mathrm{C}$, with 55% relative air humidity). 5 to 6-week old plants were transferred to either 10/$8\,^{\circ}\mathrm{C}$ (day/night), 38/$30\,^{\circ}\mathrm{C}$ or remained in 20/$18\,^{\circ}\mathrm{C}$, as control conditions. After six days, plants that were all returned to control conditions for a recovery period of 5 days.

3.2.2 Determination of photosynthetic parameters

Maximum photochemical efficiency or optimum quantum yield of photosystem II (F_v/F_m), was fluorometrically determined using a MINI-PAM Photosynthesis Yield Analyzer (Walz, `http://www.walz.com`). Plants were dark-adapted for 15 minutes before F_v/F_m measurements with illumination by application of a saturation flash. Five replicates for each treatment were done, at day 0 and on days 2, 4, 6 and 11 days of temperature treatments. The data obtained from F_v/F_m measurements in leaves were subjected to One-Way analysis of variance (One-Way ANOVA), followed by Bonferroni-Holm test to determine any significant differences in photosynthetic efficiency (Aickin and Gensler, 1996).

3.2.3 Chlorophyll quantification

Chlorophyll was extracted from $100\,\mathrm{mg}$ of tissue in $1\,\mathrm{ml}$ of 80% acetone. Total chlorophyll levels were then spectrophotometrically determined (Inskeep and Bloom, 1985). Five replicates for each treatment were done, at days 0, 6 and 11 of the temperature treatments. The data obtained from the measurements were subjected to One-Way ANOVA, followed by Bonferroni-Holm test to determine any significant differences between the various temperatures over the time course.

3.2.4 Lipid profiling

Samples were prepared and analyzed from the five replicates for each treatment, at days 0, 6 and 11 of the temperature treatments, according to Martinis et al. (2013) with small modifications. In brief, lipids were extracted from $100\,\mathrm{mg}$ of fresh tissue and suspended in $1\,\mathrm{ml}$ of tetrahydrofuran:methanol 50:50 (v/v). 5-10 glass beads ($1\,\mathrm{mm}$ in diameter) were added to the mixture and homogenized for 3 min at 30 Hz in a tissue lyser. After centrifugation (3 min, 14 000 g, and $4\,^{\circ}\mathrm{C}$), the supernatant was transferred to an HPLC vial. Lipid profiles were obtained by ultra-high pressure liquid chromatography coupled with atmospheric pressure chemical ionization-quadrupole time-of-flight mass spectrometry (UHPLC-APCI-QTOF-MS) as described in Martinis et al. (2011, 2013). Separation was performed on a reverse-phase Acquity BEH C18 column (50 x $2.1\,\mathrm{mm}$, $1.7\,\mu\mathrm{m}$) under the following conditions: solvent A=water; solvent B=methanol; 80-100% B in 3 min, 100% B for 2 min, re-equilibration at 80% B for 0.5 min. The flow rate was $0.8\,\mathrm{ml\,min^{-1}}$ and the injection volume was $2.5\,\mu\mathrm{l}$. Data were acquired using MassLynx version 4.1 (Waters), and further processed with MarkerLynx XS (Waters) to generate peak lists consisting of variables described by mass-to-charge ratio and retention time. Multivariate analysis was carried out using the statistics softwares EZinfo and Simca v.13.0.3 (Umetrics). Variables were Pareto-scaled before applying principal component analysis (PCA) and supervised partial least square discriminant analysis (PLS-DA; Eugeni Piller et al.,

2011; Martinis et al., 2011). In Pareto scaling, variables are divided by the square-root of their standard deviation as an intermediate between no scaling and dividing variables by their standard deviation alone. PLS-DA is a supervised multivariate method which takes advantage of class information. For PLS-DA models, the predictive ability and the degree of overfitting were evaluated using a leave-one-subject-out cross-validation and permutation tests with 200 random permutations. R2 and Q2 coefficient values were calculated for the original and permuted models. 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 search 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#`). Complete information regarding the identification procedure is available at `http://journal.frontiersin.org/article/10.3389/fpls.2016.00167`. For an example of the identification procedure refer to Supplemental Figure I. In addition to untargeted analysis, the UHPLC-APCI-QTOFMS method enabled the absolute quantification of several lipids for which reference standards were available. Absolute concentrations of δ-T, γ- T, α-T, α-TQ, PQ-9, PC-8, and phylloquinone (Vit K) were measured based on calibration curves obtained from pure standards. Moreover, PC-OH and PQ-OH were quantified as PC-8 and PQ-9 equivalents, respectively. Tocopherol and phylloquinone standards were obtained from Sigma-Aldrich, and PC-8 and PQ-9 standards were kindly provided by J. Kruk (Gruszka and Kruk, 2007; Kruk, 1988). The other molecules identified for which pure standards were unavailable were quantified relatively based on peak intensity measurements in the chromatograms. The two carotenoids violaxanthin and neoxanthin were measured as a sum since they could not be resolved either in the chromatographic or the mass dimensions under the conditions employed. The data obtained from the measurements were subjected to One-Way ANOVA, followed by Bonferroni-Holm test to determine any significant differences between different temperatures over the time course.

3.3 Results

3.3.1 Photosynthetic efficiency is reduced after cold temperature treatment

Chlorophyll fluorescence was measured to determine if prolonged exposure to varying temperatures ($10\,°C$ moderate cold stress, $38\,°C$ heat stress, $20\,°C$ control condition) had an impact on the photosynthetic efficiency (Fig. 3.1). Measurements of photochemical efficiency or quantum yield of photosystem II (F_v/F_m) values were carried at day 0 at $20\,°C$ and subsequently measured after 2, 4, 6 days, as well as after 5 days of recovery period at $20\,°C$. $38\,°C$ did not have a significant effect over the period of treatment. The plants exposed to $10\,°C$ showed a slight but significant reduction in F_v/F_m ($p = 0.002$) after six days and recovered after five days at control conditions ($20\,°C$).

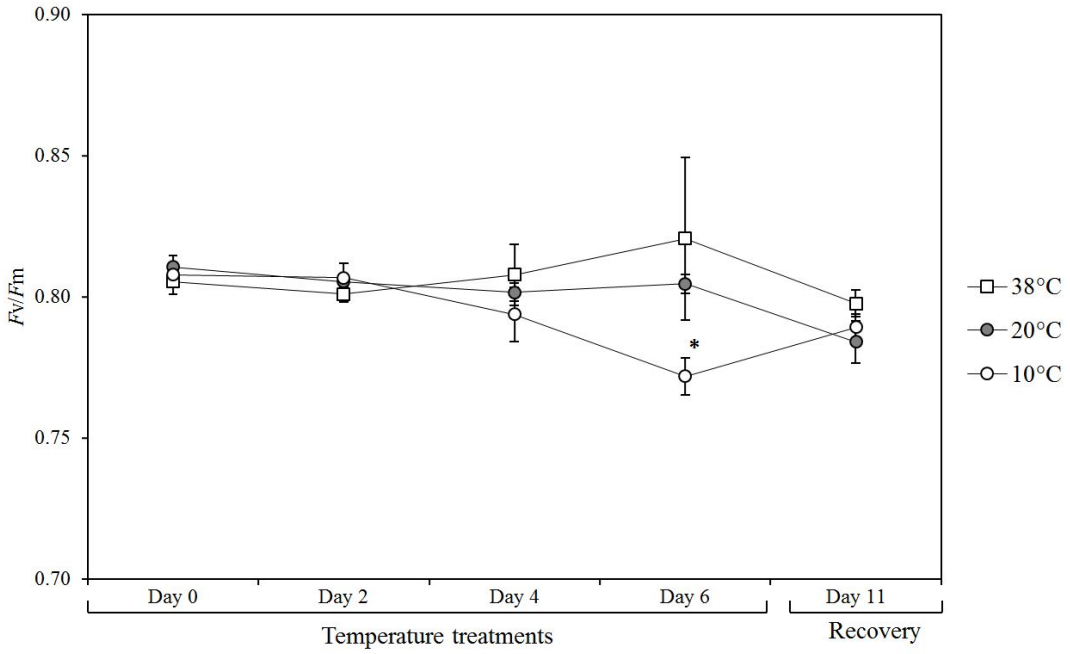

Fig. 3.1: Photosynthetic efficiency in tomato leaves over a time course of exposure to different temperatures. Values are the mean of 5 biological replicates ($n = 5$) from plants exposed to 10, 38 and $20\,°C$ (control conditions) over six days followed by five days of recovery at control temperature up to day 11. Means $\pm$ SE. Significant differences in data between temperature treatments are indicated: *, $P < 0.05$, by One-Way ANOVA.

3.3.2 Heat stress reduces chlorophyll content in tomato leaves

Total chlorophyll from tomato leaves was extracted and measured spectrophotometrically to determine differences in chlorophyll content after exposure to the three different temperatures (Fig. 3.2). A significant reduction in total chlorophyll content was detected

for tomato plants subjected to $38\,^{\circ}\mathrm{C}$ ($p = 0.0107$) for six days and after five additional days at $20\,^{\circ}\mathrm{C}$ for recovery ($p = 0.0289$) (11 days). Exposure to $10\,^{\circ}\mathrm{C}$ had no effect on chlorophyll levels.

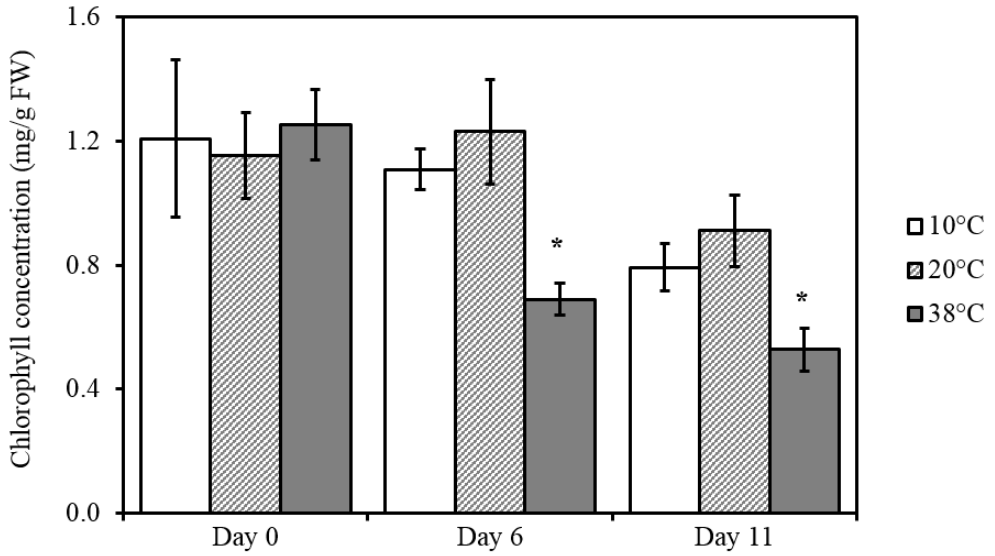

Fig. 3.2: Changes in chlorophyll content in tomato leaves exposed to different temperatures. Means ($n = 5$) $\pm$ SE. Significant differences in data between temperatures are indicated: *, $p < 0.05$, by One-Way ANOVA, followed by Bonferroni-Holm, post hoc test.

3.3.3 Untargeted lipidomics identify changes in lipid composition in tomato leaves after high temperature treatment

To determine the differences in lipid composition after exposure to the three different temperatures, we carried out untargeted lipidomics analysis (Fig. 3.3). The data obtained from total lipid extracts of leaves from each treatment were subjected to multivariate analysis to determine differences in lipid content between different temperatures. By this method, 791 markers were detected, most of which were not identified (Supplemental Table I, available online: refer to `http://journal.frontiersin.org/article/10.3389/fpls.2016.00167`). A principal component analysis (PCA) model was established to reduce data complexity according to variation in lipid content found after temperature treatments. PCA identifies and ranks major sources of variance, which allows to cluster samples based on similarities and differences, in this case, in measured lipid profiles. PCA displayed two distinct clusters when comparing the variability of lipids followed by temperature treatment tested in five biological replicates each (Fig. 3.3a). In the loadings plot (Fig. 3.3b), the most contributive features of the first principal component (PC1) were selected and characterized by a combination of tandem mass spectrometry data and consultation of databases such as LIPID MAPS (Supplemental Table I, *online*: `http://journal.frontiersin.org/article/10.3389/fpls.2016.00167`). This revealed that

prenylquinones, namely plastoquinol (PQH_2-9), plastoquinone (PQ-9) and α-tocopherol mostly contributed to the separation of the high temperature cluster, together with the galactolipid DGDG-18:3/16:0. Lower temperature treatment clusters were characterized by the presence of more unsaturated galactolipids such as MGDG-18:3/16:3 (Supplemental Figure I, available online: `http://journal.frontiersin.org/article/10.3389/fpls.2016.00167`), -18:3/18:3 and DGDG-18:3/18:3 (Fig. 3.3b). Loadings located near the center of the plot, (i.e. the vast majority) make a negligible contribution to metabolic variation.

To evaluate variations between cold treatment and control conditions data were submitted to supervised PLS-DA. In contrast to PCA, which failed to separate the two treatments, PLS-DA revealed two distinct clusters (Fig. 3.4a). Loadings from PLS-DA revealed that saturated diacylglycerols (DAG), such as DAG-18:0/18:0 and -18:0/16:0, phosphotidylethanolamines (PE), such as PE-18:2/18:2, -18:2/16:0 and saturated MGDG-18:3/16:0 were enhanced in the low-temperature samples (Fig. 3.4b). Moreover, unsaturated galactolipids, such as MGDG-18:2/18:3, -18:3/16:3, -18:3/16:1 and DGDG-18:3/18:3, as well as prenylquinones such as α-tocopherol, PQH2-9, PQ-9, PQ-OH and β-carotene contributed to the control treatment cluster (Fig. 3.4a and 3.4b).

3.3.4 High temperature increases prenylquinones in tomato leaves

To quantify the effects of different temperatures on tocopherols, δ-T (δ-tocopherol), γ-T (γ-tocopherol), α-T (α-tocopherol) and α-TQ (α-tocopherol quinone) were measured, using pure standards (Fig. 3.5). Levels of δ-T, γ-T ($p = 0.013$) and α-T ($p < 0.001$) increased under $38\,^{\circ}\mathrm{C}$ for Day 6. Levels of α-TQ, the oxidation product of α-T, appeared to increase at both $10\,^{\circ}\mathrm{C}$ and $38\,^{\circ}\mathrm{C}$, at Day 6, albeit not significantly when compared to control conditions. At Day 11, α-T ($p < 0.001$) and γ-T ($p = 0.001$) concentrations still remained significantly increased.

The effect of different temperatures on plastoquinol, plastochromanol and their relatives, PQ-9 (plastoquinone), PQH_2-9 (plastoquinol), PQ-OH (hydroxy-plastoquinone), PC-8 and PC-OH (hydroxyplastochromanol) were measured and quantified, using pure standards (Fig. 3.6a-e.). All of the compounds showed a significant increase at Day 6 at $38\,^{\circ}\mathrm{C}$ ($p < 0.001$) (Fig. 3.6) and remained significantly ($p < 0.001$) higher after five additional days of recovery under $20\,^{\circ}\mathrm{C}$ control conditions (Day 11). Phylloquinone content was quantified using a pure standard (Fig. 3.7a) but no changes was detected under any of the temperatures tested.

High temperature provokes a slight decrease in content of carotenoids in tomato leaves. Carotenoid contents were quantified after exposure to the same temperature regime as for

(a)

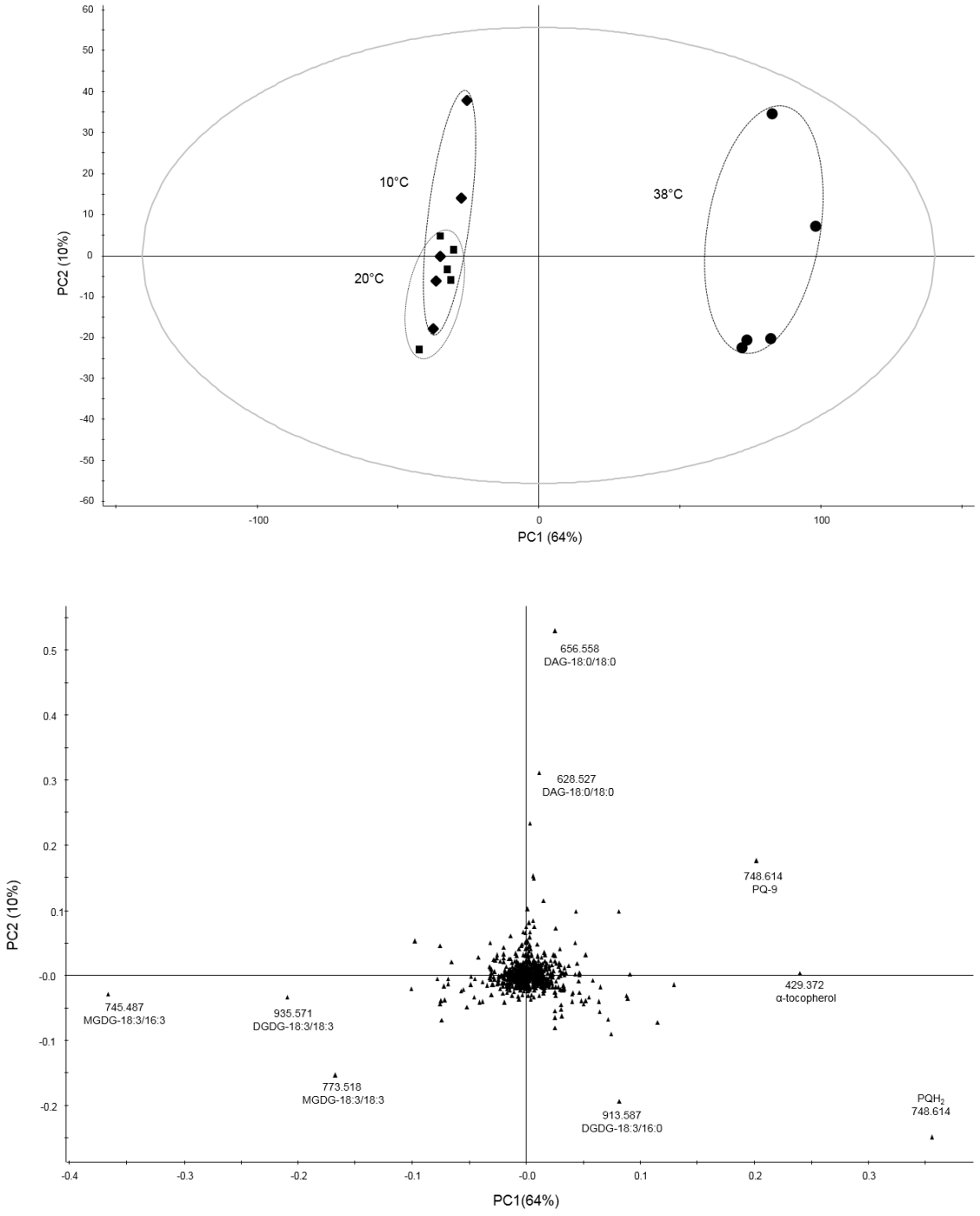

Fig. 3.3: Untargeted lipidomic profile of tomato leaves subjected to six days of low and high temperature treatments. (a) Principal component analysis (PCA) of the lipid composition of leaf samples exposed to 10, 38 and 20 °C (control conditions), over six days of temperature treatments. **(b)** Corresponding loading plots. Data were Pareto scaled prior to principal component analysis. PC1 and PC2 are first and second principal components, respectively, with their percentage of explained variance. Refer to Supplemental Table I for PCA loadings sorted according to the PC1.

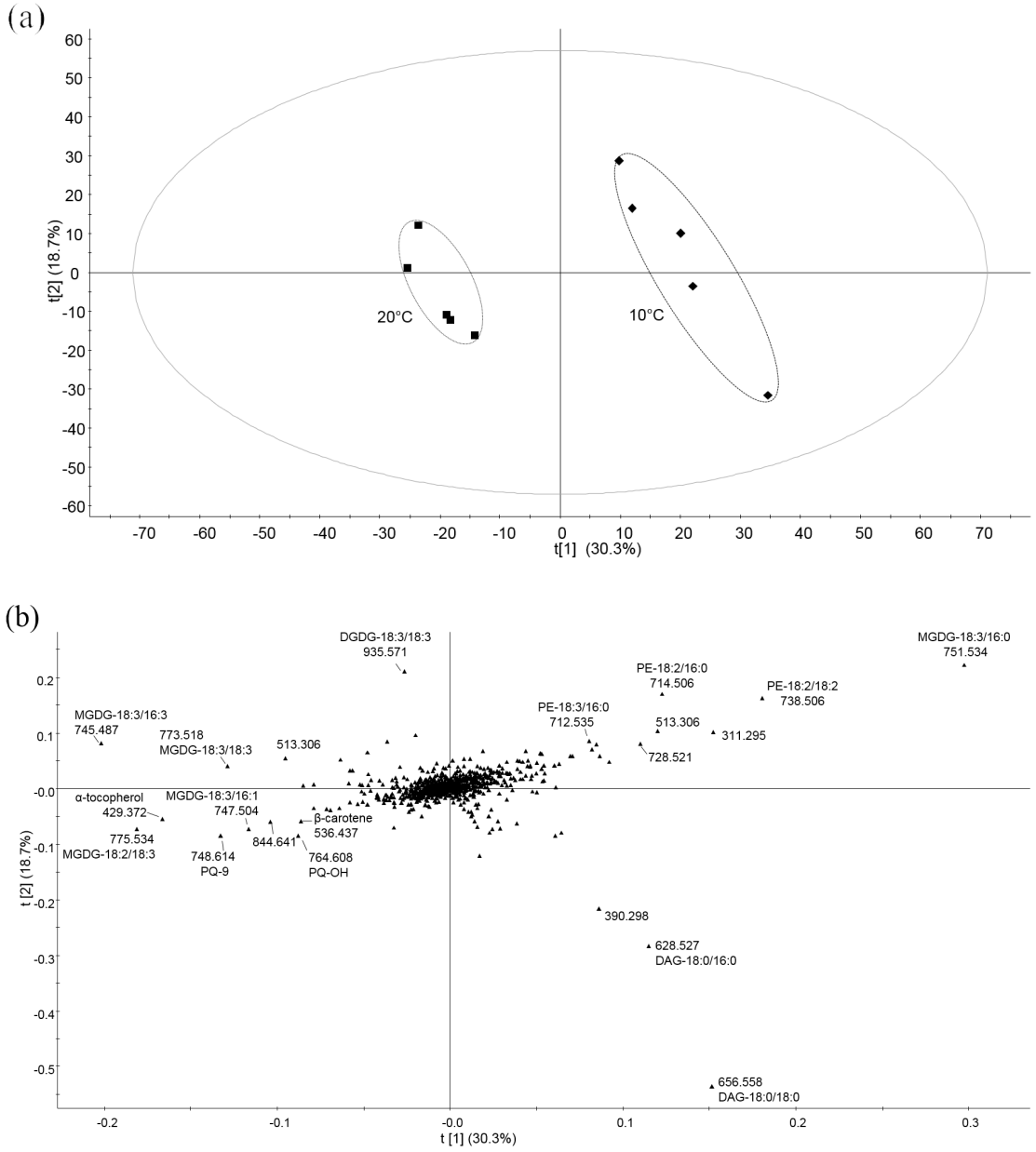

Fig. 3.4: Untargeted lipidomic profile of tomato leaves subjected to six days of low temperature treatment. (a) Partial least squares discriminant analysis (PLS-DA) of the lipid composition of leaf samples exposed to 10 and 20 °C (control conditions), over six days of temperature treatments. t[1] and t[2] are first and second latent variables, respectively, with their percentage of explained variance. Applying cross-validation on the data yielded high R2Y and Q2 coefficient values, which demonstrates the validity of the model (R2Y=0.988, Q2=0.899). **(b)** Corresponding loading plots. Data were Pareto scaled prior to PLS-DA. Refer to Supplemental Table I for PLS-DA loadings sorted according to the PC1.

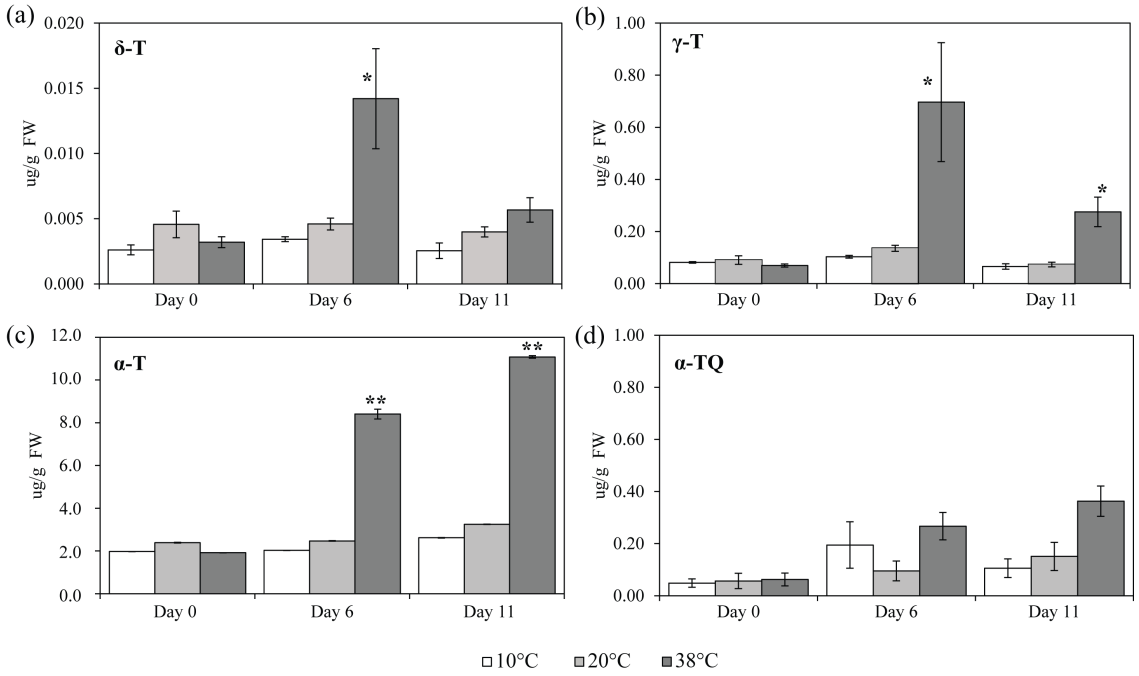

Fig. 3.5: δ**-T (**δ**-tocopherol),** γ**- T (**γ**-tocopherol),** α**-T (a-tocopherol) and** α**-TQ (a-tocopherol quinone) quantification in tomato leaves after exposure to three different temperatures. (a)** δ-T; **(b)** γ-T; **(c)** α-T; **(d)** α-TQ. Lipids were extracted from plants submitted to $10\,^{\circ}\mathrm{C}$, $38\,^{\circ}\mathrm{C}$ and $20\,^{\circ}\mathrm{C}$ for 6 days and then allowed to recover at $20\,^{\circ}\mathrm{C}$ control temperatures (Day 11); values are means $\pm$ SE, $(n = 5)$. Significant differences in data between temperatures are indicated: *, $p < 0.05$ and **, $p < 0.001$; by One-Way ANOVA, followed by Bonferroni-Holm, post-hoc test.

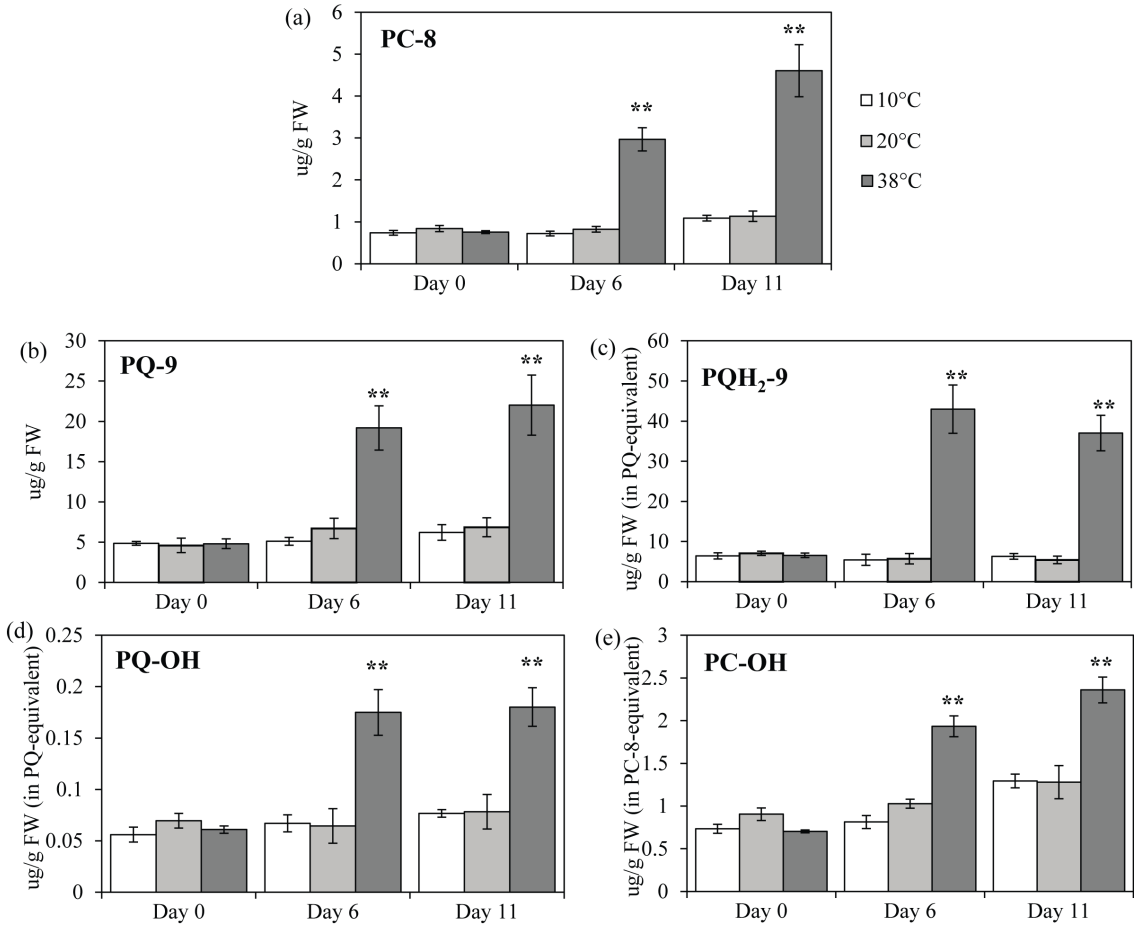

Fig. 3.6: PC-8 (plastochromanol), PQ-9 (plastoquinone), PQH_2-9 (plastoquinol), PQ-OH (hydroxy-plastoquinone), and PC-OH (hydroxy-plastochromanol) quantification in tomato leaves after temperature treatment and recovery period. (a) PC-8; **(b)** PQ-9; **(c)** PQH2-9; **(d)** PQ-OH; **(e)** PC-OH. Lipids were extracted from plants exposed to 10, 38 and 20 °C for 6 days and then allowed to recover under 20 °C control conditions for five additional days (Day 11); values are means ± SE, ($n = 5$). Significant differences in data between temperatures are indicated: *, $p < 0.05$ and **, $p < 0.001$; by One-Way ANOVA, followed by Bonferroni-Holm, post hoc test.

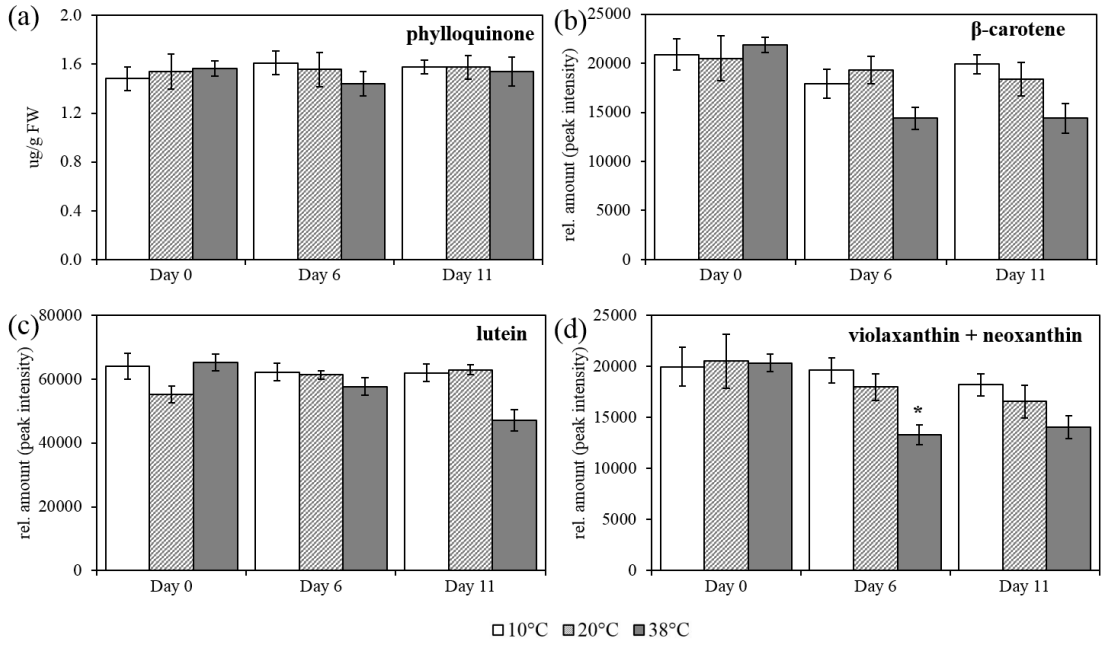

Fig. 3.7: Phylloquinone, β-carotene, lutein and combined violaxanthin+neoxanthin quantification in tomato leaves after temperature treatment and recovery period. (a) phylloquinone; **(b)** β-carotene; **(c)** lutein and **(d)** violaxanthin+neoxanthin. Lipids were extracted from plants exposed to 10, 38 and $20\,^{\circ}\mathrm{C}$ for 6 days and then allowed to recover under $20\,^{\circ}\mathrm{C}$ control conditions for 5 additional days, Day 11; values are means $\pm$ SE, ($n = 5$). Relative amount was calculated by peak intensity measurement in the chromatograms. Significant differences in data between temperatures are indicated: *, $p < 0.05$ and **, $p < 0.001$; by One-Way ANOVA, followed by Bonferroni-Holm, post hoc test.

the prenylquinones. For β-carotene, lutein, violaxanthin and neoxanthin together relative quantification was carried out by measuring the peak intensity in the chromatograms (Fig. 3.7b; 3.7c and 3.7.d; respectively). In contrast to the prenylquinones no significant changes in relative abundance of any of the compounds was observed under any of the three conditions tested except for combined violaxanthin and neoxanthin, which showed a significant decrease at Day 6 at $38\,^{\circ}\mathrm{C}$ ($p = 0.007$) (Fig. 3.5d).

