ABA-Dependent and -Independent Regulation of Tocopherol (Vitamin E) Biosynthesis in Response to Abiotic Stress in Arabidopsis

Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

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Jena

Bonn, 2019

Angefertigt mit der Genehmigung der Mathematisch-Naturwissenschaftlichen
Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn

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Tag der Promotion: 03.05.2019

Erscheinungsjahr: 2019

Acknowledgements

First, I would like to thank my supervisor Prof. Dr. Peter Dörmann for the opportunity to conduct the research for my PhD thesis in his lab at the University of Bonn.

Second, I thank Prof. Dr. Lukas Schreiber for being a great supervisor and contact person over the last three years.

With a deep sense of gratitude I want to thank Dr. Katharina Gutbrod, for her continuous help and support. I want to thank her for her time, all fruitful discussions and exchange of ideas, as well as for showing me all the analytical techniques.

I am incredibly thankful to all members of the IMBIO group. Thank you for the fantastic time I spent with you and for the close atmosphere. Helga, thank you for cheering up lunch breaks, and starting the most interesting conversations about almost everything. Biggi, thank you for being such a good friend, and constant helpful support. Jill, thank you for being the perfect lab mate, that one could ever wish to have. Andreas, thank you for being just yourself, and for providing distraction when needed.

I thank my students Svenja, Alejandra, Lisa-Christin, Martin, Stefan and Tatiana for the help in the lab. It was a great experience for me to pass on some knowledge and advice.

Special thanks go to all GRK2064 members for making this project possible, for productive und constructive discussions after various progress reports.

Finally all my appreciation goes to my awesome husband Tino, who is just everything for me.

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ABBREVIATIONS

ABA	Abscisic acid
aba	ABA-deficient mutant
abi	ABA-insensitive mutant
ABREs	ABA-responsive elements
ANOVA	Analysis of variance
AREB	ABRE-binding protein
Col-0	Columbia-0
DMPBQ	2,3-dimethyl-5-phytyl-benzoquinol
DW	Dry weight
EMS	Ethylmethanesulfonate
FLD-HPLC	Fluorescence high-pressure liquid chromatography
FW	Fresh weight
GGDP	Geranylgeranyl diphosphate
GGR	Geranylgeranyl reductase
HGA	Homogentisic acid
HPP	p-Hydroxyphenylpyruvate
HPPD	HPP dioxygenase
LC-MS	Liquid chromatography mass spectrometry
Ler	Landsberg erecta
MCSU	Molybdenum cofactor sulfurase
MPBQ	2-methyl-6-phythyl-benzoquinol
MS	Murashige and Skoog
N-	Nitrogen depleted
N+	Nitrogen sufficient
PC-8	Plastochromanol
PDP	Phytyldiphosphate
PEG	Polyethylene glycol
PMP	Phytylmonophosphate
PP2A	Protein phosphatase 2A
PP2C	Protein phosphatase 2C

PYR/PYL/RCAR	Pyrabactin resistant/ pyrabactin resistant-like/ regulatory component of ABA receptor
qPCR	Quantitative real-time RT-PCR
ROS	Reactive oxygen species
RWC	Relative water content
SD	Standard deviation
SDR	Short chain dehydrogenase/reductase
SnRK2	SNF1-related protein kinase 2
TW	Turgit weight
VTE1	Tocopherol cyclase
VTE2	Homogentisate phytyltransferase
VTE3	MPBQ methyltransferase
VTE4	γ-Tocopherol methyltransferase
VTE5	Phytol kinase
VTE6	Phytylphosphate kinase
Ws	Wassilewskija
WT	Wild type
ZEP	Zeaxanthin epoxidase

1. INTRODUCTION

Plants are exposed to and are continuously forced to adapt to ever changing environmental conditions. One of the most limiting factors for growth, development and yield of plants is water deficiency or drought (Boyer, 1982). Changes in global climate accompanied with extreme weather conditions, including drought which will occur in the future, will have an immense impact on agriculture in the coming decades (Jewell *et al.*, 2010; Melillo *et al.*, 2014). Besides drought, other abiotic stress conditions such as heat, cold, nutrient deficiency, salinity or excess of toxic metals will also limit plant productivity in agriculture, and jeopardize food security (Jenks & Hasegawa, 2005; Fedoroff *et al.*, 2010). Abiotic stress leads to an enhanced production of reactive oxygen species (ROS), which serve as secondary signals to generate further signals (Xiong *et al.*, 2002b). In plants ROS are continuously produced as byproducts of aerobic metabolic processes such as respiration and photosynthesis in mitochondria, chloroplasts, and peroxisomes (Apel & Hirt, 2004). Under physiological steady-state conditions ROS levels are in equilibrium, because of a balance between production and removal of ROS. However, apart from their function as signaling molecules in plants, ROS have the capacity to cause oxidative damage to proteins, DNA and lipids. When the equilibrium is disrupted through stress, such as drought or high temperatures, intracellular levels of ROS rise rapidly, which can have injurious effects on photosystems and thylakoid membranes (Mittler, 2002; Choudhury *et al.*, 2016). Plants increase the production of antioxidants, such as tocopherol, as an adaptive process to survive during such environmental stress conditions and to limit oxidative damage (Miret & Munné-Bosch, 2015; Boubakri *et al.*, 2016).

1.1 Occurrence and Biosynthesis of Tocochromanols in Plants

Tocopherols together with tocotrienols and plastochromanol (PC-8) belong to the group of tocochromanols, also designated as vitamin E. Tocochromanols are amphipathic molecules consisting of a hydrophobic prenyl side chain which associates with membrane lipids in the center of the bilayer, and a polar chromanol head group which is exposed to the membrane surface. Tocopherols

differ from tocotrienols only in the degree of saturation of their prenyl side chain. The α-, β-, γ-, and δ- forms of tocopherols and tocotrienols vary only in the number and position of methyl groups attached to the chromanol ring (Figure 1). Tocochromanols are synthesized by all plant species, green algae and many cyanobacteria (Grusak & DellaPenna, 1999; Horvath *et al.*, 2006). In plants, tocochromanols can be found in plastids, where they accumulate in thylakoid membranes and plastoglobules (Soll *et al.*, 1985; Vidi *et al.*, 2006). The total tocochromanol content and composition varies dependent on the species, tissue and developmental stage (Fryer, 1992; Kruk & Strzałka, 1995; Bréhélin *et al.*, 2007). In Arabidopsis, α-, β-, γ-, δ-tocopherol and PC-8 can be found.

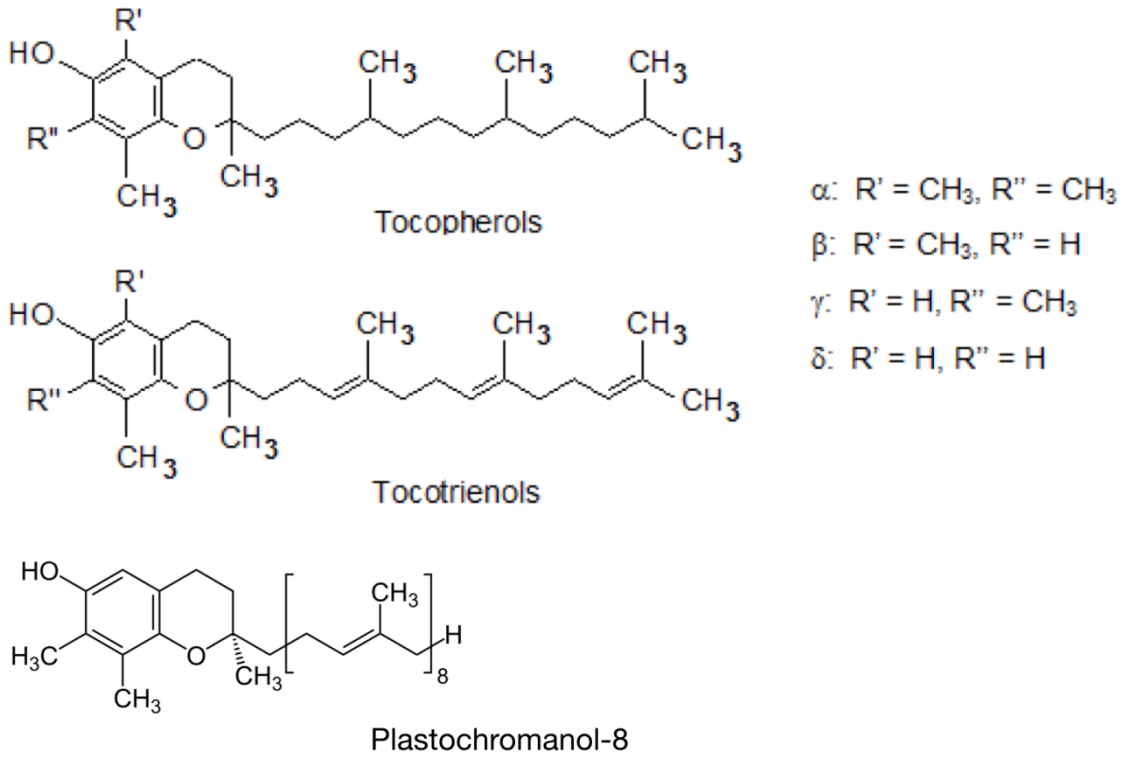


Figure 1: Chemical structures of tocopherols, tocotrienols and plastochromanol

Tocopherols, tocotrienols and plastochromanol-8 (PC-8) consist of a polar chromanol ring and a hydrophobic carbon side chain attached to the ring via the C-2 atom. Tocopherols have saturated phytyl side chains, while tocotrienols have isoprenyl side chains with three double bonds. Both tocopherols and tocotrienols occur as four forms, α-, β-, γ-, and δ, that differ from each other by the number and position of methyl groups on the chromanol ring. PC-8 is an homologue of γ-tocotrienol with a much longer chain, originating from solanesyl diphosphate [modified from http://lipidlibrary.aocs.org/Analysis/content.cfm?ItemNumber=40389 and https://de.wikipedia.org/wiki/Plastochromanol-8].

The initial step of tocopherol biosynthesis is the condensation of homogentisic acid (HGA) with phytyl diphosphate (PDP). This step is catalzyed by homogentisic acid phytyltransferase (HPT/VTE2), producing the intermediate 2-methyl-6-phythyl-benzoquinol (MPBQ) (Collakova & DellaPenna, 2003a). The MPBQ methyltransferase (VTE3) then catalyzes the methylation of MPBQ to DMPBQ (Cheng *et al.*, 2003). Next, the tocopherol cyclase (VTE1) is involved in the closure of the second ring on the head group. Using either MPBQ or DMPBQ as substrate, VTE1 produces δ-tocopherol or γ-tocopherol, respectively (Porfirova *et al.*, 2002). The last step is the methylation performed by the γ-tocopherol methyltransferase (VTE4) yielding β- or α-tocopherol (Bergmüller *et al.*, 2003) (Figure 2).

The precursor for the polar head group is HGA, which is synthesized via the chloroplastic shikimate pathway. After export from the chloroplast, the aromatic amino acid tyrosine is converted into p-hydroxyphenylpyruvate (HPP) by the tyrosine aminotransferase (TAT1) (Riewe *et al.*, 2012). Next the hydroxyphenylpyruvate dioxygenase (HPPD) converts HPP into HGA which is again imported into the chloroplast (Norris *et al.*, 1998). TAT1 and HPPD are cytosolic enzymes in Arabidopsis, but can be localized to the chloroplasts in other plants. The lipophilic isoprenyl side chain for tocopherols derives from PDP, produced in the plastid methylerythritol phosphate (MEP) pathway, from geranylgeranyl diphosphate (GGDP). Recently it was shown that PDP as precursor for the phytyl moiety in tocopherol synthesis is derived from chlorophyll breakdown. The characterization of the phytol kinase (VTE5), which phosphorylates free phytol to phytylmonophosphate (PMP) (Valentin *et al.*, 2006), and the phytylphosphate kinase (VTE6), which subsequently phosphorylates PMP to PDP (vom Dorp *et al.*, 2015), revealed that also other all phytol for tocopherol synthesis originates from chlorophyll breakdown and subsequent phosphorylation to PDP (Figure 2).

Tocopherol biosynthesis, the origin of its precursors and the key enzymes have been reviewed in detail before (DellaPenna & Pogson, 2006; Mène-Saffrané & DellaPenna, 2010; Lushchak & Semchuk, 2012; Mène-Saffrané, 2017), as well as the functional diversity of tocopherol in

photosynthetic organisms (Munné-Bosch & Alegre, 2002; Munné-Bosch, 2005; Dörmann, 2007; Maeda & DellaPenna, 2007; Falk & Munné-Bosch, 2010).

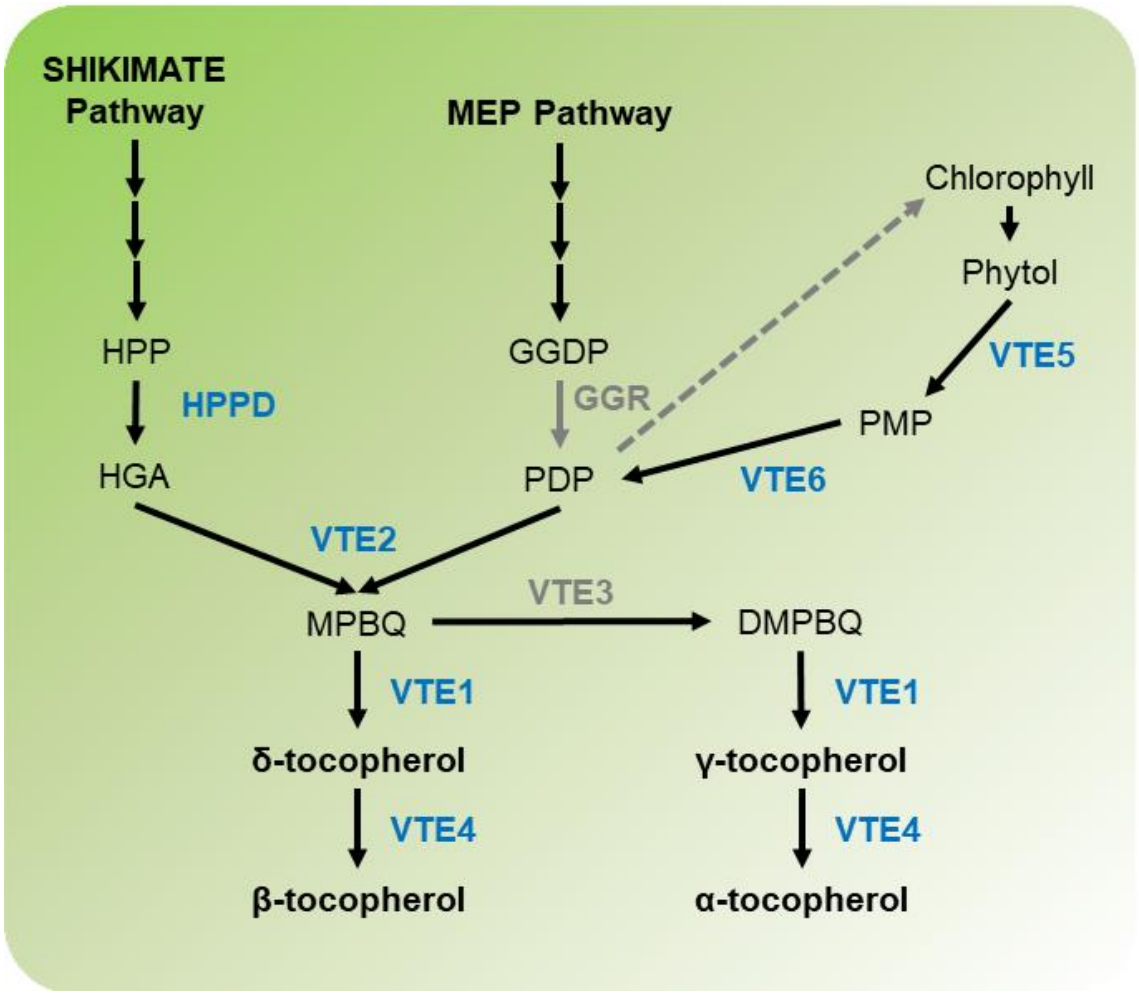


Figure 2: Tocochromanol biosynthetic pathway in Arabidopsis

The shikimate pathway provides HGA as one precursor for tocopherol synthesis. PDP as the lipid precursor is derived from free phytol released during chlorophyll degradation. Prenylation of HGA with PDP resulting in MPBQ production is the key step for tocopherol biosynthesis. Methylation of MPBQ yields DMPBQ. VTE1 converts MPBQ and DMPBQ to δ- and γ-tocopherol, respectively. Finally, VTE4 methylates δ- and γ-tocopherol to β- and α-tocopherol, respectively. The pathway and enzyme nomenclature are based on studies in Arabidopsis, as described in detail in the text. Abbreviations: DMPBQ, 2,3-dimethyl-5-phytyl-benzoquinol; GGDP, geranylgeranyl diphosphate; GGR, geranylgeranyl reductase; HGA, homogentisic acid; HPP, p-hydroxyphenylpyruvate; HPPD, HPP dioxygenase; MPBQ, 2-methyl-6-phythyl-benzoquinol; PDP, phytyldiphosphate; PMP, phytylmonophosphate; VTE1, tocopherol cyclase; VTE2, homogentisate phytyltransferase; VTE3, MPBQ methyltransferase; VTE4, γ-tocopherol methyltransferase; VTE5, phytol kinase; VTE6, phytylphosphate kinase.

1.2 Functions of Tocochromanols in Plants

In photosynthetic organisms tocochromanols function foremost as antioxidants, which deactivate photosynthesis-derived ROS and lipid peroxyl radicals, and therefore protect membrane lipids from autocatalytic peroxidation. Polyunsaturated fatty acids (PUFAs) as part of biological membrane lipids are often oxidized by ROS because of the presence of multiple double bonds, resulting in lipid radicals. Tocochromanols can quench ROS directly or scavenge radicals that result from lipid peroxidation. Tocopherol can physically quench up to 120 singlet oxygen molecules by resonance energy transfer before being degraded itself (Fahrenholtz *et al.*, 1974). In addition, tocopherol can chemically scavenge ROS and free lipid radicals by donating its phenolic hydrogen, resulting in a mixture of tocopherol radicals. These radicals might then be reduced and converted back into tocopherol by ascorbate and glutathione (Olcott & Emerson, 1937; Fryer, 1992).

Furthermore, α-tocopherol and α-tocopherol quinone were found to interact with photosynthetic electron transport in thylakoids. Here these compounds are contributing to the dissipation of excess energy and thus conferring photoprotection for the photosynthetic apparatus (Kruk & Strzałka, 1995). Due to the chemical structure of tocochromanols they strongly interact with membrane lipids suggesting that tocopherols also increase the stability of membranes (Salgado *et al.*, 1993). Tocochromanols are also essential for seed longevity and for protecting lipids from oxidation during germination and early seedling growth (Sattler *et al.*, 2004).

1.3 Abscisic Acid Structure and Function in Plants

The biochemical and physiological responses of plants to stress include the increase in the phytohormone abscisic acid (ABA) under abiotic stress (Nambara & Marion-Poll, 2005; Lee & Luan, 2012). Phytohormones are a group of signal molecules produced by plants that play fundamental roles in almost all stages of plant growth, development and responses to biotic and abiotic stresses (Davies, 2004; Verma *et al.*, 2016). Based on their structure and physiological functions phytohormones can be organized into several classes,

including ABA, auxins, cytokinins, jasmonates, salicylates, gibberellins etc. Each class has characteristic biological effects, but increasing evidence shows that multiple phytohormones can mediate physiological processes by additive, synergistic or antagonistic actions.

ABA is one of the well-investigated phytohormones, regulating numerous developmental processes such as embryo maturation, seed dormancy and germination, cell division and elongation or floral induction, as well as many adaptive stress responses to environmental cues including drought, salinity, cold and pathogen attack (Cutler *et al.*, 2010; Nambara *et al.*, 2010; Finkelstein, 2013). ABA is a sesquiterpenoid belonging to the class of isoprenoids. The molecular structure of ABA includes one asymmetric carbon atom which is important for its biological activity (Cutler *et al.*, 2010).