3.3.5 Temperature modulates saturation of galactolipids mostly present in thylakoid membranes

Fatty acid-derived lipids were profiled in tomato leaves exposed to the same temperature regime as for prenylquinones and carotenoids. Phosphatidylethanolamine (PE), diacylglycerol (DAG), MGDG, DGDG were organized according to their saturation degree (Fig. 3.8) and identified following the sn-2 position of the glycerol backbone structure (prokaryotic referring to 16C fatty acid chains of plastid origin and eukaryotic to 18C fatty acid chains of endoplasmic reticulum origin, Roughan and Slack, 1982). The DAG feeding chloroplast lipid synthesis originates from two separate pathways: the endoplasmic reticulum-localized eukaryotic pathway and the prokaryotic pathway that is located at the plastid inner envelope (Ohlrogge and Browse, 1995). Data are means of relative fold change of fatty acid-derived lipids detected relative to control ($20\,^{\circ}\mathrm{C}$) treatment at Day 6.

After six days at $38\,^{\circ}\mathrm{C}$, there was a decrease in levels of unsaturated PE-18:2/18:2 accompanied by an increase in PE-18:3/16:0. Also, there was a reduction of unsaturated MGDGs of prokaryotic origin such as MGDG-18:3/16:3 and MGDG-18:3/16:1. Moreover, there was also a significant reduction in unsaturated DGDG-18:3/16:3 together with an increase in saturated DGDG-18:3/16:0 and -18:2/16:0. In fatty acid-derived lipids of eukaryotic origin, alterations of abundance were less remarkable but still, there was a significant reduction in the abundance of highly unsaturated DGDG-18:3/18:3 and -18:3/20:3 together with an increase of MGDG-18:2/18:3 under high temperature. In contrast to high temperature exposure, MGDG-18:2/18:3 significantly decreased after low-temperature exposure, and PE-18:2/18:2 increased significantly contrasting with its decrease at high temperature.

No significant changes were observed at the different temperatures for PE-18:2/16:0; DAG-18:0/18:0 and -18:0/16:0, nor for the eukaryotic MGDG-18:3/18:3, -18:2/18:3, and DGDG-18:1/18:3 and -18:0/18:3.

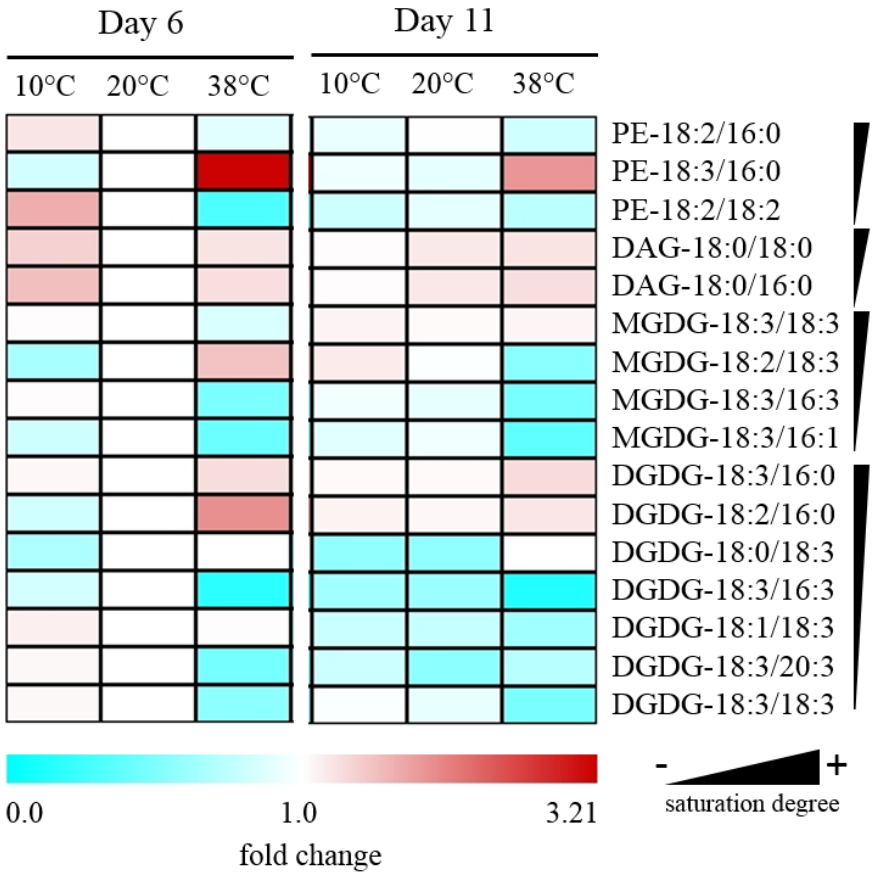

Fig. 3.8: Alteration of saturation levels of fatty acid-containing membrane lipids in tomato leaves under different temperatures. Data are means ($n = 5$) and are expressed by fold change of fatty acid-derived lipids detected relative to control (20 °C) treatment at Day 6. Phosphotidylethanolamine (PE); diacylglycerol (DAG); monogalactosyldiacylglycerol (MGDG); digalactosyldiacylglycerol (DGDG) are organized according to their saturation degree.

3.3.6 Discussion

Climate change exposes plants to increasing temperatures. Changes in temperature impact plant physiology with potentially deleterious effects. Inhibition of photosynthesis at the level of Photosystem II has been reported to occur after short exposure to high temperature (35-$40\,^{\circ}\mathrm{C}$), in a variety of species (Crafts-Brandner and Salvucci, 2000, 2002; Havaux, 1993; Law and Crafts-Brandner, 1999). Changes in temperature also have a direct influence on the physical properties of thylakoid membranes and affect electron transport dynamics (Williams, 1998). In this study, we aimed to determine the dynamics of lipid metabolism in response to high temperature ($38\,^{\circ}\mathrm{C}$) treatment and its impact on the photosynthetic efficiency of tomato plants using moderately low temperature ($10\,^{\circ}\mathrm{C}$) and $20\,^{\circ}\mathrm{C}$ as a reference. In contrast to other studies we did so at a lipidome-wide level and used statistical analysis to determine the most important changes. This approach allows to put observed changes in a wider context and gain insight on the relative importance of the observed effects. The ability of the plants to acclimate to the temperature regimes was probed by chlorophyll fluorescence measurements that give a measure of photosynthetic efficiency (Sinsawat et al., 2004) as well as photoinhibition at the level of Photosystem II that is the most thermosensitive component of the photosynthetic membrane (Berry and Björkman, 1980; Mathur et al., 2014; Srivastava et al., 1997). Chlorophyll contents was also measured as it is known to be reduced by high temperature stress (Dutta et al., 2009; Efeoglu and Terzioglu, 2009; Kumar Tewari et al., 1998). Indeed, high temperature ($38\,^{\circ}\mathrm{C}$) treatment also reduced the chlorophyll contents in tomato (Fig. 3.2). However, photosynthetic efficiency F_v/F_m was not reduced over the duration of the experiment at $38\,^{\circ}\mathrm{C}$ suggesting that the plants acclimated well. In contrast, $10\,^{\circ}\mathrm{C}$ treatment led to a reduction of F_v/F_m by around 4%. This mild photoinhibition suggested that tomato plants do not acclimate as well to low temperatures as to high temperatures.

Using non-targeted lipidomics we detected 791 molecular species in the mass spectra of total tomato leaf extracts. To determine the most important differences between the three conditions we used principle component analysis followed by the identification of compounds combining mass spectrometric information with searches in the online databases. The first principal component was dominated by the presence of prenylquinones and membrane lipids. These results indicate that the most important changes at the lipidome-wide level occur in tocopherols, plastoquinone/plastoquinone as well as their metabolites (Fig. 3.4) and in the degree of fatty acid saturation of galactolipids.

Several studies have already demonstrated that tocopherol production correlates with oxidative stress (for instance under high-light conditions), and suggested that this may reflect the ability of tocopherols to quench ROS and protect Photosystem II (DellaPenna and Pogson, 2006; Kobayashi and DellaPenna, 2008). Tocopherol biosynthesis is dependent on the availability of phytol. Recently, it has been demonstrate that phytyl diphosphate origi-

nating from chlorophyll degradation contributes significantly to tocopherol biosynthesis (Almeida et al., 2016; vom Dorp et al., 2015). It is therefore possible that the chlorophyll degradation observed at $38\,^{\circ}\mathrm{C}$ contributes directly to the increased biosynthesis of tocopherols under this condition. A temperature-dependent increase on the kinetics of prenylquinone biosynthesis at $38\,^{\circ}\mathrm{C}$ can not be excluded. However, the biosynthesis of phylloquinone as well as that of vast majority of other compounds in the samples were not affected. This suggests that there is no sweeping Arrhenius effect that would widely upregulate the synthesis of many lipid compounds (Guy et al., 2007; Kaplan et al., 2004; Ruelland and Zachowski, 2010). The high concentrations of tocopherols produced under high temperature, much like high-light, can probably be rationalized by an increased requirement for lipid antioxidants in reponse to this stress. A recent study reported a seven-fold increase in a-tocopherol in soybean that was grown at moderately high temperatures (33/$25\,^{\circ}\mathrm{C}$, Chennupati et al., 2011). Tocopherol levels in leaves subjected to $10\,^{\circ}\mathrm{C}$ treatment (in contrast to high temperature) were not significantly different from tocopherol levels detected under control temperature.

The decline in F_v/F_m in plants subjected to $10\,^{\circ}\mathrm{C}$ treatment suggests that the constant concentrations of the major prenylquinones at this temperature may be insufficient for plants to cope with this condition and, therefore, lead to photoinhibition. However, the decline in F_v/F_m was reversible indicating that no permanent damage had been inflicted (Haldimann et al., 1996). Low temperatures slow down enzymatic reactions and interfere with both antioxidant biosynthesis and regeneration leading to inhibition of photosynthesis (Allen and Ort, 2001; Jahnke et al., 1991). This may also pertain to tocopherol and explain why photoinhibition is inflicted. The unchanged concentrations of tocopherols under low-temperature treatment may also contribute to the destabilization of biophysical properties, such as the fluidity of the thylakoid membrane (Hincha, 2008).

Plastoquinone and plastoquinol were the most increased molecular species under high temperature. Plastoquinone is best known as an electron carrier in the photosynthetic electron transport chain (Amesz, 1973). However, recent reports identify plastoquinone as a lipid antioxidant functioning as a scavenger of singlet oxygen species and inhibitor of lipid peroxidation (Gruszka et al., 2008; Kruk and Trebst, 2008; Kruk et al., 2014; Mène-Saffrané and DellaPenna, 2010; Nowicka and Kruk, 2012; Nowicka et al., 2013; Olejnik et al., 1997; Szymańska and Kruk, 2010). A recent report showed that an Arabidopsis line overexpressing a gene in the biosynthetic pathway of PQ-9 SPS1 (SOLANESYL DIPHOSPHATE SYNTHASE 1) had two- to three-fold higher levels of PQ-9 and was more resistant to photooxidative stress under excess light when compared to the wild type, showing a decrease of bleaching, lipid peroxidation and PSII photoinhibition (Ksas et al., 2015). Barley seedlings exposed to 3 hours of high temperature treatment showed a reduction in the size of the photoactive plastoquinone-pool present in thylakoids, suggesting a transfer to the non-photoactive pool under temperature stress conditions (Pshybytko et al., 2008). The non-photoactive plastoquinone pool is not active in the photosynthetic electron transport

chain but participate in anti-oxidant reactions. The non-photoactive pool is contained in the plastoglobules (Ksas et al., 2015; Szymańska and Kruk, 2010). Our results showed a massive accumulation of PC-8, PQH_2-9, and PQ-9 as well as its oxidized metabolites PC-OH and PQ-OH (Fig. 3.5) under high temperature. It appears likely that the increase of PQ and metabolites under high temperature reflect the role of PQH_2-9 as a powerful membrane antioxidant rather than that of an electron carrier. Therefore, the increase of PQH_2-9 and PQ-9 may contribute mostly to the non-photoactive pool that is located in thylakoid-bound plastoglobules lipid droplets (Kruk and Trebst, 2008; Ksas et al., 2015). Carotenoids are important components in the light harvesting complexes, but have other functions, including the xanthophyll cycle in NPQ to dissipate excess excitation energy (DellaPenna, 1999; Müller et al., 2001). The interaction of carotenoids with membrane lipids may also directly influence membrane physical properties (Havaux, 1998). When carrying out targeted analysis of carotenoids, the only significant change we observed was a reduction of the sum of violaxanthin and neoxanthin after 6 days exposure to $38\,^{\circ}\mathrm{C}$. It thus appears that resistance to high temperatures does not require increased levels of carotenoids in general and that the decrease observed for xanthophylls does not have deleterious effects on NPQ and the ability to dissipate excess excitation energy.

Membrane fluidity is a function of the degree of saturation of membrane fatty acid lipids and temperature (Zheng et al., 2011). Our results confirm in tomato leaves that fatty acid saturation decreases at high temperatures and increases in the cold and show that these are amongst the most important lipidome-wide changes occurring. The most significant differences concern phosphatidylethanolamine (PE), an extraplastidic phospholipid, and mono- as well as digalactosyldiacylglyerol. Unsaturated PE-18:2/18:2 increased significantly at low-temperature contrasting with its decrease at high temperature. The prokaryotic origin galactolipids rich in unsaturated fatty acids, MGDG-18:3/16:3, -18:3/16:1 as well as DGDG-18:3/16:3, exhibited a striking drop at high temperature when compared to control and low temperature. In contrast, levels of more saturated DGDG-18:3/16:0 and -18:2/16:0 increased under high temperature treatment. These results are in agreement with reports that have shown an increase of the degree of saturation of fatty acids in Arabidopsis exposed to $36\,^{\circ}\mathrm{C}$ when compared to plants grown at $17\,^{\circ}\mathrm{C}$ (Falcone et al., 2004). The increase of saturation in membrane lipids has been shown to confer thermotolerance to Arabidopsis and tobacco plants (Kunst et al., 1989; Murakami et al., 2000; Routaboul et al., 2012).

Several unsaturated membrane lipids of eukaryotic origin decreased significantly under low temperatures, possibly because they are substrates of eukaryotic DGDG formation. The increase of unsaturated MGDG-18:2/18:3 at high temperature could be explained by a possible high temperature-induced perturbation affecting its recycling into DGDG. It has been reported that lipid saturation levels increase over time under heat stress, implying that there is a relation between the degree of saturation of leaf membrane prior to heat

stress and the ability of that plant to limit heat-induced damages during the stress period (Larkindale and Huang, 2004).

3.3.7 Acknowledgments

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3.4 Supplementary data

The Supplementary Material for this article can be found online at: `http://journal.frontiersin.org/article/10.3389/fpls.2016.00167`

The raw data used in this paper is archived at the Plant and Microbial Metabolomics Resource (PRM; URL: `http://www.metnetdb.org/PMR/experiments/?expid=259`).

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4

Down-regulation of tomato PHYTOL KINASE strongly impairs tocopherol biosynthesis and affects prenyllipid metabolism in an organ-specific manner

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Abstract

Tocopherol, a compound with vitamin E activity, is a conserved constituent of the plastidial antioxidant network in photosynthetic organisms. The synthesis of tocopherol involves the condensation of an aromatic head group with an isoprenoid prenyl side chain. The latter, phytyl diphosphate, can be derived from chlorophyll phytol tail recycling, which depends on phytol kinase (VTE5) activity. How plants coordinate the isoprenoid precursor distribution for supplying tocopherol and other prenyllipids biosynthesis in different organs is poorly understood. Here, *Solanum lycopersicum* plants impaired in the expression of two *VTE5*-like genes identified by phylogenetic analyses, named *SlVTE5* and *SlFOLK*, were characterized. Our data show that while SlFOLK does not affect tocopherol content, the production of this metabolite is over 80% dependent on SlVTE5 in tomato, both in leaves and fruits. VTE5 deficiency greatly impacted lipid metabolism, including prenylquinones, carotenoids and fatty acid phytyl esters. However, the prenyllipid profile greatly differed between source and sink organs, revealing organ-specific metabolic adjustments in tomato. Additionally, VTE5-deficient plants displayed starch accumulation and lower CO_2 assimilation in leaves associated with mild yield-penalty. Taken together, our results provide valuable insights into the distinct regulation of isoprenoid metabolism in leaves and fruits

and also expose the interaction between lipid and carbon metabolisms, which results in carbohydrate export blockage in the VTE5-deficient plants affecting tomato fruit quality.

4.1 Introduction

Tocopherols are potent lipid-soluble antioxidants synthesized only by photosynthetic organisms and together with tocotrienols are collectively referred as vitamin E (VTE) compounds (DellaPenna and Pogson, 2006; Kamal-Eldin and Appelqvist, 1996). Since plants are the major source of VTE required for human nutrition, understanding of the mechanisms underlying its synthesis and accumulation in crop species is of great interest (Fitzpatrick et al., 2012; Grusak and DellaPenna, 1999). The antioxidant function of tocopherols relies on their ability to scavenge peroxyl radicals, limiting lipid oxidation of polyunsaturated fatty acids (PUFAs) (Serbinova et al., 1991; Traber, 2008), and also singlet oxygen ($^1O_2$) (Di Mascio et al., 1990; Fukuzawa et al., 1997; Kaiser et al., 1990). In plants, light-driven photosynthetic processes are the main contributors to reactive oxygen species (ROS) production in chloroplasts owing to electron-transport chains and photosensitizing molecules such as chlorophyll (Chl) (Demmig-Adams et al., 2014; Edreva, 2005). The delicate equilibrium between ROS production and their detoxification in chloroplast, which determines damage, protection or signaling response, is controlled by a diversified ROS-scavenging system, including non-enzymatic antioxidant mechanisms (Edreva, 2005; Foyer and Noctor, 2005). Tocopherols, as part of the photoprotective machinery, are particularly involved in controlling the level of $^1O_2$ in photosystem II (PSII), and the extent of lipid peroxidation in thylakoid membranes specially under stress conditions (Miret and Munné-Bosch, 2015; Rastogi et al., 2014; Triantaphylidès and Havaux, 2009). Beyond photoprotective roles, tocopherol is also involved in seed longevity, seedling germination (Mène-Saffrané et al., 2010; Sattler et al., 2004), and photoassimilate export (Asensi-Fabado et al., 2014; Maeda et al., 2006, 2008); although, for the latter, the precise underlying mechanism remains elusive (Maeda et al., 2014).

Accumulation of tocopherol in plant tissues is a tightly controlled process and several studies determined that tocopherol levels change significantly during plant growth and development, as well as in response to environmental stimuli including high light, low temperature, salt and osmotic stress (Abbasi et al., 2007; Eugeni Piller et al., 2014; Loyola et al., 2011; Maeda et al., 2006; Munné-Bosch, 2005; Quadrana et al., 2013). Additionally, transgenic approaches have demonstrated that VTE content correlates with the expression of the biosynthesis- and recycling-related genes (reviewed by Dellapenna and Mène-Saffrané (2011)). Tocopherol synthesis occurs in plastids and requires two precursors, a prenyl side chain and a tyrosine catabolite-derived head group (Figure 4.1). The prenyl moiety phytyl diphosphate and homogentisate derived from the plastidial 2-C-methyl-D-erythritol 4-phosphate (MEP) and the shikimate pathway, respectively, are

condensed by homogentisate phytyl transferase (VTE2), the only tocopherol synthesis unique enzyme. From this precursor, the four naturally occurring tocopherol forms (α-, β-, γ- and δ-tocopherol), which vary in the methylation pattern of the chromanol ring, are synthesized via the action of methyl-phytylquinol methyl transferase (VTE3), tocopherol cyclase (VTE1), and tocopherol γ-methyl transferase (VTE4). These enzymes are also responsible for the synthesis of the other tocochromanols compounds, which not only include tocotrienols but also plastochromanol (PC-8), a product of plastoquinone (PQ-9) cyclization (Zbierzak et al., 2010).

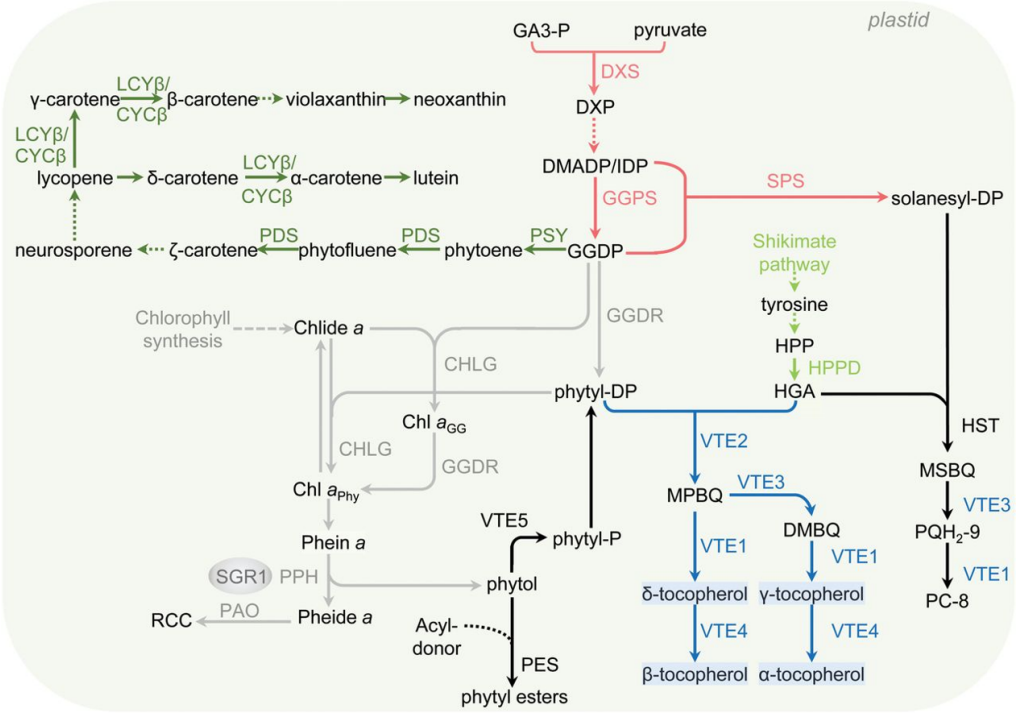

Fig. 4.1: Schematic view of tocopherol biosynthetic and related pathways. The genes are the following: 1-deoxy-D-xylulose-5-P synthase (DXS); geranylgeranyl diphosphate reductase (GGDR); 4-hydroxyphenylpyruvate dioxygenase (HPPD); homogentisate phytyl transferase (VTE2); 2,3-methyl-5-phytylquinol methyltransferase (VTE3); tocopherol cyclase (VTE1); γ-tocopherol-C-methyl transferase (VTE4); phytoene synthase (PSY); phytoene desaturase (PDS); chloroplast-specific β-lycopene cyclase (LCYβ); chromoplast-specific β-lycopene cyclase (CYCβ); chlorophyll synthase (CHLG); staygreen 1 (SGR1); pheophytinase (PPH); pheophorbide a oxygenase (PAO); phytol kinase (VTE5); farnesol kinase (FOLK); homogentisate solanesyl transferase (HST); solanesyl-diphosphate synthase (SPS). Abbreviated intermediate metabolites are: glyceraldehyde 3-phosphate (GA3-P); 1-deoxy-D-xylulose-5-P (DXP); isopentenyl diphosphate (IDP); dimethylallyl diphosphate (DMADP); geranylgeranyl-diphosphate (GGDP); hydroxyphenylpyruvate (HPP); homogentisate (HGA); chlorophyllide *a* (Chlide *a*); geranylgeranyl-chlorophyll *a* (Chl a_{GG}); phytylated-chlorophyll *a* (Chl a_{Phy}); pheophytin *a* (Phein *a*); pheophorbide *a* (Pheide *a*); red chlorophyll catabolite (RCC); 2-methyl-6-geranylgeranylbenzoquinol (MPBQ); 2,3-dimethyl-6-geranylgeranylbenzoquinol (DMBQ); 2-methyl-6-solanyl-1,4-benzoquinol (MSBQ); plastoquinol-9 (PQH$_2$-9); plastochromanol-8 (PC-8).

In addition to the *de novo* synthesis, the phytyl diphosphate precursor may also originate from Chl turnover or degradation, by the release of phytol moiety from tetrapyrrole ring.

Intriguingly, the precise identity of the tocopherol biosynthesis-related phytol hydrolase remains to be determined. In *Arabidopsis thaliana*, the absence of the known dephytylating enzymes, pheophytinase (PPH) and chlorophyllase (CLH), in triple mutants, does not alter seed tocopherol content whereas the seed-specific *PPH*-overexpressing transgenic lines exhibit modestly increased tocopherol levels (Zhang et al., 2014). The hydrolyzed phytol is sequentially phosphorylated by two enzymes, phytol kinase (VTE5) and phytyl-phosphate kinase (VTE6) (Ischebeck et al., 2006; Valentin et al., 2006; vom Dorp et al., 2015). VTE5 has been characterized in Arabidopsis where its mutant allele, *vte5*, causes a substantial reduction of the tocopherol content in seeds and to a lesser extent in leaves (Valentin et al., 2006). Furthermore, based on sequence similarity, a *locus* encoding a putative VTE5 paralog was identified in Arabidopsis, which further was characterized as a farnesol kinase (FOLK) (Fitzpatrick et al., 2011). However, its involvement in tocopherol biosynthesis was not addressed. So far, VTE5 has only been characterized in Arabidopsis, and its contribution to tocopherol content is largely unknown in other species and organs, such as in edible fleshy fruits. Moreover, the impact of VTE5 deficiency on plant metabolism remains unexplored. *Solanum lycopersicum* is an interesting model species for studying tocopherol metabolism. Besides being an important food crop worldwide, the fruits are a significant source of VTE for human diet (Chun et al., 2006). Additionally, tomato ripening, which encompasses the conversion of chloroplasts into chromoplasts, couples Chl degradation and an active MEP pathway (Seymour et al., 2013), both sources of the prenyl precursor for tocopherol biosynthesis (Almeida et al., 2015). A previous study on the regulation of tocopherol biosynthesis in this species demonstrated a strong correlation between *VTE5* mRNA levels and the contents of Chl and tocopherol in tomato leaves and fruits, suggesting the contribution of phytol recycling to tocopherol biosynthesis (Quadrana et al., 2013). Moreover, expression analysis of senescence-related tomato mutants suggested that maintenance of the *de novo* phytyl diphosphate synthesis might, at later ripening stages, compensate for the lack of Chl-derived phytol for tocopherol production in fruits (Almeida et al., 2015).

To better understand the extent of the contribution of the VTE5-dependent phytol pathway to tocopherol biosynthesis in source and sink organs, we functionally characterized *VTE5*-like genes in tomato. Tocopherol content was dramatically compromised both in leaves and fruits of *SlVTE5*-knockdown plants. By contrast, analyses of folk mutant genotype ruled out that *SlFOLK* is a major contributor to phytol kinase activity required for tocopherol biosynthesis. Additionally, VTE5 deficiency differentially impacts fatty acid phytyl ester and prenyllipid metabolism in fruits and leaves, and also have consequences in photosynthesis and sugar partitioning.

4.2 Materials and Methods

4.2.1 Plant material, growth conditions and sampling

Seeds of tomato (*Solanum lycopersicum*, cv. Micro-Tom) were obtained from the Laboratory of Hormonal Control of Plant Development (www.esalq.usp.br/tomato). The *folk-1* tomato mutant was isolated from an ethyl methanesulfonate (EMS)-mutagenized Micro-Tom collection from INRA, France. Plants were grown in a greenhouse under automatic irrigation at an average temperature of $25\,^{\circ}\mathrm{C}$, 11.5 h/13 h (winter/summer) photoperiod, and 250-350 $\mu\mathrm{mol\,m^{-2}\,s^{-1}}$ of incident photoirradiance. Source (the first fully expanded leaf) and sink (first apical leaf not fully expanded) leaves were sampled. Fruits were harvested at mature green, one day after breaker (B+1), three days after breaker (B+3) and ripe (B+6) stages at 35, 38, 40 and 43 days after anthesis, respectively. Samples were frozen in liquid N_2 and stored at $-80\,^{\circ}\mathrm{C}$. All biochemical analyses were performed in T_1 generation. For photosynthesis and yield evaluation, an independent experiment in T_2 generation was performed. Destructive harvest took place at a point where the largest possible number of fruits was ripe without visible overripening (15-week-old) (Vicente et al., 2015). At harvest time, aerial biomass was weighed and all the fruits were counted and weighed.

4.2.2 Phylogenetic analysis

For phylogenetic analysis, Blastp searches were performed using the protein sequences of *A. thaliana* VTE5 (At5g04490) and FOLK (At5g58560) as queries against the tomato genome (`http://solgenomics.net`). Homologous sequences from other plant species were retrieved by Blastp from Phytozome database (`http://phytozome.jgi.doe.gov/pz/portal.html`). *Nicotiana benthamiana* sequences were obtained from the Sol Genomics Network database (`http://solgenomics.net`). The sequences were aligned using the MUSCLE package available in the MEGA 5.0 software with default parameters (Tamura et al., 2007), and Neighbor-Joining phylogeny with 5000 bootstrap replications were created with the distances calculated according to the best model pointed by MEGA 5.0.

4.2.3 Generation of SlVTE5-RNA interference (RNAi) transgenic lines

Transgenic plants expressing a *SlVTE5*-specific intron-spliced hairpin sequence under the control of the cauliflower mosaic virus 35S promoter were obtained for RNAi-mediated silencing of Solyc03g071720 *locus*. A 237 bp fragment of *SlVTE5* was amplified by PCR using the primers RNAi-VTE5-F and RNAi-VTE5-R listed in Supplementary Table 5.S1. PCR

products were cloned into pENTR/d-TOPO vector (Invitrogen) via directional cloning, and then recombined into the binary vector pK7GWIWG2 (Karimi et al., 2002) to generate pK7GWIWG2(I)-*SlVTE5*. *Agrobacterium*-mediated transformation (strain EHA105) of *S. lycopersicum* was performed according to Pino et al. (2010). The presence of the transgene in T_0, T_1 and T_2 kanamycin-resistant plants was detected by PCR in genomic DNA using 35S-right and RNAi-VTE5-R primers (Supplementary Table 5.S1).

4.2.4 Identification of the folk-1 tomato mutant by TILLING

Mutations in *SlFOLK* were identified by screening an EMS-mutagenized tomato population (Just et al., 2013) essentially as described in Okabe et al. (2011). TILLING (Targeting Induced Local Lesions In Genomes) unlabeled external primers and internal primers 5' labeled with IRDye 700 and IRDye 800 dye are listed in Supplementary Table 5.S1. Induced point mutations were identified using the mismatch-specific endonuclease ENDO 1. Digested DNA fragments were separated on a LI-COR DNA analyser (LI-COR, USA). The mutation analysis was performed using PARSESNP (Taylor and Greene, 2003) and SIFT (Ng and Henikoff, 2003) softwares. Homozygous mutant plants were identified by sequencing of tilled M_3 family. Phenotypic characterization was performed in M_4 plants homozygous for *folk-1* allele using the corresponding segregating individuals homozygous for *FOLK* wild-type allele as control genotype.

4.2.5 qPCR analysis

RNA extraction, cDNA synthesis, and real-time quantitative PCR (qPCR) assays were performed as described by Quadrana et al. (2013). Primer sequences are listed in Supplementary Table 5.S1. qPCR reactions 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*, according to Quadrana et al. (2013). A permutation test lacking sample distribution assumptions (Pfaffl et al., 2002) was applied to detect statistical differences ($P < 0.05$) in expression ratios using the algorithms in the fgStatistics software package (Di Rienzo, 2009).

4.2.6 Leaf gas exchange and fluorescence measurements

Gas exchange and chlorophyll fluorescence parameters were evaluated in five-week-old plants using a portable open gas-exchange system incorporating infra-red CO_2 and water vapor analyzers (LI-6400XT system; LI-COR) equipped with an integrated modulated chlorophyll fluorometer (LI-6400-40; LI-COR). Reference [CO_2] was held at $400\,\mu\mathrm{mol\,mol^{-1}}$ and temperature at $25\,^{\circ}\mathrm{C}$ for all measurements. Air humidity inside the leaf chamber was

controlled to the externally measured greenhouse relative humidity (50–60%). Carbon assimilation rate (*A*), leaf stomatal conductance (g_s), leaf dark respiration (R_d) and fluorescence parameters were measured at at a photosynthetic photon flux density (PPFD) of $600\,\mu\mathrm{mol}$ PPFD $\mathrm{m}^{-2}\,\mathrm{s}^{-1}$ in the first fully expanded leaf between 10:00 h and 14:00 h. The parameters derived from Chl fluorescence, including light-adapted PSII maximum quantum efficiency (F'_v/F'_m), proportion of open PSII centers (photochemical quenching, *qP*), PSII operating efficiency (Φ_{PSII}), were calculated according to Genty et al. (1989).

4.2.7 Tocopherol, free phytol and fatty acid phytyl ester quantification

Tocopherols were extracted and measured by high performance liquid chromatography (HPLC) as previously described (Yang et al., 2011). For determination of fatty acid phytyl esters (FAPEs) and free phytol, total lipids were extracted with chloroform according to Lippold Lippold et al. (2012). Non-polar lipids were purified using chromatography on silica columns (Kieselgel 60; Merck). FAPEs were measured by direct infusion nanospray quadrupole time-of-flight tandem mass spectrometry (Q-TOF-MS/MS; Agilent 6530 Accurate Mass Q-TOF) using methanol:chloroform:300 mM ammonium acetate [665:300:35 (v/v/v); Welti et al. (2002)] as the solvent system. FAPEs were detected in the positive ion mode by neutral loss scanning for *m/z* 278.2974, a fragment characteristic for the phytol moiety. For phytol measurements, non-polar lipid fraction was silylated and then phytol quantified by gas chromatography coupled to mass spectrometry (GC-MS) as previously described (Lippold et al., 2012).

4.2.8 Prenylquinone and carotenoid profile

Prenylquinone and related compounds (α-tocopherolquinone, PQ-9, plastoquinol-9, hydroxy-plastoquinone, PC-8, hydroxy-plastochromanol, ubiquinone-10) were analyzed by a targeted analysis of lipidomic profile obtained by ultra-HPLC coupled with atmospheric pressure chemical ionization-quadrupole time-of-fight mass spectrometry (UHPLC-APCI-QTOF-MS) as described in Martinis et al. (2013) with the following modifications. Briefly, $15\,\mathrm{mg}$ of lyophilized tissue were exactly weighed and resuspended in $500\,\mu\mathrm{l}$ of tetrahydrofurane:methanol:water 42.5:42.5:15 (v/v/v). The mixture was homogenized using glass beads ($1\,\mathrm{mm}$ of diameter) for 3 min at $30\,\mathrm{Hz}$ in a tissue lyser. After two rounds of centrifugation (3 min, $14\,000\,\mathrm{g}$, and $4\,^{\circ}\mathrm{C}$), supernatants were transferred to vials. Prenyllipids were separated on a reverse-phase Acquity BEH C18 column ($50 \times 2.1\,\mathrm{mm}$, $1.7\,\mu\mathrm{m}$) under the following conditions: solvent A = water; solvent B = methanol; 80-100% B in 3 min, 100% B for 2 min, re-equilibration at 90% B for 0.5 min. The flow rate was $0.8\,\mathrm{ml\,min}^{-1}$, and the injection volume was $2.5\,\mu\mathrm{l}$. PQ-9 and PC-8 were quantifed based on calibration

curves obtained from standard compounds. Data were processed using MassLynx version 4.1 (Waters).

Carotenoids were extracted and detected as described in Almeida et al. (2015) using an Agilent 1200 Series HPLC system coupled with a Diode Array Detector on a reverse phase column [Zorbax Eclipse Plus C18 ($150\,\mathrm{mm} \times 4.6\,\mathrm{mm}$, $5\,\mu\mathrm{m}$), Agilent Technologies]. Compounds were identified at $440\,\mathrm{nm}$ by their order of elution and absorption spectra (Gupta et al., 2015), and co-migration with authentic standards (all-*trans*-lycopene, all-*trans*-β-carotene, lutein, violaxanthin, neoxanthin, and zeaxanthin). Relative quantification was performed based in chromatografic peak area normalized against sample dry weight.

4.2.9 Chlorophyll and chlorophyll catabolites

Chl and green catabolites (chlorophyllide, pheophorbide, pheophytin) were extracted from 10 mg of lyophilized tissue during 17 h at $-20\,^{\circ}\mathrm{C}$ in 90% (v/v) acetone, 10% (v/v) 0.2 M Tris-HCl, pH 8.0, pre-cooled to $-20\,^{\circ}\mathrm{C}$ ($5\,\mathrm{ml\,g^{-1}}$ initial fresh weight). After twice centrifugation (2 min, $16\,000\,\mathrm{g}$, $4\,^{\circ}\mathrm{C}$), supernatants were analyzed by HPLC as described Langmeier et al. (1993). Pigments were identified by their absorption spectra at $665\,\mathrm{nm}$. For quantification, peak areas were analyzed and referred to calibration curves built from known quantities of standard pigments (Schelbert et al., 2009).

4.2.10 Quantification of soluble sugars and starch

A $10\,\mathrm{mg}$ aliquot of the lyophilized samples were five times extracted with $1.5\,\mathrm{ml}$ of 80% ethanol at $80\,^{\circ}\mathrm{C}$ according to de Souza et al. (2013). Combined supernatants were dried under vacuum and re-suspended in 1 mL of ultrapure water. To remove pigments, an extraction with $0.5\,\mathrm{ml}$ of chloroform was performed. Alcohol-soluble sugar quantification was done by a high performance anion exchange chromatography with pulsed amperometric detection (HPAEC/PAD) (Dionex-ICS3000, Dionex). Sugar separation was carried out on a CarboPac PA1 column using isocratic elution of 150 mM NaOH with a flow rate of $1\,\mathrm{ml\,min^{-1}}$. The calibration curves were prepared using standard solutions of glucose, fructose and sucrose with a concentration range from $50\,\mu\mathrm{m}$ to $200\,\mu\mathrm{m}$.

For starch quantification, the dried insoluble material obtained after ethanol extraction was treated with α-amylase (120 U $\mathrm{ml^{-1}}$, Megazyme) from *Bacillus licheniformis* and amyloglucosidase (30 U $\mathrm{ml^{-1}}$, Megazyme) from *Aspergillus niger* according to Amaral et al. (2007). The glucose content obtained after starch hydrolysis was determined from extract aliquots of $20\,\mu\mathrm{l}$ and $50\,\mu\mathrm{l}$ for leaves and fruits, respectively, after an incubation with glucose oxidase/peroxidase and D-4-aminoantipirine (GOD/POD). Absorbance of quinoneimine dye, which is directly proportional to glucose concentration, was measured

spectrophotometrically using an ELISA-type microplate reader at $490\,\mathrm{nm}$. A standard curve was prepared using high purity glucose solution (Sigma) ranging from $2.5\,\mu\mathrm{g\,ml^{-1}}$ to $12.5\,\mu\mathrm{g\,ml^{-1}}$.

4.2.11 Trolox equivalent antioxidant capacity (TEAC) assay

The antioxidant capacity of non-polar extracts was assayed as previously described (Re et al., 1999), with minor modifications. The pre-formed radical 2,2'-azin-obis-(3-ethylbenzothiazoline-6-sulfonic acid) ($\mathrm{ABTS^{\bullet+}}$) was produced by oxidation of 7 mM ABTS with potassium persulphate (2.45 mM final concentration) dissolved in ultrapure water. The mixture was incubated in the dark at room temperature for 12–16 h before use. The $\mathrm{ABTS^{\bullet+}}$ solution was diluted with ethanol and adjusted to 0.70 ± 0.02 absorbance units at $734\,\mathrm{nm}$. A $50\,\mathrm{mg}$ aliquot of diluted extract or Trolox standard was mixed with $150\,\mu\mathrm{l}$ of diluted $\mathrm{ABTS^{\bullet+}}$ solution, and the absorbance was read at $734\,\mathrm{nm}$ after 10 min at $30\,^{\circ}\mathrm{C}$. The $\mathrm{ABTS^{\bullet+}}$ antioxidant capacity was reported as $\mu\mathrm{m}$ of TEAC per gram of sample on a dry weight basis by comparing with a Trolox standard curve (0.015–0.50 mM). Analyses were run in triplicate at two dilutions for a total of six assays per sample.

4.2.12 Transmission electron microscopy

Leaf segments were fixed at $4\,^{\circ}\mathrm{C}$ in Karnovsky's solution [2.5% glutaraldehyde, 2% (v/v) paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.2] for 24 h. After washing in buffer, the samples were post-fixed in buffered 1% (w/v) osmium tetroxide, washed, dehydrated in a graded series of acetone, and embedded in Spurr resin. The resin was polymerized at $60\,^{\circ}\mathrm{C}$. Ultrathin sections were stained with saturated uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) and observed using a Zeiss EM 900 transmission electron microscope.

4.2.13 Data analyses

Statistical analyses were performed using R statistical software (`www.r-project.org`). To determine significant differences between the transgenic lines and the control, data were analyzed by *t*-test or one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test with the level of significance set to 0.05.

4.3 Results

4.3.1 Tomato tocopherol contents are highly dependent on SlVTE5 but not on SlFOLK

By using the *A. thaliana* VTE5 protein sequence (At5g04490; Valentin et al. (2006)) as query, a survey for homologous sequences in the *S. lycopersicum* genome was performed in the Solanaceae Genomics Network (`http://solgenomics.net/`). Two *loci* were identified, Solyc03g071720 and Solyc09g018510. In order to establish the orthology relationships, a phylogenetic analysis was performed with VTE5 homologous protein sequences of 14 flowering species with completely sequenced genomes. The tree revealed two clades whose topology coincided with the established phylogenetic relationships between the analyzed species. One clade contains the Arabidopsis VTE5 protein sequence (Valentin et al., 2006) that clustered together with Solyc03g071720. The other clade groups At5g58560, an earlier proposed VTE5 paralog that was further identified as a farnesol kinase (FOLK; (Fitzpatrick et al., 2011)), together with Solyc09g018510. This analysis displayed VTE5 and FOLK proteins as sister clades, and the respective genes were named *SlVTE5* and *SlFOLK* (Supplementary Fig. 4.1). Both genes showed similar expression patterns with highest mRNA levels found in green tomato tissues (Supplementary Fig. 4.S2).

In order to gain experimental evidence regarding SlVTE5 function in the tocopherol biosynthesis, transgenic *SlVTE5*-knockdown plants were generated by RNA interference (RNAi)-mediated silencing. Out of eight primary transformants that showed reduced levels of *SlVTE5* mRNA (Supplementary Fig. 4.S3), three lines with reduction over 80% were selected for further analyses; *SlVTE5*-RNAi#1, *SlVTE5*-RNAi#7 and *SlVTE5*-RNAi#11 (Fig. 4.2A). Under normal growth conditions, these transgenic lines exhibited no evident morphological alterations and an apparently unaltered pattern of fruit degreening (Supplementary Fig. 4.S3B).

HPLC analysis revealed that down-regulation of *SlVTE5* resulted in a dramatic reduction (80-90%) of total tocopherol contents both in leaves and in fruits (Fig. 4.2B). Overall, no differences in tocopherol composition were observed (Supplementary Table 4.S2) except for the line #7 at mature green stage. Notably, we detected only traces of tocotrienols in *SlVTE5*-RNAi lines (data not shown). These results ruled out the possibility that depletion of tocopherols could be compensated by tocotrienol production in these plants.

The VTE5 deficiency in transgenic lines would be expected to increase free phytol content (Valentin et al., 2006). We therefore assayed the amount of this metabolite by GC-MS. While in mature leaves and ripe fruits of wild-type tomato plants free phytol amount ranged from $100\,\mathrm{nmol\,g^{-1}}$ DW to $190\,\mathrm{nmol\,g^{-1}}$ DW, in the counterparts from *SlVTE5*-RNAi

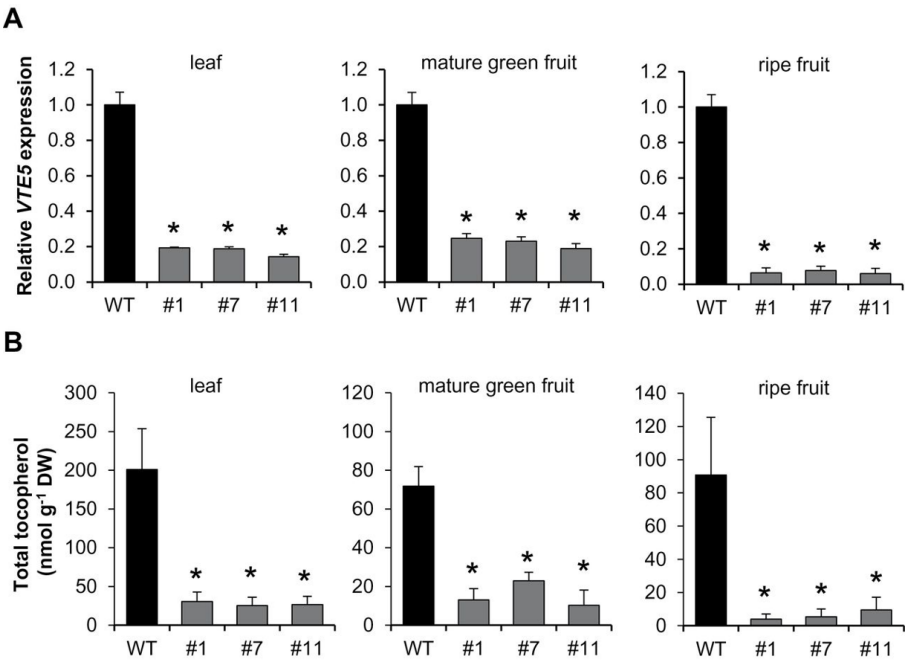

Fig. 4.2: Down-regulation of *SlVTE5* expression and tocopherol content in *SlVTE5*-RNAi transgenic lines. (A) Relative expression of *SlVTE5* gene in wild-type (WT) and *SlVTE5*-RNAi lines (#1, #7 and #11). Data are means ± SEM of five biological replicates. Asterisk denotes statistically signi?cant differences (permutation test, P < 0.05). **(B)** Total tocopherol was measured in leaves, mature green and ripe fruits of *SlVTE5*-RNAi lines. Data represent the mean ± SD of five biological replicates. Asterisk denotes significant differences between the wild-type (WT) and the transgenic lines (ANOVA/Dunnett's test, $P < 0.05$). DW, dry weight.

lines this prenyl alcohol accumulated four to five times more (Fig. 4.3). Interestingly, the molar amount of free phytol that accumulated in leaves was of the same order of magnitude as the reduction observed in total tocopherol contents. Strikingly, the increase in the amounts of free phytol in transgenic ripe fruits was 10 times higher than the decrease in tocopherol (Supplementary Table 4.S3), suggesting differential regulation in response of phytol metabolism perturbations in source and sink tomato organs.

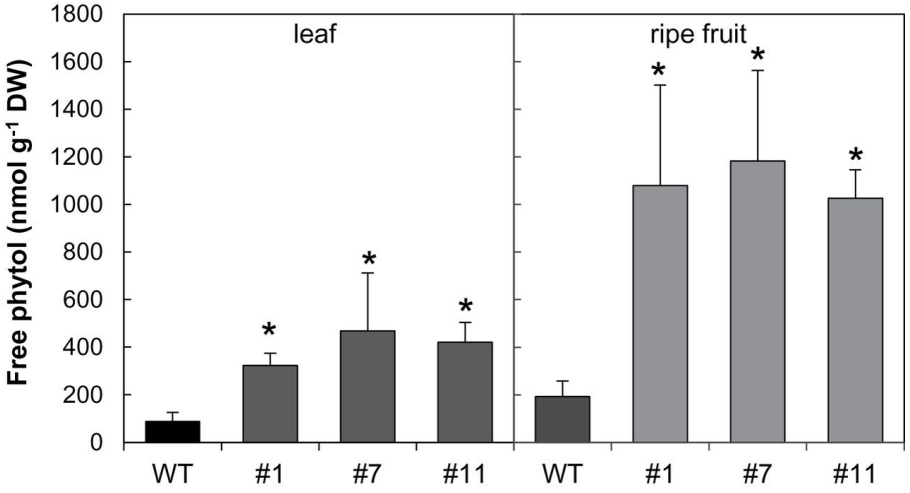

Fig. 4.3: Free phytol content in leaves and ripe fruits of the *SlVTE5*-RNAi transgenic lines. Data represent the mean ± SD of at least three biological replicates. Asterisk denotes significant differences between the wild-type (WT) and the transgenic lines (ANOVA/Dunnett's test, $P < 0.05$). DW, dry weight.

Due to sequence similarity between the tomato phosphatidate cytidylyltransferases proteins SlVTE5 and SlFOLK, and the lack of a complete functional characterization of the latter, the putative impact of SlFOLK on tocopherol metabolism was also explored. In this case, a TILLING-based molecular screening was applied to identify a loss-of-function mutation in *SlFOLK* using an EMS-mutagenized tomato collection. Among the identified mutants, one, named *folk-1*, displayed a G to A substitution disrupting the 3' splicing site of intron 4. Sequence analyses of *folk-1* cDNA from homozygous mutant plants revealed that this lesion led to the use of a cryptic splicing site in intron 4, producing a mRNA that lacks exon 4 and contains a fragment of intron 4 (Supplementary Fig. 4.S4A). This abnormal spliced transcript of *FOLK*, which is the only isoform detected in the mutant, contains an in-frame premature stop codon that presumably leads to a truncated protein (Fig. 4.4A, Supplementary Fig. 4.S4A, B). mRNA harboring premature termination codons can be recognized by the RNA surveillance machinery as aberrant; these transcripts may be targeted by the nonsense-mediated decay pathway being rapidly degraded (Filichkin et al., 2015). Expression analysis by qPCR showed the amount of the abnormal mRNA in *folk-1* corresponded to only 10% of the fully spliced transcript found in control plants (Supplementary Fig. 4.S4C). Tocopherol levels and composition in plants homozygous for the *folk-1* allele were much the same as in control plants, suggesting a small, if any, contribution of SlFOLK to tocopherol biosynthesis both in leaves and fruits (Fig. 4.4B,

Supplementary Table 4.S2). Having demonstrated the major role of *VTE5* in tomato tocopherol metabolism, we further performed a comprehensive phenotypic characterization of *SlVTE5*-RNAi lines.

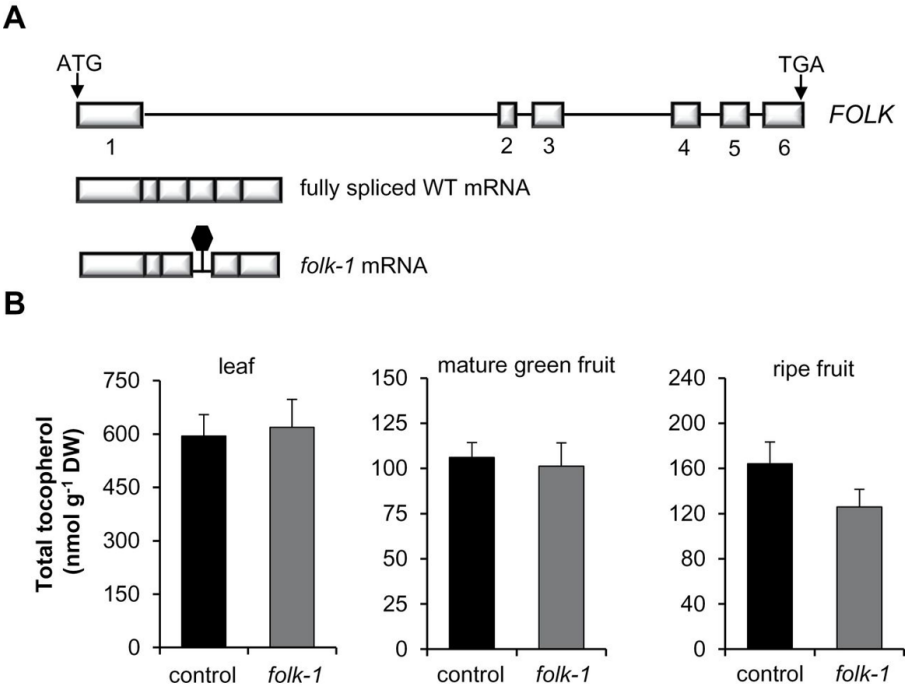

Fig. 4.4: Total tocopherol content in *folk-1* mutant. (A) Diagram showing *SlFOLK* gene and fully spliced mRNA found in wild-type (WT) and abnormal spliced mRNA found in *folk-1* mutant. Boxes and solid lines represent exons and introns, respectively. The premature stop codon is indicated by a black hexagon. **(B)** Total tocopherol was measured in leaves, mature green and ripe fruits of M4 plants homozygous for *folk-1* allele. The corresponding segregating individuals homozygous for *FOLK* WT allele were used as control. Data represent the mean ± SD of five biological replicates. No significant differences were observed (Student's t-test, $P > 0.05$). DW, dry weight.

4.3.2 Down-regulation of SlVTE5 boosted phytyl ester synthesis in leaves

Free phytol can be esterified directly with fatty acids derived from activated acyl groups. Fatty acid phytyl esters (FAPEs) increase during stress-associated Chl degradation (e.g. nitrogen deprivation) and senescence (Gaude et al., 2007; Lippold et al., 2012). To address the question whether the increased phytol levels affect FAPE contents in the *SlVTE5*-RNAi lines, the level of these compounds was measured by direct infusion Q-TOF MS/MS. Notably, *SlVTE5* knockdown resulted in a dramatic increase of FAPE content up to 10-fold in leaves. In contrast, fruits from *SlVTE5*-RNAi lines exhibited levels of FAPE identical to those of wild-type plants (Fig. 4.5). Besides the total amount, FAPE composition was also highly affected in transgenic leaves (Fig. 4.6A). With the exception of palmitic (16:0) and linolenic (18:3) acids, analysis of profiles showed that the contribution of the different

acyl chains was not proportional to the increment in total FAPE content observed in *SlVTE5*-RNAi lines. In particularly, FAPEs containing oleic acid (18:1), hexadecatrienoic acid (16:3), and medium-chain fatty acids (10:0, 12:0, 14:0) exhibited a reduction in their relative content, while FAPEs containing stearic acid (18:0) and linoleic acid (18:2) became the predominant forms, increasing at least two-fold in transgenic leaves compared to wild-type. In *SlVTE5*-RNAi fruits the FAPE composition at mature green and ripe stages remained almost unchanged (Fig. 4.6 B,C).

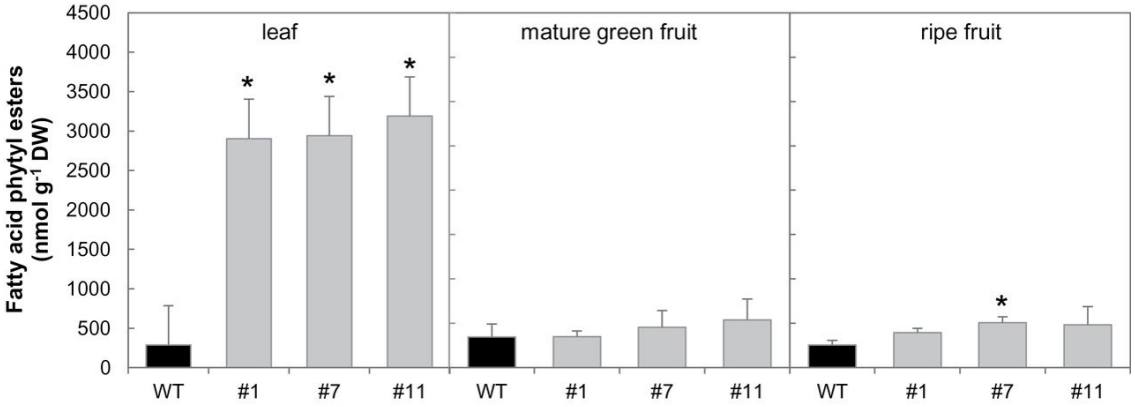

Fig. 4.5: Total fatty acid phytyl ester (FAPE) content in *SlVTE5*-RNAi transgenic lines. Data represent the mean ± SD of at least three biological replicates. Asterisk denotes significant differences between the wild-type (WT) and the transgenic lines (ANOVA/Dunnett's test, $P < 0.05$). DW, dry weight.

4.3.3 Chlorophyll content is not affected in SlVTE5-RNAi lines

To examine whether *SlVTE5* knockdown affects Chl metabolism, we determined Chl *a*, Chl *b* and pheophytin *a* (Phein *a*) levels in leaves and fruits at three different ripening stages by HPLC. The contents of these compounds were largely unaltered in both tested organs, suggesting that accumulation of phytol did not significantly affect Chl and Phein *a* levels in tomato (Fig. 4.7).

4.3.4 SlVTE5 knockdown alters prenyllipid metabolism in fruits

In addition to tocopherols, the plastidial antioxidant network includes a variety of prenyllipids derived from the MEP isoprenoid pathway with strong antioxidant properties, such as carotenoids, prenylquinones (PQ-9) and other tocochromanols (e.g. PC-8) (Nowicka et al., 2013). To investigate whether tocopherol deficiency in *SlVTE5*-RNAi lines is compensated by any other non-enzymatic antioxidant mechanism, we performed a comprehensive profiling of prenyllipids (Table 4.1). For determination of prenylquinone and their derivative compounds, a targeted analysis of lipidomic profile obtained by UHPLC-QTOF MS method was performed, whereas carotenoids were quantified by HPLC. In leaves and ripe fruits of the transgenic plants, depletion of tocopherol was accompanied by a decrease

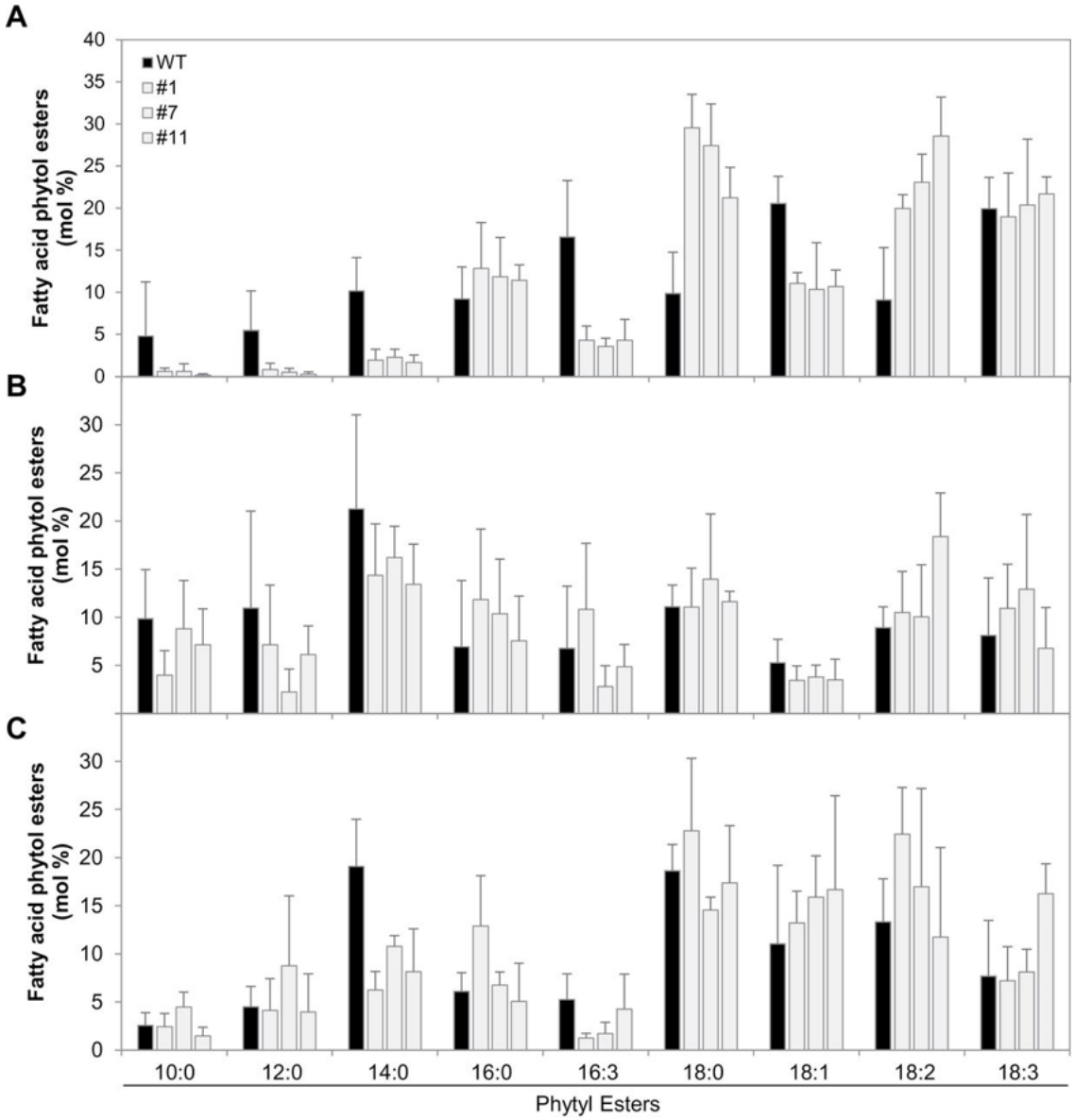

Fig. 4.6: Molecular species composition of fatty acid phytyl esters (FAPEs) in *SlVTE5*-RNAi transgenic lines. FAPEs were measured in leaves (A), mature green (B) and ripe fruits (C). Data represent the mean ± SD of at least three biological replicates

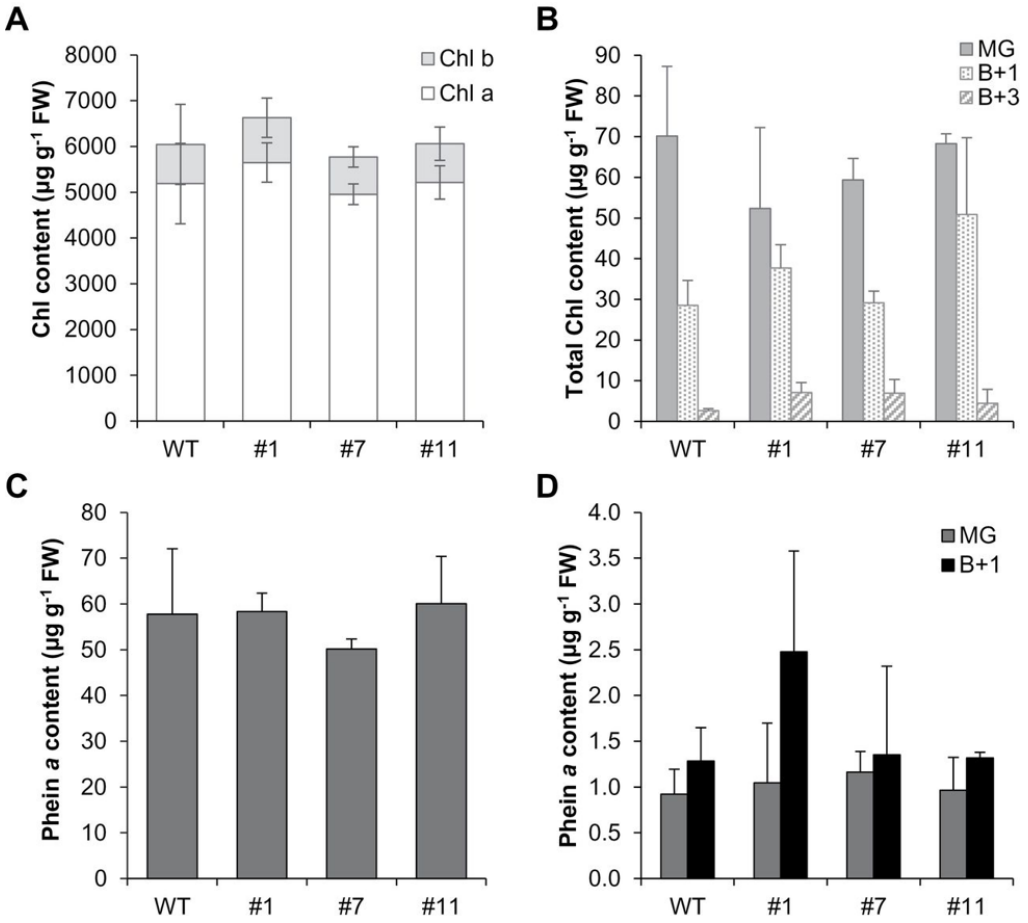

Fig. 4.7: Chlorophyll (Chl) and pheophytin *a* (Phein *a*) content in *SlVTE5*-RNAi transgenic lines. **(A,C)** Quantification of Chl and Phein *a* in leaves. **(B,D)** Quantification of Chl and Phein a in fruits at mature green (MG), breaker+1 (B+1) and breaker+3 (B+3) stage. Data represent the mean ± SD of at least three biological replicates. No significant differences were observed (ANOVA/Dunnett's test, $P > 0.05$). FW, fresh weight.

of α-tocopherolquinone, an oxidized intermediate of the tocopherol redox cycle; yet, the level of this metabolite remained unchanged in mature green fruits. Remarkably, the levels of photosynthetic electron carrier phylloquinone (vitamin K), another product of phytyl diphosphate-dependent biosynthesis, did not change between *SlVTE5*-RNAi lines and wild-type control.