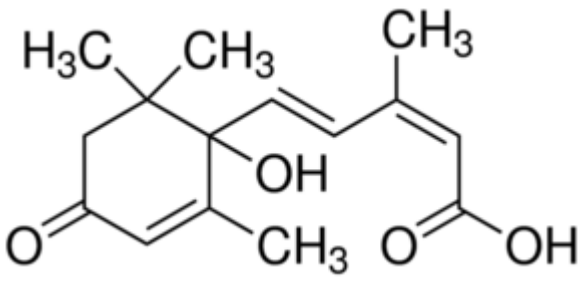


Figure 3: Chemical structure of abscisic acid

(+)-Abscisic acid, S-5-(1-hydroxy-2,6,6-trmethyl-4-oxocyclohex-2-en-1-yl)-3-methyl-(2Z,4E)-pentadienoic acid), is a 15-C sesquiterpenoid with one asymmetric carbon atom (C-1), having a cyclohexene ring with one keto and one hydroxyl group, and a side chain with a terminal carboxylic acid group. Natural (+)-ABA carries the C-1 in S configuration. The double bond at position 2 of the side chain can occur in *cis* (Z) or *trans* (E) configuration. Only the 2cis (2Z) isoform is biologically active.

Abiotic stress, such as drought or cold, activates the production of ABA through induction of ABA biosynthetic genes (Xiong & Zhu, 2003; Shinozaki & Yamaguchi-Shinozaki, 2007), triggering the expression of various genes for specific biochemical responses. Promoter analyses of the ABA-responsive genes revealed the existence of several *cis*-regulatory elements, known as ABA-responsive elements (ABREs) (Shinozaki & Yamaguchi-Shinozaki, 2007). Such an ABA-specific motif has been identified in the promoter regions of the

vitamin E biosynthesis genes *OsHPPD*, *OsγTMT* and *OsMPBQMT1* in rice (Chaudhary & Khurana, 2009), suggesting that the tocopherol biosynthesis might be regulated by ABA. In addition regression analyses revealed a positive correlation between contents of ABA and those of tocochromanols (Fleta-Soriano & Munné-Bosch, 2017). Moreover, Ghassemian and co-workers provided the molecular evidence of such a relationship, showing that ABA-treated Arabidopsis seedlings had increased levels of tocopherol and tocopherol biosynthesis gene transcripts (Ghassemian *et al.*, 2008).

1.4 ABA-Biosynthesis and ABA-Deficient Mutants

ABA deficient mutants of Arabidopsis have been isolated which contain reduced amounts of ABA and are characterized by reduced seed dormancy and increased tendency to wilt after exposure to water deficiency (Koornneef *et al.*, 1982; Léon-Kloosterziel *et al.*, 1996). The first mutant was *aba1-6* with Col-0 genetic background which is derived from EMS mutagenesis. In this mutant the gene of the zeaxanthin epoxidase (ZEP) carries a G to A mutation at nucleotide position 478, causing a change from glycine to serine at the amino acid 160 (Niyogi *et al.*, 1998; Barrero *et al.*, 2005). The zeaxanthin epoxidase catalyzes the first reaction from zeaxanthin to violaxanthin via the intermediate antheraxanthin. Mutants impaired in ZEP accumulate zeaxanthin and contain severely reduced levels of ABA, especially after stress (Xiong *et al.*, 2002a; Nambara & Marion-Poll, 2005). Therefore, the *ZEP* gene has also been designated *ABA1* (ABA DEFICIENT 1, AT5G67030). Due to the block in ZEP activity, ABA and all precursors and conjugates of ABA should be absent from or decreased in amount in *aba1* mutant plants.

The next mutant is *aba2* for which different allelic lines were isolated (*aba2-1* and *aba2-4*) of the *ABA2* gene (ABA DEFICIENT2, AT1G52340). *ABA2* belongs to the short chain dehydrogenase/reductase (SDR) family. ABA2 encodes an enzyme catalyzing the conversion from xanthoxin to abscisic aldehyde (AB aldehyde), which is already a functional precursor of ABA.

Mutants of ABA2 are expected to lack abscisic aldehyde and ABA. Since it is a single gene in Arabidopsis, *aba2* null mutants are reported to contain reduced ABA levels, but they still have residual ABA amounts. The reason for

this is not understood (González-Guzmán *et al.*, 2002; Nambara & Marion-Poll, 2005). The EMS-induced mutant allele *aba2-1* (Col-0) harbors a G to A exchange at position 1464 that results in a serine to asparagine replacement at position 264 (Léon-Kloosterziel *et al.*, 1996; González-Guzmán *et al.*, 2002). The sequence of the second EMS-induced allele *aba2-4* (Col-0) is unknown. It was originally named *sis4* (sugar-insensitive 4) (Laby *et al.*, 2000).

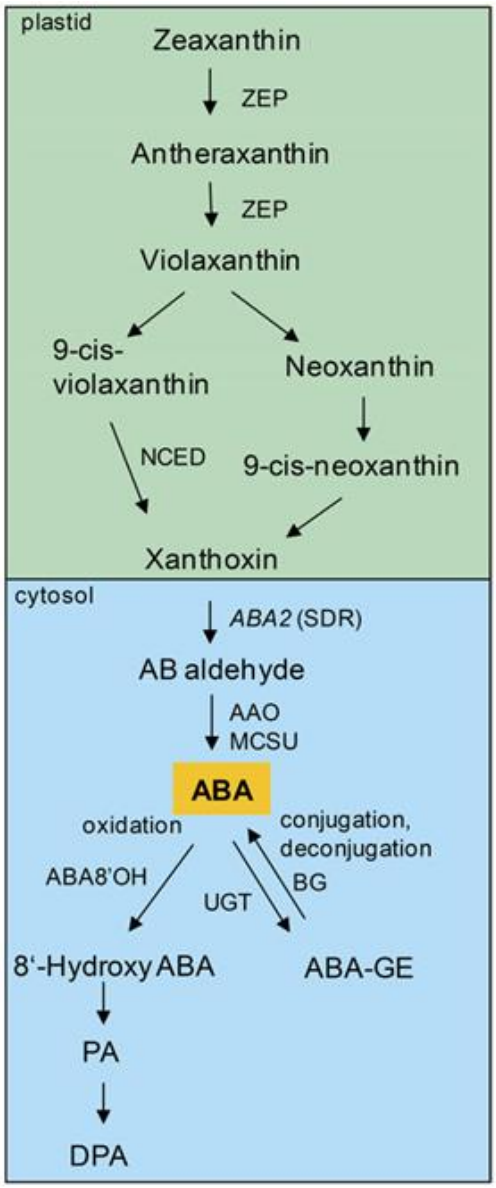


Figure 4: ABA Biosynthesis and catabolism

The first reaction is catalyzed in the plastids by the zeaxanthin epoxidase (ZEP, ABA1) from zeaxanthin to violaxanthin via the intermediate antheraxanthin. The final plastid-localized steps are the conversion to neoxanthin by ABA4, isomerization of either violaxanthin or neoxanthin to their 9-cis-isomers, and finally the rate-limiting cleavage by 9-cis-epoxycarotenoid dioxygenase (NCED) to xanthoxin. The short-chain alcohol dehydrogenase (SDR) ABA2 catalyzes the conversion from xanthoxin to abscisic aldehyde (AB aldehyde). The final step is performed by the abscisic aldehyde oxidase (AAO) together with a molybdenum cofactor (MoCo), synthesized by the molybdenum cofactor sulfurase (MCSU, ABA3). The two major catabolic pathways are: (1) hydroxylation of ABA over the unstable 8'-Hydroxy-ABA that is isomerized to phaseic acid (PA), and (2) esterification of ABA to ABA-glucose ester (ABA-GE) [modified after (Seiler *et al.*, 2011)].

The final step of ABA biosynthesis is the oxidation of the aldehyde group of abscisic aldehyde to the carboxylic acid. In Arabidopsis, this step is catalyzed by abscisic aldehyde oxidase (AAO3) which requires a molybdenum cofactor (MoCo) for its catalytic activity (Seo *et al.*, 2000). Molybdenum cofactor sulfurase (*MCSU*) is the gene responsible for the synthesis of the MoCo required for ABA synthesis. Therefore, *MSCU* is also named *ABA3* (ABA DEFICIENT3, AT1G16540) A mutation in ABA3 leads to ABA deficiency, characterized by the typical phenotype that is known for ABA-deficiency (Léon-Kloosterziel *et al.*, 1996; Bittner *et al.*, 2001; Xiong *et al.*, 2001). In this work, the EMS-induced allele *aba3-1* in the Col-0 background was investigated. This allele harbors a change from glycine to glutamic acid at the amino acid position 496 (Léon-Kloosterziel *et al.*, 1996; Bittner *et al.*, 2001). The second allele *aba3-2* carries a mutation which introduces a stop codon after amino acid 387. These mutants were acquired from the Ler ecotype via gamma radiation. The other steps of the ABA biosynthesis and catabolism have also been investigated in detail and reviewed elsewhere (Xiong & Zhu, 2003; Nambara & Marion-Poll, 2005; Finkelstein, 2013).

1.5 ABA Signaling Pathway and ABA-Insensitive Mutants

In addition to ABA deficient mutants, ABA-insensitive (*abi*) mutants were previously isolated. Mutants that are ABA insensitive have been identified by selecting for germination in the presence of exogenous ABA. These *abi* mutants (like *aba* mutants) also have decreased seed dormancy (Koornneef *et al.*, 1984; Finkelstein & Somerville, 1990). In the ABA signal transduction network, there are several classes of ABA response regulators such as transcription factors, protein kinases and phosphatases, RNA processing enzymes, and proteins that metabolize or respond to secondary messengers.

The ABA response network starts with the perception of ABA through receptor proteins. The group of receptors encompasses the PYR/PYL/RCAR-family (pyrabactin resistant/ pyrabactin resistant-like/ regulatory component of ABA receptor), whose proteins can directly bind ABA (Gonzalez-Guzman *et al.*, 2012). If ABA is bound to one of the PYR/PYL/RCAR receptors, they bind and inhibit the group A protein phosphatases (PP2Cs) (Melcher *et al.*, 2009). The

PP2C phosphatases are negative regulators of ABA signaling, and so the assumed function of the PYR/PYL/RCAR proteins is to inhibit PP2C activity and relieving the negative input into the signaling pathway. This controls ABA signaling over mechanisms such as regulation of activity or proteolytic degradation. One target of PP2C is the family of protein kinases SnRK2 (SNF1-related protein kinase 2), which are inactivated by dephosphorylation in the absence of ABA. In Arabidopsis the SnRK2 protein kinase family consists of 10 members. Several of these have been found to be major kinases involved in ABA signaling after the analysis of single or multiple mutants (Fujii & Zhu, 2009; Fujii *et al.*, 2011). Furthermore, PP2Cs may link ABA perception with the control of various effectors involved in stress tolerance (Umezawa *et al.*, 2009). SnRK2s play an important role as positive components in ABA signaling. In the presence of ABA, phosphorylated SnRK2s accumulate, which then activate or inactivate downstream transcriptions factors such as ABI3, ABI4 or ABI5. The bZIP transcription factor ABI5 (ABA-INSENSITIVE 5) functions in the core ABA signaling pathway regulating seed germination and early seedling growth (Finkelstein, 1994; Skubacz *et al.*, 2016). ABI3 (ABA-INSENSITIVE 3) encodes a B3-family transcription factor which is also involved in seed development and maturation, additionally through co-regulation of ABI5 (Finkelstein & Somerville, 1990; Skubacz *et al.*, 2016). The AP2-domain transcription factor ABI4 (ABA-INSENSITIVE 4) also is involved in this co-regulating network and functions during seed germination under abiotic stress together with ABI3 and ABI5. Besides, the ABA response also includes rapid physiological changes, such as stomatal closure. For example the membrane-resident anion channel SLAC1 plays a central role in guard cells during this process (Negi *et al.*, 2008; Vahisalu *et al.*, 2008). Also the inward-rectifying potassium channel KAT1 is another target of SnRK2. While SLAC1 is activated through phosphorylation, KAT1 is inhibited through phosphorylation by SnRK2 promoting stomatal closure in both cases (Sato *et al.*, 2009). ABA accumulation under stress conditions also induces the expression of many ABA-responsive genes, which are characterized by the presence of ABA-responsive elements (ABREs) in the promoter regions. ABRE-binding proteins (AREBs; synonymous: ABRE-binding

factors, ABFs) bind to the ABRE promoter elements and regulate expression of ABA-responsive genes. The AREBs/ABFs belong to the bZIP transcription factors (Jakoby *et al.*, 2002). Downstream genes include many late embryogenesis abundant (LEA) class genes, further transcriptions factors and PP2C. This closes the negative feedback loop of ABA response to abiotic stress (Merlot *et al.*, 2001; Cutler *et al.*, 2010; Yoshida *et al.*, 2010; Umezawa *et al.*, 2010; Finkelstein, 2013) (Figure 5).

Until now 14 members of the PYR/PYL/RCAR receptors are known in Arabidopsis. Therefore several multiple mutants were generated to study the response of the receptors due to ABA and abiotic stress (Gonzalez-Guzman *et al.*, 2012; Keereetaweep *et al.*, 2015). The *pyr1pyl2pyl4pyl5pyl8* pentuple mutant was used in this work. It has a reduced growth and severe germination defect in comparison to its Col-0 wild type. Recently also *pyl* duodecuple and quattuordecuple mutants have been generated and characterized. While the quattuordecuple mutant was severely impaired in growth and failed to produce seeds, the duodecuple mutant was further characterized to be defective in growth, flower development, and seed production. In addition the duodecuple mutant is extremely insensitive to ABA effects on seed germination, seedling growth, stomatal closure, osmotic regulation and leaf senescence (Zhao *et al.*, 2018). Compared to the pentuple mutant used during this work, the duodecuple mutant shows even stronger growth defects under normal growth conditions, but does not seem to be more ABA-insensitive than the pentuple mutant.

The EMS-induced *abi2-1* mutant is deficient in the *ABI2* gene (ABA INSENSITIVE 2, AT5G57050). This allele harbors a change from glycine to aspartic acid at position 168 originated from the Ler wild type (Koornneef *et al.*, 1984). The *ABI2* gene belongs to the group A PP2Cs and represents one of the major players in ABA signaling. So far, only ABI1 and ABI2 have been reported as homologous PP2Cs involved in ABA signal transduction in Arabidopsis, even though the PP2C family contains 9 members in Arabidopsis and belongs to the larger group of protein serine/threonine phosphatases with diverse regulatory functions (Cohen, 1989; Leung *et al.*, 1997; Merlot *et al.*, 2001).

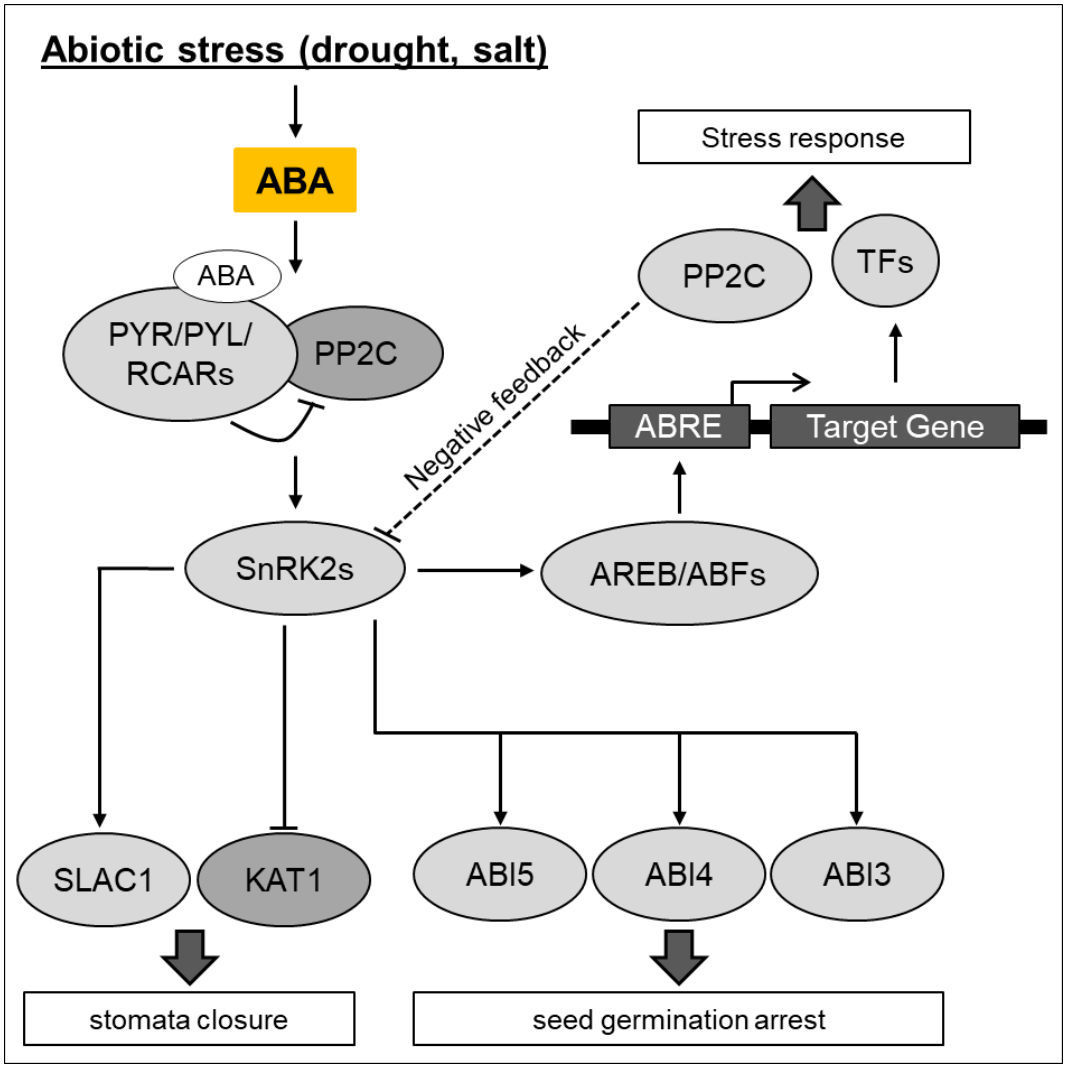


Figure 5: ABA signaling network in response to abiotic stress

Model of the core ABA signaling pathway interactions. Abbreviations and explanations are described in detail in chapter 1.5. Arrows show activation, bars indicate repression.

The mutant allele *abi3-1* was is also in Ler background. The *abi3-1* plant carries a change from aspartic acid to asparagine at position 580 in the B3-type transcription factor ABI3 (Koornneef *et al.*, 1984). Two abi4 mutant alleles (AT2G40220) have been investigated, both generated from the Col-0 ecotype. First, the gamma-radiation induced *abi4-1* allele which has a frame shift at the amino acid position 157 resulting in an early stop codon of the AP2-domain transcription factor ABI4 (Finkelstein, 1994). Second, the EMS-induced *abi4-102* allele, carrying a change which introduces a stop codon after amino acid 102 instead of tryptophan. For the *ABI5* gene, the allele *abi5-1*, originating from

the Wassilewskija (Ws) ecotype, was included. The *abi5-1* allele contains a G to T transition, introducing a stop codon at amino acid position 88 of the bZIP transcription factor ABI5 (Finkelstein, 1994; Lopez-Molina & Chua, 2000).

1.6 Goals

Tocopherols are radical scavenger and antioxidants which accumulate in response to abiotic stress such as drought. Tocopherol accumulation is accompanied by an increase of the phytohormone ABA, which serves as a signaling molecule in various stress reactions. While the biosynthesis of tocopherol has been in the focus of numerous studies, little is known about the regulation of this pathway. Since tocopherol and ABA increase alongside during abiotic stress, it was assumed for a long time that the two pathways might be linked. The aim of this project was to study the involvement of ABA in the regulation of tocopherol biosynthesis during abiotic stress in the model organism *Arabidopsis thaliana*. To address this central question, two strategies will be followed: (1) Investigation of ABA biosynthesis mutants and (2) investigation of ABA-signal-transduction mutants. The first strategy is to test mutants that interrupt the ABA synthesis. If ABA itself, ABA precursors or ABA conjugates directly affect tocopherol biosynthesis, the amount of tocopherol should be decreased in the mutants under stress, in comparison to the wild type. Therefore, several ABA-deficient mutants (*aba*) will be analyzed. The second strategy is to test mutants that are impaired in one step of the ABA signaling network. If a transcription factor, activated by the ABA signal transduction network, would activate tocopherol biosynthesis, the amount of tocopherol should be decreased in the mutants under stress, compared to the wild type. Therefore, several ABA-insensitive mutants (*abi*), which are known to be a part of the ABA signaling cascade, will be analyzed. The *aba* and *abi* mutants will be grown under drought stress on soil, on medium containing PEG8000 or in nitrogen-deficient medium, or under high light stress. Mutants will furthermore be challenged by growth on ABA containing medium. The regulation of tocopherol synthesis by ABA will be followed by measuring tocochromanols and by recording expression of key tocopherol biosynthesis gens.