The presence of the reduced and oxidized forms of PQ-9 in wild-type tomato leaves has already been reported (Jones et al., 2013), and our data showed that PC-8 is also detected in *S. lycopersicum*, in both leaves and fruits (Supplementary Fig. 4.S5). PC-8 was less abundant than tocopherols in wild-type tomato leaves as described for other species (Kruk et al., 2014). In fruits, however, the amount of these tocochromanols was similar (Fig. 4.2 and Supplementary Fig. 4.S5).

The comparison of prenyllipid profiles between wild-type and *SlVTE5*-knockdown leaves revealed that PQ-9 forms (PQ-9, PQH_2-9 and PQ-OH) remained unchanged. In contrast, a reduction to 50% of PC-8 content was observed in *SlVTE5*-RNAi lines, which was accompanied by lower levels of its oxidation product, PC-OH (Table 4.1). In sharp contrast, the prenylquinone pool of transgenic fruits was significantly increased. PQ-9 levels were >2-fold higher in the *SlVTE5*-RNAi lines, although the reduced form PQH_2-9 were unchanged. Intriguingly, the levels of the mitochondrial prenylquinone UQ-10 were also $\sim$ 2-fold increased in *SlVTE5*-RNAi fruits. In addition, PC-8 accumulated up to 2-fold in both mature green and ripe fruits of the transgenic lines (Table 4.1). These results suggest that the pool of PQ-9 and PC-8 contributes to fulfilling antioxidant function in the transgenic fruits.

Carotenoid contents in leaves and mature green fruits remained largely unaltered in the *SlVTE5*-RNAi lines (Table 4.1). Although no differences in visual appearance were identified (Supplementary Fig. 4.S3B), at the ripe stage, silenced fruits exhibited 30% less lycopene than those of wild-type plants, which was also accompanied by an equivalent reduction in levels of lycopene precursors (phytoene, phytofluene and ζ-carotene).

Considering the differences observed in prenyllipid profiles, the total antioxidant capacity between *SlVTE5*-RNAi and wild-type tomato plants were evaluated in non-polar extracts by the Trolox equivalent antioxidant capacity (TEAC) assay. Transgenic ripe fruits showed a reduction in TEAC values in lines #1 and #11 (Supplementary Fig. 4.S6). These results emphasize the role of tocopherol and/or carotenoids in antioxidant protection, since the increase in prenyllipid contents exhibited in the fruits of the *SlVTE5*-RNAi lines did not compensate the TEAC values up to those observed in wild-type plants.

Table 4.1: Changes in prenyllipids contents in leaves and fruits of *SlVTE5*-RNAi transgenic lines compared with wild-type.

Prenyllipids		*SlVTE5*-RNAi		
(relative amounts)	WT	#1	#7	#11
Leaf				
α-TQ	1.00 ± 0.18	$\mathbf{0.23 \pm 0.03}$	$\mathbf{0.22 \pm 0.08}$	$\mathbf{0.24 \pm 0.01}$
PC-8	1.00 ± 0.30	$\mathbf{0.52 \pm 0.19}$	$\mathbf{0.54 \pm 0.19}$	$\mathbf{0.36 \pm 0.07}$
PQ-9	1.00 ± 0.18	0.83 ± 0.15	0.87 ± 0.20	$\mathbf{0.63 \pm 0.12}$
PQH_2-9	1.00 ± 0.66	1.27 ± 0.59	1.02 ± 0.43	0.86 ± 0.37
PC-OH	1.00 ± 0.21	$\mathbf{0.65 \pm 0.18}$	0.72 ± 0.24	$\mathbf{0.51 \pm 0.11}$
PQ-OH	1.00 ± 0.20	1.01 ± 0.26	1.04 ± 0.27	0.70 ± 0.19
UQ-10	1.00 ± 0.24	0.83 ± 0.23	0.81 ± 0.12	0.81 ± 0.10
phylloquinone	1.00 ± 0.16	0.87 ± 0.12	0.84 ± 0.12	0.81 ± 0.12
β-carotene	1.00 ± 0.07	0.89 ± 0.04	0.91 ± 0.02	0.91 ± 0.07
lutein	1.00 ± 0.07	0.97 ± 0.06	0.96 ± 0.03	0.95 ± 0.03
violaxanthin/neoxanthin	1.00 ± 0.06	0.97 ± 0.06	1.00 ± 0.02	0.99 ± 0.06
Mature green fruit				
α-TQ	1.00 ± 0.15	1.11 ± 0.48	1.20 ± 0.47	1.13 ± 0.10
PC-8	1.00 ± 0.15	$\mathbf{1.89 \pm 0.26}$	$\mathbf{1.78 \pm 0.05}$	$\mathbf{1.88 \pm 0.19}$
PQ-9	1.00 ± 0.15	$\mathbf{2.11 \pm 0.46}$	$\mathbf{2.22 \pm 0.42}$	$\mathbf{2.22 \pm 0.26}$
PQH_2-9	1.00 ± 0.30	0.77 ± 0.23	1.24 ± 0.36	1.16 ± 0.36
PC-OH	1.00 ± 0.42	0.89 ± 0.34	1.10 ± 0.30	1.02 ± 0.29
PQ-OH	1.00 ± 0.26	1.31 ± 0.49	$\mathbf{1.74 \pm 0.47}$	1.52 ± 0.32
UQ-10	1.00 ± 0.13	$\mathbf{2.13 \pm 0.24}$	$\mathbf{1.76 \pm 0.28}$	$\mathbf{1.93 \pm 0.34}$
phylloquinone	1.00 ± 0.27	1.23 ± 0.18	1.25 ± 0.32	1.32 ± 0.16
β-carotene	1.00 ± 0.22	0.96 ± 0.16	0.92 ± 0.15	1.00 ± 0.16
lutein	1.00 ± 0.22	0.98 ± 0.10	1.09 ± 0.11	$\mathbf{1.31 \pm 0.15}$
violaxanthin/neoxanthin	1.00 ± 0.34	0.85 ± 0.16	0.85 ± 0.07	1.28 ± 0.20
Ripe fruit				
α-TQ	1.00 ± 0.25	$\mathbf{0.54 \pm 0.04}$	$\mathbf{0.64 \pm 0.17}$	$\mathbf{0.64 \pm 0.15}$
PC-8	1.00 ± 0.15	$\mathbf{2.29 \pm 0.34}$	$\mathbf{1.67 \pm 0.20}$	$\mathbf{2.07 \pm 0.05}$
PQ-9	1.00 ± 0.17	$\mathbf{2.40 \pm 0.54}$	$\mathbf{2.24 \pm 0.20}$	$\mathbf{1.76 \pm 0.26}$
PQH_2-9	1.00 ± 0.29	1.55 ± 0.39	1.77 ± 0.55	1.92 ± 1.06
PC-OH	1.00 ± 0.27	1.26 ± 0.51	1.35 ± 0.46	0.99 ± 0.42
PQ-OH	1.00 ± 0.07	2.27 ± 1.03	2.24 ± 0.80	2.10 ± 0.94
UQ-10	1.00 ± 0.14	$\mathbf{2.40 \pm 0.38}$	$\mathbf{1.96 \pm 0.53}$	$\mathbf{2.13 \pm 0.24}$
phylloquinone	1.00 ± 0.18	1.11 ± 0.18	1.32 ± 0.50	1.41 ± 0.05
phytoene	1.00 ± 0.09	$\mathbf{0.68 \pm 0.11}$	$\mathbf{0.72 \pm 0.19}$	$\mathbf{0.62 \pm 0.10}$
phytofluene	1.00 ± 0.14	$\mathbf{0.72 \pm 0.13}$	$\mathbf{0.66 \pm 0.14}$	$\mathbf{0.64 \pm 0.08}$
neurosporene	1.00 ± 0.22	0.73 ± 0.24	0.97 ± 0.18	$\mathbf{0.60 \pm 0.20}$
ζ-carotene	1.00 ± 0.20	$\mathbf{0.44 \pm 0.17}$	$\mathbf{0.33 \pm 0.19}$	$\mathbf{0.39 \pm 0.18}$
lycopene	1.00 ± 0.06	$\mathbf{0.78 \pm 0.14}$	$\mathbf{0.70 \pm 0.08}$	$\mathbf{0.74 \pm 0.13}$
β-carotene	1.00 ± 0.06	0.87 ± 0.20	0.86 ± 0.16	0.90 ± 0.14
lutein	1.00 ± 0.13	1.07 ± 0.13	1.02 ± 0.09	1.19 ± 0.25

Data were normalized to sample dry weight and expressed relative to wild-type (WT) in each tissue. Values are represented as means $\pm$ SD. Terms in bold indicate a statistically significant difference by ANOVA/Dunnett's test ($P < 0.05$). α-tocopherolquinone (α-TQ), plastoquinone-9 (PQ-9), plastoquinol-9 (PQH_2-9), hydroxy-plastoquinone (PQ-OH), plastochromanol-8 (PC-8), hydroxy-plastochromanol (PC-OH), ubiquinone-10 (UQ-10).

4.3.5 VTE5 deficiency affects the expression of tocopherol metabolism-related genes

The biochemical profile described above showed that *SlVTE5* knockdown results in an adjustment in prenyllipid and FAPE metabolism in an organ-specific manner. In order to understand whether these changes could be associated with differential gene expression regulation, mRNA levels of genes encoding proteins involved in MEP, shikimate, tocochromanol, carotenoid and Chl metabolism (Almeida et al., 2015; Lira et al., 2014; Quadrana et al., 2013), as well as in prenylquinone and FAPE synthesis were measured by the qPCR (Fig 4.1). Genes that showed significantly different mRNA levels in at least two transgenic *SlVTE5*-RNAi lines and, when applicable, the third followed the same trend are shown in Fig. 4.8. The complete set of data is shown in Supplementary Table 4.S4.

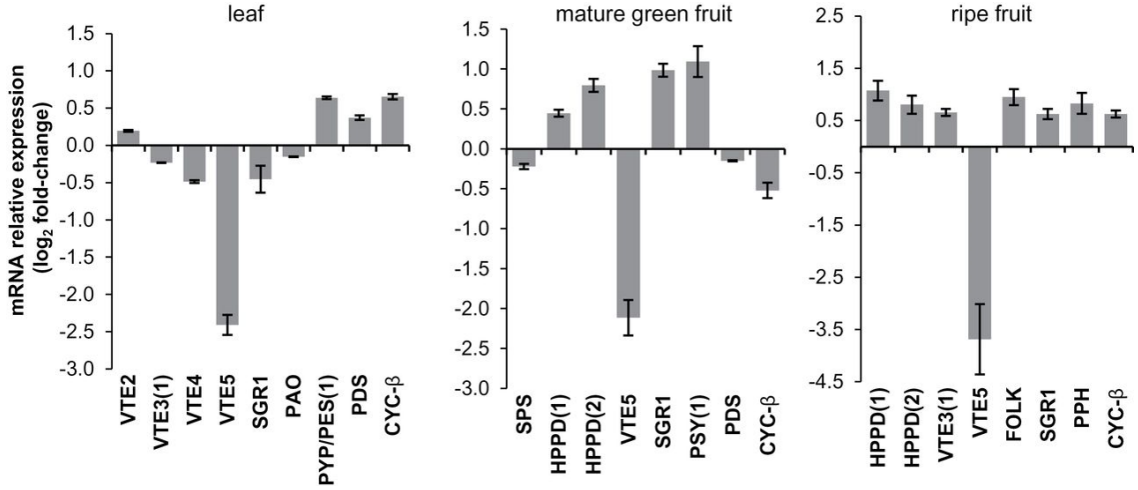

Fig. 4.8: Changes in gene expression levels of some key isoprenoid-metabolism related genes resulting from *SlVTE5* down-regulation in both leaves and fruits.The mRNA amount of the enzyme encoding genes showed in Figure 4.1 were quantified. Expression data are means ± SEM of three biological replicates of log2-fold changes compared to the corresponding organ of the wild-type control. Only genes that showed signi?cantly different mRNA levels in *SlVTE5*-knockdown lines are shown (permutation test, $P < 0.05$). For simplicity, solely data from *SlVTE5*-RNAi#7 were represented. The complete data set is available in Supplemental Table 4.S4.

Genes of tocochromanol biosynthesis did not exhibit a consistent expression tendency in leaves of *SlVTE5*-RNAi lines. In fruit, the elevated expression of *HPPD(1)* and *HPPD(2)* is consistent with the increased prenylquinone content in silenced plants. Moreover, in transgenic ripe fruit, *VTE3(1)* up-regulation also coincided with the higher content of PC-8 and PQ-9. Intriguingly, the expression of the gene encoding the solanesyl-diphosphate synthase (SPS), which catalyzes the production of the PQ-9 or PC-8 prenyl side chain, was decreased in transgenic mature green fruits, suggesting a negative feedback regulatory mechanism.

In leaves, an apparent reduction in the Chl degradation pathway was observed in *SlVTE5*-RNAi lines, as indicated by a down-regulation of *STAY-GREEN1 (SGR1)* and *PHEOPHORBIDE A OXYGENASE (PAO)* expression. Although Chl contents remained invariant, the observed transcriptional down-regulation may reflect a response to phytol accumulation. This scenario contrasts with that observed in fruits where *SGR1* was up-regulated in transgenic plants compared to wild-type. Additionally, ripe fruit of *SlVTE5*-RNAi lines showed higher mRNA levels of *PHEOPHYTINASE (PPH)*.

Regarding carotenoid biosynthesis, only certain genes showed significant changes in their mRNA levels in leaves and mature green fruits of the *SlVTE5*-RNAi lines, although the biochemical profiles of these compounds remained unaltered when compared to wild-type. In contrast, in ripe fruits, the increased levels of *CHROMOPLAST-SPECIFIC β-LYCOPENE CYCLASE (CYC-β)* transcripts could account for the reduction in lycopene and its immediate precursors verified in the transgenic lines.

Finally, the *PALE YELLOW PETAL 1 (PYP1)* gene, the ortholog of the *Arabidopsis PHYTYL ESTER SYNTHASE1 (PES1)* (Ariizumi et al., 2014), showed increased levels of transcripts in the leaves of the *SlVTE5*-RNAi lines, in agreement with the higher FAPE contents observed in these organs. Thus, the analysis of transcriptional profiles reinforces the specific regulation occurring in leaves and fruits.

4.3.6 Measurements of carbohydrate metabolism, photosynthesis and yield parameters suggest carbon export impairment in *SlVTE5*-knockdown plants

To evaluate whether the observed tocopherol deficiency in *SlVTE5*-RNAi lines affects carbon fixation and partitioning, the starch and soluble sugar content, photosynthetic performance, and yield parameters were assessed. Leaves of 5-week-old transgenic plants showed up to 4.5-fold increase in starch content accompanied by a 20% decrease in sucrose levels compared to wild-type control at the middle of light period (Fig. 4.9). Concomitantly, the carbon assimilation rates were reduced in *SlVTE5*-RNAi lines, while the efficiency of photosystem II (PSII) activity (Φ_{PSII}) displayed moderate reduction (Fig. 4.10). The chloroplast ultrastructure was mostly preserved in *SlVTE5*-knockdown plants (Supplementary Fig. 4.S7).

In agreement with carotenoid profiling and gene expression pattern, transgenic plants displayed a delay in fruit development and ripening as indicated by the frequency of red and green fruits, as well as, the red fruit yield compared to control genotype at harvest time (Table 4.2). Moreover, they displayed a yield penalty evidenced by a modest reduction

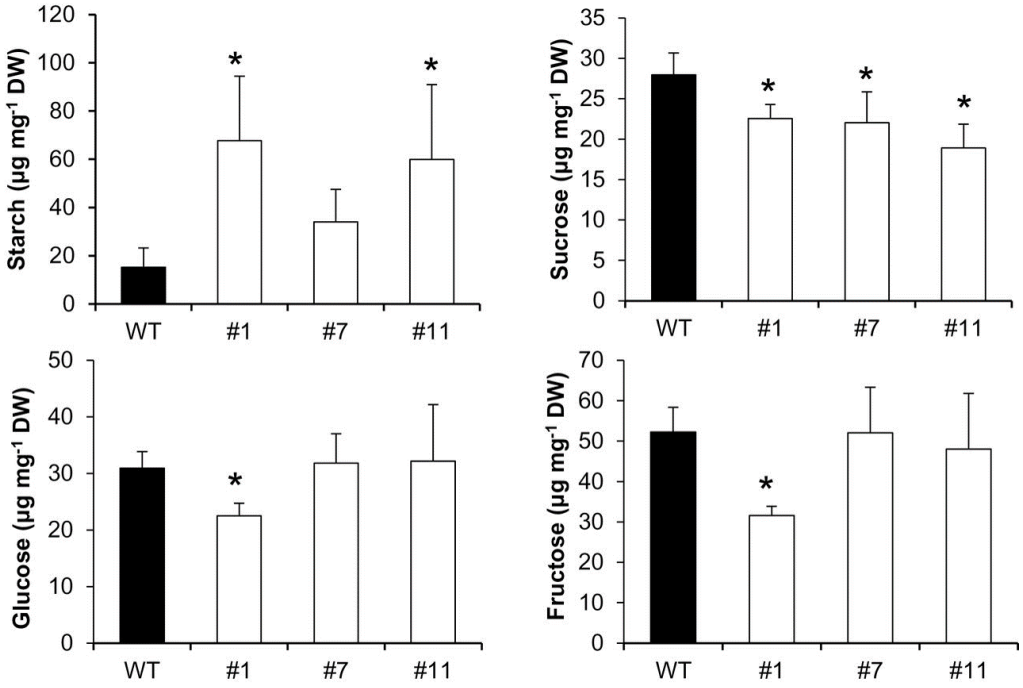

Fig. 4.9: Starch and soluble sugar levels in source leaves in *SlVTE5*-RNAi transgenic lines. First-fully expanded leaves were harvested from 5-week-old plants at the middle of the light cycle. Starch is given in μg glucose equivalents. Data are means $\pm$ SD of five biological replicates. Asterisk denotes significant differences between the wild-type (WT) and the transgenic lines (ANOVA/Dunnett's test, $P < 0.05$). DW, dry weight.

in the harvest index. These results suggest that efficiency in photosynthate partitioning is compromised by the tocopherol deficiency in transgenic lines.

4.4 Discussion

Several reports have dissected tocopherol biosynthesis, identified quantitative trait loci (QTLs) that determine VTE accumulation and characterized regulatory mechanisms that control the tocopherol biosynthetic pathway Almeida et al., 2011; Dellapenna and Mène-Saffrané, 2011; Martinis et al., 2013, 2014; Quadrana et al., 2013, 2014; Zhang et al., 2013; Zhang et al., 2014. One well-defined metabolic constraint is the availability of phytyl diphosphate precursor for tocopherol biosynthesis, which can be derived from *de novo* biosynthesis *via* MEP pathway and from Chl phytol tail recycling Ischebeck et al., 2006; Quadrana et al., 2013; Zhang et al., 2013. The phytol hydrolysis of Chl is the primary source of prenyl chain for tocopherol biosynthesis in Arabidopsis seeds (Valentin et al., 2006). However, functional analysis of the phytol salvage pathway has been limited to this species. In this study, we investigated the contribution of VTE5-mediated phytyl diphosphate synthesis for tocopherol production and its impact on plant physiology in tomato. The comprehensive metabolite profiling, expression analyses and evaluation of

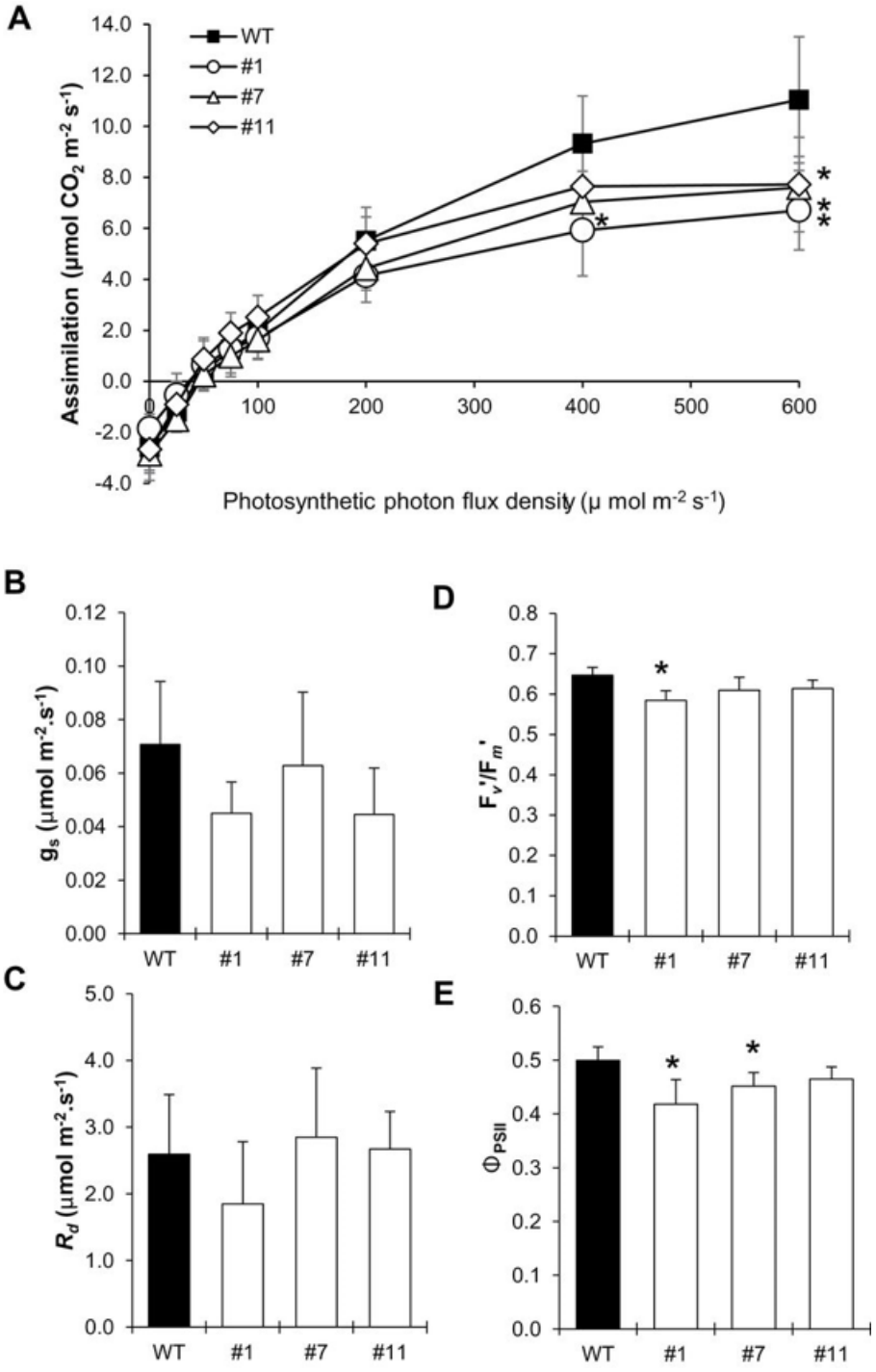

Fig. 4.10: Gas-exchange and PSII efficiency parameters in *SlVTE5*-RNAi transgenic lines. (A) The response of carbon assimilation (A) to light intensity. **(B)** Leaf stomatal conductance (g_s). **(C)** Leaf dark respiration (R_d). **(D)** Light-adapted PSII maximum quantum efficiency (F'_v/F'_m). **(E)** PSII operating efficiency (ϕ_{PSII}). Data correspond to measurements in the first fully expanded leaf of 5-week-old plants and represent the means $\pm$ SD of five biological replicates. Asterisk denotes significant differences between the wild-type (WT) and the transgenic lines (ANOVA/Dunnett's test, $P < 0.05$).

Table 4.2: Yield associated-traits of *SlVTE5*-RNAi transgenic lines.

Trait	WT	*SlVTE5*-RNAi		
		#1	#7	#11
Number of total fruits	36.2 ± 5.8	37.4 ± 4.7	40.8 ± 4.3	35.8 ± 1.5
Frequency red fruits (%)	63.9 ± 6.9	$\mathbf{49.6 \pm 7.8}$	54.0 ± 11.5	53.3 ± 8.8
Frequency green fruits (%)	37.3 ± 6.9	$\mathbf{50.4 \pm 7.8}$	46.0 ± 11.5	46.7 ± 8.8
Vegetative plant weight (g FW)	30.0 ± 8.3	34.3 ± 8.0	39.4 ± 6.9	34.0 ± 7.3
Total yield per plant (g FW)	114.4 ± 17.2	115.9 ± 11.3	125.9 ± 28.8	101.6 ± 16.9
Harvest index	0.79 ± 0.02	0.77 ± 0.04	$\mathbf{0.76 \pm 0.02}$	$\mathbf{0.75 \pm 0.03}$
Red yield /aerial biomass ratio	0.57 ± 0.08	$\mathbf{0.47 \pm 0.11}$	$\mathbf{0.50 \pm 0.09}$	0.52 ± 0.15

Vegetative plant weight was determined by weighing only the vegetative tissue (after harvesting the fruits) without the root. Harvest index was calculated as the ratio between total fresh yield per plant (red and green fruit mass) and aerial biomass (total yield + vegetative plant weight). Values indicate the means $\pm$ SD of phenotypic values ($n = 5$) determined for 15-week-old plants. Statistically significant differences between the wild-type (WT) control and transgenic lines are indicated in bold (Student's *t*-test, $P < 0.05$). FW, fresh weight.

photosynthetic parameters performed in tomato *SlVTE5*-knockdown plants allowed to gain insights into the interactions between phytol recycling, lipid and carbon metabolisms, exposing distinct metabolic adjustment in source and sink organs.

Even with an active phytyl diphosphate *de novo* synthesis, down-regulation of *SlVTE5* dramatically reduces tocopherol content in leaves and fruits in comparison with the wild-type genotype. These data demonstrated that in tomato, tocopherol production is mostly dependent on the Chl-linked pathway for phytyl diphosphate synthesis in both vegetative and reproductive organs. Furthermore, these findings suggest that *SlVTE5* is the main—if not sole—contributor to VTE5 activity. The analysis of the tomato *folk-1* mutant suggested that *SlFOLK* is not involved in reactivation of free phytol.

Coincident with *SlVTE5* down-regulation, free phytol, the substrate of the phytol kinase reaction, accumulates in leaves of transgenic lines. Interestingly, the amount of free phytol accumulated corresponds to the decrease in tocopherol content, suggesting that a large proportion of phytol-diphosphate derived from the phytol phosphorylation pathway is used for tocopherol biosynthesis in tomato. Furthermore, FAPEs are strongly increased in the *SlVTE5*-RNAi lines mature leaves, although the Chl levels remain unchanged. FAPEs are plastogobule compounds that represent a class of stress-induced lipids in higher plants, which may act as plastidial transient sinks for the deposition of fatty acids and phytol (Gaude et al., 2007). Since the phytol moiety of FAPEs is mostly derived from Chl degradation (Lippold et al., 2012), our findings raise an intriguing issue concerning the origin of the phytol that sustains FAPE synthesis in *SlVTE5*-knockdown plants. One possible explanation may be that perturbations on phytol metabolism caused by the lack of VTE5 activity promote an increase in Chl turnover in transgenic lines. In leaves, the increase of steady-state phytol levels were channeled to FAPE synthesis, while in fruits it remained

as free form. Alternatively, the origin of FAPE-associated phytol might be explained by the impairment in catabolism, which in plants involves the production of phytenoyl-CoA in chloroplasts that is further degraded by α-oxidation in peroxisomes and mitochondria similar to that described in animals (Araujo et al., 2011). Notably, phylloquinone levels, another phytyl diphosphate chain-containing molecule, were unchanged both in leaves and fruits, indicating that VTE5-dependent phytol pathway does not affect vitamin K synthesis in tomato. This result coincides with the previous observation of feeding experiments of Arabidopsis seedlings with radiolabelled phytol (Ischebeck et al., 2006).

Even under permissive growing conditions, VTE5-tocopherol deficiency not only impacts lipid profile but also carbon metabolism and photosynthesis, having consequences on the tomato plant physiology. *SlVTE5*-RNAi lines showed higher starch accumulation in mature leaves that correlated with lower CO_2 assimilation rate and the reduction in PSII operating efficiency. This scenario suggests carbohydrate-mediated feedback inhibition rather than a direct impact of tocopherol deficiency on photosynthetic capacity (Adams et al., 2013; Asensi-Fabado et al., 2014). Moreover, a subtle reduction of the number of harvestable fruits and harvest index were observed. These results could be indicative of sugar export blockage from leaves towards sink organs in *SlVTE5*-knockdown tomato plants. Photoassimilate export impairment mediated by tocopherol deficiency has been reported in literature such as in potato *VTE1*-RNAi lines (Asensi-Fabado et al., 2014; Hofius et al., 2004) and *vte2 A. thaliana* during low-temperature adaptation (Maeda et al., 2006, 2008), where carbon accumulation was verified in source leaves at the end of light period. How tocopherol influences photoassimilate partitioning has not been precisely addressed yet. However, Song et al. (2010) provided robust genetic evidence that alterations in extra-plastidic lipid metabolism are upstream of the defect in photoassimilate export in VTE-deficient plants, which is mediated by fatty acid desaturases (FADs). In particularly, it was reported that VTE depletion led to increase linoleic acid (18:2) content and reduced level of linolenic acid (18:3). Consistent with this, one of the acyl groups that mostly contribute to the total FAPEs increase in the chloroplasts of the *SlVTE5*-RNAi lines was 18:2. Additionally, our lipidomic data revealed that the plastidial digalactosyldiacylglycerol (DGDG), which mainly consist of pairs including 18:3 species, were reduced in leaves of *SlVTE5*-RNAi lines compared to wild-type (Supplementary Table 4.S5), resembling the lipid alterations previously reported (Maeda et al., 2006, 2008). It has been proposed that changes in membrane lipid composition as a result of tocopherol deficiency might affect the properties of the secretory membrane systems (Maeda et al., 2008, 2014). Since tomato has been described as an apoplastic phloem loader (Muller et al., 2014), we could speculate that the alteration of endomembrane vesicle formation affects the sucrose efflux mediated by SWEET proteins, which have been described as key players for phloem transport (Chen et al., 2012; McCurdy and Hueros, 2014). Alternatively, the carbon export impairment observed in *SlVTE5*-knockdown tomato plants could be the result of the interaction between lipid and sugar metabolism by a yet unidentified mechanism probably involving sugar sensing proteins, as proposed by Asensi-Fabado et al. (2014).

VTE5 deficiency triggered different metabolic responses in fruits compared to those described for leaves, reflecting the intrinsic physiological differences between organs and their corresponding plastids. First, fruits of *SlVTE5*-knockdown plants accumulated phytol in the free form rather than channeled into synthesis of FAPEs. This might be explained by the lowered availability of acyl-donors inherent to fruit-specific lipid metabolism (Domínguez et al., 2010). Secondly, the observation that non-tocopherol prenylquinone pool, including PQ-9 and PC-8, is increased in fruits of *SlVTE5*-knockdown plants suggests that a regulatory compensation mechanism between the tocopherol and prenylquinone pathways exists in this organ. The ability of plastoquinol (PQH_2-9), ubiquinol (UQH_2-10), the reduced forms of PQ-9 and UQ-10, respectively, and PC-8 to scavenge ROS and inhibit lipid peroxidation have been demonstrated before (Kruk and Trebst, 2008; Nowicka et al., 2013; Rastogi et al., 2014). Moreover, PQ-9 and PC-8 have already been associated with inhibition of lipid peroxidation and $^1O_2$ scavenging in VTE-deficient Arabidopsis *vte2* mutants (Mène-Saffrané and DellaPenna, 2010). Likewise, in tomato leaves, the reduction of PQ-9 content by virus-induced gene silencing approach resulted in increased tocopherol and UQ-10 levels (Jones et al., 2013). Interestingly, the favorable biochemical environment for the PQ-9 and PC-8 accumulation is supported not only by reduced flux through tocopherol competing pathway but also by the *HPPD* and *VTE3* up-regulation in this organ. Finally, *SlVTE5*-RNAi ripe fruits exhibited perturbations in carotenoid pathway. The reduced amount of lycopene and its biosynthetic precursors can be explained by the higher transcripts levels of *SGR1* found at mature green and ripe stages. Besides having an important role in the regulation of plant Chl degradation and senescence Hörtensteiner, 2009, SGR1 also regulates lycopene and β-carotene biosynthesis by direct interaction with PSY(1), thereby inhibiting its activity (Luo et al., 2013). Simultaneously, the failure of coordinate transcriptional repression of the CYC-β in *SlVTE5*-knockdown ripe fruits could also account for the reduced abundance of acyclic carotenoids. The amounts of β-carotene and lutein in transgenic fruits were similar to those in wild-type at the expense of the preceding carotenoids. Additionally, it has been demonstrated that the level of *PPH* transcripts decreases during tomato fruit ripening (Lira et al., 2014); however, *SlVTE5*-RNAi ripe fruits displayed higher levels of *PPH* transcripts than wild-type. Thus, the comprehensive analysis of the biochemical and transcriptional data together with the results of the yield experiment indicates that the ripe profiled transgenic fruits are indeed less ripe than wild-type ones. The reduction in carbohydrate export described above might be in part responsible for the delay in fruit development and ripening, resulting in a reduced proportion of mature fruits at harvest time in *SlVTE5*-knockdown plants.

The results presented here clearly show that in tomato VTE biosynthesis is largely dependent on the salvage pathway for phytyl diphosphate synthesis rather than the *de novo* synthesis from the MEP pathway in both leaves and fruits. VTE5 deficiency affected lipid metabolism evidenced by the abundance and composition of FAPEs in leaves and prenylquinones in fruits. Together, these results exposed the complexities of the metabolic regulation that emerge from isoprenoid pathway network, which involves a tight control

between precursor supply and utilization highly dependent on the plastid type. Moreover, our data highlighted the cross-talk between lipid and carbon metabolism mediated by tocopherol that resulted in the impairment of carbon export in *SlVTE5*-knockdown tomato plants compromising fruit development and ripening.