2. MATERIAL AND METHODS

2.1 Chemicals

(+) *cis*, *trans*-Abscisic acid	Olchemin, Olomouc, Czech Republic
2H6-(+) cis, trans-Abscisic acid	Olchemin, Olomouc, Czech Republic
MS salts including vitamins	Duchefa, Haarlem, Netherlands
Phytoagar	Duchefa, Haarlem, Netherlands
PEG8000	Roth, Karlsruhe, Germany
Sodium hypochlorite (NaClO)	Carl Roth, Karlsruhe, Germany
tert.-Butylmethylether	VWR, Darmstadt, Germany
Triton X-100	Carl Roth, Karlsruhe, Germany

2.2 Plant Material

The Arabidopsis ABA-synthesis mutants *aba1-6*, *aba2-1*, *aba2-4*, *aba3-1* and *aba3-2* as well as the ABA-insensitive mutants *abi2-1*, *abi3-1*, *abi4-1*, *abi4-102*, and *abi5-1* were obtained from the Nottingham Arabidopsis Stock Centre (NASC). The ABA-insensitive *pyr1pyl2pyl4pyl5pyl8* pentuple mutant was provided by Pedro L. Rodriguez (Instituto de Biologia Molecular y Celular de Plantas, Valencia, Spain). The three *Arabidopsis thaliana* ecotypes Columbia-0 (Col-0), *Landsberg erecta* (*Ler*) and Wassilewskija (Ws) were used as wild type controls. A detailed description of the mutants is provided in chapter 1.4 and 1.5.

2.3 Plant Growth Conditions and Abiotic Stress Treatments

2.3.1 Seed Surface Sterilization and Seedling Growth

For all experiments Arabidopsis seeds were surface sterilized by a first washing step with 95% ethanol, followed by a treatment with sterilization solution (sodium hypochlorite/ deionized water (1:1, v/v)) containing 0.1% Triton-X-100 for about 10 min under continuous shaking. The sterilization solution was discarded and seeds were washed three times with sterile deionized water. Then, surface-sterilized seeds were sown on Murashige and Skoog (MS) medium plates, composed of 1x MS basalt salts, 2% sucrose, 10 mM MES and

0.7% phyto agar (pH 6.0, adjusted with 1M KOH). Plates were sealed with fabric tape and stratified in the dark at 4°C for at least 24 h. After stratification the plates were incubated in plant growth chambers under a 16 h light/8 h dark cycle at 22°C and 150 µmol m^{-2} s^{-1}, until further use.

2.3.2 Drought Stress on Soil

After 14 days of growth on MS plates seedlings were transferred to soil with three seedlings each pot. 10 x 7.5 cm pots were filled with 150 g soil (Einheitserde Type Topf 1.5, Gebrüder Patzer, Sinntal, Germany), soaked with water supplemented with 0.15% ProPlant (Arysta Life Science, Düsseldorf, Germany) and 0.1% boric acid. On soil, plants grew in climate chambers also under a 16 h light/8 h dark cycle at 22°C and 150 µmol m^{-2} s^{-1}, with about 55% air humidity. During another 14 days of growth, the soil was kept well-watered. Drought stress was initiated by withholding water for up to 10 days. Plants grown for control conditions were well-watered during this time.

2.3.3 Osmotic Stress on PEG8000

For osmotic stress on polyethylene glycol (PEG) containing medium, Arabidopsis seeds were surface sterilized, sown and germinated as described in section 2.2.1. Arabidopsis plants were grown for 3 weeks with transferring seedlings every 7 days to new MS plates, ensuring continuous and similar nutrient availability. After 21 days of growth, 10 to 15 plantlets were transferred to MS plates with Gelrite™ containing either no, 10% or 20% PEG8000 (w/v). Osmotic stress was performed for up to 9 days. During normal growth and stress treatment, plants were kept in plant growth cupboards (Percival) under a 16 h light/8 h dark cycle at 22°C and 150 µmol m^{-2} s^{-1}.

2.3.4 PEG8000-Containing Medium

For plates containing no PEG, 0.3% Gelrite™ (Duchefa, Haarlem, Netherlands) was added directly to the liquid MS medium before autoclaving. The MS medium was composed of 1xMS basalt salts, 2% sucrose and 10 mM MES (pH 6.0, adjusted with 1 M KOH). After autoclaving, the medium was poured into 145 x 20 mm petri dishes. Preparation of medium containing either 10% or 20% PEG8000 (w/v) required a split-up of the medium. Therefore, 1 L of liquid MS

medium, composed exactly as described above, was divided equally to two flasks. Into one flask the required amount of Gelrite™ was added (6 g/L for 10% PEG and 8g/L for 20% PEG, respectively). After autoclaving, the flask containing the Gelrite was stored at 60-80°C in a water bath until further use. The other flask containing a magnetic stirrer was filled under a sterile bench with the required amount of PEG8000 (Roth, Karlsruhe, Germany). The medium was stirred until the PEG8000 was completely dissolved. Then, the PEG-containing solution was also stored in a water bath at 60-80°C for at least 30 minutes. When both solutions reached the same temperature, the PEG solution was mixed with the Gelrite-containing medium under the sterile bench. Medium was directly poured into 145 x 20 mm petri dishes (Figure 6).

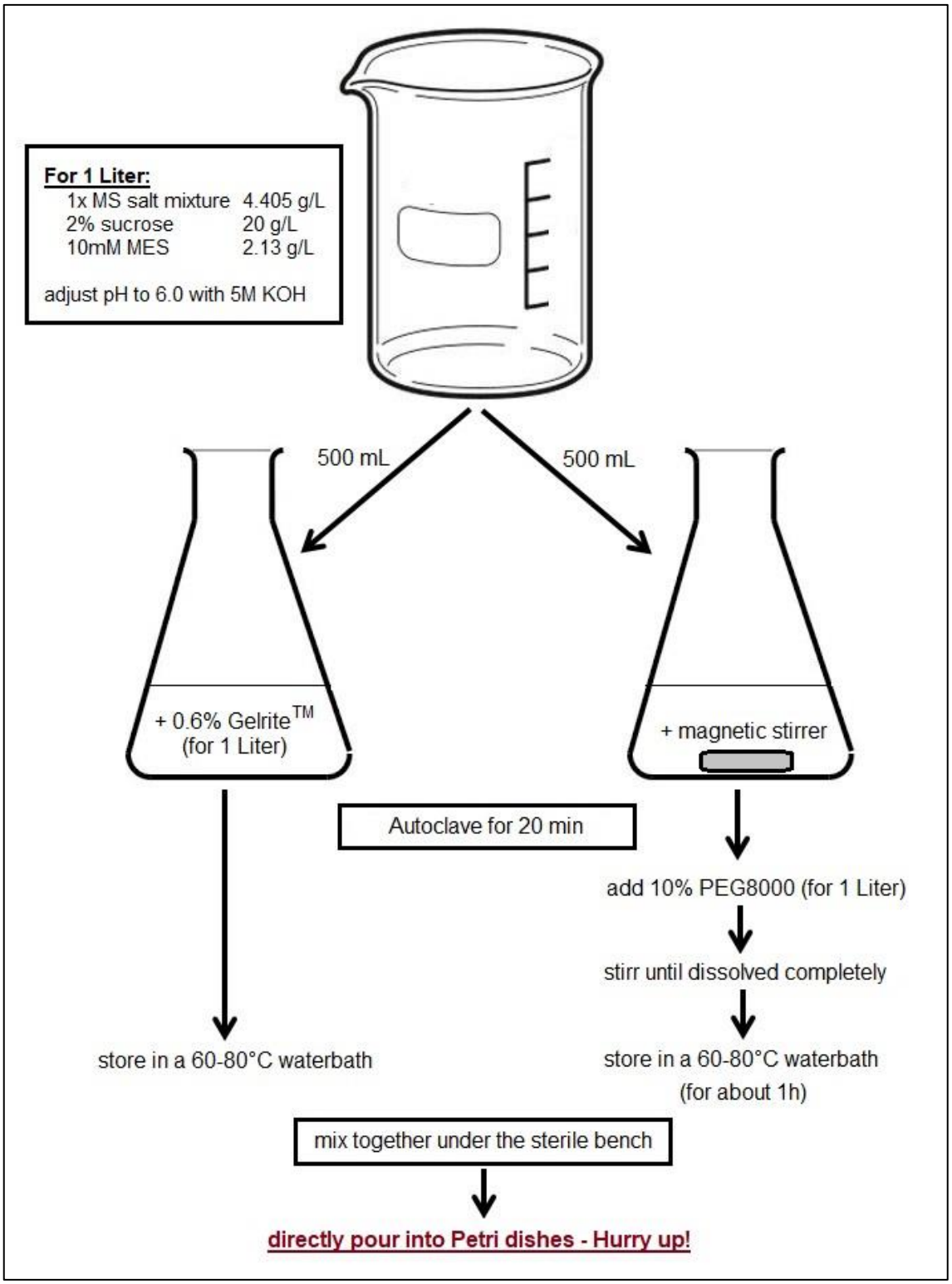


Figure 6: Scheme of the protocol for the preparation of PEG8000-containing solidified medium

Flow chart outlining the key steps of the protocol to prepare MS medium containing PEG8000. Concentrations for Gelrite™ and PEG8000 are exemplified here for preparing medium with 10% PEG (w/v). For a detailed description of the protocol see chapters 2.3.4 and 3.2.

2.3.5 ABA Treatment

At first Arabidopsis seeds were surface sterilized, sown and germinated as described in section 2.3.1 and plants were grown for 3 weeks on MS plates with transferring seedlings every 7 days to new MS plates, ensuring continuous and similar nutrient availability. After 21 days of growth, 10 to 15 plantlets were transferred to MS plates containing different concentrations of (+) *cis*, *trans*-abscisic acid (ABA; Olchemin, Olomouc, Czech Republic). Plants were exposed to ABA for 9 days while kept in plant growth chambers under a 16 h light/8 h dark cycle at 22°C and 150 µmol m^{-2} s^{-1}.

2.3.6 Nitrogen Deficiency

For nitrogen deficiency experiments, Arabidopsis seeds were surface sterilized, sown and germinated as described above in section 2.2.1. After 2 weeks plants were transferred to synthetic media composed of 0.7% phyto agar, 1% sucrose, 2.5 mM KNO_3, 1 mM $MgSO_4$, 1 mM $Ca(NO_3)_2$, 1 mM KH_2PO_4, 1 mM NH_4NO_3, 25 µM Fe-EDTA, 35 µM H_3BO_3, 7µM $MnCl_2$, 0.25 µM $CuSO_4$, 0.5 µM $ZnSO_4$, 0.1 µM Na_2MoO_4, 5 µM NaCl and 5 nM $CoCl_2$ (pH 6.0, adjusted with 1M KOH) for nitrogen sufficient (N+) conditions. For nitrogen deprivation, NH_4NO_3 was omitted from the medium, $Ca(NO_3)_2$ was exchanged by $CaCl_2$ and KNO_3 was exchanged by KCl (Gaude *et al.*, 2007). In all experiments plants were kept 14 days on either nitrogen sufficient or depleted medium, in plant growth cupboards under a 16 h light/8 h dark cycle at 22°C and 150 µmol m^{-2} s^{-1}.

2.3.7 High Light Treatment

High light experiments were performed as described by Eugeni Piller et al. (2014). Arabidopsis seeds were surface sterilized, sown and germinated as described above in section 2.3.1. Plants were grown for 2 weeks on MS plates in plant growth chambers before transfer onto soil. Pots (10 x 7.5 cm) were prepared with soil, soaked with water supplemented with 0.15% ProPlant (Arysta Life Science, Düsseldorf, Germany) and 0.1% boric acid. On soil, plants grew in climate chambers again under a 16 h light/8 h dark cycle at 22°C and 150 µmol m^{-2} s^{-1}, with about 55% air humidity. During another 3 weeks of growth the soil was kept well-watered. For high light experiments plants were

then exposed to 500 µmol m^{-2} s^{-1} light intensity still under a 16 h light/8 h dark cycle for 4 days or 8 days. As control, plants were kept under the previous normal light conditions (Eugeni Piller *et al.*, 2014).

2.4 Relative Water Content

The relative water content of Arabidopsis leaves was calculated according to the following equation (Sade *et al.*, 2015):

$$RWC = \frac{(FW - DW)}{(TW - DW)} * 100$$

The fresh weight (FW) is obtained by weighing a leaf or small plantlet, at the same time point as the material is harvested for biochemical measurements. This leaf or plantlet is floated for 24 h in 5 mM $CaCl_2$ and then the turgid weight (TW) is determined. For the dry weight (DW) determination, the leaf or plantlet is dried for 24 h at 60°C and weighed.

2.5 Chlorophyll Analysis

Leaf chlorophyll content was quantified spectrophotometrically (Porra *et al.*, 1989). Leaves or plantlets were harvested and directly frozen in liquid nitrogen. After homogenization chlorophyll was extracted with 80% acetone and adsorption was measured at 646.6 nm, 663.6 nm and 750 nm. The amount of chlorophyll a and b was calculated with the following equations, and referred to the leaf area:

$$Chl\ a = 12.25 * (A663.6 - A750) - 2.55 * (A646.6 - A750)$$

$$Chl\ b = 20.31 * (A646.6 - A750) - 4.91 * (A663.6 - A750)$$

2.6 Tocopherol Analysis

For the extraction of tocopherol about 100 mg of leaf or plantlet material was collected, frozen in liquid nitrogen and homogenized. For total lipid extraction ground tissue was suspended in 1 mL diethylether. As internal standard 500 ng tocol was added, followed by 300 µL 1M KCl. Phase separation was achieved by centrifugation. The upper organic phase was collected. To extract remaining lipids from the aqueous phase 1 mL diethylether was added two times. Every time the upper organic phase was collected, and all organic phases combined. Diethylether was dried under nitrogen flow and the residue dissolved in n-

hexane for quantification of tocopherol by fluorescence high-pressure liquid chromatography (FLD-HPLC). Samples were analyzed by chromatography on a normal phase LiChrospher 100 diol, 5 µM, 250 x 4 mm column (KNAUER, Berlin, Germany) column with fluorescence detection (Agilent 1100 HPLC System). As isocratic running solvent n-hexane/tertiary butylmethyl ether (96:4, v/v) was used. Fluorescence was recorded with an excitation at 290 nm and an emission at 330 nm [modified after (Kanwischer *et al.*, 2005)].

2.7 Gene Expression Analysis by qPCR

Total RNA was extracted from Arabidopsis leaf material following the protocol of the NucleoSpin® RNA Plant kit (MACHEREY-NAGEL, Düren, Germany). Frozen samples were ground using the Precellys® 24 homogenisator (PeqLab/VWR, Darmstadt, Germany). In the final step of the protocol, only 50 µL of RNase-free water was used to elute the RNA from the column, after another 10-15 min incubation time prior to centrifugation. Removal of gDNA was included within the RNA extraction kit as an on-column DNA digest. The RNA integrity was analyzed spectrophotometrically by Nanodrop (ND-1000 V3.8.1, Thermo Scientific) and confirmed with gel electrophoresis. RNA was stored at -20°C until further use. For cDNA synthesis, 1 µg of total RNA from each RNA extraction was converted into cDNA using the First Strand cDNA Synthesis Kit according to the manufacturer's instructions (#K1622, Thermo Scientific, Waltham, USA). qPCR was performed with the 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, USA). The cDNAs were diluted with ddH_2O to a concentration of 5 ng/µL and used for qPCR. The qPCR reactions contained 2 µL diluted cDNA, 250 nM forward and reverse primers and 4 µL my-Budget 5x EvaGreen® QPCR-Mix II (ROX) (Bio-Budget, Krefeld, Germany) in a 20-µL reaction. The list of primers is provided in Table 1. The qPCR protocol started with a first stage at 50°C for 2 min. The initial denaturation stage was at 95°C for 10 min, followed by 40 cycles of amplification (95°C for 15 s and 60°C for 1 min) and a final elongation stage. Finally a dissociation curve was generated to check for specificity of the amplification. Each assay included two technical and three biological replicates and a no-template control. Gene expression data was normalized against the reference gene PP2A

(PROTEIN PHOSPHATASE 2A) according to the ΔΔCt method (Livak & Schmittgen, 2001). Final results represent the relative gene expression of selected tocopherol biosynthesis genes normalized to the expression of *PP2A* and referred to the gene expression under the respective control conditions. Data was depicted on a logarithmic scale. Thus, values at "1" depict that transcript abundance of this gene was the same as in the control. Values above "2" mean that the gene was transcribed 2-times more often than under control conditions.

Table 1: Synthetic Oligonucleotides for qPCR Analyses

Oligonucleotides were ordered from IDT Genomics (Leuven, Belgium).

Number	Primer-Name	AGI Code	Sequence 5' > 3'
bn2109	VTE6-qPCR-fw	AT1G78620	ATTGGTTCAAGTGCTGCTGG
bn2110	VTE6-qPCR-rev	AT1G78620	AGCTCCCTCTGTTCCTCTTG
bn2111	VTE1-qPCR-fw	AT4G32770	ATTTGCGATGATGGCCGTAC
bn2112	VTE1-qPCR-rev	AT4G32770	AACTCAAACCTTTCACCGCC
bn2113	VTE2-qPCR-fw	AT2G18950	CCCTATCTTCCATTGGCATCA
bn2114	VTE2-qPCR-rev	AT2G18950	CAATCCACCCAAGCCAGAA
bn2115	HPPD-qPCR-fw	AT1G06570	AGGGACGTTGCTTCAAATCTTC
bn2116	HPPD-qPCR-rev	AT1G06570	CAAAACCACCACATCCTCCA
bn2119	VTE4-qPCR-fw	AT1G64970	GCTTTTATGACCCTGATTCTTCTGT
bn2120	VTE4-qPCR-rev	AT1G64970	CACACCCAACATCCACTACTTTCT
bn2125	VTE5-qPCR-fw	AT5G04490	ATGGCAGCAACCTTACCTCT
bn2126	VTE5-qPCR-rev	AT5G04490	ACGTCATGCAACAGCGAATT
bn2903	PP2A-qPCR-fw	AT1G13320	TAACGTGGCCAAAATGATGC
bn2904	PP2A-qPCR-rev	AT1G13320	GTTCTCCACAACCGCTTGGT
bn2963	RD29A-qPCR-fw	AT5G52310	GTTACTGATCCCACCAAAGAAGA
bn2964	RD29A-qPCR-rev	AT5G52310	GGAGACTCATCAGTCACTTCCA

2.8 ABA Analysis

For ABA measurements, 50 mg leaf material was harvested at the indicated time points and flash-frozen in liquid nitrogen. Frozen samples were ground using the Precellys® 24 homogenizer (PeqLab/VWR, Darmstadt, Germany). Phytohormones were extracted following the instructions from Pan and

coworkers with minor changes (Pan *et al.*, 2010). After extraction, phytohormones were dissolved in 0.1 mL methanol/H_2O (1:1 (v/v), +0.1% formic acid) and separated on a reverse-phase C18 Gemini HPLC Column (5 µm particle size, 150 x 2.00 mm, Phenomenex) for HPLC-ESI-MS/MS analysis. Quantification of ABA by mass spectrometry was carried out using a QTRAP 6500+ LC-MS/MS system (Sciex, Darmstadt, Germany) with a Tubo V ion source and an Agilent 1260 Infinity quaternary pump. Phytohormones were detected in the negative ion mode with the following instrument settings: curtain gas, 25 psi; collision gas, medium; ion spray voltage, -4500 V; ion source temperature, 150°C; nebulizer and heater gas, 25 psi. The parameters for multiple reaction monitoring (MRM) transitions were: declustering potential, -80 V; entrance potential, -10 V; cell exit potential, -8 V; collision energy, 30V. Data evaluation was carried out using the MultiQuant 3.0.2 software. For quantification of ABA, the peak areas of the chromatograms resulting from MRMs were compared to those of the internal standard d_6-ABA (Pan *et al.*, 2010).