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4.6 Supplementary data

Table 4.S1: Primers used for each experiment.

Experiment	Primer name[a]	Primer Sequence (5'-3')	
		Foward	Reverse
qPCR	DXS(1)	CAGGACTGGTGTGGTTTCAG	GGGATAGTTCACAGTGTCC
	GGPS(2)	GTTGATTCATGGGGTCAAGC	CAAATCGCCTTTTCAGCTACG
	GGDR	CAGAGACGCTCGCTAAGG	GCTTCAGAGTCTGTCCGATATC
	HPPD(1)	CCAGGGCAGGGGATATACTG	CTCCTTCCTCGTTTTTCAGC
	HPPD(2)	CCAGGCGTGTGAAGAATTG	CGATCTAAACAGCTCAGAG
	VTE2	CAATTCCAGTTCCTGCTGAG	CCTCCAACATGCTCTTGCGTG
	VTE3(1)	CTTGACCAATCTCCTCATC	GCACGCCTTTCCTCCAGG
	VTE3(2)	GCTAAGGCTAGGCAGAAGGAG	CAGGCAACCCCACCTATGG
	VTE1	CGAACTCCTCATAGCGGGTATC	CACGCCAGTAAACCGAGGC
	VTE4	CAGATCATCGTGCTGCTCAG	CCTCTCTGCTTGTACAGGAC
	VTE5	CGTATCAGGACGGGCTCGC	TCACCACCACACATCATTGCTAATG
	FOLK	CTATGAGCCGATTTGGAGACC	GAACCTCCTGCCAACAATGTC
	SGR1	GCAAAGAACTCCCTGTGGTT	CCCACCAGAAGAAGATGAGG
	CHLG	CCAATTCCTTCAGGTGCGGT	CCCACCAAGGCAAGCTGATA
	PPH	TATGGAGGGAGCAAGTACGC	TGGAGGGCAGAGGAAAAGTAC
	PAO	TCAGAAGTGGGTGATATGGA	TATCCCCGTCATACACCTTA
	HST	GCTGCTAACTTGGTGCTC	GATCCTAGCACAGTCCCACG
	SPS	GTGGTTGCGGATGACCTACTTA	CTTCTGTGATTTGTGGTGAGTTCC
	PSY(1)	CGATGGTGCTTTGTCCGATAC	CTCATCAACCCAACCGTACC
	PSY(2)	GCATCACACATAACTCCACAAGC	CGCATTCCTTCAACCATATCTCTG
	PDS	CGTTCCGTGCTTCTCCGC	CTAGAACATCCCTTGCCTCCAG
	LCYβ	GCACCCACATCAAAGCCAGAG	GCCACATGGAGAGTGGTGAAG
	CYCβ	TTGACTTAGAACCTCGTTATTGG	AACAGTTCCCTTTGTCATTATCT
	PYP/PES(1)	ACAGGACACAACTCCAACC	TAACCATCGCCATCTTCAGTG
	PES(2)	CGAAGAGAGGGAAAAATGCCTGTG	GCTGCCATCCTGACAAATTCAGAC
	CAC	CCTCCGTTGTGATGTAACTGG	ATTGGTGGAAAGTAACATCATCG
	EXPRESSED	GCTAAGAACGCTGGACCTAATG	TGGGTGTGCCTTTCTGAATG
SlVTE5-RNAi lines	RNAi-VTE5	CACCATGCAAGCTTTGGTGTGTG	CTAATAAGCAAAAGCCAATGATGCTAC
	35S-right	CCCACTATCCTTCGCAAG	
TILLING SlFOLK screening	external	TCATCTTTTAGAAGTGATATCCTAACC	ACACAATCCTGTGTTAGACTAACACAT
	internal	GAACAGAAAGCTTGTTCATATTAGCAT	ACATTGCCCATTTTCAGTAAGCATTTT

[a] 1-deoxy-D-xylulose-5-P synthase (DXS); geranylgeranyl pyrophosphate synthase (GGPS); geranylgeranyl diphosphate reductase (GGDR); 4-hydroxyphenylpyruvate dioxygenase (HPPD); homogentisate phytyl transferase (VTE2); 2,3-dimethyl-5-phytylquinol methyltransferase (VTE3); tocopherol cyclase (VTE1); γ-tocopherol-C-methyl transferase (VTE4); phytol kinase (VTE5); farnesol kinase (FOLK); chlorophyll synthase (CHLG); staygreen 1 (SGR1); pheophytinase (PPH); pheophorbide a oxygenase (PAO); homogentisate solanesyl transferase (HST); solanesyl-diphosphate synthase (SPS); phytoene synthase (PSY); phytoene desaturase (PDS); chloroplast-specific β-lycopene cyclase (LCYβ); chromoplast-specific β-lycopene cyclase (CYCβ); pale yellow petal/phytyl ester synthase (PYP/PES); clathrin adaptor complex medium subunit (CAC).

Table 4.S2: Tocopherol content and composition of *SlVTE5*-RNAi transgenic lines and *folk-1* mutant.

	α-tocopherol	β-/γ-tocopherol	δ-tocopherol	Total tocopherol
leaf				
WT	176.1 ± 51.4	24.9 ± 2.8	nd	201 ± 52.6
SlVTE5-RNAi#1	**28.7 ± 12.5**	**2.0 ± 1.9**	nd	**30.7 ± 12.1**
SlVTE5-RNAi#7	**21.2 ± 9.1**	**4.2 ± 1.7**	nd	**25.5 ± 10.7**
SlVTE5-RNAi#11	**22.1 ± 9.5**	**4.5 ± 1.5**	nd	**26.6 ± 10.9**
control	587.5 ± 60.0	6.8 ± 0.7	0.1 ± 0.1	594.4 ± 60.8
folk-1	611.7 ± 78.0	6.8 ± 1.0	0.2 ± 0.1	618.7 ± 79.1
Mature green fruit				
WT	53.6 ± 7.3	18.2 ± 4.1	nd	71.8 ± 10.1
SlVTE5-RNAi#1	**2.2 ± 3.4**	**11.0 ± 6.0**	nd	**13.1 ± 5.7**
SlVTE5-RNAi#7	**20.5 ± 6.2**	**2.5 ± 2.4**	nd	**23.0 ± 4.4**
SlVTE5-RNAi#11	**6.5 ± 7.3**	**3.7 ± 0.6**	nd	**10.3 ± 7.8**
control	103.8 ± 7.7	2.2 ± 0.6	0.1 ± 0.1	106.1 ± 8.3
folk-1	98.4 ± 11.9	2.8 ± 0.9	0.1 ± 0.1	101.3 ± 12.9
Ripe fruit				
WT	44.6 ± 25.3	43.8 ± 17.8	2.4 ± 2	90.8 ± 34.7
SlVTE5-RNAi#1	**3.2 ± 1.9**	**0.7 ± 1.5**	nd	**3.9 ± 3.2**
SlVTE5-RNAi#7	**3.3 ± 1.1**	**1.9 ± 3.3**	**0.2 ± 0.4**	**5.3 ± 4.7**
SlVTE5-RNAi#11	**6.3 ± 5.4**	**3.0 ± 5.1**	**0.3 ± 0.6**	**9.6 ± 7.6**
control	147.5 ± 11.1	15.9 ± 7.6	0.9 ± 0.4	164.2 ± 19.1
folk-1	115.8 ± 11.3	9.7 ± 4.1	0.4 ± 0.2	125.9 ± 15.6

Tocopherol content is showed as nmol. g^{-1} dry weight. The corresponding M4 segregating individuals homozygous for FOLK WT allele were used as control. Statistically significant differences between the wild-type (WT) and transgenic lines are indicated in bold terms (ANOVA/Dunnett's test, $P < 0.05$). nd, not detected.

Table 4.S3: Moles of prenyllipids found in *SlVTE5*-RNAi transgenic lines.

		Prenyllipid (nmol g^{-1} DW)					
		PC-8	PQ-9	PQH_2-9	tocopherol	FAPE	free phytol
Leaf	WT	57	653	322	200	290	90
	SlVTE5-RNAi [a]	**27**	508	339	**25**	**3000**	**400**
Mature green	WT	87	33	20	71	350	nm
	SlVTE5-RNAi [a]	**160**	**72**	21	**15**	450	nm
Ripe	WT	75	56	15	90	260	193
	SlVTE5-RNAi [a]	**150**	**120**	26	**6**	460	**1095**

[a] Mean value obtained from three transgenic lines. Significant differences between wild-type (WT) and transgenic lines are indicated in bold (ANOVA/Dunnett's test, $P < 0.05$). Plastochromanol-8 (PC-8), oxidized plastoquinone-9 (PQ-9), plastoquinol-9 (PQH_2-9), fatty acid phytyl ester (FAPE). nm, not measured.

Table 4.S4: Transcriptional profile of genes encoding isoprenoid metabolism-related enzymes.

	Leaf				Mature green fruits				Ripe fruits			
	WT	*SlVTE5*-RNAi#1	*SlVTE5*-RNAi#7	*SlVTE5*-RNAi#11	WT	*SlVTE5*-RNAi#1	*SlVTE5*-RNAi#7	*SlVTE5*-RNAi#11	WT	*SlVTE5*-RNAi#1	*SlVTE5*-RNAi#7	*SlVTE5*-RNAi#11
DXS(1)	1.00 ± 0.06	1.14 ± 0.15	1.12 ± 0.05	**1.53 ± 0.15**	1.00 ± 0.09	0.87 ± 0.09	0.87 ± 0.09	**0.58 ± 0.06**	1.00 ± 0.13	0.94 ± 0.18	**0.70 ± 0.07**	0.68 ± 0.14
GGPS(2)	1.00 ± 0.14	0.75 ± 0.08	1.06 ± 0.07	**1.80 ± 0.34**	1.00 ± 0.08	**1.43 ± 0.07**	1.23 ± 0.24	1.07 ± 0.07	1.00 ± 0.09	0.99 ± 0.06	**1.67 ± 0.22**	1.10 ± 0.17
GGDR	1.00 ± 0.07	0.84 ± 0.07	0.91 ± 0.04	**1.48 ± 0.20**	1.00 ± 0.17	0.96 ± 0.02	1.09 ± 0.14	**0.66 ± 0.06**	1.00 ± 0.27	1.21 ± 0.18	1.16 ± 0.16	0.68 ± 0.22
SPS	1.00 ± 0.12	**0.59 ± 0.06**	0.90 ± 0.04	0.92 ± 0.09	1.00 ± 0.10	**0.61 ± 0.02**	0.86 ± 0.13	**0.53 ± 0.05**	1.00 ± 0.11	1.20 ± 0.15	**1.36 ± 0.11**	1.17 ± 0.24
HST	1.00 ± 0.01	1.14 ± 0.12	1.03 ± 0.06	**1.37 ± 0.08**	1.00 ± 0.09	0.91 ± 0.09	1.20 ± 0.09	**0.75 ± 0.08**	1.00 ± 0.22	0.93 ± 0.12	**2.10 ± 0.26**	0.83 ± 0.13
HPPD(1)	1.00 ± 0.08	**0.70 ± 0.08**	**0.79 ± 0.01**	1.08 ± 0.05	1.00 ± 0.03	**1.28 ± 0.03**	**1.36 ± 0.13**	**1.09 ± 0.01**	1.00 ± 0.10	**1.38 ± 0.17**	**2.10 ± 0.37**	1.03 ± 0.17
HPPD(2)	1.00 ± 0.14	**0.52 ± 0.08**	0.80 ± 0.05	1.16 ± 0.05	1.00 ± 0.06	**1.74 ± 0.28**	**1.73 ± 0.18**	**1.25 ± 0.04**	1.00 ± 0.11	**1.55 ± 0.19**	**1.75 ± 0.37**	1.22 ± 0.21
VTE1	1.00 ± 0.06	1.06 ± 0.07	1.06 ± 0.03	**1.22 ± 0.02**	1.00 ± 0.10	0.98 ± 0.09	1.00 ± 0.03	**0.77 ± 0.05**	1.00 ± 0.19	1.11 ± 0.10	**1.57 ± 0.11**	0.98 ± 0.07
VTE2	1.00 ± 0.02	1.08 ± 0.06	**1.14 ± 0.07**	**1.28 ± 0.04**	1.00 ± 0.02	0.91 ± 0.10	**1.05 ± 0.02**	**0.83 ± 0.02**	1.00 ± 0.20	0.97 ± 0.10	1.26 ± 0.12	0.86 ± 0.08
VTE3(1)	1.00 ± 0.02	0.91 ± 0.07	**0.85 ± 0.02**	**0.96 ± 0.00**	1.00 ± 0.07	0.99 ± 0.04	1.08 ± 0.08	0.90 ± 0.02	1.00 ± 0.11	**1.23 ± 0.08**	**1.58 ± 0.16**	**1.39 ± 0.13**
VTE3(2)	1.00 ± 0.03	**0.73 ± 0.04**	**0.80 ± 0.03**	1.00 ± 0.02	1.00 ± 0.04	**0.84 ± 0.04**	1.01 ± 0.05	**0.71 ± 0.03**	1.00 ± 0.15	1.09 ± 0.03	**1.70 ± 0.27**	1.13 ± 0.10
VTE4	1.00 ± 0.09	0.86 ± 0.08	**0.71 ± 0.03**	**0.83 ± 0.02**	1.00 ± 0.10	0.86 ± 0.06	1.04 ± 0.09	**0.70 ± 0.02**	1.00 ± 0.10	0.89 ± 0.06	1.01 ± 0.10	1.10 ± 0.09
VTE5	1.00 ± 0.07	**0.19 ± 0.00**	**0.19 ± 0.01**	**0.14 ± 0.01**	1.00 ± 0.07	**0.25 ± 0.03**	**0.23 ± 0.02**	**0.19 ± 0.03**	1.00 ± 0.04	**0.06 ± 0.02**	**0.08 ± 0.01**	**0.06 ± 0.00**
FOLK	1.00 ± 0.09	0.92 ± 0.10	0.91 ± 0.04	1.07 ± 0.04	1.00 ± 0.07	0.86 ± 0.07	1.00 ± 0.07	**0.72 ± 0.04**	1.00 ± 0.14	**1.34 ± 0.11**	**1.93 ± 0.31**	1.28 ± 0.12
SGR1	1.00 ± 0.42	**0.56 ± 0.09**	**0.73 ± 0.29**	**0.54 ± 0.14**	1.00 ± 0.29	**1.83 ± 0.25**	**1.98 ± 0.16**	1.14 ± 0.10	1.00 ± 0.05	**1.55 ± 0.08**	**1.54 ± 0.25**	1.27 ± 0.15
CHLG	1.00 ± 0.01	**0.86 ± 0.04**	0.90 ± 0.07	**1.11 ± 0.04**	1.00 ± 0.02	0.97 ± 0.09	**1.17 ± 0.07**	**0.75 ± 0.05**	1.00 ± 0.12	1.16 ± 0.12	**1.88 ± 0.22**	1.04 ± 0.13
PAO	1.00 ± 0.04	0.77 ± 0.17	**0.90 ± 0.02**	**0.75 ± 0.07**	1.00 ± 0.12	**1.37 ± 0.09**	1.23 ± 0.11	0.97 ± 0.03	1.00 ± 0.01	1.04 ± 0.10	1.13 ± 0.07	1.04 ± 0.03
PPH	1.00 ± 0.06	0.79 ± 0.16	0.90 ± 0.02	0.84 ± 0.09	1.00 ± 0.05	1.07 ± 0.11	0.81 ± 0.09	**0.71 ± 0.03**	1.00 ± 0.17	**1.39 ± 0.07**	**1.78 ± 0.43**	1.20 ± 0.28
PYP/PES(1)	1.00 ± 0.10	**1.48 ± 0.21**	**1.56 ± 0.05**	**1.92 ± 0.05**	1.00 ± 0.14	0.96 ± 0.08	1.06 ± 0.15	**0.66 ± 0.05**	1.00 ± 0.11	1.50 ± 0.33	1.11 ± 0.01	0.92 ± 0.17
PES(2)	1.00 ± 0.11	1.11 ± 0.14	1.16 ± 0.10	1.09 ± 0.06	1.00 ± 0.01	1.14 ± 0.08	**1.25 ± 0.09**	1.02 ± 0.05	1.00 ± 0.12	1.02 ± 0.10	1.20 ± 0.08	1.09 ± 0.13
PSY(1)	1.00 ± 0.10	**0.37 ± 0.04**	**0.58 ± 0.07**	1.19 ± 0.12	1.00 ± 0.12	**1.89 ± 0.31**	**2.13 ± 0.37**	**1.30 ± 0.07**	1.00 ± 0.06	**1.41 ± 0.15**	**0.79 ± 0.08**	1.12 ± 0.13
PSY(2)	1.00 ± 0.03	**0.44 ± 0.05**	**0.58 ± 0.02**	1.10 ± 0.13	1.00 ± 0.04	**0.84 ± 0.05**	0.92 ± 0.07	0.87 ± 0.15	1.00 ± 0.07	**1.45 ± 0.21**	1.07 ± 0.08	1.07 ± 0.27
PDS	1.00 ± 0.08	**1.22 ± 0.05**	**1.29 ± 0.10**	**1.37 ± 0.02**	1.00 ± 0.06	**0.79 ± 0.04**	0.90 ± 0.04	**0.72 ± 0.05**	1.00 ± 0.05	1.01 ± 0.15	0.84 ± 0.22	1.09 ± 0.08
LCY-β	1.00 ± 0.06	**0.65 ± 0.03**	**0.70 ± 0.04**	1.00 ± 0.07	1.00 ± 0.05	1.00 ± 0.03	1.12 ± 0.12	**0.86 ± 0.06**	1.00 ± 0.05	1.16 ± 0.11	**0.85 ± 0.06**	0.81 ± 0.11
CYC-β	1.00 ± 0.09	**2.22 ± 0.27**	**1.57 ± 0.08**	**2.61 ± 0.29**	1.00 ± 0.08	**0.54 ± 0.08**	**0.70 ± 0.13**	**0.79 ± 0.06**	1.00 ± 0.05	**1.34 ± 0.14**	**1.54 ± 0.16**	**1.61 ± 0.16**

Values represent means from at least three biological replicates. The expression data shown represent fold-change compared to control wild-type (WT) in the respective organ. Significant differences were determined according to a permutation test (P<0.05) and indicate in bold. 1-deoxy-D-xylulose-5-P synthase (DXS); geranylgeranyl pyrophosphate synthase (GGPS); geranylgeranyl diphosphate reductase (GGDR); 4-hydroxyphenylpyruvate dioxygenase (HPPD); tocopherol cyclase (VTE1); homogentisate phytyl transferase (VTE2); 2,3-dimethyl-5-phytylquinol methyltransferase (VTE3); γ-tocopherol-C-methyl transferase (VTE4); phytol kinase (VTE5); farnesol kinase (FOLK); staygreen 1 (SGR1); chlorophyll synthase (CHLG); pheophytinase (PPH); pheophorbide a oxygenase (PAO); pale yellow petal/phytyl ester synthase (PYP/PES); homogentisate solanesyl transferase (HST); solanesyl-diphosphate synthase (SPS); phytoene synthase (PSY); phytoene desaturase (PDS); chloroplast-specific β-lycopene cyclase (LCYβ); chromoplast-specific β-lycopene cyclase (CYCβ).

Table 4.S5: Changes in fatty acid-derived lipids in leaves of *SlVTE5*-RNAi transgenic lines compared with wild-type.

		SlVTE5-RNAi		
Relative amounts	WT	# 1	#7	#11
MGDG-18:3/16:3	1.00 ± 0.06	0.92 ± 0.07	0.93 ± 0.03	0.96 ± 0.03
MGDG-18:3/18:3	1.00 ± 0.05	0.93 ± 0.08	**0.91 ± 0.02**	0.97 ± 0.03
DGDG-18:3/18:3	1.00 ± 0.05	**0.90 ± 0.07**	**0.89 ± 0.03**	**0.91 ± 0.04**
DGDG-18:3/16:0	1.00 ± 0.04	**0.90 ± 0.09**	**0.89 ± 0.06**	0.96 ± 0.03
DGDG-18:2/16:0	1.00 ± 0.08	1.09 ± 0.13	1.00 ± 0.07	0.92 ± 0.03
MGDG-18:2/18:3	1.00 ± 0.05	0.99 ± 0.13	0.91 ± 0.09	0.88 ± 0.01
DGDG-18:3/16:3	1.00 ± 0.07	**0.84 ± 0.09**	**0.88 ± 0.06**	**0.88 ± 0.05**
MGDG-18:3/16:1	1.00 ± 0.10	0.98 ± 0.14	0.95 ± 0.10	0.99 ± 0.09
DGDG-18:1/18:3	1.00 ± 0.02	0.93 ± 0.06	0.92 ± 0.05	**0.85 ± 0.06**
DGDG-18:3/20:3	1.00 ± 0.15	0.78 ± 0.21	0.81 ± 0.11	**0.76 ± 0.04**
DGDG-18:0/18:3	1.00 ± 0.11	0.85 ± 0.12	0.85 ± 0.16	**0.75 ± 0.06**
PE-18:2/16:0	1.00 ± 0.08	1.08 ± 0.04	1.07 ± 0.08	1.10 ± 0.10
PE-18:3/16:0	1.00 ± 0.06	0.92 ± 0.13	0.92 ± 0.20	0.92 ± 0.11
PE-18:2/18:2	1.00 ± 0.27	1.19 ± 0.17	1.11 ± 0.18	**1.38 ± 0.20**
DAG-18:0/18:0	1.00 ± 0.17	0.93 ± 0.25	0.79 ± 0.10	0.82 ± 0.22
DAG-18:0/16:0	1.00 ± 0.16	0.93 ± 0.26	0.77 ± 0.09	0.83 ± 0.23
DAG	1.00 ± 0.17	0.93 ± 0.25	0.79 ± 0.09	0.82 ± 0.22

Data were normalized to sample dry weight and expressed relative to wild type (WT) in each tissue. Values are represented as means ± SD. Terms in bold indicate a statistically significant difference by ANOVA/Dunnett's test ($P < 0.05$). Monogalactosyldiacylglycerol (MGDG); digalactosyldiacylglycerol (DGDG); phosphotidylethanolamine (PE); diacylglycerol (DAG).

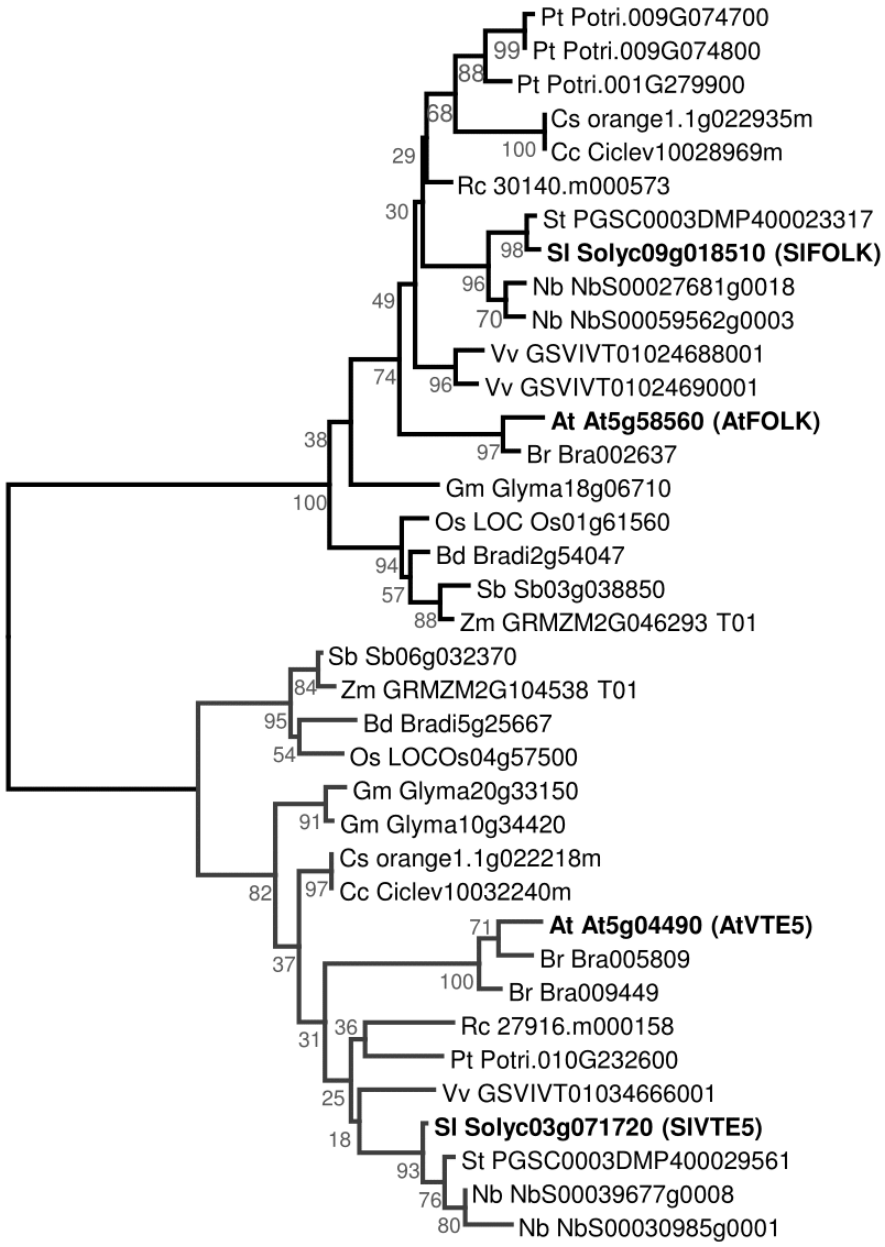

Fig. 4.S1: Phylogenetic analysis VTE5 and FOLK proteins. Neighbor-joining phylogeny of VTE5 and FOLK homologs in flowering plants, which were identified by Blastp searches using AtVTE5 as the query against the Phytozome database (`http://www.phytozome.net`). Br, *Brassica rapa*; Bd, *Brachypodium distachyon*; Cc, *Citrus clementina*; Cs, *Citrus sinensis*; Pt, *Populus trichocarpa*; Nb, *Nicotiana benthamiana*; St, *Solanum tuberosum*; Sb, *Sorghum bicolor*; Zm, *Zea mays*; Os, *Oryza sativa*; Vv, *Vitis vinifera*; Rc, *Ricinus communis*. Gene numbers following names are based on those listed at Phytozome, with the exception of Arabidopsis ID.

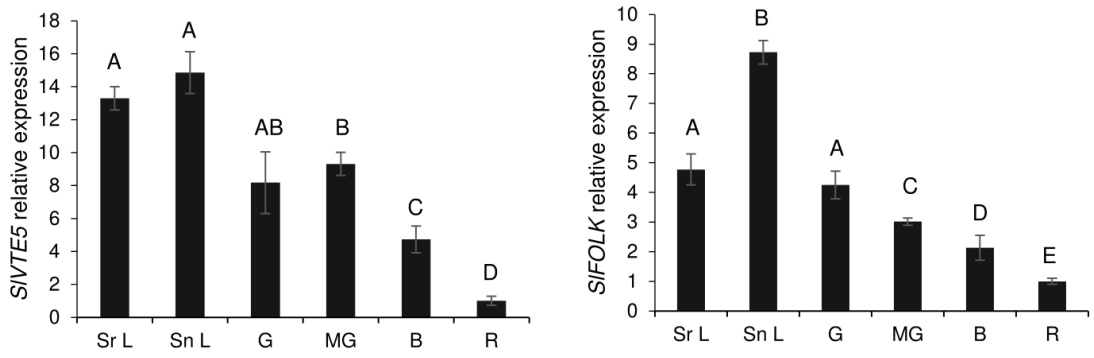

Fig. 4.S2: Expression of *SlVTE5* and *SlFOLK*. Relative expression were measured by qPCR in source (SrL) and sink (SnL) leaves, green (G), mature green (MG), breaker (B) and ripe (R) fruits in at least three biological replicates. The means were calculated from two technical replicates and normalized against R fruit samples. Statistically significant differences (permutation test, $P < 0.05$) are indicated with different letters.

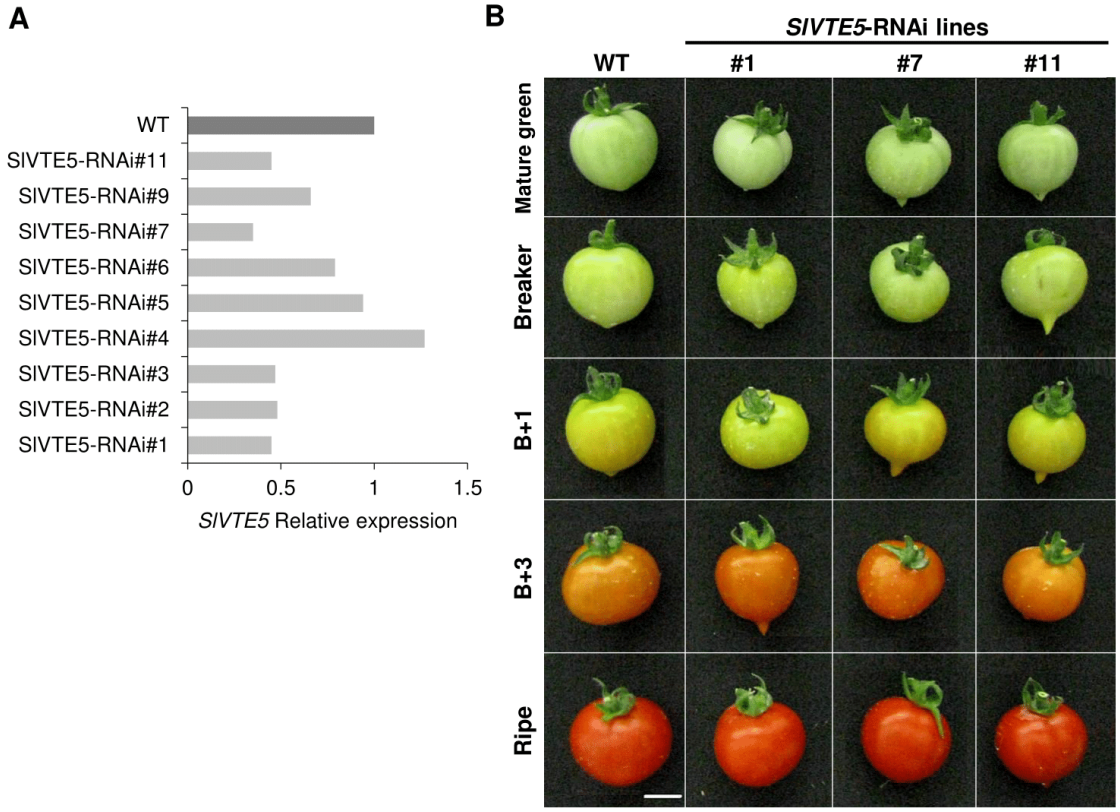

Fig. 4.S3: Screening of primary transformants and fruit phenotype of *SlVTE5*-RNAi transgenic lines. (A) *SlVTE5* mRNA levels in source leaves from T_0 transformants. Bars represent the mean of three technical replicates. Values were expressed as relative abundance of mRNA compared to wild-type (WT). **(B)** Representative T_1 fruits of *SlVTE5*-RNAi transgenic lines at mature green, breaker, one day after breaker (B+1), three days after breaker (B+3) and ripe (B+6) stages. Scale bar = 1 cm.

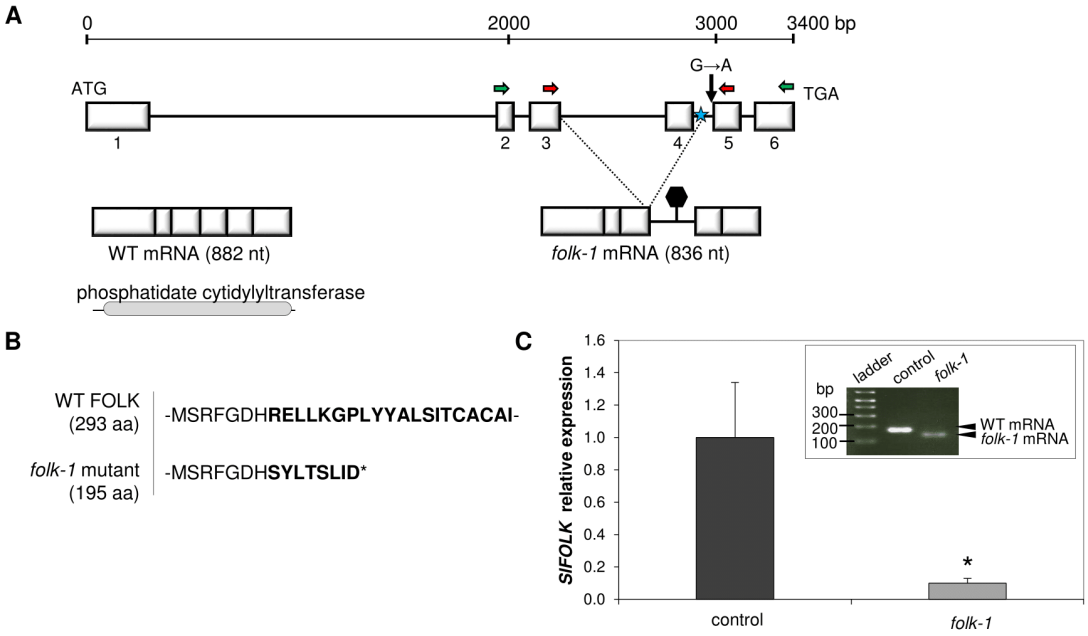

Fig. 4.S4: Analysis of *folk-1* mutation. **(A)** Schematic diagram of *SlFOLK* (Solyc09g018510) splicing pattern showing the fully spliced wild-type (WT) and *folk-1* mutant mRNA. The open boxes and solid lines represent exons and introns, respectively. The green arrows indicate the position of the primers used for TILLING screening and the red arrows indicate intron-flanking primers used for qPCR assay. The position of the G to A substitution (G2976A) is indicated in the 3' consensus splicing site of intron 4. The star denotes the 3' cryptic splicing site. The *folk-1* mutant mRNA generates a premature stop codon indicated by a black hexagon, which presumably affects the phosphatidate cytidylyltransferase domain. **(B)** Partial amino acid sequences deduced from WT and abnormal cryptic site transcripts. Frameshift in predicted amino acid sequence is indicated in bold. (C) qPCR analysis of *SlFOLK* expression in leaves of M4 plants homozygous for *folk-1* allele. The corresponding segregating individuals homozygous for *FOLK* wild-type allele were used as control. Data are means $\pm$ SEM of three biological replicates. Asterisk denotes statistically significant differences (permutation test, $P < 0.05$). The insets shows agarose gel electrophoresis of *SlFOLK* PCR amplicons obtained from control and *folk-1* plants. Nt, nucleotide; bp, base pair; aa, amino acid.