2.9 Statistical Analysis

Data analysis and statistical tests were performed with OriginPro9 (OriginLab Corporation, Northampton, USA). Normal distribution of the data was tested with the Shapiro-Wilk test. Since all data was normally distributed it was tested for statistical significance of differences between mean values obtained from plants grown under different abiotic stress treatments at a significance level of 0.05 with analysis of variance (Fisher LSD).

3. METHODICAL ASPECTS

3.1 Experimental Approaches for Drought Stress

Different experimental approaches of exposing plants to water deficit can be employed to investigate drought stress in a defined manner. The most obvious way is to apply drought to plants growing on soil in pots by withholding water or by high-frequency deficit irrigation (Puértolas *et al.*, 2017). Drought stress on soil is near to field conditions and it is applicable for different plants in all developmental stages (Kawaguchi *et al.*, 2004; Guo *et al.*, 2009; Fleta-Soriano *et al.*, 2015; Abid *et al.*, 2016). One caveat is that Arabidopsis mutants showing poor growth are difficult to handle in the soil drought experiment. Another obstacle can be if the heterogeneity of field soil shall be represented in the pot experiment, because soil in the field is composed of many different types of soil such as sand, clay etc. On the other hand, the soil used for the pot experiments was derived from a mixture of standard soil with vermiculite which result in a homogenous, stable soil. In any case, water deficit in soil is difficult to apply, because of the large number of parameters to consider (e.g. field capacity, bulk density, soil pH, electrical conductivity, available nutrients, air humidity, air condition etc.). Also, the water potential of the soil is difficult and expensive to monitor. In addition the stress conditions on soil are often troublesome to separate from each other, since a water deficit also can lead to high salinity or infections with pests and insects, interfering with the ongoing experiment. In addition to soil heterogeneity and differences in water evaporation from the pots, another issue is the different kinetics of rosette leaf desiccation. It has been shown that the old leaves of the rosette lose water first and start to become senescent, while the young, innermost leaves are still green and turgid. Therefore, leaf sampling becomes a critical point during drought experiments.

Another method is applying drought in hydroponic cultures. Desiccation can be stimulated by changing the osmotic potential in the nutrient solution after addition of e.g. PEG, or by removing the water and exposing the roots to air (Conn *et al.*, 2013; Horn *et al.*, 2013). As on soil, hydroponics can be applicable for all species and nearly all developmental stages. Again, poorly growing

mutants are difficult to handle in hydroponics. One advantage over drought stress on soil is that in hydroponic cultures the conditions are more stable, because they are well-defined and better reproducible. Hydroponic growing conditions represent is an artificial system and there are still many parameters that have to be considered, as the air humidity, aeration of the solution etc. Also hydroponics can be laborious, expensive and difficult to set up.

In 2000 van der Weele and co-workers published another possible method to expose plants to conditions related to drought stress. This system employs tissue culture using PEG-infused agar plates (van der Weele *et al.*, 2000). Tissue culture applications have the advantage over the other previous introduced methods that they are sterile (no plant infections), stable, controllable, always well-defined and easy to set up. In total there are fewer parameters to consider and it is possible to separate different types of stress conditions. Also it is cheaper than hydroponics and space-saving in comparison to potted plants (van der Weele *et al.*, 2000; Verslues *et al.*, 2006). On the other hand it is not suitable for all species and all developmental stages but sufficient for Arabidopsis, in particular for plant whose growth is compromised. And one could still argue that it is an artificial system.

In hydroponics as well as in tissue culture, the water potential of plant culture solutions can be decreased by introducing the high-molecular-weight solute polyethylene glycol (PEG). PEG is a neutral polymer available in a wide range of molecular weights. It is highly soluble in water and not toxic. Due to these properties PEG has been widely used to impose water stress on plants, by decreasing the water potential of the rooting medium and with that the water potential of the plant (Büssis & Heineke, 1998; Bajji *et al.*, 2000; Fan *et al.*, 2016; Frolov *et al.*, 2017).

It was shown that PEG does not enter the plant due to its large molecular weight (Lagerwerff *et al.*, 1961). Low molecular weight forms of PEG pass through the plant system without undergoing any breakdown of its basic structural unit (Lawlor, 1970). The PEG polymers of high molecular weight are considered to block the pathway of water movement, reducing water absorption and causing desiccation of the plant. PEG molecules above a critical size cause progressive blocking of the pathway of transpiration, either by adhering to the

cellular matrix of the wall space, cutting the effective area of the lumen or by causing a blockage at constrictions in the pathway. The size of the PEG8000 polymers is larger than the interstices of the cell wall, so they cannot pass into the cell (Carpita *et al.*, 1979). This makes PEG8000 the ideal osmoticum for simulating water stress in a controlled manner in hydroponics and tissue culture.

3.2 Establishment of a Protocol for PEG8000-Containing Medium

Due to concerns about equal distribution of the PEG polymers in the medium following the PEG-infused protocol by van der Weele et al. (2000) an alternative protocol to introduce a high-molecular-weight PEG (PEG8000) into plant culture medium was established, as described in detail in section 2.3.4.

This protocol describes the preparation of solidified medium plates with different concentrations of PEG8000 (no PEG, 10% and 20%). This protocol can also be used for other concentrations of PEG resulting in various different water potentials (Table 2). Since the commonly used phyto agar does not solidify in the presence of PEG, Gelrite™ (i.e. Gelrite^{TM}, Duchefa, Haarlem, Netherlands) was used instead. Gelrite™ has different characteristics compared with phyto agar. It is a gelling polymer solidifying very fast when getting cool. Once solidified it cannot be reheated again. Additionally Gelrite™ still solidifies very fast in the presence of PEG. This can be controlled with split-up prepared medium, and keeping the medium at higher temperatures before mixing. Also it should be noted that the addition of PEG increases the volume of the medium, therefore larger flasks should be used with higher concentrations of PEG. Different concentrations of Gelrite™ were tried with five different concentrations of PEG8000 and the water potential was measured using the WP4C Water Potential Meter (METER Group, USA) (Table 2).

Table 2: Combinations for PEG8000-containing media

Amount of Gelrite™ and PEG8000 to obtain different PEG-containing media, with corresponding water potentials. Values of water potential are given as means ± SD of at least five independent replicates (n=5). Different letters indicate significant differences between means at a significance level of 0.05 in One-Way-ANOVA (Fisher LSD). Please note that the absolute value for the water potential of PEG-free medium is ~-0.40 MPa, due to the presence of MS salts and agar (ddH_2O has a water potential of 0 MPa). The value of -0.4 MPa for PEG-containing medium presented in Fujii et al. (2011) presumably refers to the difference of water potential between PEG-containing and PEG-free medium.

	Gelrite	PEG8000	Water Potential (MPa)
No PEG	3 g/L	-	- 0.40 ± 0.05 a
5% PEG8000	4.5 g/L	50 g/L	- 0.43 ± 0.03 a
10% PEG8000	6 g/L	100 g/L	- 0.55 ± 0.07 b
15% PEG8000	7 g/L	150 g/L	- 0.64 ± 0.03 c
20% PEG8000	8 g/L	200 g/L	- 0.95 ± 0.06 d
25% PEG8000	9 g/L	250 g/L	- 1.21 ± 0.07 e

3.3 Drought Stress Parameters

When investigating drought stress it is unlikely that a single measurement is adequate to provide an all-encompassing assessment of drought. Because there is a multitude of parameters involved in drought response, ranging from cellular metabolism to changes in growth rates and crop yields. Drought triggers a wide variety of plant responses such as (1) physiological changes including the relative water content (RWC) (Weatherley, 1950), changes in transpiration rates or water-use efficiency due to alterations in stomatal conductance (Farquhar & Sharkey, 1982) (Steduto, 1996), (2) morphological changes due to alterations in leaf size, stem extension or root proliferation (Nonami, 1998; Farooq *et al.*, 2009) and (3) biochemical changes such as accumulation of compatible solutes (e.g. free proline) (Smirnoff & Cumbes, 1989), photosynthetic pigments (Havaux, 1998), and generation of reactive oxygen species (Mittler, 2002; Tripathy & Oelmüller, 2012) or secondary metabolites (Yordanov *et al.*, 2000; Farooq *et al.*, 2009).

Besides other physiological changes the relative water content (RWC) is an important parameter describing the plant water status, and it is a measure for the metabolic activity in tissues. The RWC is related to water uptake through the roots as well as water loss through transpiration. During drought stress the RWC decreases, which has already been noted for a variety of plants, such as wheat, maize, barley or tomato (Matin *et al.*, 1989; Nayyar & Gupta, 2006; Sade *et al.*, 2009). Measurement of the RWC can be assessed by a reliable and simple method, which is easier compared to other techniques for measuring physiological changes during drought stress (Smart & Bingham, 1974).

In this work the RWC was measured to display the water status of *Arabidopsis thaliana* ecotype Col-0 plants grown either on PEG8000-containing medium or exposed to drought stress on soil. The RWC indeed decreased due to osmotic stress with PEG8000. On medium containing no PEG the RWC was about 70% with minor variations. The RWC was not higher, presumably because the medium without PEG8000 has already a water potential of -0.4 MPa, due to the presence of Gelrite (3g/L), sucrose (2%) and salts (1x MS). Plants exposed to 10% or 20% PEG8000 had a reduced RWC of about 45% and 40% in leaves, respectively. The reduced water potential in the medium consequently leads to a reduced RWC in plant leaves. The RWC measured in plants that were treated with PEG8000 had a much wider variation (Figure 7 A).

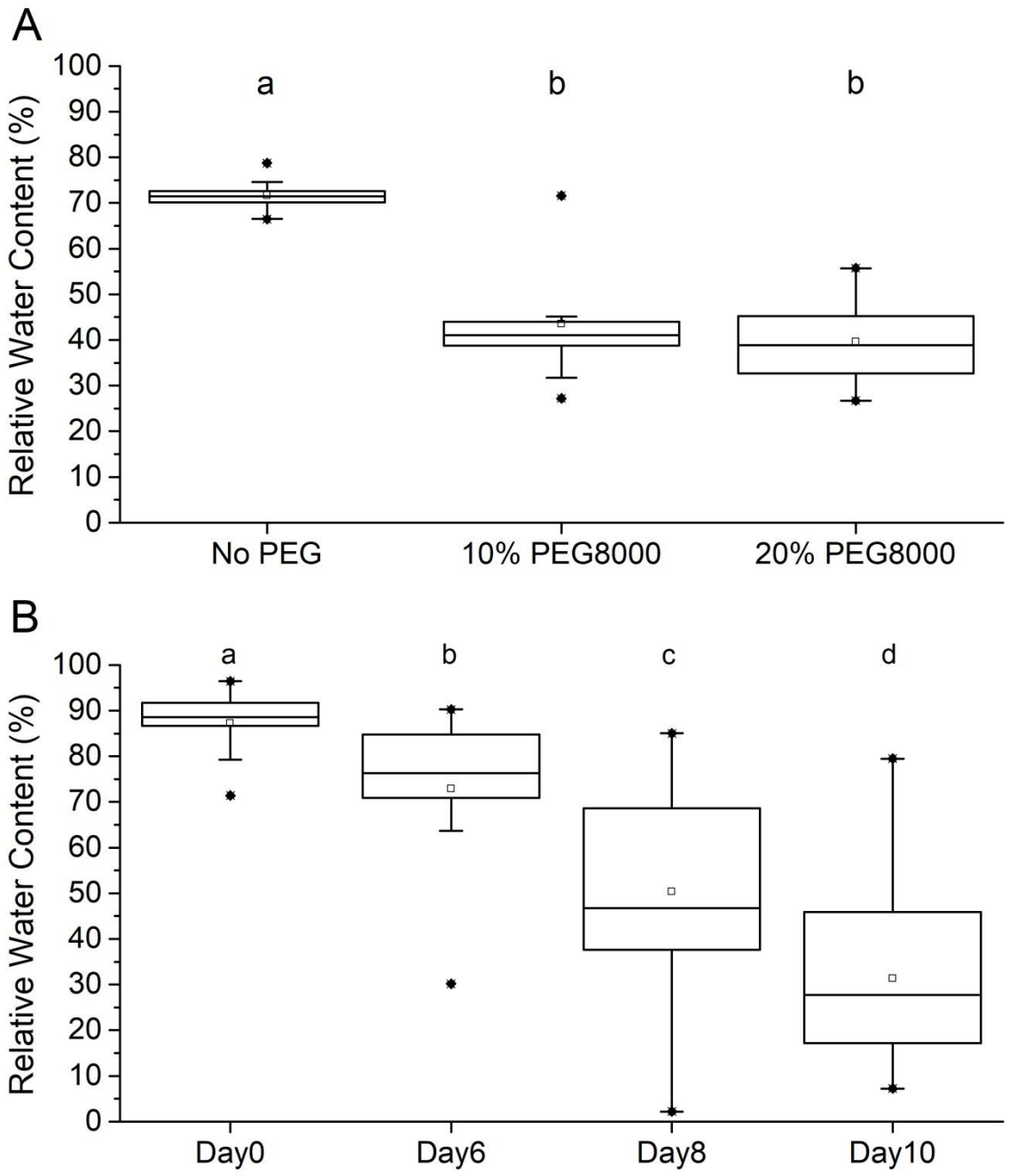


Figure 7: Relative water content of *Arabidopsis thaliana* ecotype Col-0 plants grown on PEG8000-containing medium or exposed to drought stress on soil

Relative water content (RWC) of Col-0 plants grown (A) on MS medium containing no, 10% or 20% PEG8000 (w/v) for 9 days, or (B) on soil were water was withheld for 0, 6, 8 or 10 days. The boxes range from 25 to 75 percentiles. The square in the box represents the mean value. The whiskers range to the outliers. Each box represents 20 measured leaves (n=20). Different letters indicate significant differences between means at a significance level of 0.05 in One-Way-ANOVA (Fisher LSD).

In contrast, plants grown on soil had about 90% of RWC prior to water-stress, due to much higher water availability. The RWC decreased to 70% after 6 days of withholding water and further to 50% and even 30% after 8 and 10 days, respectively. On soil, the RWC also showed a greater variation the longer the stress treatment lasted (Figure 7 B). Drought stress on soil seemed to have a much stronger impact on the plant water status than the osmotic stress imposed by PEG8000, in this particular experimental set-up. But as mentioned before, drought stress on soil is less controllable than under tissue culture conditions, resulting in the large variations of RWCs.

One of the most drought-sensitive processes is the cell growth, due to the reduction in turgor pressure. Under severe water deficiency, cell elongation and expansion is inhibited by interruption of the water flow. This causes impaired mitosis, which thereupon is leading to a reduced number of leaves per plant and a reduction of individual leaf size (Nonami, 1998).

Growth inhibition was particularly observed for the Arabidopsis Col-0 plants grown on PEG8000 medium. Plants grown on MS medium without PEG8000 looked healthy and similar to each other. Osmotic stress originating from 10% PEG8000 caused strong growth retardation. The plants are smaller and appear darker. In addition, the individual plants showed a strong variation of size and chlorophyll content. Some plants are larger and darker, others are smaller and pale green with some yellow leaves. Morphological changes caused by 20% PEG8000 treatment are similar to those on 10% PEG8000. The plants are smaller and darker than the control plants, but even smaller than the ones from medium containing 10% PEG8000. The size and the color are also variable between individual plants (Figure 8).

Drought stress on soil caused different morphological changes (Figure 8). First of all plants were larger on soil compared to plants grown on MS plates, because they grew in a non-restricted environment. After 6 days of withholding water, the above-ground plant organs still appeared healthy and viable. The only difference was the desiccation of the top layer of the soil, compared with day 0. Withholding water for 8 days already had an immense impact on leaf structure. Leaves were wilted and yellowish and they looked smaller, probably due to the loss of water. After 10 days without watering, the leaves looked

worse. All yellowish leaves were completely wilted and dried-out. Leaves which were still green were also wilted and curled (Figure 8).

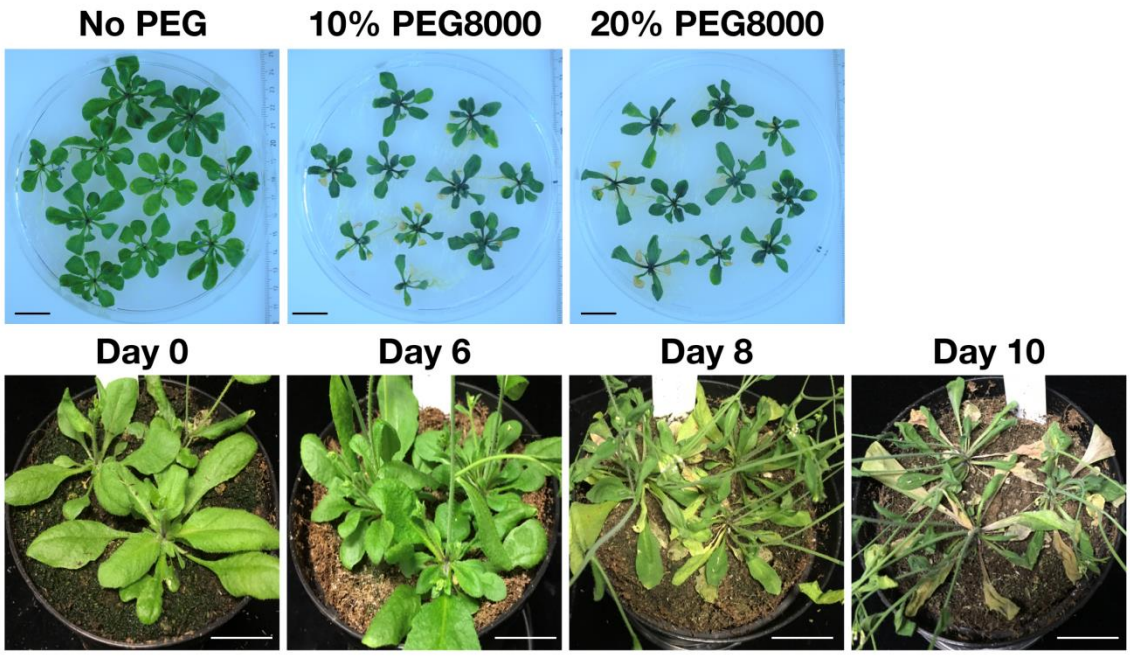


Figure 8: *Arabidopsis thaliana* Col-0 plants grown on PEG8000-containing medium or exposed to drought stress on soil

Photos of Col-0 plants grown on MS medium containing no, 10% or 20% PEG8000 (w/v) for 9 days, or on soil where water was withheld for 0, 6, 8 or 10 days. Bars = 2 cm.

Environmental stresses such as drought also have direct impact on the photosynthetic apparatus. Photosynthesis is affected by drought because all major mechanisms including the thylakoid electron transport, the carbon reduction cycle and the stomatal control of the CO_2 influx are disturbed (Yordanov *et al.*, 2000; Anjum *et al.*, 2011). One of the first responses to water deficiency is stomatal closure which leads to the decline of the photosynthesis rate. In addition stomata-independent mechanisms such as changes in chlorophyll synthesis or structural reorganization in chloroplasts can decrease photosynthetic activity under drought stress (Chaves *et al.*, 2009; Farooq *et al.*, 2009; Lisar *et al.*, 2012). Chlorophyll is the key pigment for photosynthesis and together with the other photosynthetic pigments, chlorophyll is important for harvesting light energy. The chlorophyll content is positively correlated with the photosynthetic rate. Therefore changes in chlorophyll levels can have

detrimental effects on plant performance under stress. Decreased, unchanged or increased chlorophyll levels have been reported in many species, depending on the duration and severity of drought (Kyparissis *et al.*, 1995; Zhang & Kirkham, 1996; Munné-Bosch *et al.*, 2001). Although the decrease in chlorophyll content of leaves under drought stress has been described in several reports, many studies indicated that a higher chlorophyll content, reminiscent of a stay-green trait, is associated with improved yield and water use efficiency (Borrel *et al.*, 2000; Li *et al.*, 2006).

Changes in the chlorophyll content are different in response to osmotic stress or drought stress (Figure 9). While osmotic stress imposed by PEG8000 results in an increase in chlorophyll content, drought stress with plants on soil leads to chlorophyll degradation. In both cases the control conditions do not show a change in chlorophyll level over time. When PEG8000 is added to the medium the chlorophyll content is significantly increased after 9 days of treatment. Also 20% PEG8000 has a greater impact, resulting in even higher chlorophyll levels than with 10% PEG8000 in the medium. In the beginning of the experiment the chlorophyll levels of all three conditions are similar to each other. After 4 days the trend of chlorophyll accumulation started in PEG8000 treated plants. While the standard deviations are relatively moderate under control conditions, they are increased the longer the PEG8000 treatment lasted, showing that plants exposed to stress do not behave all in the same way (Figure 9 A).

Drought stress on soil showed the more commonly expected chlorophyll decrease in response to water deficit. While well-watered plants had almost no change in chlorophyll content over time, the chlorophyll content decreased significantly after 6, 8 and 10 days of drought. In the beginning there was no difference between the chlorophyll levels of well-watered or water-stressed plants. Only with progressing stress treatment the changes in chlorophyll became visible. Again, the variations between individual pots and plants are higher under stress conditions and higher the longer stress was applied (Figure 9 B).

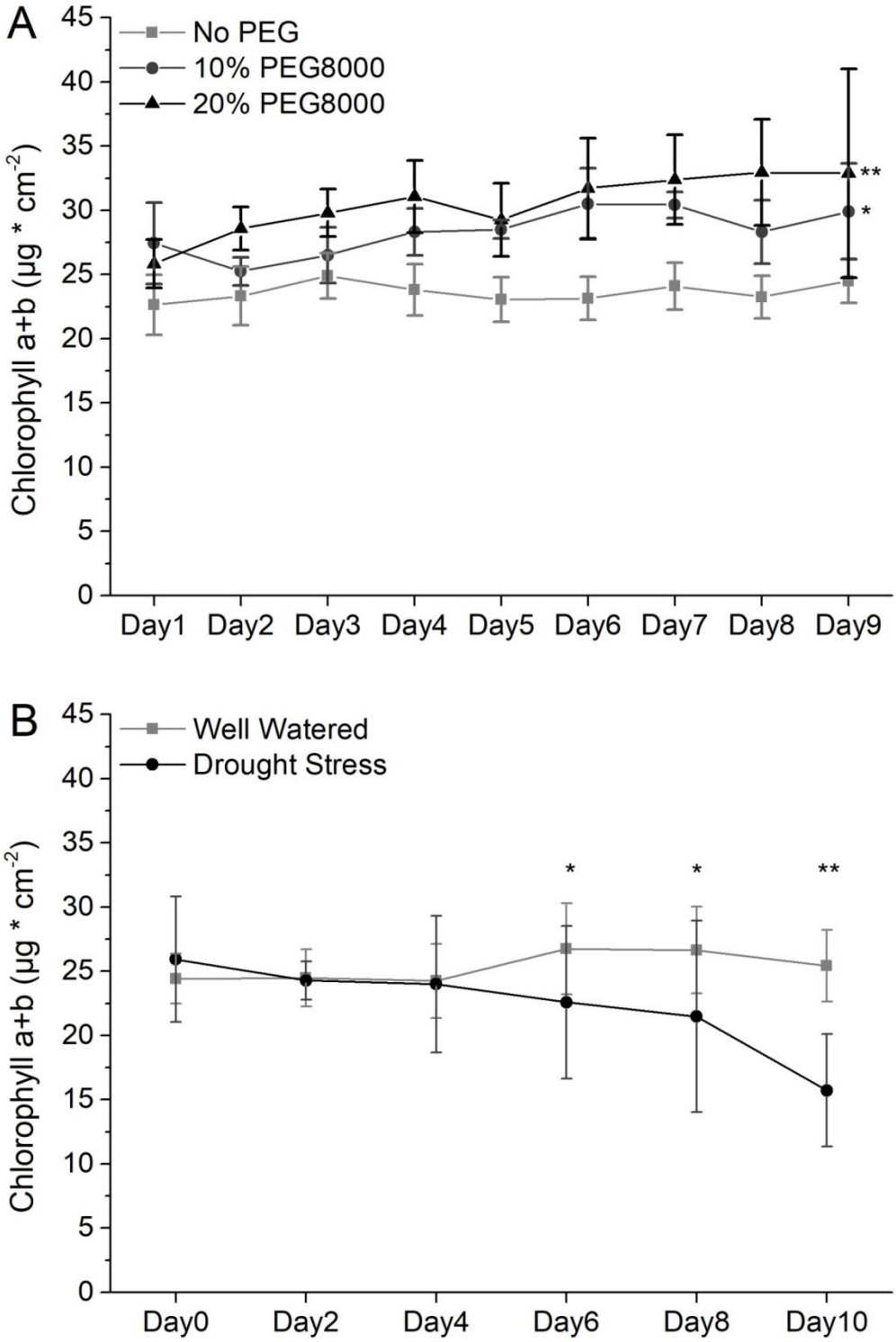


Figure 9: Chlorophyll content of *Arabidopsis thaliana* Col-0 plants grown on PEG8000-containing medium or exposed to drought stress on soil

Chlorophyll a+b content of Col-0 plants grown (A) on MS medium containing no, 10% or 20% PEG8000 (w/v), or (B) on soil were water was withhold for 0,2,4,6,8 or 10 days. Asterisks indicate significant differences between means of the control and the stress treatments in One-Way-ANOVA (Fishers LSD; *P<0.05, **P<0.01).

Taken together, the results of the physiological changes (indicated by RWC) and the morphological changes demonstrate that PEG8000 treatment in tissue culture has similar effects on Arabidopsis Col-0 plants as drought stress on soil. In the two experimental set-ups, the water content of the leaves decreased and the plants showed obvious signs for water stress. The RWC were decreased in similar ways under PEG treatment and drought stress on soil. However, chlorophyll contents did not change in the same ways, indicating that these experimental set-ups are distinct. While PEG treatment is mainly osmotic stress predominantly sensed by the root system, drought stress on soil is not only a water deficit stress but also nutrient deficiency and/or salt stress because of the water loss. While in the tissue culture system with PEG8000 on plates, air humidity stays constantly high around 100%, the air humidity for plants on soil is around 55%. Many parameters important to consider during drought stress on soil, such as soil pH, bulk soil density, or water potential of the soil etc., have not been monitored. At the same time, the PEG8000 treatments were performed always exactly in the same way, ensuring stable and well-defined conditions.

Another biochemical change triggered by drought stress is the generation of reactive oxygen species (ROS), including oxygen ions, free radicals and peroxides. The enhanced production of ROS is one of the earliest responses of plant cells to biotic and abiotic stresses, such as water-stress (Bhattacharjee, 2005). In this scenario, ROS act as secondary messengers activating subsequent defense reactions in plants. Under “non-stress” conditions, ROS are produced as natural byproducts of the normal metabolism of oxygen and have important cell signaling roles. During environmental stress, such as drought, the levels of ROS increase dramatically, known as the oxidative burst, resulting in oxidative damage of DNA, proteins and lipids (Apel & Hirt, 2004). Since ROS, such as O_2^-, H_2O_2 or •OH, are highly reactive, they can seriously damage plant cells by directly attacking membrane lipids and initiating autocatalytic lipid peroxidation leading to membrane instability and eventually cell death (Mittler, 2002). Plants have many antioxidant enzymes as a defense system (e.g. catalase or superoxide dismutase), which keep the balance between ROS production and decomposition. These enzymes interact in a

network to avoid injuries of active oxygen, thus guaranteeing normal cellular function (Møller *et al.*, 2007). Non-enzymatic antioxidants, such as glutathione, ascorbate, carotenoids or tocopherol, complete this defense system to maintain the integrity of membranes under oxidative stress (Ahmad *et al.*, 2010). In cooperation with other antioxidants, tocopherol plays an important role in controlling ROS levels (mainly O_2^- and •OH) in photosynthetic membranes and in scavenging lipid radicals thereby preventing lipid peroxidation (Fryer, 1992; Munné-Bosch, 2005). Enhanced levels of tocopherol are correlated with enhanced production of ROS. Therefore, the determination of tocopherol can be taken as a measure for monitoring plant stress status.

3.4 Reference Values

When investigating drought stress, the question about the correct reference value arises. Especially for the measurements of tocopherol under drought stress, it was not clear which parameter is best to refer to. In literature, tocopherol is mostly referred to the fresh weight (FW) of the plant material, taken prior to lipid extraction or freezing (Porfirova *et al.*, 2002; Collakova & DellaPenna, 2003b; Kanwischer *et al.*, 2005; Eugeni Piller *et al.*, 2014; vom Dorp *et al.*, 2015). It is difficult to argue whether the FW is appropriate, since plant material loses weight in form of water under drought conditions. Consequently, the FW does not remain constant in samples from well-watered and water-stressed plants. Another option for the reference value is the dry weight (DW). For tocopherol measurements, the DW cannot be obtained from the exact same samples, because the fresh material is used for the extraction. Therefore, the DW needs to be taken from separately harvested samples. Then, with the help of a regression curve, the ratio can be calculated to convert FW into the DW. But the DW has also a drawback because water stress often leads to lignification of the cell walls (Ros Barceló, 1997; Vincent *et al.*, 2005; Lee *et al.*, 2007; Moore *et al.*, 2008; Le Gall *et al.*, 2015). When the cell loses water during drought, the cell pressure decreases and the leaves curl and bend. To prevent this, the plant produces lignin to support the cell walls, resulting in a higher DW of drought-stressed plants. Therefore, the DW is also not fully independent from drought.

Another reference value is the leaf area. Under water deficit, cell elongation is inhibited due to the osmotically driven interruption of the water flow from the xylem to the surrounding elongating cells (Nonami, 1998). Thus, leaves of water stressed plants are smaller at the same age, compared to well-watered plants. But the leaf size of viable stressed plants does not decrease or shrink, because of the stability of the cell walls, keeping the leaves in shape. Therefore, the leaf area is an independent reference value, while the FW is decreasing, because of water loss, and the DW is increasing, because of lignification.

To study the effects of different reference values, the tocochromanol contents after exposure to osmotic or drought stress were referred to the FW, the DW or the leaf area (Figure 10). All measurements were performed with Arabidopsis Col-0 plants grown under osmotic stress imposed by PEG8000 or under drought stress on soil by withholding water. The tocochromanol content related to FW increases after exposure to 10% and 20% PEG8000 compared to control plants. Tocochromanol contents related to the DW show a decrease in the presence of PEG8000, because of the DW of stressed plants increases compared to the DW of control plants, as deduced from repeated DW measurements. When referring tocochromanol amounts to the leaf area, the content increases similarly to tocochromanol values related to FW.

Another interesting trend was observed for the tocochromanol measurements under drought stress on soil. The tocochromanol content referred to FW of Col-0 leaves increased with withholding water. The longer the water was withhold the more tocopherol was accumulated. But when referred to the DW the tocopherol content accumulated until 8 days of withholding water, and then slightly decreased until day 10. Using the leaf area as reference value illustrated that tocochromanol increases with water-stress, similar to tocochromanol contents related to FW. Therefore, the tocochromanol increase related to FW or leaf area was similar when plants were exposed to PEG8000, The tocochromanol increase related to FW was more drastic as compared to leaf area when plants were exposed to water deficit on soil, presumably because the leaves lost some water during drought stress on soil, but not when grown on PEG8000 medium.

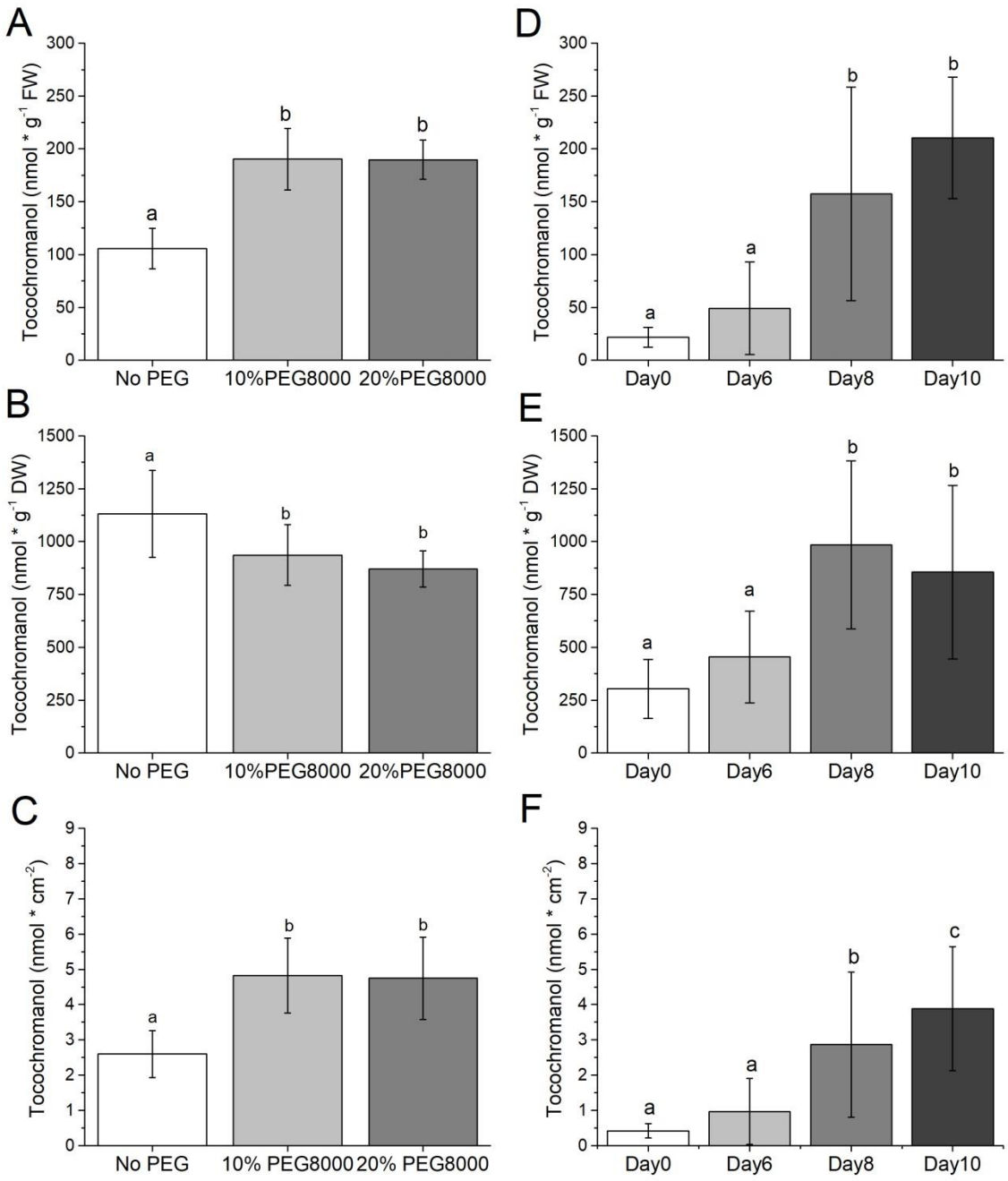


Figure 10: Tocochromanol accumulation of Arabidopsis Col-0 plants referred to fresh weight, dry weight and leaf area

Tocochromanol content in leaves of Col-0 plants grown either (A,B,C) on MS medium containing no, 10% or 20% PEG8000 (w/v) for 9 days, or (D,E,F) on soil were water was withhold for 0, 6, 8 or 10 days. Tocochromanol amounts are referred to either (A+D) the fresh weight, (B+E) the dry weight or (C+F) the leaf area. Bars represent means ± SD of at least 20 biological replicates (n=20-35). Different letters indicate differences between means at a significance level of 0.05 in One-Way-ANOVA (Fisher LSD).

4. RESULTS

4.1 ABA MUTANTS UNDER ABIOTIC STRESS

4.1.1 *aba* and *abi* Mutants under Drought and Osmotic Stress

Drought stress experiments on soil were performed with *aba* and *abi* mutants. In all mutants, tocopherol increased under drought conditions (Figure 11 A). In some mutants, the tocopherol accumulation even exceeded the one in the corresponding wild types, e.g. *aba2-1*, *abi4-1* or *aba3-2*. Drought stress on soil was especially difficult to control. Thus, the next step was to try another experimental set-up which allows easier control of stress conditions. The growth of wild type plants on PEG-containing medium was chosen as an alternative protocol to perform water deficit experiments (chapter 3.3). Therefore, all *aba* and *abi* mutants were grown under 10% or 20% PEG8000. The results show that in all mutants, the content of tocopherol increased after growth on medium containing 10% or 20% PEG8000 (Figure 11 B). But three mutants, *abi4-1*, *abi4-102* and *abi2-1*, showed reduced amounts of tocopherol in comparison to their corresponding wild types. In the *abi4-1* and *abi4-102* mutants, the tocopherol content was significantly decreased when plants were grown on control medium or 10% or 20% PEG8000. The *abi2-1* mutants had similar tocopherol levels as the Ler wild type in control conditions, but reduced amounts at 10% and 20% PEG8000.

The total tocochromanol content includes the sum of all tocopherols and plastochromanol (PC-8). Arabidopsis leaves contain four tocopherols and PC-8. The most prominent one is α-tocopherol, accounting for about 80-90% of the total tocochromanol. PC-8 accounts for 5-20%. Minor amounts of β-, γ-, or δ-tocopherol varying between 0.1-5% are also present.

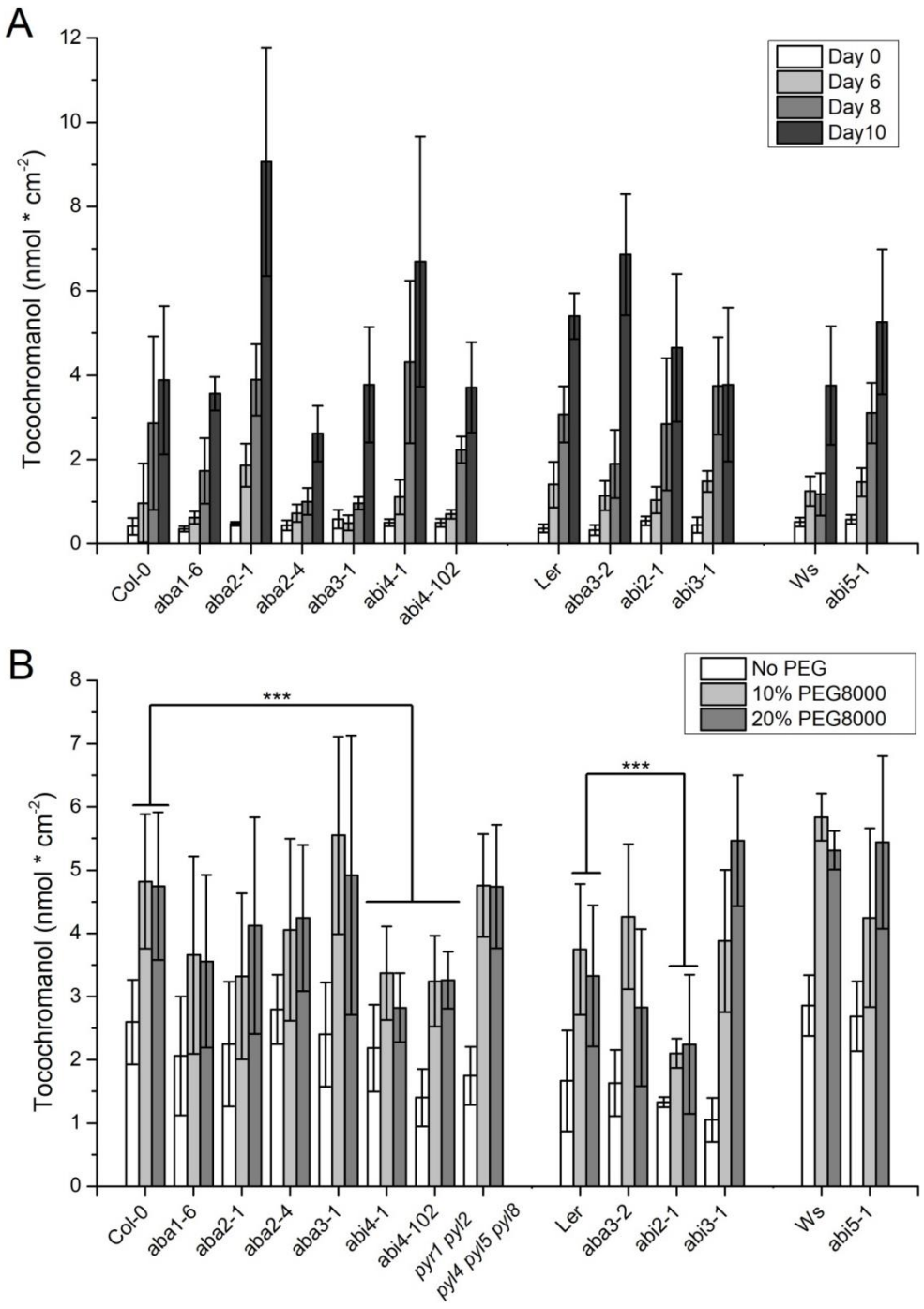


Figure 11: Tocochromanol accumulation of ABA-deficient and ABA-insensitive mutants exposed to drought stress on soil or grown on PEG8000-containing medium

Tocochromanol content in leaves of Arabidopsis ABA-deficient and ABA-insensitive mutants, with their corresponding wild types, grown (A) on soil were water was withhold for 0, 6, 8 or 10 days, or (B) on MS medium containing no, 10% or 20% PEG8000 (w/v) for 9 days. Bars represent mean values ± SD of at least 5 independent biological replicates (n=5-30). Asterisks indicate significant differences between means of the corresponding WT and the mutant under various stress treatments in One-Way-ANOVA (Fishers LSD; ***P<0.001).