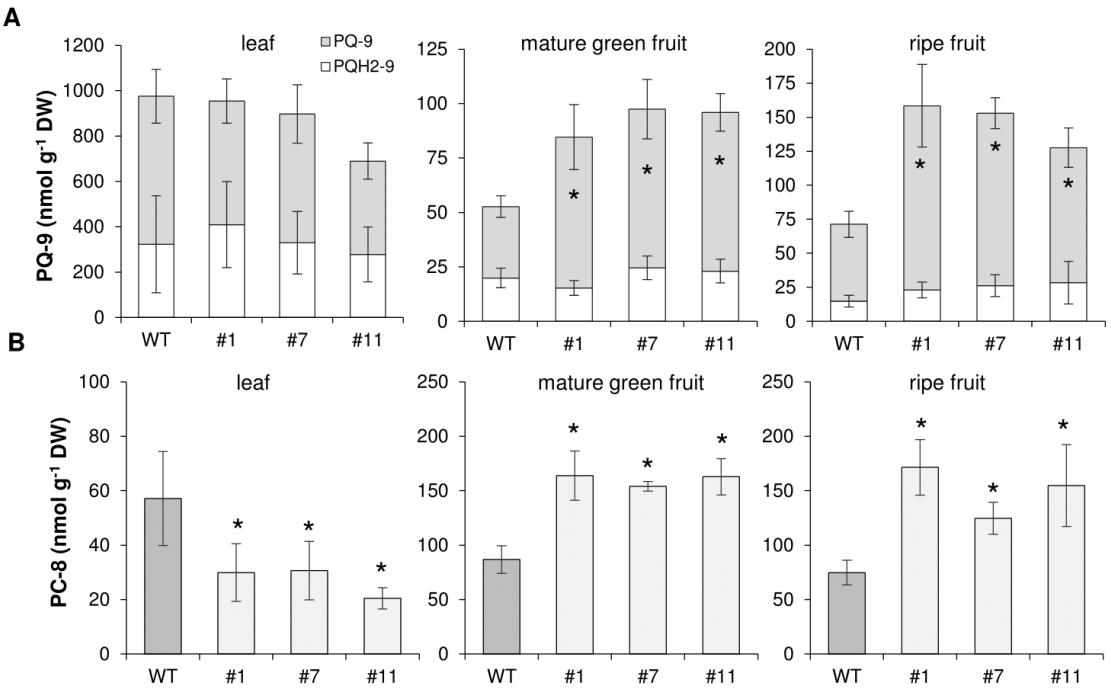

Fig. 4.S5: Plastoquinone (PQ-9) and plastochromanol (PC-8) levels in *SlVTE5*-RNAi transgenic lines. Prenylquinone were measured in leaves, mature green and ripe fruits of SlVTE5-RNAi lines. **(A)** Total PQ-9 content; bars indicate the fraction of oxidized (PQ-9) and reduced (PQH_2-9) forms. **(B)** PC-8 content. Data represent the mean $\pm$ SD of five biological replicates. Asterisk denotes significant differences between the wild-type (WT) and the transgenic lines (ANOVA/Dunnett's test, $P < 0.05$). DW, dry weight.

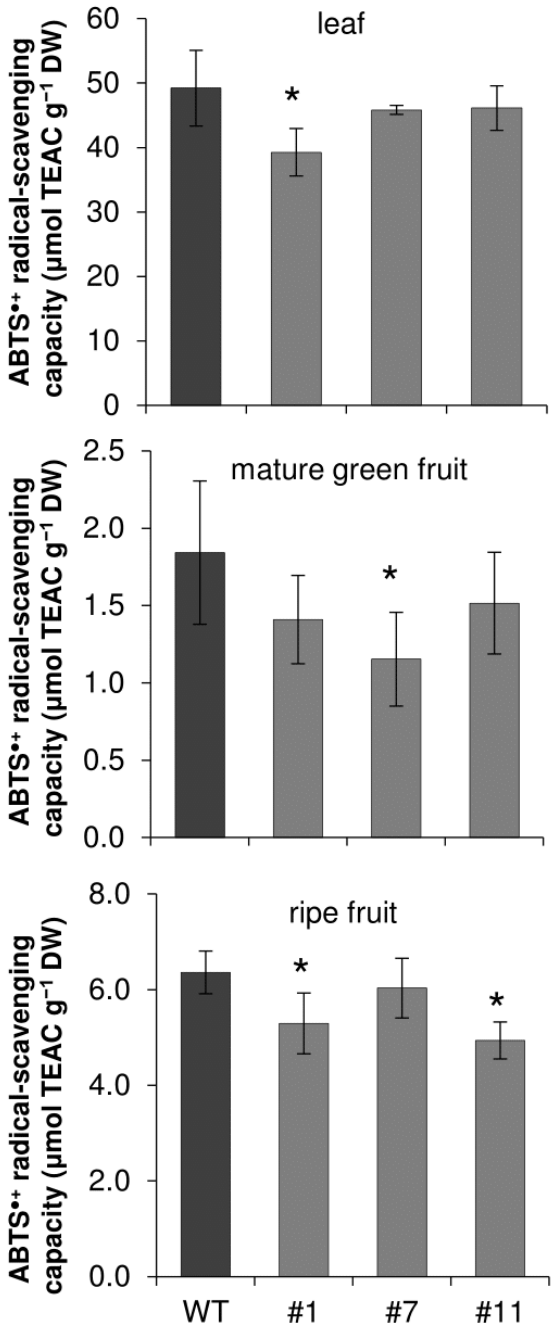

Fig. 4.S6: Trolox equivalent antioxidant capacity (TEAC) in leaves and fruits of *SlVTE5*-RNAi transgenic lines. Data represent the mean ± SD of five biological replicates. Measurements are from three technical replicates. Asterisk denotes significant differences between the wild-type (WT) and the transgenic lines (ANOVA/Dunnett's test, $P < 0.05$). DW, dry weight.

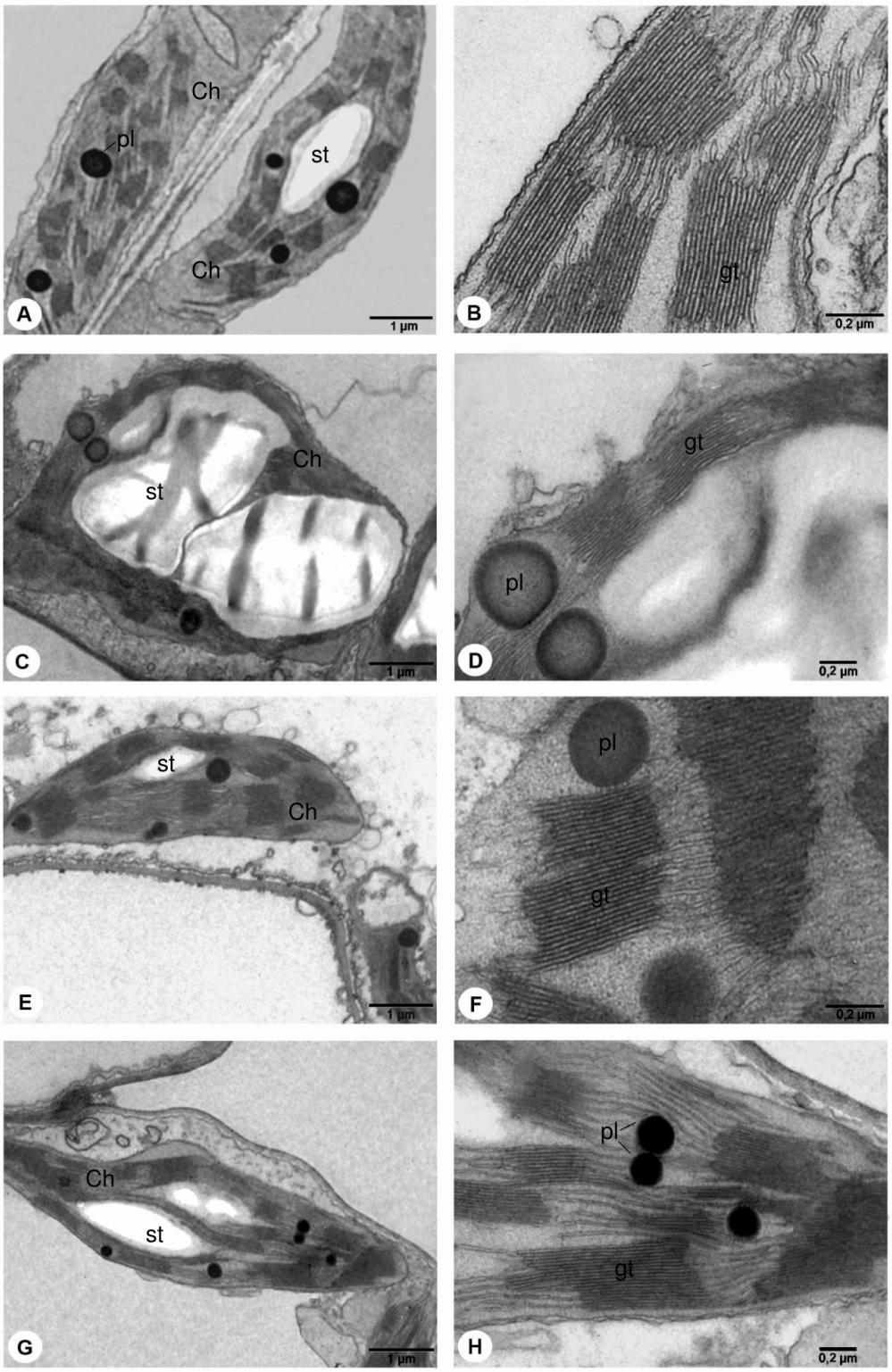

Fig. 4.S7: Chloroplast ultrastructure resulting from *SlVTE5* downregulation. Transmission electron micrographs of the first leaflet from the first fully expanded leaf at the middle of daylight (16-week-old plant). (A,B) wild-type, (C,D) *SlVTE5-RNAi*#1, (E,F) *SlVTE5-RNAi*#7 and (G,H) *SlVTE5-RNAi*#11. Ch: chloroplast; st: starch granule; pl: plastoglobule; gt: grana thylakoids.

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5

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

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Article in preparation

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 against combined high temperature and high light stress. Among several hundred metabolites, the two most strongly upregulated compounds were α-tocopherol and plastoquinone/-ol. Both are well-known lipid antioxidants and contribute to the protection of photosystem II against photodamage under environmental stress. To address the protective function of tocopherol, a RNAi line (*vte5*) with decreased expression of VTE5 and reduced levels of α-tocopherol was selected. VTE5 encodes phytol kinase that acts in the biosynthetic pathway of tocopherols. *vte5* suffered strong photoinhibition and photobleaching when exposed to combined 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. Therefore, VTE5 protects against combined high light and high temperature stress and does so by supporting α-tocopherol production.

5.1 Introduction

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

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 which act as antioxidants, preventing lipid peroxidation and photoinhibition by quenching reactive oxygen species (ROS) under high light intensities (Gruszka et al., 2008; Ksas et al., 2015; Mène-Saffrané and DellaPenna, 2010; Miret and Munné-Bosch, 2015; Munné-Bosch and Alegre, 2002; Nowicka and Kruk, 2012; Rastogi et al., 2014; Triantaphylidès and Havaux, 2009).

Tocopherols (collectively known as Vitamin E) contribute to seed longevity, seedling development and protection of the photosynthetic apparatus against oxidative stress (Mène-Saffrané and DellaPenna, 2010; Sattler et al., 2004) and their synthesis is increased under these conditions (Collakova and DellaPenna, 2003; Eugeni Piller et al., 2012; Loyola et al., 2011; Maeda et al., 2006; Munné-Bosch, 2005; Quadrana et al., 2013; Spicher et al., 2016). Yet, conditions under which tocopherols are essential are currently not known. The synthesis of tocopherol requires the condensation of the aromatic homogentisate ring derived from the plastidial shikimate pathway and a phytol diphosphate chain. The majority of the phytol required is salvaged from chlorophyll catabolism rather than synthesized de novo (Almeida et al., 2016; vom Dorp et al., 2015). The salvage pathway requires phytol kinase (VTE5) and phytyl-phosphate kinase (VTE6) converting free phytol into phytyl diphosphate and *vte5* and *-6* mutant plants accumulate less tocopherol (Almeida et al., 2016; Ischebeck et al., 2006; Valentin et al., 2006; vom Dorp et al., 2015). Apart from tocopherol, phytol may also be converted into fatty acid phytyl esters (FAPEs) (Gaude et al., 2007; Ischebeck et al., 2006; Lippold et al., 2012). This detoxifies free phytol that 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 hight temperature and high light stress.

5.2 Materials and Methods

5.2.1 Plant material

A tomato VTE5-knockdown transgenic line (*SlVTE5*-RNAi#1, herein *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 SlVTE5 gene (Solyc03g071720). Wild-type (WT, *Solanum lycopersicum*, cv. MT) and *vte5* plants were grown in soil under conditions referred to as control ($250\,\mu\mathrm{mol\,m^{-2}\,s^{-1}}$ of light, 16-h light/8-h dark, at $20/18\,^{\circ}\mathrm{C}$, with 55% relative air humidity).

5.2.2 Stress treatments

5 to 6-week old plants were either kept under standard growth conditions referred to as control, or subjected for 6 days to stress treatments, such as HL ($800\,\mu\mathrm{mol\,m^{-2}\,s^{-1}}$, at $20/18\,^{\circ}\mathrm{C}$,16-h light/8-h dark), HT ($250\,\mu\mathrm{mol\,m^{-2}\,s^{-1}}$ of light,$38/30\,^{\circ}\mathrm{C}$, 16-h light/8-h dark), and a combination of HL and HT ($800\,\mu\mathrm{mol\,m^{-2}\,s^{-1}}$ of light, at $38/30\,^{\circ}\mathrm{C}$, 16-h light/8-h dark).

5.2.3 Transmission electron microscopy

Transmission electron microscopy was used to analyze the chloroplast ultrastructure of WT and *vte5* and compare their ultrastructure under HL, HT and a combination of both HT+HL stress treatments. Leaf segments from tomato plants exposed for 6 continuous days to stress treatments (day 6 after 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 tetraoxide at $20\,^{\circ}\mathrm{C}$. 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 and heated for 24 h at $60\,^{\circ}\mathrm{C}$ to allow solidification and embedding of the leaf section in the resin. Ultrathin sections of Sigma $90\,\mathrm{nm}$ 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 Reynold's lead citrate (Reynolds, 1963). Ultrathin sections were observed with a Philips CM-100 electron microscope operating at Sigma $60\,\mathrm{kV}$.

5.2.4 Determination of photosynthetic parameters

The ratio of maximum photochemical efficiency or optimum quantum yield of photosystem II (*F*v/*F*m), electron transport rate (ETR) and non-photochemical quenching (NPQ) were fluorometrically determined using a MINI-PAM Photosynthesis Yield Analyzer (Walz, `http://www.walz.com`). Plants were dark-adapted for 15 min before *F*v/*F*m measurements with illumination by application of a saturation flash. Measurements were done in the green photosynthetic tissue. At least four replicates for each treatment were performed, at day 0, as a baseline measurement before application of stress, and at day 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).

5.2.5 Untargeted lipid profiling

After six days of the HT, HL, and combined high-temperature and HL treatment, at least four replicates for each treatment of leaf material were harvested for untargeted lipid analysis (Martinis et al., 2013). Lipids were extracted from Sigma $100\,\mathrm{mg}$ of fresh leaf tissue using Sigma $1\,\mathrm{ml}$ of tetrahydrofuran:methanol 50:50 (v/v) according to Spicher et al. (2016). After centrifugation, the supernatant was directly analysed by ultrahigh pressure liquid chromatography (UPLC™, Waters) coupled to quadrupole time of flight mass spectrometry (Synapt G2, Waters) through an APCI interface. To evaluate variations in the wild type and the *vte5*, features were extracted from lipidomics data using Markerlynx XS (Waters) mean-centred and Pareto-scaled before applying principal component analysis analysis (PCA) Eugeni Piller et al., 2011; Martinis et al., 2011. The changes in lipid content after stress treatments was 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 search 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#`).

5.2.6 Targeted lipid profiling: prenylquinone, carotenoid and glycolipids profile

Absolute concentrations of delta-tocopherol (δ-T), gamma-tocopherol (γ-T), alpha-tocopherol (α-T), alpha-tocopherolquinone (α-TQ), plastoquinone (PQ-9), plastochromanol (PC-8),

and phylloquinone (Vit K) were measured based on calibration curves obtained from standards as described in Martinis et al. (2013). Moreover, plastoquinol (PQH_2-9), hydroxy-plastoquinone (PQ-OH) and hydroxy-plastochromanol (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 and PC-8 were obtained by purification from spinach leaves and flaxseed oil, respectively. PQ was extracted from entire leaves in acetone and further purified by normal phase open-column chromatography followed by reverse phase semi-preparative HPLC. PC-8 was obtained by saponification of the oil followed by partition with a hexane:ethylacetate mixture. The resulting extract was then separated by open column chromatography and semi-preparative HPLC as for PQ isolation. A detailed description of the purification process is given in supplementary material and method (Figure 5.S5). The two carotenoids violaxanthin and neoxanthin were measured as a sum since they could not be resolved either in 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 Bonferroni–Holm test comparisons vs. control in WT, to determine any significant differences between different temperatures and light conditions over the time course.

5.2.7 Free phytol and fatty acid phytyl ester quantification

Total lipids were extracted from approx. 20 mg of lyophilized leaf tissue with diethylether and 300 mM ammonium acetate. Afterwards fatty acid phytyl esters (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 diethylether (`www.cyberlipid.org`): FAPEs were eluted with *n*-hexane:diethylether 99:1 (v/v), while free phytol was eluted with *n*-hexane:diethylether 92:8 (v/v). FAPEs and free phytol were analyzed as previously described (vom Dorp et al., 2015).

5.2.8 qPCR 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 5.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). 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, 2009).

5.2.9 Isolation of thylakoid membranes and Hill reaction

Thylakoid membranes were prepared from $50\,\mathrm{g}$ of fresh leaves from 18-day-old pea plants, which were grown under control conditions with minor modifications Fitzpatrick and Keegstra, 2001; Kummerová et al., 2006, 2008; Smith et al., 2003. The Hill reaction was adapted and carried out to measure the direct effect of phytol on the photosynthetic primary reaction at level of PSII. $4\,\mathrm{ml}$ of total reaction mixture contained $0.03\,\mu\mathrm{M}$ of sodium salt solution of 2,6-dichloro-phenol-indophenol (redox system - DCPIP), 20% of dimethyl sulfoxide (DMSO, Sigma-Aldrich) and 0.5 and Sigma $1\,\mathrm{mg}$ thylakoid membranes in suspension buffer. Phytol (97%, mixture of isomers) was purchased from Sigma-Aldrich and suspended in DMSO at a final concentration of Sigma $10\,\mu\mathrm{M}$. Phytol was used to obtain final concentrations of $500\,\mu\mathrm{M}$ and $50\,\mu\mathrm{M}$. The reactions were incubated for 5 minutes and then exposed for 6 minutes to an irradiance of 200-250 $\mu\mathrm{mol\,m^{-2}\,s^{-1}}$ at room temperature. A $1\,\mathrm{ml}$ aliquot was removed at 3 and 6 minutes, centrifuged for 1 min at 13200 rpm and measured spectrophotometrically at Sigma $600\,\mathrm{nm}$. Photosynthetic activity was determined by measuring DCPIP reduction using an UltraViolet-Visible Spectroscopy (UV/VIS) Ultrospec 3100 pro (Amersham Biosciences) in 1 cm visible cuvettes. Measurements were repeated at least three times at each time point.

5.3 Results

5.3.1 Untargeted lipidomics demonstrated changes in lipid composition in tomato leaves after combined high light and HT

To determine variations in the lipid composition after exposure to HL, HT, and the combination of HT+HL in wild type tomato plants (WT), we carried out untargeted lipidomics analysis (Figure 5.1). Lipid extracts were isolated from fresh leaf tissue and analyzed by ultra-high pressure liquid chromatography coupled with atmospheric pressure chemical ionization-quadrupole time-of-flight mass spectrometry (UHPLC-APCI-QTOF-MS). Principal component analysis (PCA) identified four distinct clusters when comparing control to the different stress conditions (Figure 5.1A). In the loadings plot (Figure 5.1B), 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

(PQH_2-9) 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 as well as HL clusters were specifically separated from the other clusters represented by MGDG-18:3/16:3, -18:3/18:3 and DGDG-18:3/18:3 (Figure 5.1B). Loadings located near the center of the plot, (i.e., the vast majority) make a negligible contribution to metabolic variation.

5.3.2 *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 HL, HT and HT+HL conditions. HL treatment alone had little effect on wild type (WT) and *vte5* plants. Under HT treatment, no differences were apparent between WT and *vte5* or when compared to control conditions. While WT plants developed a mild bleaching phenotype only in the oldest leaves after 6 days under HL+HT, *vte5* plants were extensively photobleached and developed chlorotic leaves. The chlorosis became visible after 2 days under HL+HT. The visible phenotype after 4 days is shown in Supplementary Figure 5.S1.

5.3.3 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 Chl fluorescence (Supplementary Figure 5.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 5.3). In *vte5* a strong and increasing reduction of the photochemical efficiency of photosystem II (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 5.3, 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 non-photochemical quenching (NPQ) under HL+HT. However, NPQ in WT began to recover at day 6 of HT+HL whereas *vte5* improved only during the 5 day recovery period under control conditions. Electron transport rate (ETR) in WT under 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.

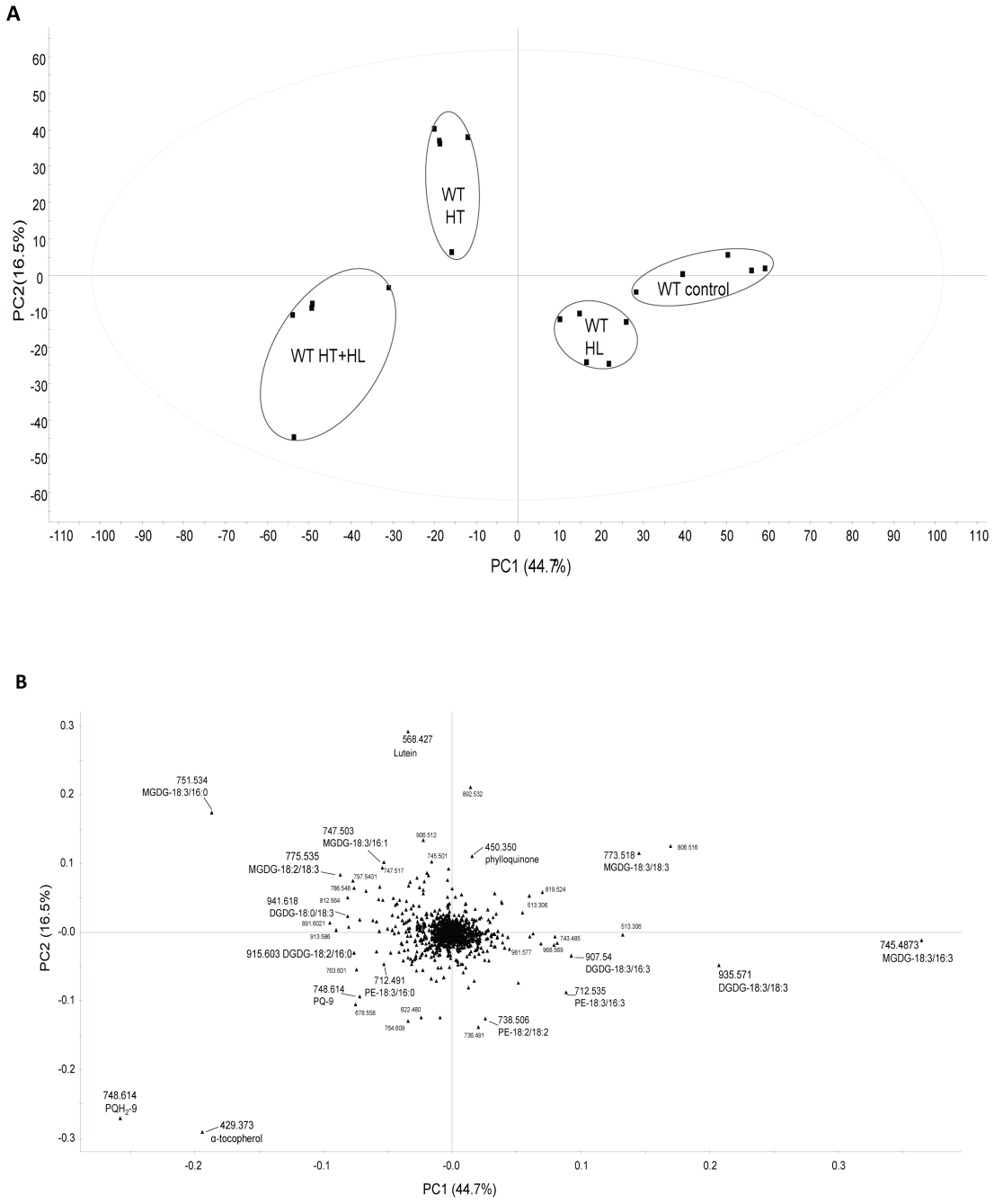


Fig. 5.1: Untargeted lipidomics profile of WT tomato leaves subjected to 6 days of HL, HT and the combination of HT+HL stress treatments. (A) Principal component analysis (PCA) of the lipid composition of WT leaf samples exposed to HL, HT and a combination of HT+HL for 6 days. PC1 and PC2 are first and second principal components, respectively, with their percentage of explained variance. **(B)** Corresponding loading plots. Data were mean-centred and Pareto scaled before principal component analysis.

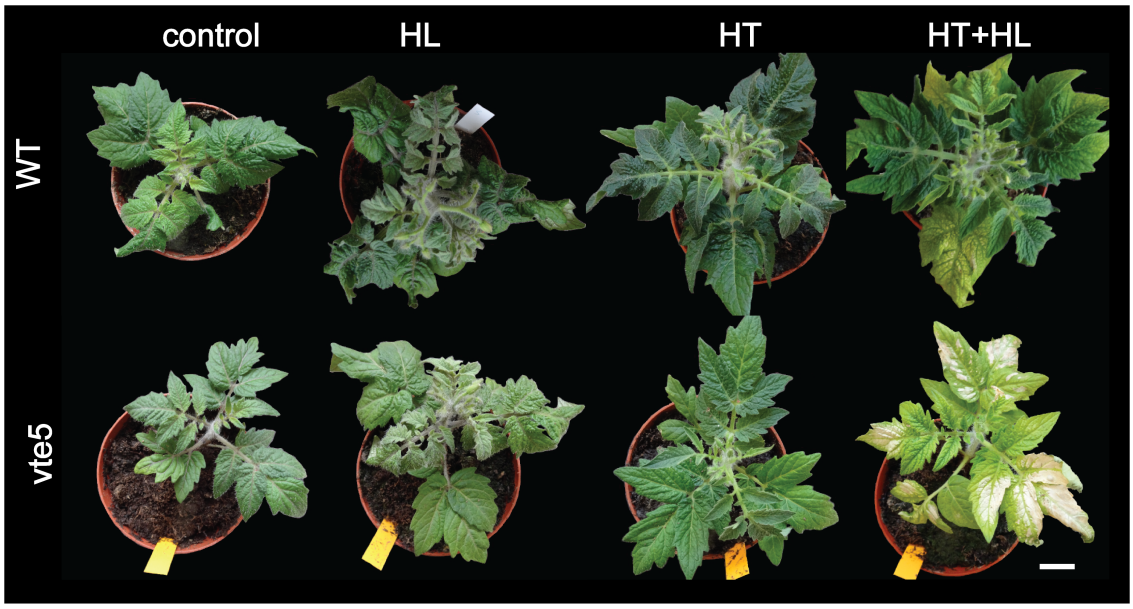


Fig. 5.2: Visible phenotypes of WT and vte5 after six days under control, HL, HT and combined HT+HL conditions. Scale bar: 3 cm.

5.3.4 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 their impact on chloroplast ultrastructure (Fig 5.4, showing representative images). Under control conditions, WT (Fig 5.4A) and *vte5* (Fig 5.4B) chloroplasts were very similar. Under HL conditions, chloroplasts in both WT and *vte5* contained large starch granules (Fig 5.4C and5.4D) and also larger plastoglobules appeared, particularly in *vte5*. The *vte5* plastoglobules were surrounded by a non-osmiophilic ring appearing to contain additional, smaller globular structures (Fig 5.4D). Under HT treatment highly stacked thylakoids were observed and large plastoglobules surrounded by non-osmiophilic rings appeared in both WT and *vte5* (Fig 5.4E and 5.4F). Under HL+HT, WT chloroplasts contained large plastoglobules with non-osmiophilic rings and thylakoids appeared disorganized and swollen ((Fig 5.4G). *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 5.4H).