Next, the percentage distribution of the individual tocochromanols (in mol%) was calculated. In wild type Col-0, a change in the relative distribution was observed in response to drought stress on soil. While the total amount of all tocopherols increased after withholding water for several days, the proportion of α-tocopherol declined from 90% to 65% after 10 days of drought stress. The proportion of γ-tocopherol increased from 0.9% to 21% and δ-tocopherol amounts to almost 9% after 10 days of drought, while not detected under control conditions. The relative amount of PC-8 decreased from 6% to 4% with drought stress. The content of β-tocopherol changed the least during drought stress on soil (Figure 12 A).

For all mutant and wild type lines, this tendency was observed under drought stress on soil, except for some notable differences. The plants of the *aba3-1* mutant have in total much higher proportions of α-tocopherol, with only little change in percentage distribution during stress. In *aba3-1* mutants, β- and δ-tocopherol were not detectable at all. The *aba1-6* and *aba2-4* mutants did not show this strong decline in α-tocopherol percentage, and δ-tocopherol was only detected for *aba1-6* mutants, after 10 days of drought stress (Figure 12 A).

When plants were grown on synthetic MS plates and treated with PEG8000, the distribution of tocopherols was different to the one described for plants grown on soil. The most obvious difference was the absence of a decline in the percentage of α-tocopherol in response to PEG8000 stress. The relative amounts of α-tocopherol were between 70-80% during all conditions on synthetic medium. PC-8 was still the second most abundant tocochromanol with 10-20%, followed by γ-tocopherol with again 10-20% and β-tocopherol with 0.1-2%. Only minor amounts of δ-tocopherol were detected. Contrary to the distribution on soil, the percent proportion of α-tocopherol was increased under PEG8000 treatment. The percent decrease of PC-8 and the increase of γ-tocopherol were comparable to the drought experiment on soil, just less drastic. δ-Tocopherol was only detected in minor amounts with almost no change during PEG8000 application, which was also different from the data observed with soil-grown plants. The proportion of β-tocopherol was higher though, but also unchanged during stress (Figure 12 B).

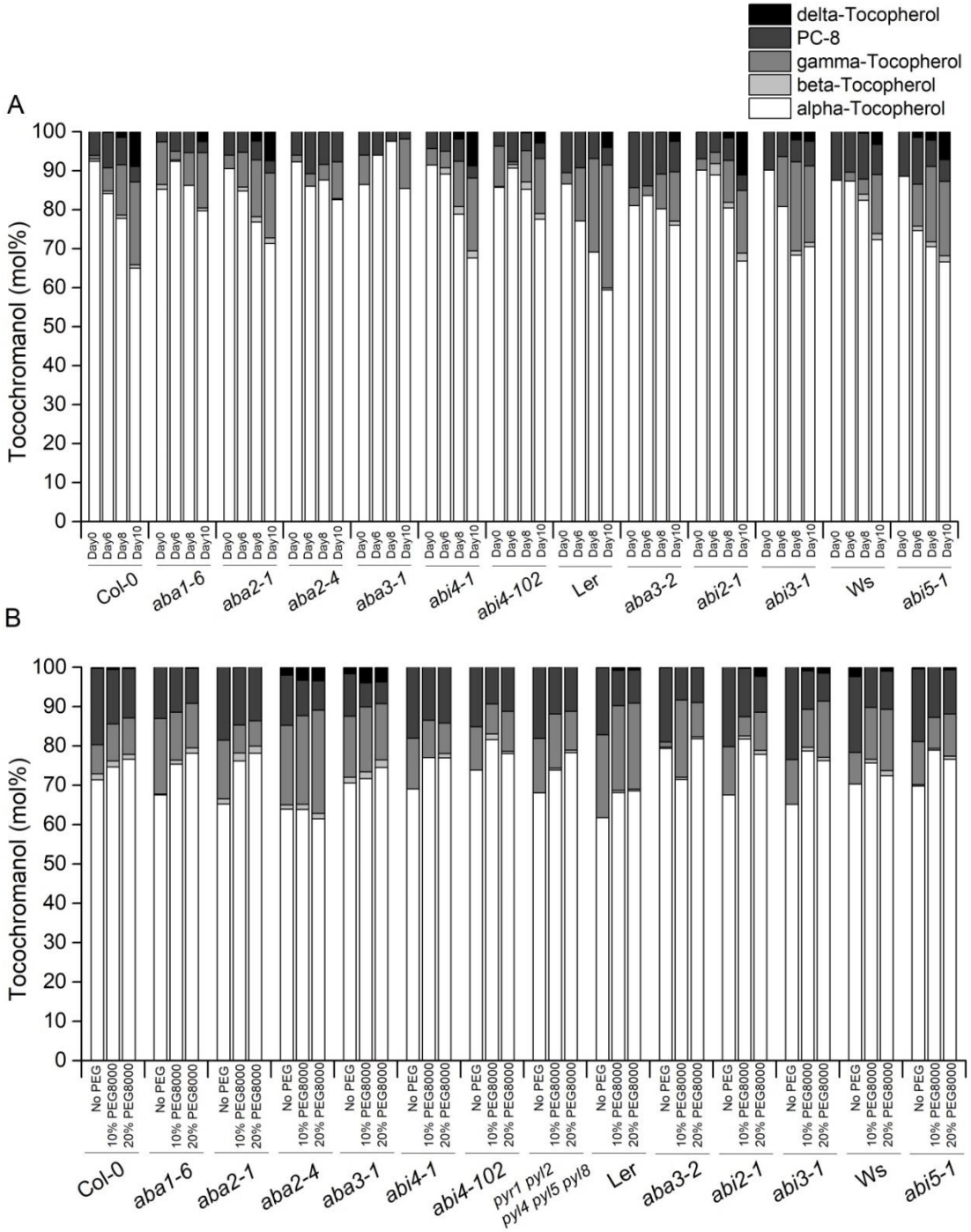


Figure 12: Percent distribution of tocochromanol in ABA-deficient and ABA-insensitive mutants grown on PEG8000-containing medium or exposed to drought stress on soil

Percent distribution of α-, β-, γ-, δ- tocopherol and plastochromanol (PC-8) in leaves of Arabidopsis ABA-deficient and ABA-insensitive mutants, with their corresponding wild types, grown (A) on soil were water was withhold for 0, 6, 8 or 10 days, or (B) on MS medium containing no, 10% or 20% PEG8000 (w/v) for 9 days. Bars represent mean values of at least 5 independent biological replicates (n=5-30).

Again, here the mutants show almost the same relative percent distributions of tocochromanols as their corresponding wild types. However, the mutants *aba2-4* and *aba3-1* are different. Plants of the *aba2-4* mutant have lower α-tocopherol proportions, but higher γ-tocopherol proportions of up to 26%. In the *aba2-4* and *aba3-1* mutants, high amounts of δ-tocopherol were found under all conditions compared to the wild type. The plants of the Ler wild type also have high proportions of γ-tocopherol, while the mutants of with Ler background show tocopherol distributions which are highly related to Col-0 (Figure 12 B).

4.1.2 *aba* and *abi* Mutants under Nitrogen Deficiency

Based on the results obtained from drought stress and osmotic stress applications, nitrogen deprivation, another abiotic stress well known for its strong effect on tocopherol synthesis, was tested. Nitrogen deficiency causes chlorotic stress in plants which leads to chlorophyll breakdown (Gaude *et al.*, 2007). When chlorophyll is degraded, phytol is hydrolyzed from chlorophyll (Peisker *et al.*, 1989; Hörtensteiner, 1999). Free phytol is toxic to plants and therefore plants have developed mechanisms to metabolize phytol into other less toxic compounds such as phylloquinone (vitamin K), phytyl esters or tocopherol (Rise *et al.*, 1989; Ischebeck *et al.*, 2006; Lippold *et al.*, 2012; vom Dorp *et al.*, 2015). As a result, a large amount of tocopherol is synthesized under nitrogen deficiency. This led to the hypothesis that the amount of tocochromanol might be strongly reduced in ABA mutants grown on nitrogen-depleted medium, in case that ABA plays a role in the tocopherol synthesis during chlorophyll breakdown.

The results show that all *aba* and *abi* mutants accumulate tocochromanol when grown on nitrogen-depleted medium, to a similar extent as the corresponding wild types. The *aba2-4* mutant showed an even stronger accumulation of tocochromanol, already under nitrogen-sufficient conditions. Also the pentuple mutant *pyr1pyl2pyl4pyl5pyl8* showed more tocochromanol accumulation during nitrogen deficiency. Only the plants of the *aba3-1* mutant had less tocopherol under control and stress conditions, but still they accumulated tocopherol under nitrogen-depleted conditions (Figure 13 A).

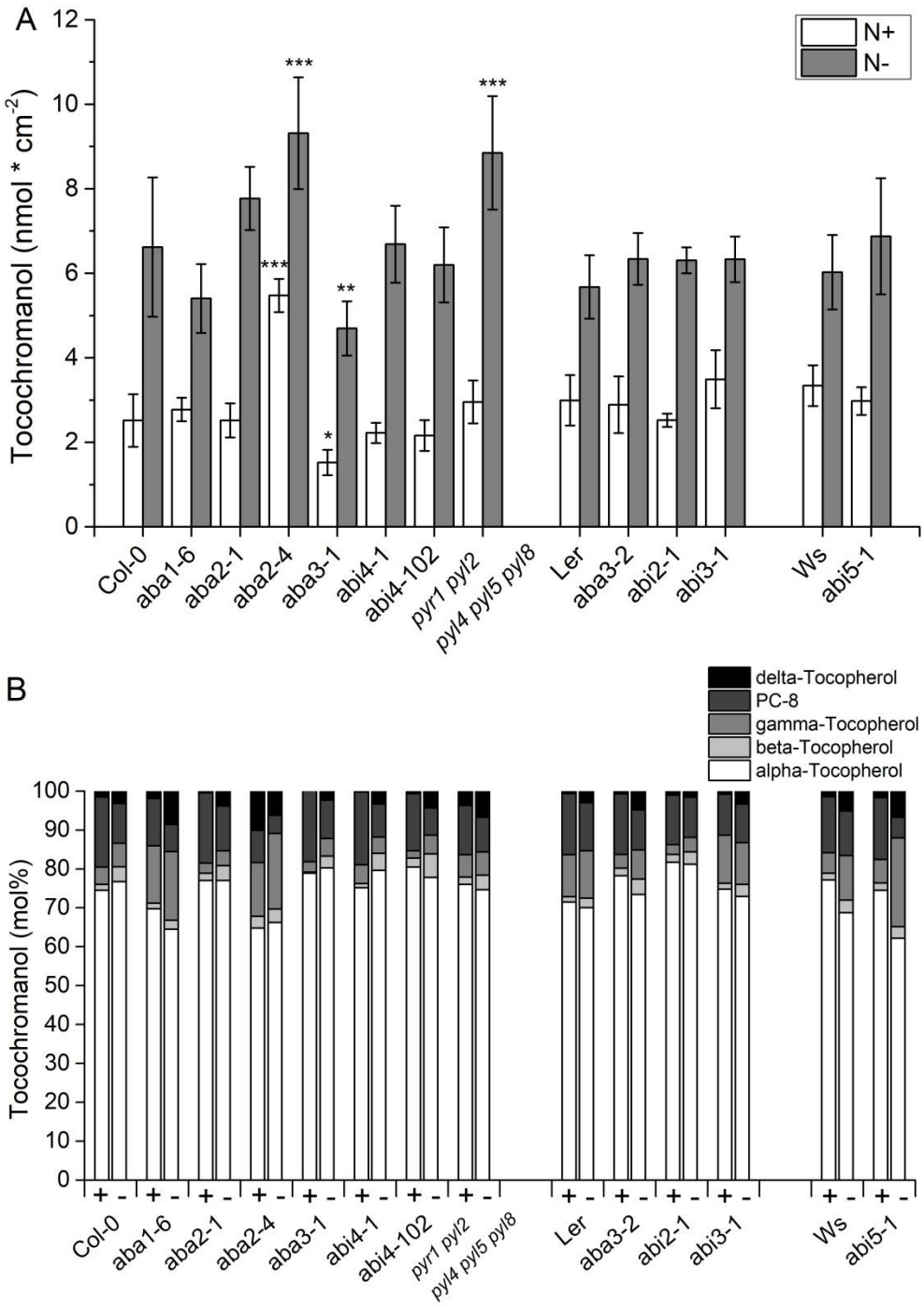


Figure 13: Tocochromanol accumulation and percent distribution in ABA-deficient and ABA-insensitive mutants grown under nitrogen deficiency

(A) Tocochromanol content and (B) percent distribution of α-, β-, γ-, δ- tocopherol and plastochromanol-8 (PC-8) in leaves of Arabidopsis ABA-deficient and ABA-insensitive mutants, with their corresponding wild types, grown on nitrogen sufficient (N+) or depleted (N-) medium. Bars represent mean values (± SD) of 5 biological replicates (n=5). Asterisks indicate significant differences between means of the corresponding WT and the mutant under N+ or N- in One-Way-ANOVA (Fishers LSD; *P<0.05, **P<0.01, ***P<0.001).

The relative distribution of the tocochromanols was not changed between the ABA-deficient and –insensitive mutants compared to their respective wild types after growth on N- medium. The distribution of the tocochromanols was comparable to that of plants grown on MS plates with or without PEG8000, with major amounts of α-tocopherol in leaves followed by smaller fractions of PC-8, β-, γ-, and δ-tocopherol. The percent composition of tocochromanols did not change in the wild types or in the mutants when the plants were grown with or without nitrogen. Only the amount of δ-tocopherol was higher in the nitrogen-deprived plants than on nitrogen sufficient conditions (Figure 13 B).

4.1.3 Tocochromanol Content in Seeds of *aba* and *abi* Mutants

Seeds of Arabidopsis contain about 10-fold more total tocopherol than leaves when measured per FW (Grusak & DellaPenna, 1999). The main function of tocopherols in vegetative and reproductive tissue is to scavenge and quench ROS and lipid oxidation products and thus stabilize membrane lipids. Tocopherols are required for seed longevity and for preventing lipid peroxidation during seed dormancy, germination and early seedling development (Sattler *et al.*, 2004). To study whether ABA is involved in the regulation of tocopherol biosynthesis during seed maturation, the tocochromanol content was measured in independent seed batches of the *aba* and *abi* mutant lines. High amounts of tocochromanols were measured in seeds of the *aba* and *abi* mutants. The seeds of the mutants *aba1-6* and *aba3-1* contain even more tocopherol compared to wild type Col-0 seeds (Figure 14 A).

In seeds of Arabidopsis, the percentage distribution of the tocochromanols is different from leaves. Whereas in leaves α-tocopherol is most prominent, seeds mainly contain γ- tocopherol (85-90%) with only little amounts of α-, δ-tocopherol (1.5-4% each) and PC-8 (5-9%) (Shintani & DellaPenna, 1998). β-Tocopherol is hardly detected. The exact same relative distribution of tocopherol compounds was detected in all mutants and wild types (Figure 14 B).

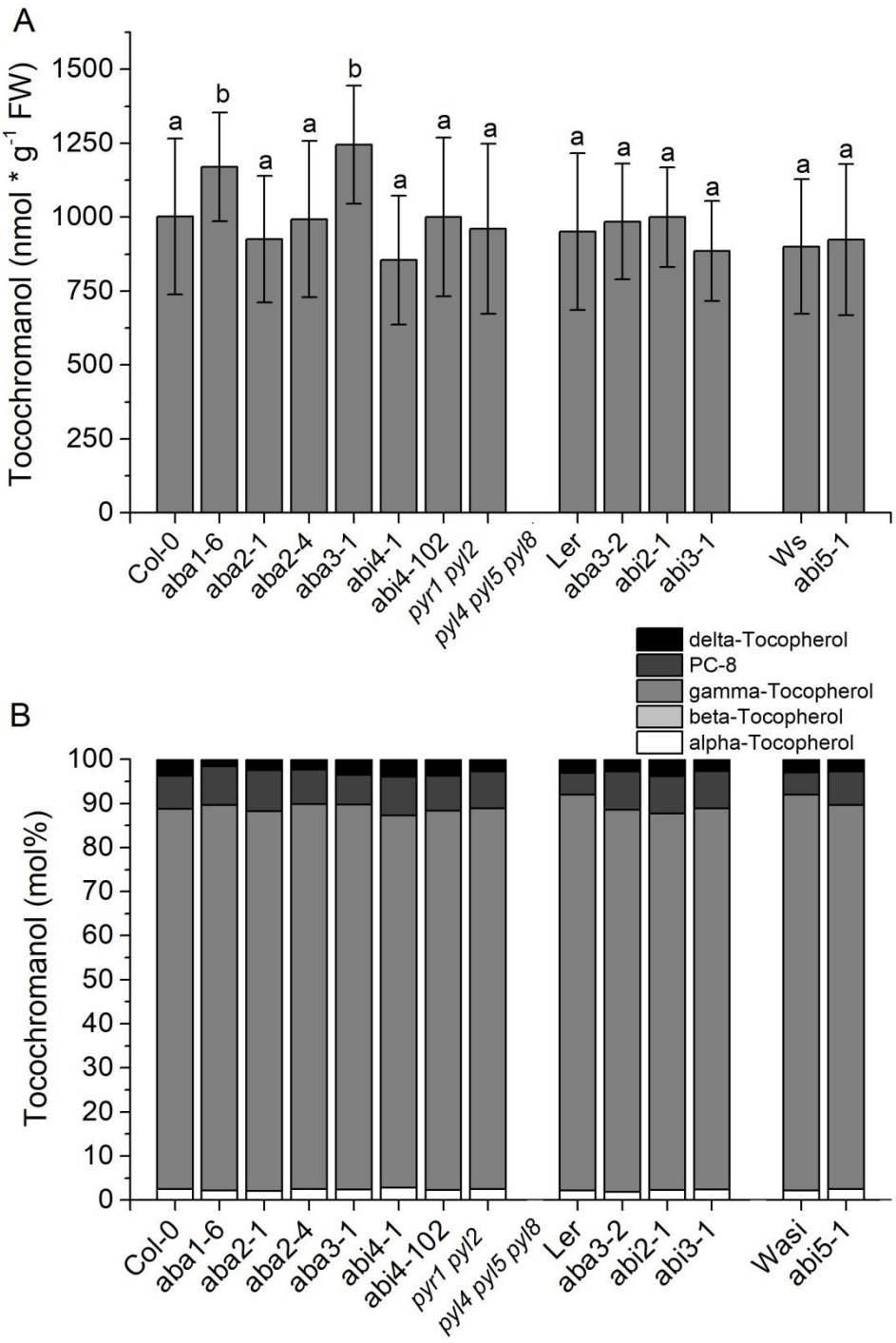


Figure 14: Tocochromanol accumulation and percentage distribution in seeds of ABA-deficient and ABA-insensitive mutants

(A) Tocochromanol content and (B) percentage distribution of α-, β-, γ-, δ- tocopherol and plastochromanol (PC-8) in seeds of Arabidopsis ABA-deficient and ABA-insensitive mutants, with their corresponding wild types. Bars represent mean values ± SD of at least 6 independent biological replicates (n=6-20). One replicate was obtained from about 20 mg of one seed batch. Different letters indicate significant differences between means at a significance level of 0.05 in One-Way-ANOVA (Fisher LSD).