5.3.5 Stress treatments change prenylquinone and carotenoid metabolism

Knockdown of *vte5* results in tocopherol-deficiency (Almeida et al., 2016). Under control conditions, α-tocopherol content in *vte5* was one third of that in WT and other tocopherols displayed similar ratios (Fig 5.5). 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 stress

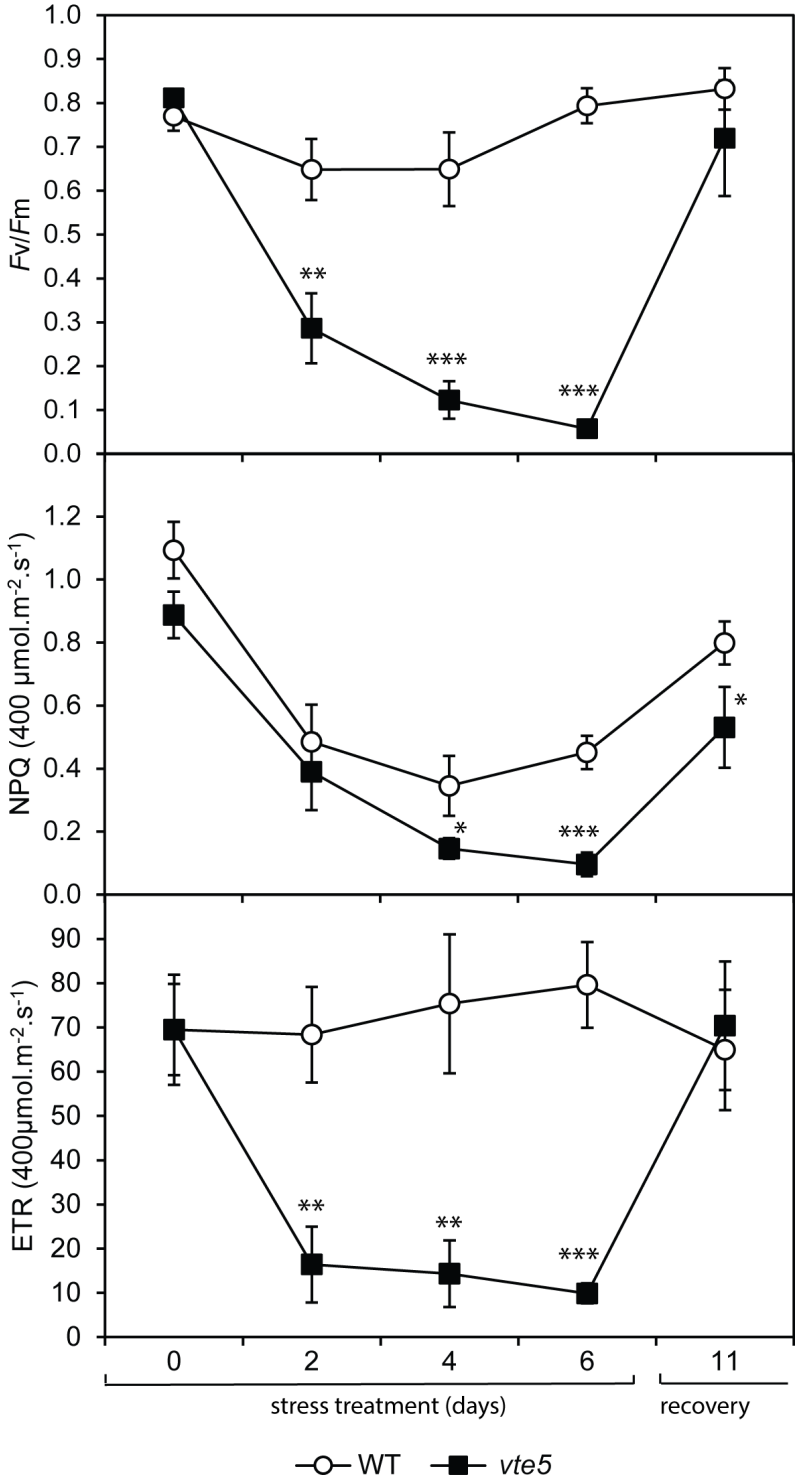


Fig. 5.3: Photosynthetic parameters in WT and *vte5* over a time course of exposure to the combination of HT+HL stress. The photochemical efficiency of photosystem II (F_v/F_m), electron transport rate (ETR) and non-photochemical quenching (NPQ) values are the mean of at least 4 biological replicates ($n = 4$) from plants exposed to HT+HL ($820\,\mu\mathrm{mol\,m^{-2}\,s^{-1}}$, 16-h light/8-h dark, at 38/30 °C) for 6 days followed by 5 days of recovery at control temperature up to day 11. Means ± SE. The asterisks indicate significant differences between the WT and *vte5* subjected to HT+HL (One-ANOVA, followed by Holm-Sidak, *posthoc* test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

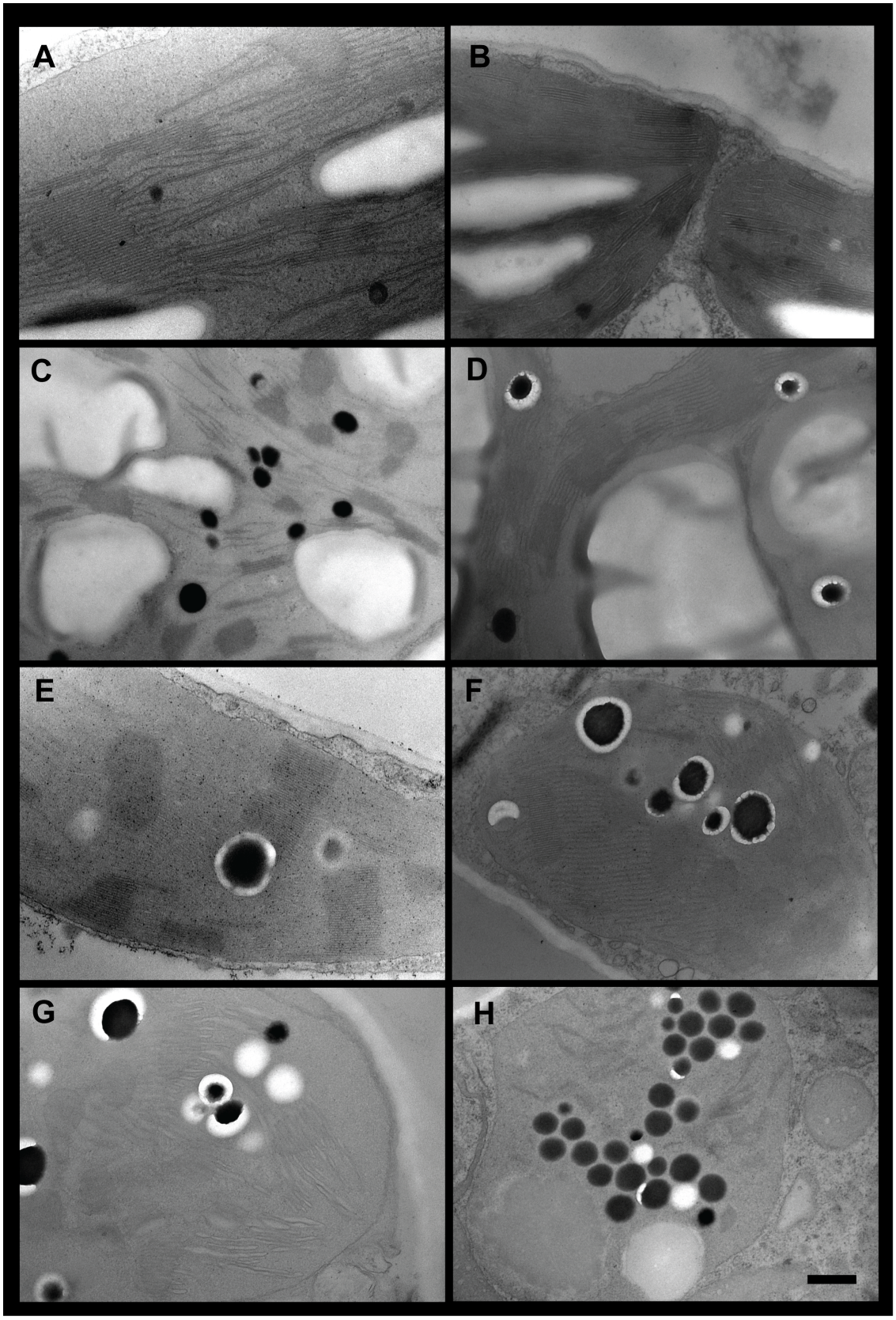


Fig. 5.4: Changes in chloroplast ultrastructure under control and HL, HT and combined HT+HL stress. Transmission electron micrographs of WT (A, C, E, G) and vte5 (B, D, F, H) leaves upon 6 days of control (A, B), HL (C,D), HT (E,F), and combined HL+HT(G,H) treatments. Scale bar: 500nm.

WT plants showed a striking 2.8-fold increase in α-tocopherol. This indicates that high light and high temperature have additive effects on α-tocopherol accumulation in WT. In *vte5*, α-tocopherol also increased in an additive manner under HT+HL, but it was only around half of that observed in WT (Fig 5.5). Also, *vte5* showed a 3-fold increase in α-tocopherolquinone (α-TQ) when compared to WT under combined stress conditions (Fig 5.5).

α-T (α-tocopherol), γ-T (γ-tocopherol), δ-T (δ-tocopherol), and α-TQ (α-tocopherol quinone) 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-QTOFMS. Values are means of at least 4 biological replicates $\pm$ SE. The asterisks indicate significant differences between the WT (control) and the stress treatments, including vte5 (One-Way ANOVA, followed by Holm-Sidak, posthoc test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

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 (PC-OH) were also measured (Fig 5.6). PQH_2-9 and PQ-OH concentrations under all conditions were similar in WT and *vte5*. PQ-9 was similar under control conditions and single stress, but PQ-9 was increased 1.5-fold in *vte5* when compared to the WT under combined HT+HL stress (Fig 5.6). PC-8 was reduced by around half in *vte5* under HT and HT+HL while PC-OH was similar. Phylloquinone concentrations were not strongly affected in *vte5* and WT under any of the conditions (Fig 5.6).

No significant changes in β-carotene and Violaxanthin+Neoxanthin concentrations were observed under any conditions when comparing WT to *vte5* (Supplementary Figure 5.S3). However, lutein increased 1.5- and 1.3-fold under HT in WT and *vte5* with respect to control conditions.

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

Quantification of fatty acid phytyl esters 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 5.7). 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.

Fatty acid phytyl ester (FAPE) content in WT and vte5 after exposure to control, HL, HT and combined HT+HL stresses. WT and vte5 were subjected to control or stress conditions for 6 days. Data represent the mean$\pm$SE of at least three biological replicates.

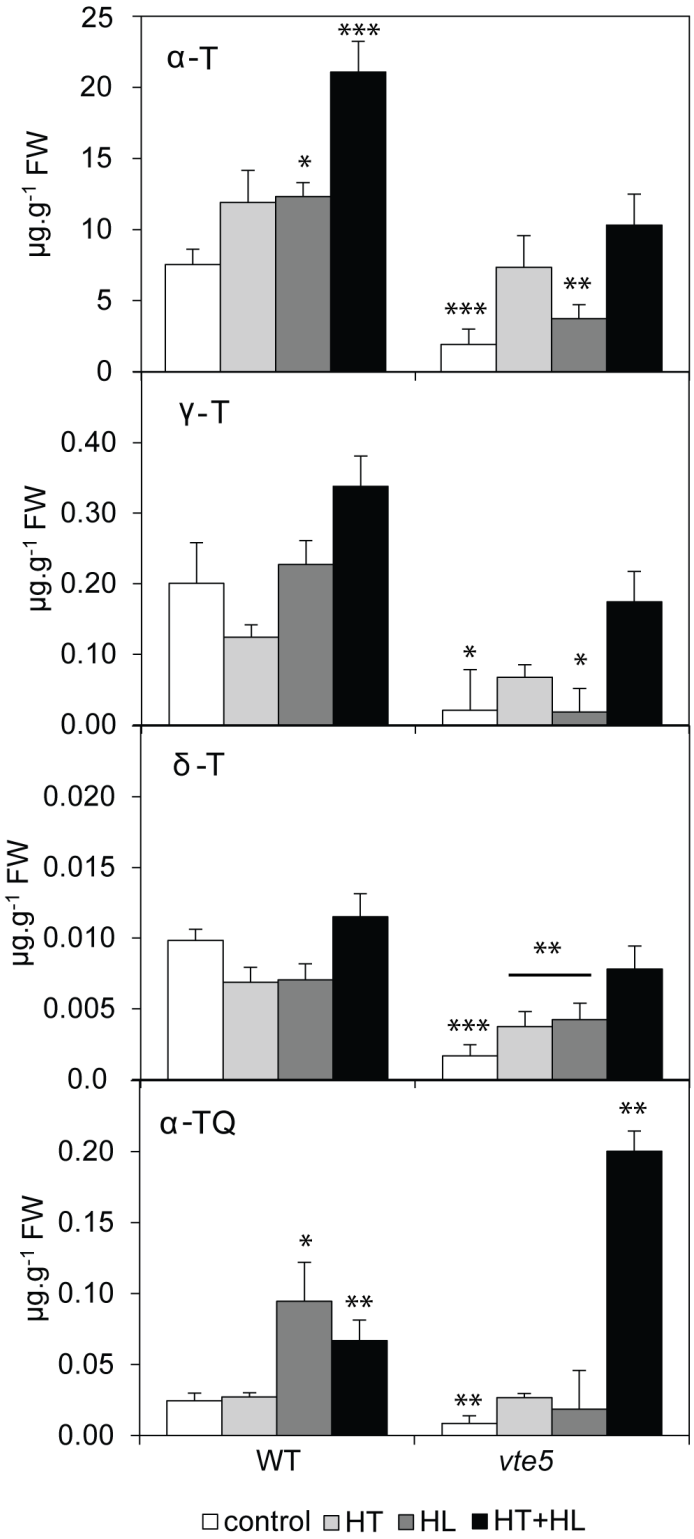


Fig. 5.5: **α-T (α-tocopherol), γ- T (γ-tocopherol), δ-T (δ-tocopherol), and α-TQ (α-tocopherol quinone) after exposure to control, HL, HT and combined HT+HL stresses**. Lipids were extracted from WT and *vte5* plants subjected to control or stress conditions for 6 days; Lipids were analyzed by UHPLC-QTOFMS. Values are means of at least 4 biological replicates $\pm$ SE. The asterisks indicate significant differences between the WT (control) and the stress treatments, including *vte5* (One-Way ANOVA, followed by Holm-Sidak, *posthoc* test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

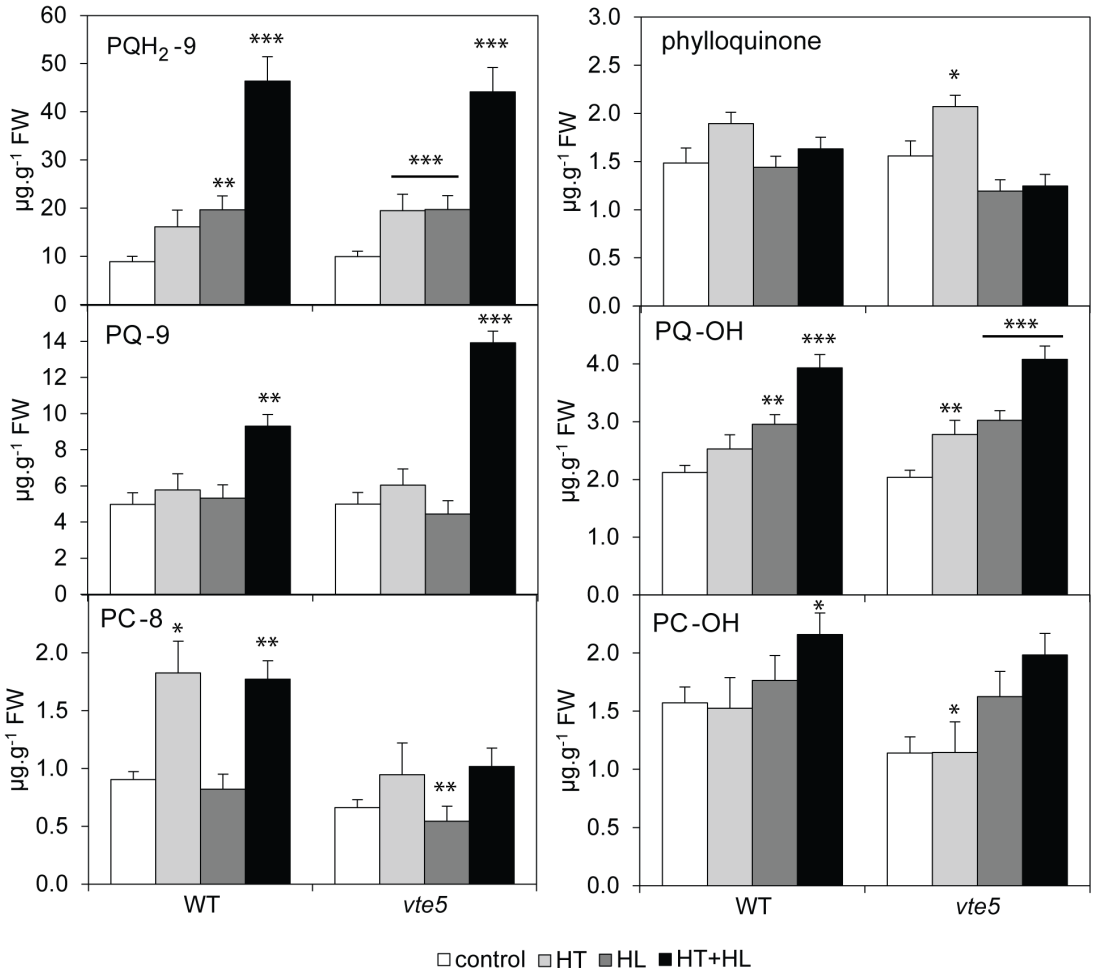


Fig. 5.6: Plastoquinone, plastoquinone-derived quinones and phylloquinone after exposure to control, HL, HT and combined HT+HL stresses. Lipids were extracted from WT and *vte5* plants subjected to control or stress conditions for 6 days; lipids were analyzed by UHPLC-QTOFMS. Values are means of at least 4 biological replicates ± SE. The asterisks indicate significant differences between the WT (control) and the stress treatments, including *vte5* (One-Way ANOVA, followed by Holm-Sidak, *posthoc* test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

The asterisks indicate significant differences between the WT (control) and the stress treatments, including vte5 (One-Way ANOVA, followed by Holm-Sidak, posthoc test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

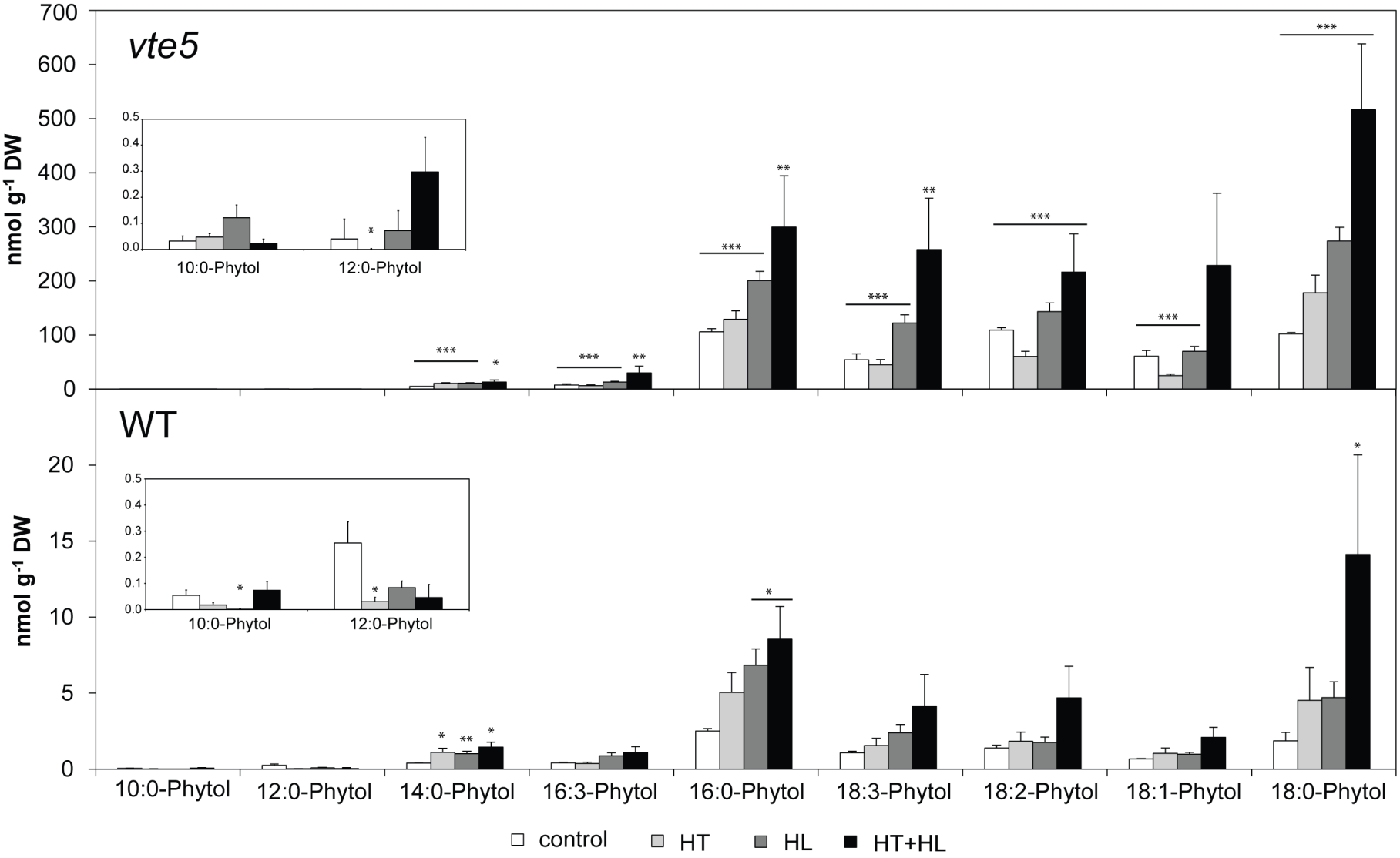


Fig. 5.7: Fatty acid phytyl ester (FAPE) content in WT and *vte5* after exposure to control, HL, HT and combined HT+HL stresses. WT and *vte5* were subjected to control or stress conditions for 6 days. Data represent the mean ± SE of at least three biological replicates. The asterisks indicate significant differences between the WT (control) and the stress treatments, including *vte5* (One-Way ANOVA, followed by Holm-Sidak, *posthoc* test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

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

vte5 plants accumulate free phytol in the leaf (Fig 5.7)(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.

Free phytol content in WT and vte5 after exposure to control, HL, HT and combined HT+HL stresses.

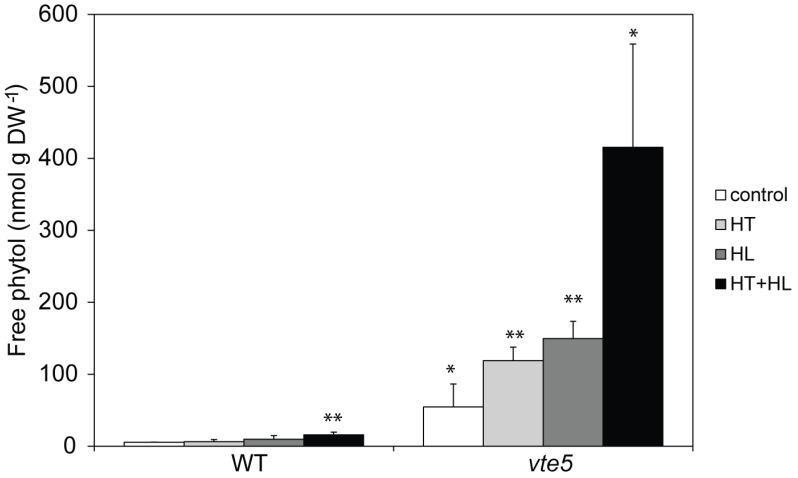


Fig. 5.8: Free phytol content in WT and *vte5* after exposure to control, HL, HT and combined HT+HL stresses. Data represent the mean ± SE of at least four biological replicates. The asterisks indicate significant differences between the WT (control) and the stress treatments, including *vte5* (One-Way ANOVA, followed by Holm-Sidak, *posthoc* test, *, $p < 0.05$; **, $p < 0.01$).

5.3.8 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 under HT+HL in *vte5*. To determine whether phytol may exert toxicity via perturbation of the thylakoid membrane and of the photosynthetic electron transport, we carried out the Hill reaction in the presence of increasing concentrations of phytol. The Hill reaction is a method to measure photosynthetic electron transport in isolated thylakoid membranes (*Pisum sativum*) at the level of PSII (Fig 5.7). In the Hill reaction, the synthetic electron acceptor DCPIP (2,6-dichlorophenolindophenol) is reduced which can be measured spectrophotometrically. In the absence of phytol, DCPIP reduction was arbitrarily set to 100% reduction; in a negative control experiment in the

absence of thylakoids, DCPIP reduction did not occur and was set to 0%. The effets of the presence of two concentrations of phytol ($50\,\mathrm{uM}$ and $500\,\mu\mathrm{M}$) on the Hill reaction were measured. The phytol concentrations were calculated to be 50 and 500 times as high as those measured *in vivo* in *vte5* leaves under HT+HL. Two separate experiments were carried out using isolated thylakoids corresponding to either $0.5\,\mathrm{mg}$ or $1\,\mathrm{mg}$ of chlorophyll per reaction. The Hill reaction was only significantly inhibited at $500\,\mu\mathrm{M}$ phytol.

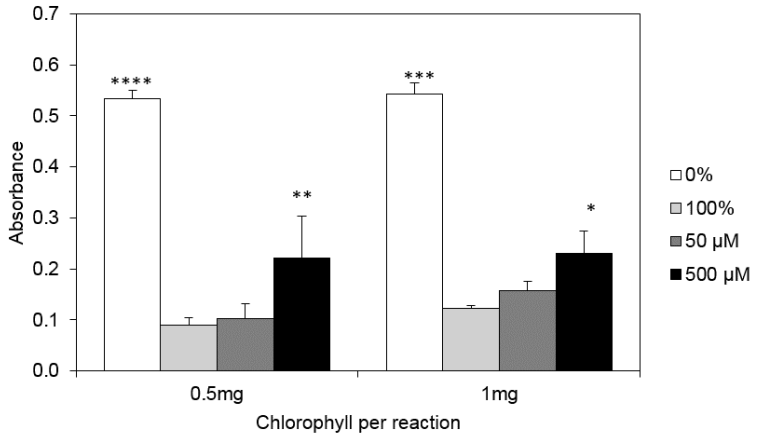


Fig. 5.9: Assessment of phytol toxicity on photosynthetic electron transport in isolated thylakoid membranes. Photoreduction of DCPIP (2,6-dichlorophenolindophenol) was measured in the presence of $50\,\mathrm{and}\,500\,\mu\mathrm{M}$ phytol. 0% reduction relates to the negative control in the absence of both thylakoids and phytol. 100% refers to the Hill reaction in the absence of phytol. Absorbance was measured after 6 minutes of incubation. Values are mean ± SE, ($n = 3$). Significant differences in data between assays in each time point are indicated: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ by One-Way ANOVA, followed by Holm-Sidak, *posthoc* test.

5.3.9 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 MEP, carotenoid, prenylquinone, Chl and phytol metabolism (Almeida et al., 2016; Lira et al., 2016) were measured by qPCR (Supplementary Figure 5.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 HL as well as HT conditions the expression patterns of genes encoding enzymes involved in tocochromanol, prenylquinone, chlorophyll degradation, carotenoid, and FAPE metabolic pathways (Supplementary Figure 5.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. How-

ever, 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 upregulated in *vte5* under combined HT+HL. In contrast *VTE1* expression was much lower in *vte5* than the transcript levels found in WT under the same stress conditions. The low *VTE1* transcription level correlates 1) with the accumulation of α–TQ, indicating insufficient activity in the redox recycling of α–TQ by VTE1 and 2) with the reduced levels of PC-8 that were observed in *vte5* (Fig 5.6). In WT as well as *vte5*, geranylgeranyl reductase (*GGDR*) mRNA abundance was diminished under all stress conditions, potentially provoking a reduction of chlorophyll synthesis. Higher expression of the plastoglobule-associated enzyme PES (phytol ester synthase) was observed to similar degrees under HL as well as HL+HT in both WT and *vte5*. However, only in *vte5* a dramatic increase of FAPEs was actually observed. This suggests that the transcriptional regulation had only a limited role in diverting the metabolic flux into the FAPE biosynthetic pathway.

5.3.10 Discussion

Photosynthetic metabolism is among the primary processes that are strongly affected by environmental perturbations (Chaves et al., 2009; Lichtenthaler and Burkart, 1999; Mathur et al., 2014; Tezara et al., 1999). 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 multi-stresses connected to climate change. Only few studies have investigated the impact of simultaneous HL and HT on the photosynthetic apparatus of plants (Buchner et al., 2014; Faik et al., 2016; Gerganova et al., 2016; Krause et al., 2015; Qiu and Lu, 2003; Quiles, 2006; Streb et al., 2003).

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 plastochromanol. Both tocopherols and plastoquinone have been implicated in resistance to high light. A recent study that carefully dissected the distinct roles of tocopherols and plastoquinone indicated a predominant role for plastoquinone under high light (Ksas et al., 2015). This came as somewhat of a surprise as plastoquinone is normally associated with electron transport and the role of a lipid antioxidant is usually attributed to tocopherols. Our experiments aimed at determining 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 to HT and 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 been correlated with oxidative stress in the past (Havaux et al., 2005; Kobayashi and DellaPenna, 2008). Our own recent work suggested a correlation of the increase of α-tocopherol and plastoquinone with the normal functioning of the photosynthetic apparatus under HT stress (Spicher et al., 2016). That study however was 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 the phytol kinase (VTE5) is silenced. VTE5 which salvages phytol from chlorophyll degradation, contributed to around 70% of the tocopherol biosynthesis. *vte5* plants were highly sensitive to combined HT+HL but not the single stresses, HT+HL resulting in extensive photobleaching (Fig 5.2). This phenotype was likely brought about by photoinhibition as apparent from the severe reduction of *F*v/*F*m in *vte5* (Fig 5.3). The tolerance of WT plants but not *vte5* to HT+HL suggested that tocopherol metabolism holds the key to HT+HL resistance in tomato.

When comparing WT and *vte5* prenyllipid profiles under HT+HL, tocopherols were diminished in *vte5* in agreement with the defect in the tocopherol salvage pathway. In contrast, PQH_2-9 (the reduced form of plastoquinone) was unchanged, and PQ-9 (the oxidized form of plastoquinone) was even increased in *vte5*. This increase in PQ-9 is probably due to the 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 diminished under HT+HL. However, PC-8 is present at far lower concentrations than the oxidized and reduced forms of plastoquinone and α-tocopherol, and therefore unlikely to contribute to HT+HL resistance in a major way. A possible explanation for the diminished concentration of PC-8 is the observed downregulation of VTE1 in the vte5 background. This downregulation under HT+HL may also be responsible to the accumulation of α–TQ, an α-tocopherol oxidation product which 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? α-tocopherol partitions into the hydrophobic phase of a membrane lipid bilayer. 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). Reduction of membrane fluidity may therefore contribute to the adaptation of the thylakoid membrane to high temperatures. Although the membrane stabilizing effect has not been shown in artificial membranes having thylakoid lipid composition, it is tempting to propose that the protective function under HT+HL lies in the combination of α-tocopherol's anti-oxidant 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 (Almeida et al., 2016; Lippold et al., 2012). The FAPE content rose significantly in response to HL, HT, and HT+HL in both *vte5* and WT but in far more dramatic fashion in *vte5* (Fig 5.7). FAPE synthesis is boosted by the high levels of free phytol due to the increase in chlorophyll catabolism and simultaneous lack of *VTE5* activity. But not only FAPE increased, also free phytol reached much higher levels in *vte5* than in WT. Under HT+HL the levels were particularly high and this can probably be attributed to the 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 (Löbbecke and Cevc, 1995; Sikkema et al., 1995). In contrast, FAPE (lacking detergent-like structure) are not considered toxic and in addition they are sequestered in plastoglobules (Fig 5.4) (Gaude et al., 2007; Lippold et al., 2012). Indeed, plastoglobule numbers visibly increased in *vte5* under HT+HL (Fig 5.4). 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 as high as 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. Alltogether the evidence presented here indicates that tocopherols more than any other of the metabolites analyzed are responible for tolerance to combined high temperature and high light stress in tomato.

5.3.11 Acknowledgements

The authors are grateful to the University of Neuchâtel and Swiss National Science Foundation (Grants 31003A_156998 and IZEBZ0_143169).

5.4 Supplementary data

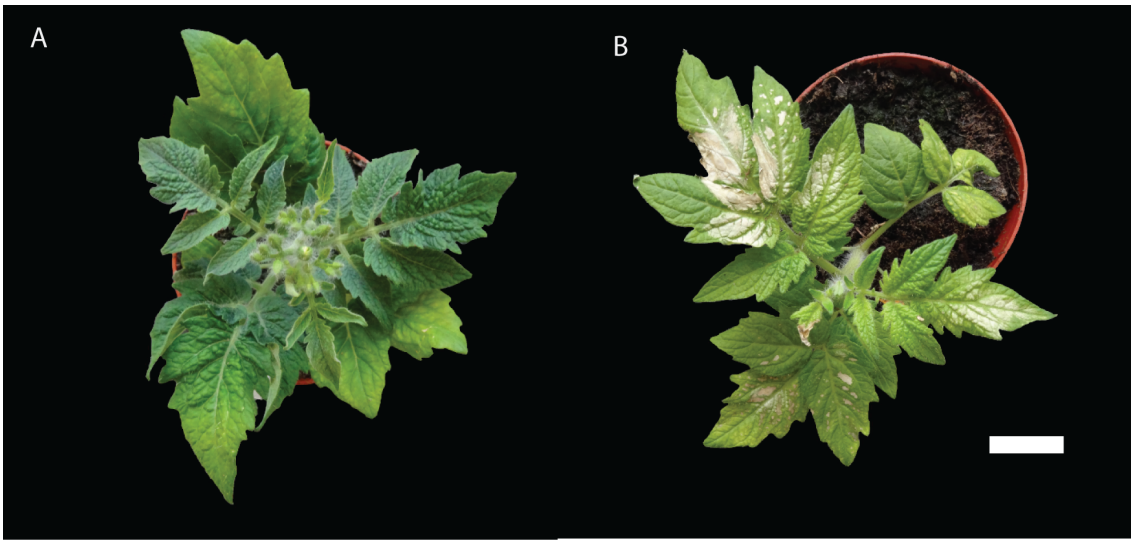


Fig. 5.S1: Phenotype MT and *vte5* after 4 days of combined HL and HT. Scale bar: 3cm.

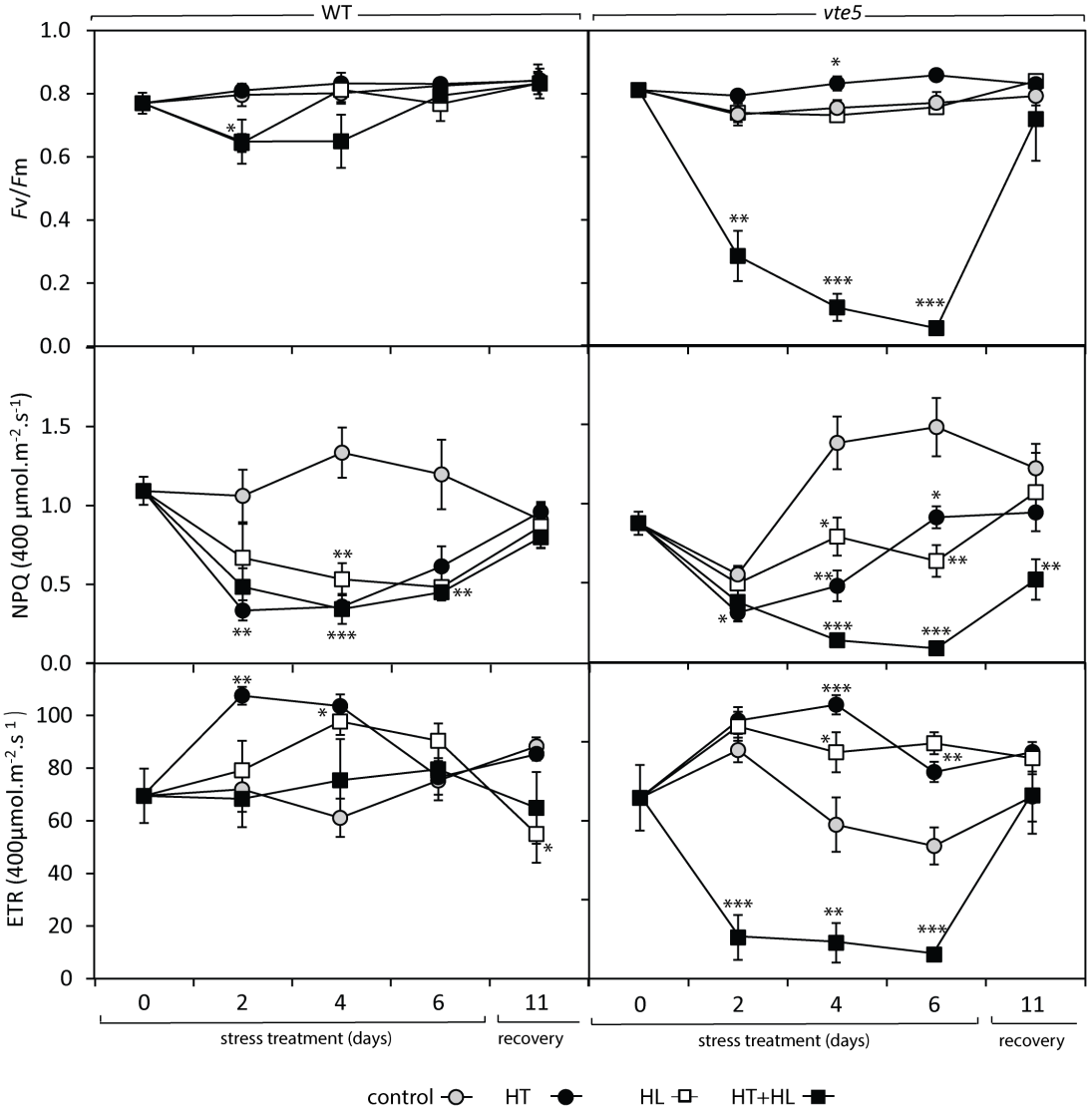


Fig. 5.S2: Chlorophyll fluorescence measurements. Photosynthetic efficiency in tomato leaves, WT and *vte5*, over a time course of exposure to different stress treatments. The ratio of maximum photochemical efficiency or optimum quantum yield of photosystem II (F_v/F_m), electron transport rate (ETR) and non-photochemical quenching (NPQ) values are the mean of at least 4 biological replicates ($n = 4$) from plants exposed to control conditions ($250\,\mu\mathrm{mol\,m^{-2}\,s^{-1}}$ of light, 16-h light/8-h dark, at 20/18 °C), HT($250\,\mu\mathrm{mol\,m^{-2}\,s^{-1}}$ of light, 16-h light/8-h dark, at 38/30 °C), HL ($820\,\mu\mathrm{mol\,m^{-2}\,s^{-1}}$, 16-h light/8-h dark, at 20/18 °C), and combination of both stresses HT+HL ($820\,\mu\mathrm{mol\,m^{-2}\,s^{-1}}$, 16-h light/8-h dark, at 38/30 °C conditions for 6 days followed by 5 days of recovery at control temperature up to day 11. Means ± SE. The asterisks indicate significant differences between the control and the stress treatments for each time point (One-Way ANOVA, followed by Holm-Sidak, *posthoc* test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

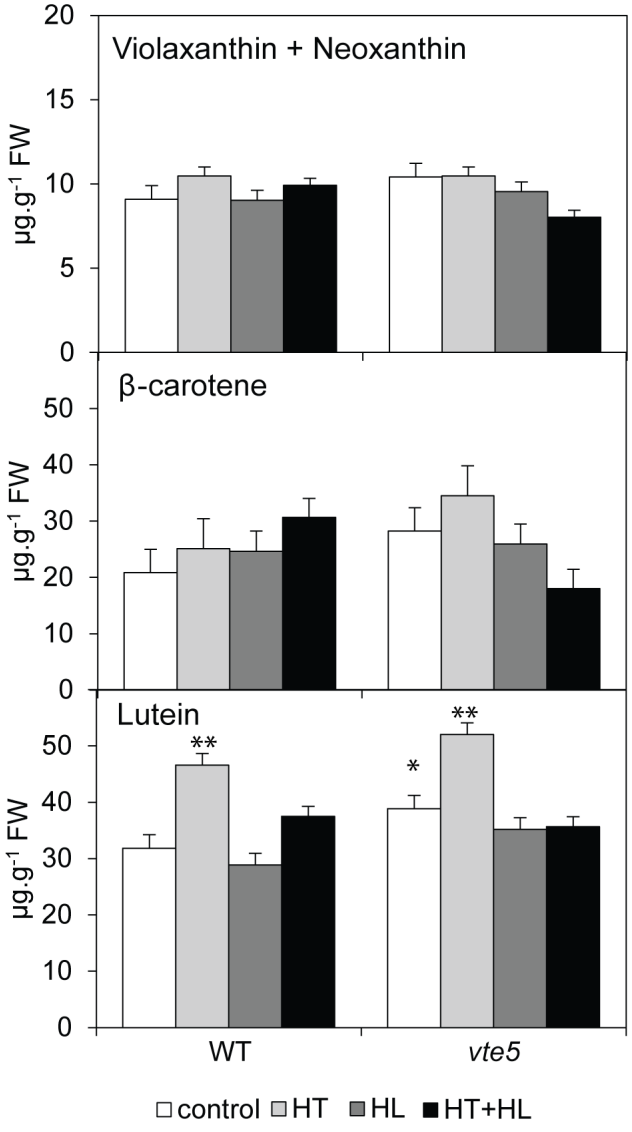


Fig. 5.S3: Violaxanthin + Neoxanthin, β-carotene, Lutein in tomato leaves after exposure to control, high light, high temperature and a combination of both HT+HL stresses. Carotenoids were extrated from WT and *vte5* plants subjected to control conditions, high temperature (HT), high light (HL), or combined HT+HL stress conditions for 6 days; Carotenoids were analyzed by UHPLC-QTOFMS. Values are means of at least 4 biological replicates $\pm$ SE. The asterisks indicate significant differences between the WT (control) and the stress treatments, including vte5 (One-Way ANOVA, followed by Holm-Sidak, *posthoc* test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

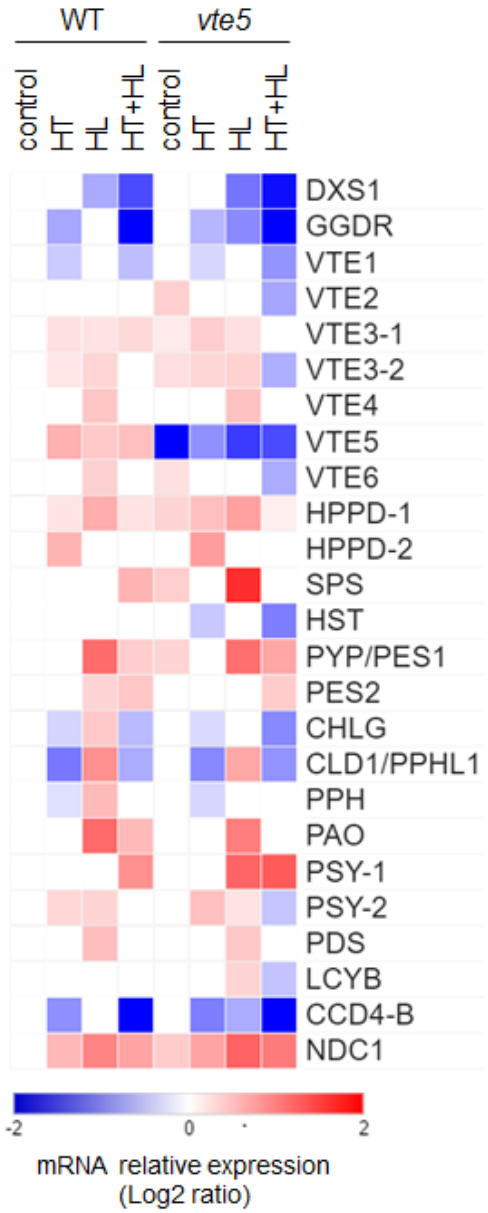


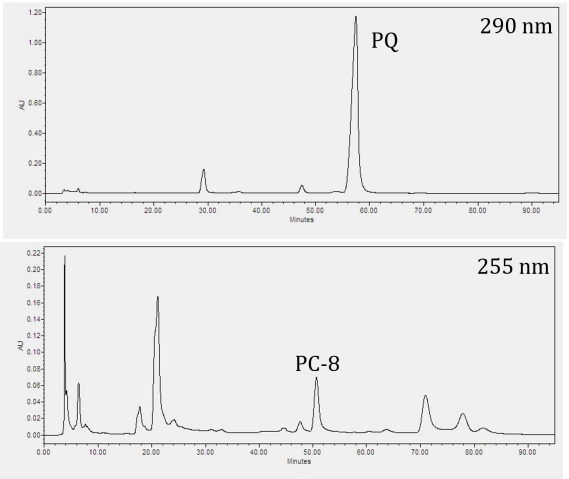
Fig. 5.S4: Expression of genes encoding isoprenoid metabolism-related enzymes. The transcript abundance shown compares to WT control genotype of at least three biological replicates. The $\log_2$ of the expression ratios is indicated by a color scale where red and blue indicate statistically significant decreases and increases of transcript abundance ($p < 0.05$), respectively. 1-deoxy-D-xylulose-5-P synthase (DXS); geranylgeranyl diphosphate reductase (GGDR); tocopherol cyclase (VTE1); homogentisate phytyl transferase (VTE2); 2,3-methyl-5-phytylquinol methyltransferase (VTE3); γ-tocopherol-C-methyl transferase (VTE4); phytol kinase (VTE5); phytyl-phosphate kinase (VTE6); 4-hydroxyphenylpyruvate dioxygenase (HPPD); solanesyl-diphosphate synthase (SPS); homogentisate solanesyl transferase (HST); chlorophyll synthase (CHLG); chlorophyll dephytylase (CLD1/PPHL1); pheophytinase (PPH); pheophorbide *a* oxygenase (PAO); phytoene synthase (PSY); pale yellow petal/phytyl ester synthase (PYP/PES); phytoene desaturase (PDS); chloroplast-specific β-lycopene cyclase (LCYβ); naD(P)H dehydrogenase C1 (NDC1); carotenoid cleavage dioxygenase 4 (CCD4/ NCED4).

Table 5.S1: Primers used for each experiment.

Experiment	Primer name[a]	Tomato locus	Primer Sequence (5'-3') Foward	Reverse
qPCR	DXS(1)	Solyc01g067890	CAGGACTGGTGTGGTTTCAG	GGGATAGTTCACAGTGTCC
	GGDR	Solyc03g115980	CAGAGACGCTCGCTAAGG	GCTTCAGAGTCTGTCCGATATC
	HPPD(1)	Solyc07g045050	CCAGGGCAGGGGATATACTG	CTCCTTCCTCGTTTTTCAGC
	HPPD(2)	Solyc05g041200	CCAGGCGTGTGAAGAATTG	CGATCTAAACAGCTCAGAG
	VTE2	Solyc07g017770	CAATTCCAGTTCCTGCTGAG	CCTCCAACATGCTCTTGCGTG
	VTE3(1)	Solyc09g065730	CTTGACCAATCTCCTCATC	GCACGCCTTTCCTCCAGG
	VTE3(2)	Solyc03g005230	GCTAAGGCTAGGCAGAAGGAG	CAGGCAACCCCACCTATGG
	VTE1	Solyc08g068570	CGAACTCCTCATAGCGGGTATC	CACGCCAGTAAACCGAGGC
	VTE4	Solyc08g076360	CAGATCATCGTGCTGCTCAG	CCTCTCTGCTTGTACAGGAC
	VTE5	Solyc03g071720	CGTATCAGGACGGGCTCGC	TCACCACCACACATCATTGCTAATG
	VTE6	Solyc07g062180	AGCACAAGCATCAGTGTCTG	AAGAAAGCAGCCGCAATACC
	CHLG	Solyc09g014760	CCAATTCCTTCAGGTGCGGT	CCCACCAAGGCAAGCTGATA
	PPH	solyc01g088090	TATGGAGGGAGCAAGTACGC	TGGAGGGCAGAGGAAAAGTAC
	PAO	Solyc11g066440	TCAGAAGTGGGTGATATGGA	TATCCCCGTCATACACCTTA
	HST	Solyc03g051810	GCTGCTAACTTGGTGCTC	GATCCTAGCACAGTCCCACG
	SPS	Solyc07g061990	GTGGTTGCGGATGACCTACTTA	CTTCTGTGATTTGTGGTGAGTTCC
	PSY(1)	Solyc03g031860	CGATGGTGCTTTGTCCGATAC	CTCATCAACCCAACCGTACC
	PSY(2)	Solyc02g081330	GCATCACACATAACTCCACAAGC	CGCATTCCTTCAACCATATCTCTG
	PDS	Solyc03g123760	CGTTCCGTGCTTCTCCGC	CTAGAACATCCCTTGCCTCCAG
	LCYβ	Solyc04g040190	GCACCCACATCAAAGCCAGAG	GCCACATGGAGAGTGGTGAAG
	PYP/PES(1)	Solyc01g098110	ACAGGACACAACTCCAACC	TAACCATCGCCATCTTCAGTG
	PES(2)	Solyc02g094430	CGAAGAGAGGGAAAAATGCCTGTG	GCTGCCATCCTGACAAATTCAGAC
	CAC	Solyc06g061150	CCTCCGTTGTGATGTAACTGG	ATTGGTGGAAAGTAACATCATCG
	EXPRESSED	Solyc07g025390	GCTAAGAACGCTGGACCTAATG	TGGGTGTGCCTTTCTGAATG
	NDC1	Solyc03g043750	TGCTGGTTGGAATTTGTGGG	CCATCAGTGAAACTTGGTGAAACAG
	CCD4A	Solyc08g075480	GTGGGGTAGTGAGTAGACATCC	ACGATCCCTGATAACTTAGGTGG
	CCD4B	Solyc08g075490	GAAGACAGGAATGGTGAGC	CTATTACTTTTGGCATAGGACCC
	CLD1/PPHL1	Solyc02g062610	GATTTGGTGCTTCTGCCTTTC	GCTGTTTCTTCAGTTCCTTC

[a] 1-deoxy-D-xylulose-5-P synthase (DXS); geranylgeranyl diphosphate reductase (GGDR); 4-hydroxyphenylpyruvate dioxygenase (HPPD); homogentisate phytyl transferase (VTE2); 2,3-dimethyl-5- phytylquinol methyltransferase (VTE3); tocopherol cyclase (VTE1); γ-tocopherol-C-methyl transferase (VTE4); phytol kinase (VTE5); phytyl-phosphate kinase (VTE6); chlorophyll synthase (CHLG); pheophytinase (PPH); pheophorbide *a* oxygenase (PAO); homogentisate solanesyl transferase (HST); solanesyl-diphosphate synthase (SPS); phytoene synthase (PSY); phytoene desaturase (PDS); chloroplast-specific β-lycopene cyclase (LCYβ); pale yellow petal/phytyl ester synthase (PYP/PES); clathrin adaptor complex medium subunit (CAC); naD(P)H dehydrogenase C1 (NDC1); carotenoid cleavage dioxygenase 4 (CCD4/ NCED4); chlorophyll dephytylase (CLD1/PPHL1).

Fig. 5.S5: Supplementary material and method: PQ and PC purification. Plastoquinone (PQ) was isolated from about $200\,\mathrm{g}$ fresh spinach leaves according to a protocol derived from Malferrari and Francia (2014). Leaves were extracted in $1\,\mathrm{L}$ acetone under continuous stirring for 2 h followed by re-extraction in $300\,\mathrm{mL}$. The extract was filtered using filter paper and the resulting solution was evaporated to dryness and reconstituted in $10\,\mathrm{mL}$ of petroleum ether. The concentrated extract was fractionated by open column chromatography on acidic alumina (Sigma-Aldrich) using increasing concentrations of diethylether in petroleum ether (0, 0.2, 2, 4, 8, 12, 16, 20, 24, 28% v/v). The 8% diethylether fraction containing PQ was evaporated, reconstituted in 0.5 mL THF:water (85:15, v/v) and injected in semi-preparative HPLC (see below). Plastochromanol (PC-8) was purified from flaxseed oil using a protocol adapted from Siger et al. (2014). To 2 g of oil were added $0.5\,\mathrm{g}$ of pyrogallol, $20\,\mathrm{mL}$ ethanol and $2.5\,\mathrm{mL}$ KOH 60%. The mixture was heated at $80\,^{\circ}\mathrm{C}$ for 30 min, after which $50\,\mathrm{mL}$ of NaCl 1% was added. Once at room temperature, $50\,\mathrm{mL}$ of hexane:ethylacetate (9:1) were added and the resulting mixture was agitated for 30 min at 300 rpm. The two phases were then separated and the upper phase was washed twice with water. The organic phase was collected and dried with Na_2SO4, filtered and evaporated to dryness. The dried extract was reconstituted in $10\,\mathrm{mL}$ petroleum ether and chromatographied as described above for plastoquinone. The 12% diethylether fraction containing plastochromanol was evaporated to dryness and reconstituted in $0.5\,\mathrm{mL}$ THF:water (85:15, v/v) for injection in semi-preparative HPLC. Final purification of both PQ and PC-8 was achieved by reverse phase semi-preparative HPLC. The semi-preparative system was composed of a 1525 EF pump (Waters) and a dual wavelength UV detector (2487, Waters) equipped with a semi-preparative UV cell (path length 3 mm). An XTerra MS C18 column (19 x 150 mm, $5\,\mu\mathrm{M}$) thermostated at $60\,^{\circ}\mathrm{C}$ in a heated water bath was employed. Gradient conditions were as follows: solvent A was water and solvent B was methanol; initial conditions 90% B, 0-40.0 min 90-100% B, 40-77 min 100% B. Both prenyllipids were purified in one single injection of $5\,\mu\mathrm{L}$. The flow rate was set to $8.0\,\mathrm{mL\,min^{-1}}$. Detection was performed at 255 and Sigma $290\,\mathrm{nm}$ and fractions were collected every minute in 13x100 mm glass tubes using a Gilson FC203B fraction collector. An aliquot of the fractions corresponding to PQ and PC-8 was diluted 200-fold and re-injected in UHPLC-QTOFMS for confirmatory analysis. The remaining solutions were evaporated to dryness, yielding $2.0\,\mathrm{mg}$ of PQ and Sigma $1.2\,\mathrm{mg}$ of PC-8. The purity (>95%) of the standards was assessed by comparison of the signal intensities with those of authentic standards provided by Jerzy Kruk (Gruszka and Kruk, 2007; Kruk, 1988).



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6 General conclusion

The results of this thesis provide new insights into the mechanisms of resistance of the photosynthetic apparatus to abiotic stress. Given the rapidity and severity of climate change on the planet, the understanding of the mechanisms of resistance of the photosynthetic apparatus to temperature and light dual-stress is highly significant (Maheswari et al., 2012; Pretty et al., 2010). I studied how tomato plants regulate lipid metabolism to this effect. The new found role for α-tocopherol in conferring resistance of the photosynthetic apparatus to high light and high temperature stress appears extremely important. α-tocopherol accumulation and biosynthesis in crop plants, beyond their physiological importance in photosynthetic plants, are also essential for human health. In this thesis, we use a crop model system, the tomato plant, which may allow a more rapid translation of the findings into agricultural systems.

Since the characterization of their proteome (Austin II et al., 2006; Lundquist et al., 2012; Vidi et al., 2006; Ytterberg et al., 2006), the involvement of plastoglobules in a variety of essential metabolic pathways has been established, e.g. those of α-tocopherol, phylloquinone and carotenoids. Moreover, plastoglobules have been shown to play additional roles such as detoxification of chlorophyll and galactolipid catabolic products i.e. the storage and conversion of fatty acids and phytol into phytyl esters (Besagni and Kessler, 2013; Gaude et al., 2007; Ischebeck et al., 2006; Lippold et al., 2012; Rottet et al., 2015).

In this context, we presented a review (Chapter 2) of how NAD(P)H dehydrogenase C1 (NDC1) (Eugeni Piller et al., 2011), tocopherol cyclase (VTE1), activity of bc1 complex-like kinases (ABC1K1 and -K3, ABC1K1/K3) (Lundquist et al., 2013; Martinis et al., 2013, 2014), all located in plastoglobules, are involved in the biosynthesis and metabolism of tocopherol and phylloquinone. In phylloquinone metabolism, NDC1 together with MenG is required for the final methylation step of the 2-phytyl-1,4-naphthoquinone (Phytyl-NQ) precursor (Eugeni Piller et al., 2011; Fatihi et al., 2015; Lohmann et al., 2006). Moreover, ABC1K1 is necessary for the elevated accumulation of tocopherol accumulation under high light stress (Lundquist et al., 2013; Martinis et al., 2013, 2014). Also, tocopherol redox recycling requires VTE1, ABC1K1/K3 and NDC1 (Eugeni Piller et al., 2014; Kobayashi and DellaPenna, 2008; Martinis et al., 2013, 2014; Mène-Saffrané and DellaPenna, 2010; Porfirova et al., 2002). Lastly, ABC1K1/K3 are also involved in the regulation of plastochromanol synthesis (Eugeni Piller et al., 2014; Lundquist et al., 2012; Martinis et al., 2013, 2014; Mène-Saffrané and DellaPenna, 2010).

As it is shown here, tomato plants exposed to temperature stress exhibit a massive increase of α-tocopherol and plastoquinone/ol, suggesting that these molecules contribute to membrane protection under high temperature stress (Chapter 3). Secondly, in collaboration with our colleagues we demonstrated that, through perturbation of phytol kinase, α-tocopherol biosynthesis in tomato is strongly diminished (Chapter 4). Third, phytol kinase, via its function in α-tocopherol synthesis, is essential for resistance to combined high temperature and light stress in tomato plants (Chapter 5).

Chloroplast lipid metabolism in tomato plants seems to be utterly well suited to high temperature conditions. We demonstrated how prenylquinone biosynthesis in tomato plants responds to high temperature ($38\,^{\circ}\mathrm{C}$) in comparison to mild ($20\,^{\circ}\mathrm{C}$) and moderately cold temperature ($10\,^{\circ}\mathrm{C}$) (Chapter 3). By employing lipidomic-based methods, which allow the simultaneous and rapid profiling of prenylquinones and carotenoids in plant extracts (Martinis et al., 2011), we carried out non-targeted lipidomics analysis to determine changes at the level of membrane composition. This powerful method was developed in collaboration with the Neuchâtel Platform of Analytical Chemistry (NPAC) and to our knowledge, is one of the first to allow simultaneous analysis of different lipid types at the level of the whole membrane system.

Chlorophyll fluorescence parameters were measured to determine photosynthetic efficiency. The fluorescence parameter *F*v/*F*m is a sensitive indicator of maximum efficiency of the photosystem II, allowing to assess damage to the photosystem II (Hendrickson et al., 2004). No effects on maximum efficiency of the photosystem II under high temperature were observed. However, a slight inhibition at moderately cold temperatures was detected. This indicates that the tomato plants resist better to high temperature stress than to cold temperatures. Interestingly, the lipidomics results confirmed that tocopherol and the degree of saturation of fatty acids were increased under high temperatures. Our results showed that the thylakoid membrane is remodeled with regard to fatty acid saturation in galactolipids and lipid antioxidant concentrations under high temperature stress.

Surprisingly, hardly any change was observed for carotenoids. It should be noted, however, that carotenoids are structural constituents of the light harvesting complexes. Besides, they have functions in non-photochemical quenching (NPQ) as dissipators of excitation energy in the xanthophyll cycle (Choudhury and Behera, 2001; DellaPenna, 1999; Frank and Cogdell, 1996; Gruszecki and Strzałka, 2005; Mimuro and Katoh, 1991; Müller et al., 2001; Shumskaya and Wurtzel, 2013). Davison et al. (2002) showed that Arabidopsis plants overexpressing the *chy*B gene that encodes β-carotene hydroxylase exhibited a two-fold increase in the xanthophyll pool size. The protection against stress was attributed to zeaxanthin, as transformed plants showed reduced levels of anthocyanins and lipid peroxidation in conditions of high light and temperature. However, no prenylquinones were measured in that study. Furthermore, carotenoids have been widely connected to protection against photo-oxidative damage (Cogdell and Frank, 1987; Falk and Munné-

Bosch, 2010; Lichtenthaler, 1999; Strzałka et al., 2003; Zhu et al., 2010). Even though a later study from Havaux and Tardy (1996) suggested that zeaxanthin also plays a role in protecting thylakoid membranes specifically from the effects of heat stress, the heat stress treatments were applied in leaf discs for a short duration of time (15 minutes). One could speculate that even though carotenoids are known antioxidants, resistance to high temperatures in tomato plants does not require an increase of overall carotenoid levels. This is in agreement with the lack of an increase in the maximum extent of NPQ in constitutive zeaxanthin-accumulating Arabidopsis mutants (Hurry et al., 1997; Pogson et al., 1998).

Moreover, my results demonstrated that plastoquinone is strongly upregulated under high temperatures. It appears very likely that plastoquinone, like tocopherol, contributes to protecting membranes under high temperature. Ksas et al. (2015) demonstrated resistance to photooxidative stress under excessive light in an Arabidopsis line overexpressing the SPS1 (SOLANESYL DIPHOSPHATE SYNTHASE 1) gene of the PQ-9 biosynthetic pathway. Under high light stressthis line had two- to three-fold higher levels of PQ-9 and showed a decrease of bleaching, lipid peroxidation, and the photoinhibition PSII (Ksas et al., 2015). Our data in tomato plants strongly suggest that massively increased concentrations of α-tocopherol and plastoquinone are necessary for protection against high temperature stress and proper function of the photosynthetic apparatus.

The understanding of the regulation of α-tocopherol metabolism is crucial for targeting specific genes for improvement in crop plants. Prior to this study, a systematic in silico study of all genes involved in tocopherol biosynthesis in tomato was carried out based on prediction and similarity to Arabidopsis orthologues (Almeida et al., 2011). By a reverse genetics approach, we evaluated the contribution of two enzymes, phytol kinase (VTE5) and farnesol kinase (FOLK), to tocopherol synthesis in different tomato organs. *vte5* silenced plants had strongly reduced levels of tocopherol. *vte5* plants are an attractive model to study the impact of tocopherol reduction in tomato plants, as the mutation does not interfere with PQ-9 content in leaves, which would disrupt the electron transport chain. We investigated how changes in tocopherol levels affect the levels of other related compounds and antioxidants (Chapter 4). Phytol kinase phosphorylates phytol that is released during chlorophyll degradation. Phytol kinase is involved in tocopherol synthesis in Arabidopsis seeds and to some extent also in leaves (Valentin et al., 2006). Downregulation of VTE5 dramatically reduces tocopherol content and also alters lipid metabolism, which ultimately affects carbon partitioning in tomato plants. However, prenyl alcohol kinase activity of VTE5 has not been yet demonstrated for phytol or any other related prenyl alcohols. Therefore, this could be interesting to pursue in future studies.

Our results provide a comprehensive analysis of prenyllipids and membrane lipids, as well as the analysis of the antioxidant capacity of different organs and a number of physiological parameters in wild type and mutant tomato plants. Tocopherol synthesis in tomato is

highly dependent on phytol originating from chlorophyll degradation, which applies, not only to active photosynthetic tissues but also to fruit during the ripening process. This is in contrast with Arabidopsis, in which the VTE5 pathway has been claimed to be more important in seed than the photosynthetic tissue (Valentin et al., 2006). Moreover, even though FOLK was identified in this study through phylogenetic analysis as a potential phytol kinase candidate, it turned out not to contribute to phytol phosphorylation and tocopherol synthesis. Finally, VTE5 was characterized as the major enzyme providing phosphorylated phytol for tocopherol synthesis in tomato (Chapter 4).

We investigated the effects of combined high temperature and high light stress and the role of α-tocopherol role as a potent antioxidant (Chapter 5). Under the combined stress, as well as under high temperature and high light stress alone, we observed a striking increase in tocopherol as well as plastoquinone in wild-type tomato plants. To address the question of whether tocopherol is required for resistance to the combined stress, we used *vte5* RNAi lines (Almeida et al., 2016). The tocopherol concentrations in this mutant were up to 70% lower than in wild-type plants. Under the combined stress, the *vte5* plants exhibited strong photobleaching as well as photoinhibition. This indicates that plants lacking normal tocopherol levels have lethally impaired resistance to the combined stress. So far, this is the first indication ever that tocopherol has an essential function under any condition. Tocopherols are potent lipid-soluble antioxidants and their levels are known to increase during stresses, which would place a significant demand on providing large quantities of phytyl-diphosphate for the prenyltransferase in tocopherol synthesis. Under combined high light and high temperature stress, the perturbed VTE5 activity does not only alter tocopherol synthesis but also has a substantial impact on other prenyllipid metabolic pathways. It causes a massive increase of of free phytol and fatty acids phytyl ester (FAPEs). The implication of two plastoglobule-localized acyl transferases (PES1 and PES2) in thylakoid disassembly during senescence and nitrogen starvation has been reported (Gaude et al., 2007; Lippold et al., 2012). PES1 and PES2 esterified either diacylglycerol or phytol, released from galactolipids and chlorophyll, with free fatty acids (FA), resulting in triacylglycerols and FAPE (Lippold et al., 2012).

Plastoglobules alter their protein composition in response to stress or developmental transitions. Plastoglobules can also act as a transient sink sequestering significant amounts of phytol. *vte5* mutant plants under combined stress exhibited larger numbers and size of plastoglobules than the wild type. In electron micrographs of chloroplast and plastoglobule ultrastructure catabolic events within the chloroplast were evident. The visual appearance by electron microscopy correlated with the dramatic loss of photosynthetic activity seen in the *vte5* mutant line. To exclude that high accumulation of phytol (a long chain alcohol that can exert detergent-like effects) in the *vte5* plants was the main cause for the catabolism of photosynthetic tissue (Löbbecke and Cevc, 1995; Sikkema et al., 1995), we measured the impact of high concentrations of phytol on electron transport in isolated thylakoid membranes (Hill reaction). Even though highest phytol levels in the experiment exceeded

in vivo levels more than 500 fold, only a comparatively mild impact on electron transport was detected. In summary, the data indicate that VTE5 protects against combined high light and high temperature stress and do so by supporting α-tocopherol production.

6.1 Future directions

The photosystems are composed of protein complexes that are embedded in the thylakoid membrane. These photosynthetic protein complexes consist of the two photosystems (PSII and PSI), the light-harvesting complex II (LHCII), the cytochrome b6f and the ATP synthase. These complexes strongly interact with the thylakoid lipids, and it is suggested that alterations in the composition of lipids that form photosynthetic membranes affects their stability, function and conformation resulting in altered supercomplexes (Dörmann, 2013; Kanervo et al., 1997; Kobayashi et al., 2007; Nevo et al., 2012; Pribil et al., 2014; Rochaix et al., 2012). Further analysis of the photosynthetic complexes and supercomplexes in tomato, wild-type (WT) and perturbed phytol kinase (*vte5*) line would allow us to understand how the lack of α-tocopherol under the different stress conditions affect the structure and dynamics of photosynthetic complexes and also their protein components.

The inability of *vte5* to salvage phytol resulting from chlorophyll degradation into the α-tocopherol biosynthesis leads to the accumulation of free phytol and FAPE biosynthesis (Almeida et al., 2016; Lippold et al., 2012). *vte5* plants were sensitive to the tested combination of stress which led to strong photobleaching and photoinhibition. α-tocopherol in the membrane lipid layer, besides its antioxidant functions, physically stabilizes membrane structure (Arora et al., 2000; Wang and Quinn, 2000). The increase of chlorophyll catabolism under the photobleaching conditions suggests that higher quantities of chlorophyll intermediates are accumulated. The identification and quantification of these intermediates, which can act as photosensitizers, may give information on the chlorophyll turnover in *vte5* under different stresses (Hörtensteiner and Kräutler, 2011; Sakuraba et al., 2012; Schelbert et al., 2009). The reduced content of α-tocopherol may impact the thylakoid membrane stability, which under conditions of combined stress, lead to the high chlorophyll catabolism observed in *vte5* coupled with an increase of ROS production.

Another possibility to be explored is the perturbation of other genes related to the pathway of phytol recycling in the α-tocopherol biosynthesis pathway in tomato plants (Kobayashi and DellaPenna, 2008; Sattler et al., 2003; Schelbert et al., 2009; Soll et al., 1985). A couple of examples would be: the production of pheophytinase (*PPH*) mutants that could give us insights into the phytol salvage pathway from chlorophyll degradation into α-tocopherol production, or phytyl ester synthase (*PES*) mutants that could also give insights on the phytol conversion into FAPEs, following different stress conditions.

Plastoglobules are an important site for metabolism of essential metabolites inside the chloroplast. It appears that plastoglobules provide a favorable chemical environment for the biosynthesis and metabolism of lipophilic vitamins, e.g. α-tocopherol (Vitamin E) and phylloquinone (Vitamin K).

The lipid and proteomic analysis of plastoglobules isolated from tomato plants through membrane fractionation could give insights into their functions in tomato species. Moreover, the characterization of unknown plastoglobule proteins (Vidi et al., 2006; Ytterberg et al., 2006) and the analysis of plastoglobule proteomes of plants growing under various stresses in combination with modern lipid profiling methods will bring valuable insights into new approaches to create resistant crop lines. Modification of factors regulating Vitamin E and K, for instance, may lead to new ways to increase the nutritional value of crop species. This approach may also result in the identification of yet new candidate proteins and advance our understanding of the involvement of plastoglobules in yet other lipid metabolic pathways.

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