4.1.4 ABA Content in Leaves of *aba* and *abi* Mutants

Results of the ABA measurements by LC-MS showed that three *aba* mutants had significantly reduced amounts of ABA in leaves in comparison to their corresponding wild type. Compared to the ABA content in wild type Col-0, the mutants, *aba1-6* and *aba2-1* had less than a third of the ABA amount. The *aba3-2* mutant showed about half of the amount as the Ler wild type. The other two ABA-deficient mutants, *aba2-4* and *aba3-1*, had comparable ABA levels as the Col-0 wild type. ABA-insensitive mutants were also included in the measurements. Most *abi* mutants had similar ABA amounts as their corresponding wild types. However, abi3-1 might contain more ABA, albeit this difference was not significant at P<0.05.

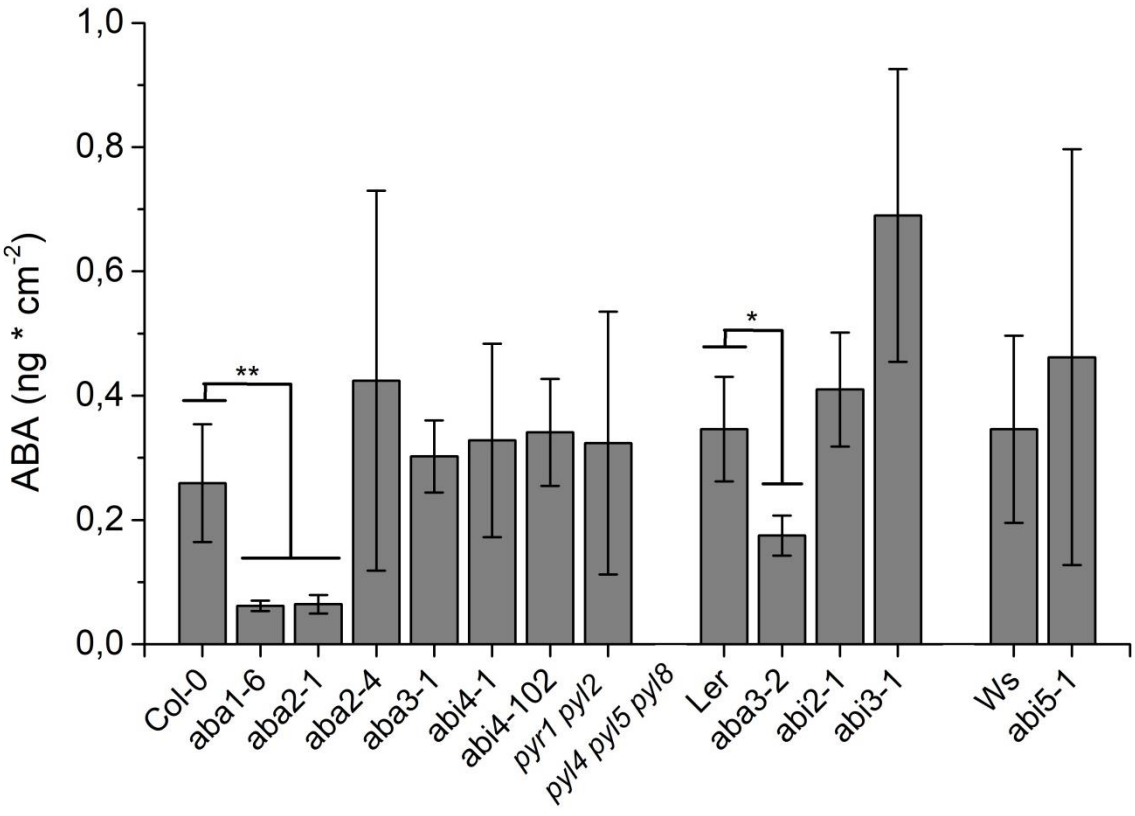


Figure 15: ABA Content of ABA-deficient and ABA-insensitive mutants

The amount of ABA was measured in leaves of Arabidopsis ABA-deficient and ABA-insensitive mutants, with their corresponding wild types. Bars represent mean values ± SD of 5 biological replicates (n=5). Asterisks indicate significant differences between means of the corresponding WT and the mutant in One-Way-ANOVA (Fishers LSD; *P<0.05, **P<0.01).

4.2 WILD TYPE RESPONSE TO DIFFERENT ABIOTIC STRESS

All results presented so far indicate that tocopherol can increase in Arabidopsis in an ABA-independent way. Tocopherol biosynthesis is clearly induced by drought stress and osmotic stress (PEG8000), but ABA does not seem to be the only key regulator. Next, the response of the wild type under various abiotic stresses was evaluated. To this end, only Arabidopsis Col-0 plants were investigated after exposure to five different abiotic stress treatments. Samples were taken from leaves of plants grown during the different stress experiments for the measurement of (1) tocochromanol by fluorescence HPLC, (2) gene expression using qPCR and (3) ABA by HPLC-MS/MS.

4.2.1 Morphological Changes in Response to Abiotic Stress

First, the morphological changes of the Arabidopsis Col-0 plants in response to different abiotic stresses were investigated. The first three stress applications were performed in tissue culture using PEG8000, exogenous ABA or nitrogen deficiency.

Osmotic stress induced by PEG8000 led to small, dark green plants. The resulting morphological changes have already been described in detail (chapter 3.3). Independent from the concentration of PEG8000, some plants also had some yellow, senescent leaves, while other plants appeared to have a darker almost purple color, probably due to the accumulation of anthocyanins (Figure 16 a-c).

Application of exogenous ABA also led to morphological changes comparable to those of the PEG8000 stressed plants. Addition of 50 µM ABA resulted in growth retardation of all plants. Control plants grown without exogenous ABA have very similar sizes, but plants grown with 50 µM ABA are smaller and differ in size. Leaves of ABA-treated plants are smaller and curled. When grown on medium with 150 µM ABA, plants suffer even more. They are also retarded in growth and leaves start to get yellow and senescent. Some leaves showed a darker color and became purple, presumably because of the synthesis of anthocyanins (Figure 16 d-f). Next, plants were grown on nitrogen sufficient or nitrogen depleted medium for a time of 14 days. While plants grown

on nitrogen sufficient conditions look as healthy as plants grown under the other control conditions, plants grown under nitrogen deficiency suffer from chlorosis and are pale green. Some leaves start to turn yellow and senescent at the tips. A detailed observation reveals some residual green spots on the pale leaves, typical for chlorotic stress in Arabidopsis plants (Figure 16 g+h).

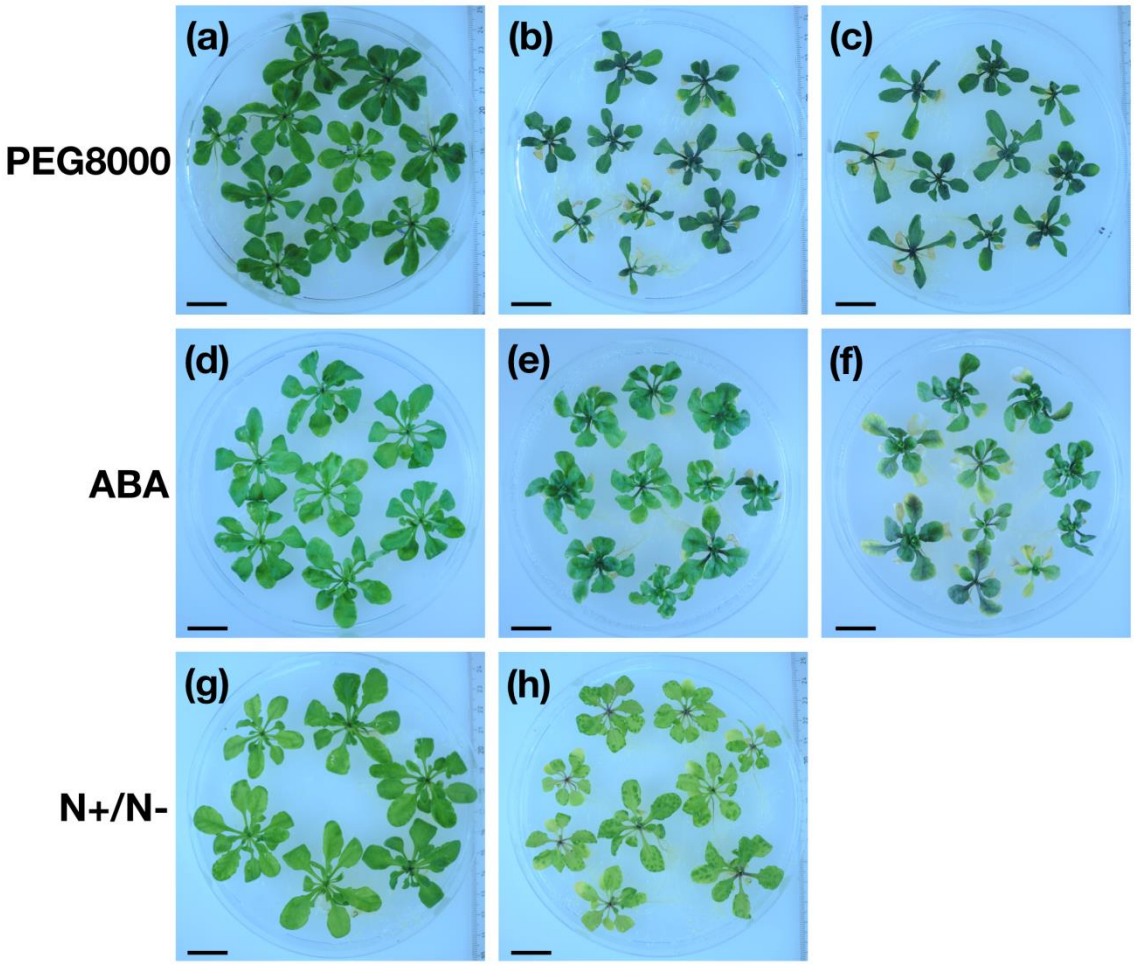


Figure 16: Arabidopsis Col-0 plants grown under stress on synthetic medium

Photos of Arabidopsis Col-0 plants grown on synthetic MS medium for various stress treatments. Medium contained either (a) no PEG, (b) 10% PEG8000 (w/v), (c) 20% PEG8000 (w/v), (d) no ABA, (e) 50 µM ABA, (f) 150 µM ABA, (g) sufficient nitrogen or (h) deficient nitrogen. Bars = 2 cm.

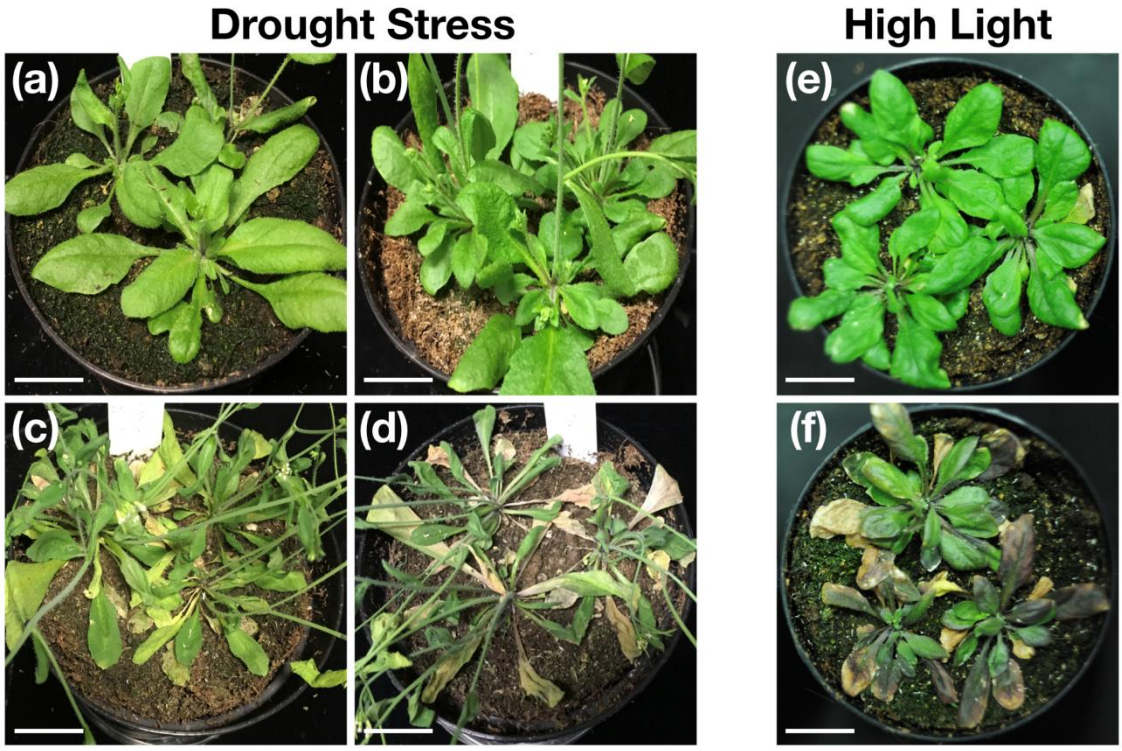


Figure 17: Arabidopsis Col-0 plant grown on soil

Photos of Arabidopsis Col-0 plants grown on soil and under drought stress through withholding water, or high light stress. Plants depicted on the left are stressed by withholding water for (a) 0 days, (b) 6 days, (c) 8 days or (d) 10 days. On the right, plants were grown for 8 days under a light intensity of (e) 150 µmol m^{-2} s^{-1} or (f) 500 µmol m^{-2} s^{-1}. Bars = 2 cm.

The other two stress applications were performed on soil, by withholding water for up to 10 days or applying high light stress for 8 days. Compared to plants grown on synthetic medium, it is obvious that the plants are larger, because they grew in a non-restricted environment. The morphological changes of the drought stressed plants have already been described (chapter 3.3). In summary, water deficit on soil results in stronger morphological changes than osmotic stress imposed by PEG8000. After 6 days of withholding water, the plants look still similar to the control, because only the upper part of the soil started to dry out. After 8 days the leaves are severely wilted and yellow, and after 10 days the plants were completely wilted and dried out (Figure 17 a-d).

High light induced other morphological changes. Under control light conditions (150 µmol m^{-2} s^{-1}), the plants looked healthy and green. Under high light treatment (500 µmol m^{-2} s^{-1}) for 8 days, most leaves turned purple, probably due to the accumulation of anthocyanins. Some leaves also became

senescent resulting in yellow, brown colored and wilted tissue. Younger leaves in the center of the rosette sometimes stayed green on the adaxial side, while the abaxial leaf surface also was deep purple (Figure 17 e+f).

4.2.2 Changes in Tocopherol Content in Response to Abiotic Stress

The previous results showed that wild type Col-0 plants accumulate tocochromanol after exposure to (A) PEG8000, (B) ABA, (C) nitrogen-depleted medium, (D) drought stress on soil or (E) high light treatment (Figure 18 A, B, C, D, E, respectively). The plants had twice as much tocopherol after exposure to medium containing 10% or 20% PEG8000 compared with control plants (Figure 18 A). ABA treatment increased tocopherol in a concentration-dependent manner. At the concentration of 150 µM ABA, the tocochromanol level increased to the double amount compared with control conditions (no ABA) (Figure 18 B). Under nitrogen deficiency, the level of tocochromanol is almost 3-fold higher than in plants grown under nitrogen sufficient conditions (Figure 18 C). The three stress conditions with PEG8000, ABA or nitrogen deficiency were all performed in tissue culture with plants grown on synthetic medium. In all three experiments, the medium composition of control plates was slightly different, but the control plants still showed the same tocopherol content. It is interesting to note that tocopherol contents of tissue culture grown (control) plants were higher than for control plants grown on soil.

Drought stress on soil had the strongest effect on tocopherol biosynthesis. After 10 days of withholding water, the tocopherol content increased almost by 10-fold compared to the control conditions. This strong increase is in particular due to the fact that tocopherol in plants grown on soil is lower than in plants grown in tissue culture. The final total tocochromanol content was comparable to the other stresses. Tocopherol continued to increase as long as the water was withheld until day 10 when the experiment was terminated because the plants started to die (Figure 18 D).

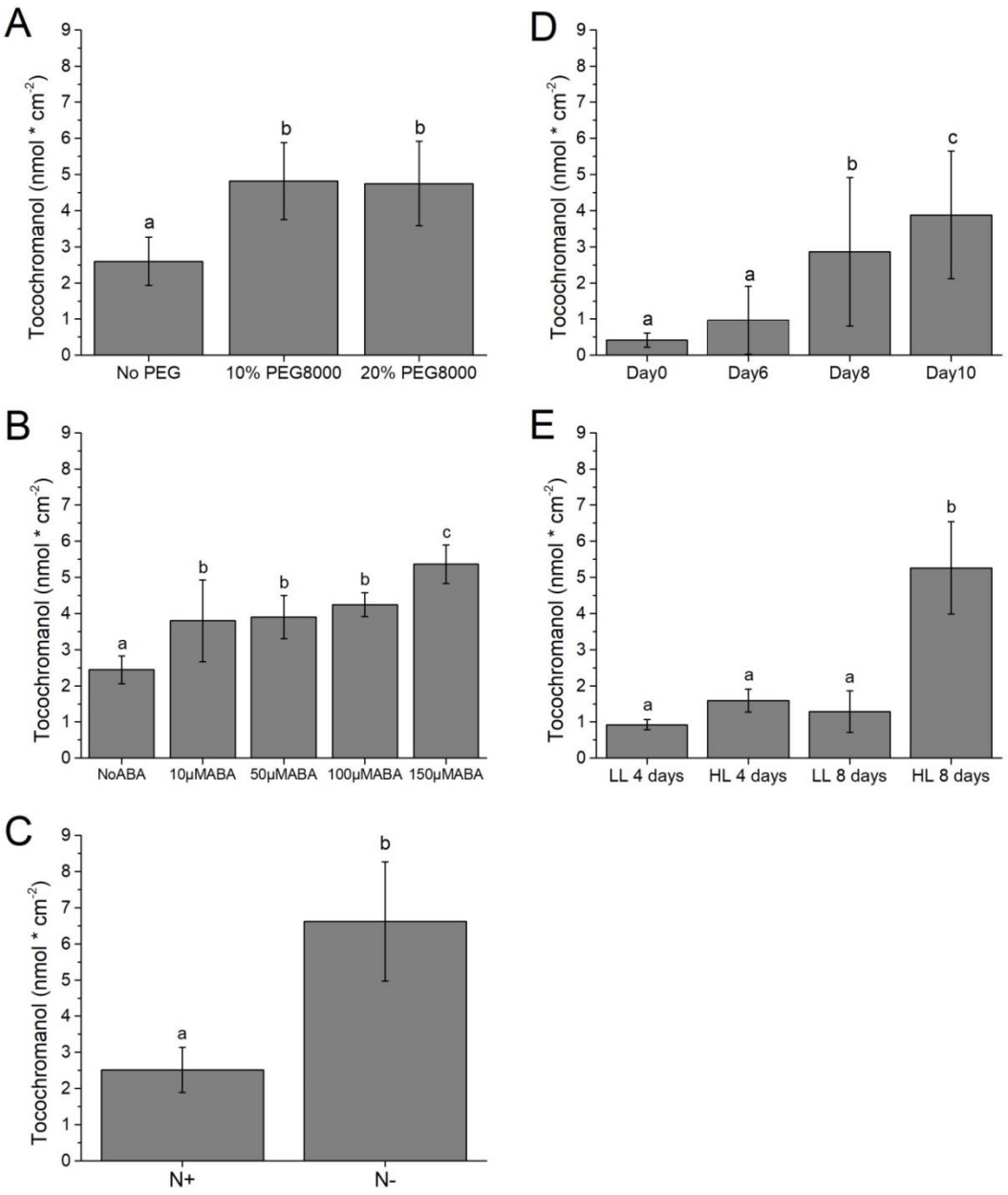


Figure 18: Tocochromanol accumulation of Arabidopsis Col-0 in response to abiotic stress

Tocochromanol contents in leaves of Arabidopsis Col-0 after treatment with (A) PEG8000, (B) exogenous ABA, (C) nitrogen deficiency, (D) drought stress on soil and (E) high light. Bars represent mean values ± SD of at least 5 biological replicates (n=5-30). Different letters indicate significant differences between means at a significance level of 0.05 in One-Way-ANOVA (Fisher LSD).

Similarly, high light imposed a strong accumulation of tocochromanol. While after 4 days of high light stress, the tocopherol content did not increase significantly, the amount of tocochromanol was 5-fold higher after 8 days of high

light exposure of Arabidopsis wild type Col-0 plants (Figure 18 E). Notable is also that the biological variation increases with application of any kind of abiotic stress, showing that even under highly controlled experimental conditions, plants differ in their reaction to stress.

The percent amounts of the five tocochromanols, α-, β-, γ-, δ- tocopherol and PC-8, vary dependent on growth conditions. Growing Col-0 plants in tissue culture on synthetic medium resulted in higher relative amounts of PC-8. Thus the relative amount of α-tocopherol is lower in plants grown on medium than in plants grown on soil. In plants grown with or without PEG8000, the relative amount of α-tocopherol is between 70-80%. The second most abundant tochromanol is PC-8 with 10-20%, followed by γ-tocopherol with 10-20% and β-tocopherol with 0.1-2%, while only minor amounts of δ-tocopherol were detected. Under PEG8000 treatment, the proportion of α-tocopherol increased, while the amounts of PC-8 and γ-tocopherol decreased. β-Tocopherol and δ-tocopherol proportions are unchanged during osmotic stress.

After exogenous ABA application, α-tocopherol remained the most abundant tocochromanol, and it increased only slightly with higher concentrations of ABA. PC-8 represents the second most abundant tocochromanol, but decreased strongly with higher ABA amounts. The fractions of γ-tocopherol and δ-tocopherol increased with higher amounts of ABA, while the fraction of β-tocopherol stayed again unchanged.

Plants grown on nitrogen sufficient or depleted medium had similar distributions of tocochromanols as control plants and plants grown with PEG8000. The main component was α-tocopherol, and PC-8 represents the second most abundant component, followed by minor amounts of γ-, β- and δ-tocopherol. While α-, β-, γ- and δ-tocopherol increased slightly under nitrogen deprivation, the relative amount of PC-8 decreased. In the experiments with ABA application and nitrogen deficient medium, δ-tocopherol was detected in increasing amounts, but it was detected only in minor amounts in plants grown on medium with or without PEG8000. The proportions of other tocochromanols are similar in all three tissue culture experiments.

When grown on soil, Arabidopsis plants showed a different distribution of tocochromanols. α-Tocopherol is still the most prominent compound with about 80-90% of the total amount, under control conditions. With up to 10%, PC-8 is the second most abundant tocochromanol. β-, γ-, or δ-tocopherol are only detected in minor amounts varying between 0.1-5%, dependent on the growth conditions.

Drought stress on soil through withholding water caused the most drastic change in tocochromanols distributions of all abiotic stresses. After 10 days of drought stress, the proportion of α-tocopherol declined from 90% to 65%. While the proportion of γ-tocopherol increased from 0.9% to 21% and δ-tocopherol amounted to almost 9% after 10 days of withholding water, while it was undetectable under control conditions. The proportion of PC-8 on the other hand decreased from 6% to 4% with continuing stress. The content of β-tocopherol changed the least during drought stress on soil.

Also the exposure to high light changed the proportions of the tocopherol compounds. After 8 days of treatment, the proportion of α-tocopherol declined to around 75% and PC-8 from 7% to 3%. While the amount of β-tocopherol was also unchanged during high light treatment, the amounts of γ-tocopherol and δ-tocopherol increased to 13% and 4%, respectively (Figure 19).

Plant water status was measured by recording RWC for all treatments to differentiate the effects of abiotic stress. As mentioned in chapter 3.3, the RWC of plant leaves after treatment with 10% or 20% PEG8000 was reduced, as well as after withholding water on soil. The other three stress treatments, application of exogenous ABA, nitrogen deficiency and high light stress, did not alter the RWC. In each stress condition, the RWC of leaves was similar to that of the control conditions.

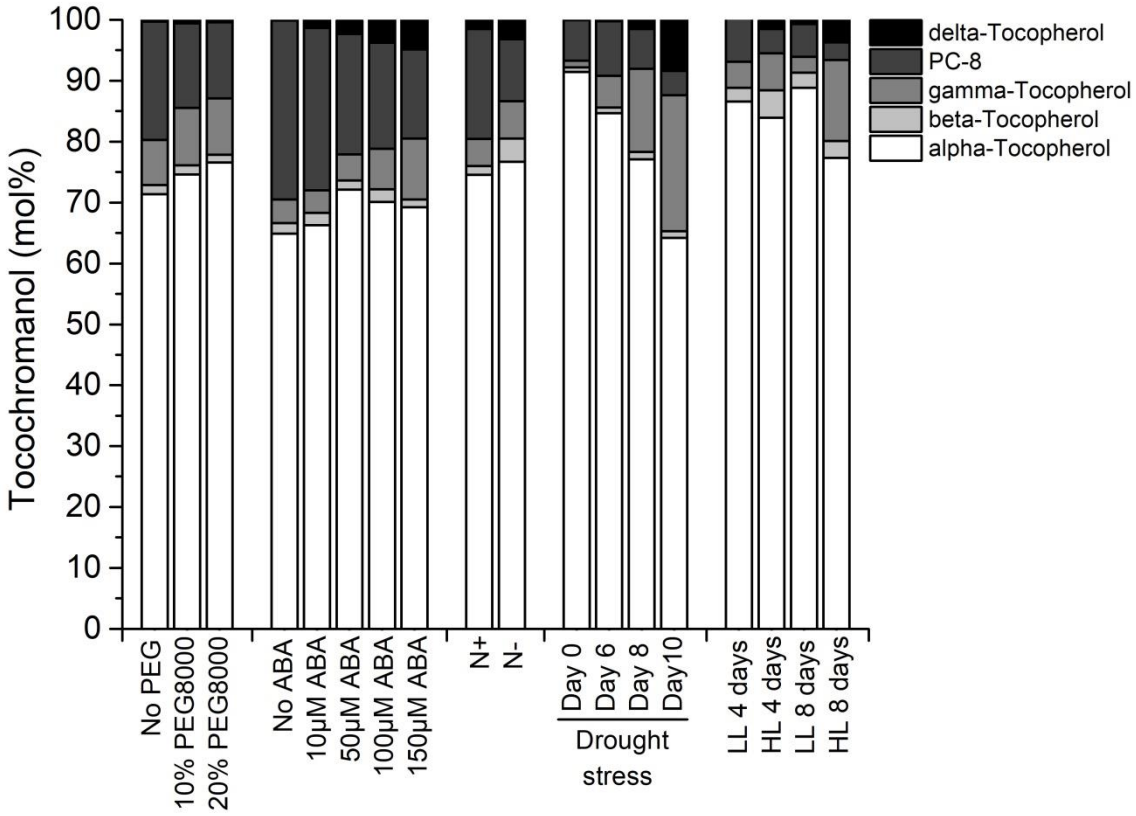


Figure 19: Percent distribution of tocochromanols in Arabidopsis Col-0 in response to abiotic stress

Percent distribution of α-, β-, γ-, δ- tocopherol and plastochromanol-8 (PC-8) in leaves of Arabidopsis Col-0 after treatment with (1) PEG8000, (2) exogenous ABA, (3) nitrogen deficiency, (4) drought stress on soil and (5) high light. Bars represent mean values of at least 5 biological replicates (n=5-30).

The chlorophyll content of leaves was also measured for all stress treatments. As described previously in chapter 3.3 the chlorophyll content increased after treatment with 10% and 20% PEG8000, while it decreased under drought stress on soil (Figure 20 A+D). Application of exogenous ABA had no effect on the chlorophyll content independent from the concentration. Even though some leaves of the plants grown on 150 µM ABA became yellow, there was no significant change in chlorophyll content (Figure 20 B). High light treatment caused only a minor decrease in chlorophyll after 8 days. 4 days of high light treatment did not change the chlorophyll content at all (Figure 20 E). Nitrogen deficiency caused the strongest decline in chlorophyll content. Here the amount of chlorophyll decreased even more severely than during drought stress (Figure 20 C).

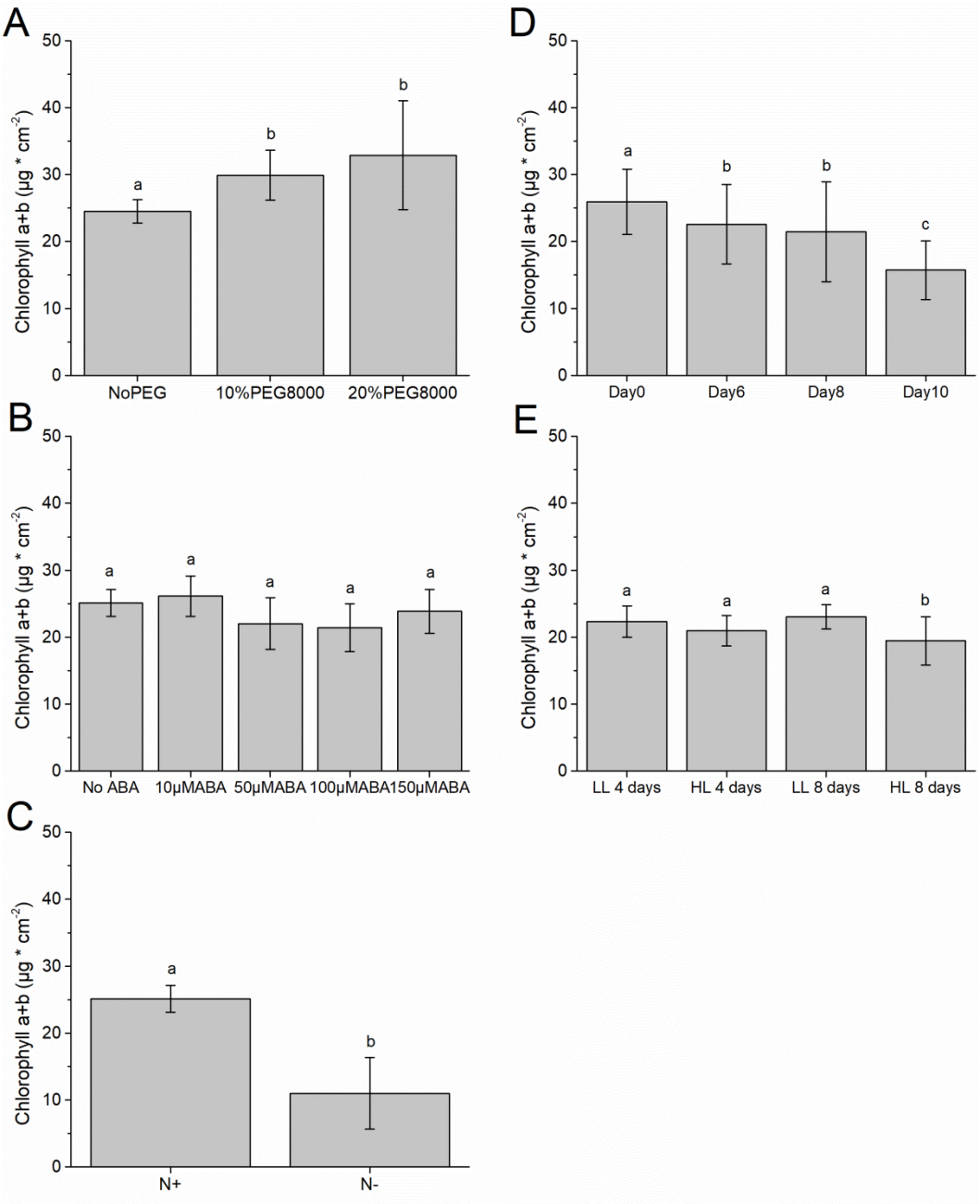


Figure 20: Chlorophyll content of Arabidopsis Col-0 in response to abiotic stress

Chlorophyll a+b content in leaves of Arabidopsis Col-0 after treatment with (A) PEG8000, (B) exogenous ABA, (C) nitrogen deficiency, (D) drought stress on soil and (E) high light. Bars represent mean values ± SD of at least 5 biological replicates (n=5-30). Different letters indicate significant differences between means at a significance level of 0.05 in One-Way-ANOVA (Fisher LSD).

4.2.3 Relative Expression of Tocopherol Genes under Abiotic Stress

Next, the relative gene expression was analyzed in leaves of plants grown under the same abiotic stress experiments used for tocochromanol measurements. Samples were harvested at the exact same time point as for the tocopherol measurements to reveal the actual gene expression while plants are still stressed.

Osmotic stress induced by PEG8000 induces the expression of the tocopherol biosynthesis genes *VTE2*, *HPPD* and *VTE6*. The expression of *VTE2* and *VTE6* also slightly depends on the PEG8000 concentration, indicated by higher relative expression under 20% PEG8000. At the same time, the expression of *HPPD* shows a lesser increase after treatment with 20% PEG8000. In addition to the tocopherol biosynthesis genes, the ABA-dependent reference gene *RD29A* was also included in the qPCR analyses. *RD29A* gene expression is strongly induced by ABA. Exposure to 10% and 20% PEG8000 resulted in an increase in expression of RD29A by 8 and 16-fold higher than under control conditions, respectively. This indicates that ABA plays a role during PEG8000 stress. Expression of *VTE1* and *VTE5* seems to be decreased under PEG8000 treatment, but the extent of decrease is low with large error bars (Figure 21 A).

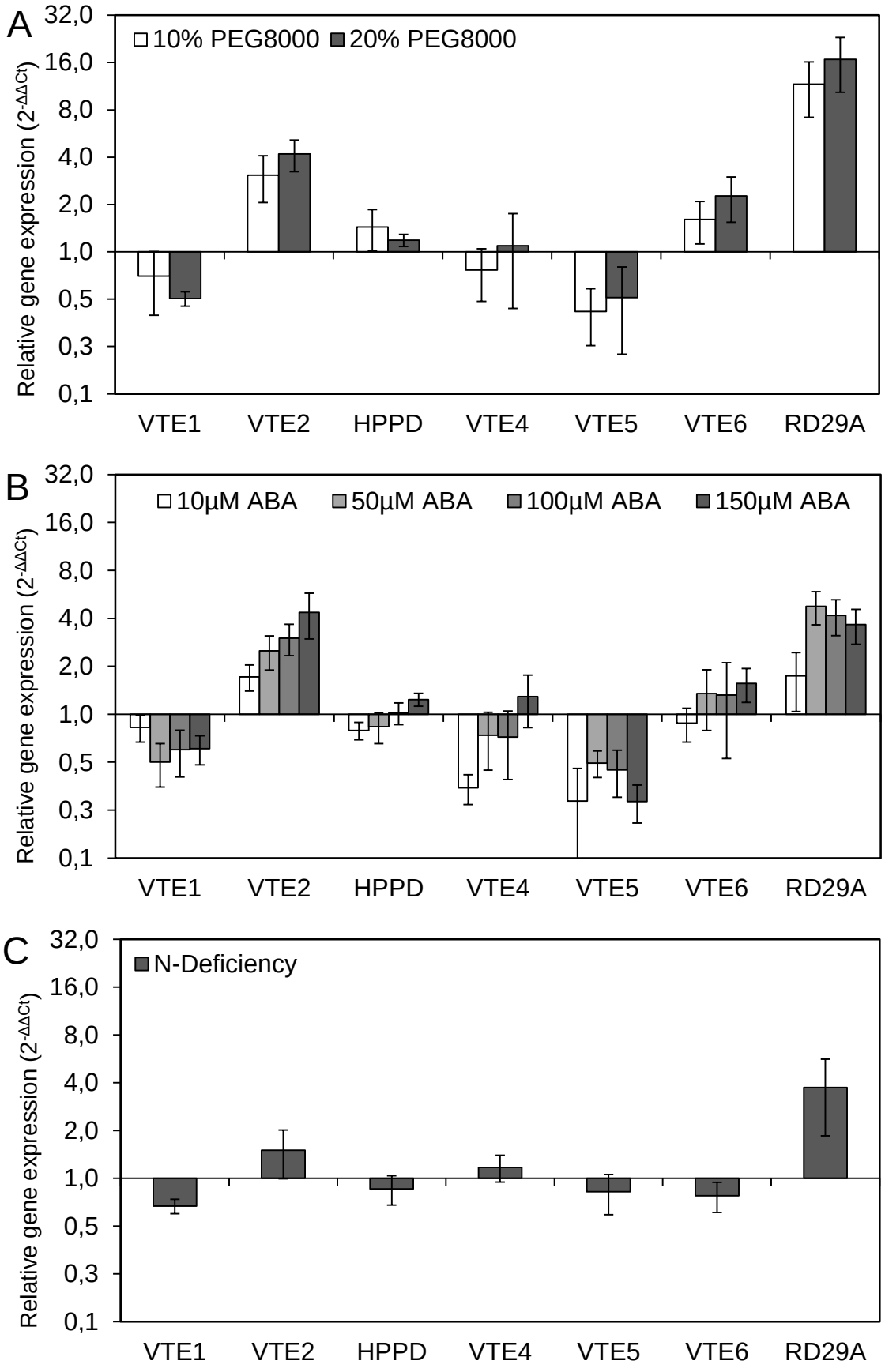
ABA application stimulated the expression of *VTE2* and *VTE6* in a concentration-dependent manner. Similarly, the expression of *RD29A* was increased in an ABA dependent way. The expression of *HPPD* and *VTE4* was induced only under the highest concentrations of ABA (150 µM) and rather decreased under low concentrations. In addition, the expression of *VTE1* and *VTE5* was decreased under ABA treatment. Taken together, tocopherol synthesis gene expression in response to PEG8000 and ABA treatment showed similar patterns, except for the higher induction of *HPPD* after PEG8000 treatment (Figure 21 B).

Nitrogen deficiency on the other hand shows a completely different pattern. Overall, the expression of none of the genes is strongly induced. The expression of *VTE2* and *VTE4* is slightly induced, but less than 2-fold. Remarkably, *RD29A* is induced by nitrogen deficiency, even though the

induction is relatively low in comparison to the induction by PEG8000 or ABA (Figure 21 C).

Under drought stress on soil, the *RD29A* expression is highest when comparing all five abiotic stress treatments. The expression is more than 16-fold higher than in well-watered plants. Under drought stress, ABA presumably accumulates to very high amounts resulting in activation of RD29A transcription. Drought stress also induces the expression of *VTE1*, *VTE2*, *HPPD* and *VTE6*. The increase in *VTE*2 and *VTE6* expression is similar as under PEG8000 or ABA treatment. But *HPPD* is much stronger induced than during the other abiotic stresses, and induction results in expression to a level comparable to VTE2 expression under drought. Withholding water also affects the transcription of *VTE1*, while *VTE5* again is down regulated (Figure 21 D).

The most outstanding gene expression pattern was obtained with plants exposed to high light. Contrary to the other abiotic stresses, high light induces expression of all tocopherol synthesis genes analyzed, while transcription of the *RD29A* gene is rather decreased. There are differences in the gene expression after 4 and 8 days of high light treatment. For example, *VTE2* is decreased after 4 days while strongly increased after 8 days. *HPPD* and *VTE4* are higher expressed after longer exposure to high light. The opposite trend was found for *VTE1*, *VTE5* and *VTE6*. The expression was also higher for all genes compared to the other stresses (Figure 21 E).


A
10% PEG8000
20% PEG8000
Relative gene expression (2-ΔΔCt)
32,0
16,0
8,0
4,0
2,0
1,0
0,5
0,3
0,1
VTE1
VTE2
HPPD
VTE4
VTE5
VTE6
RD29A
B
10µM ABA
50µM ABA
100µM ABA
150µM ABA
Relative gene expression (2-ΔΔCt)
C
N-Deficiency
Relative gene expression (2-ΔΔCt)

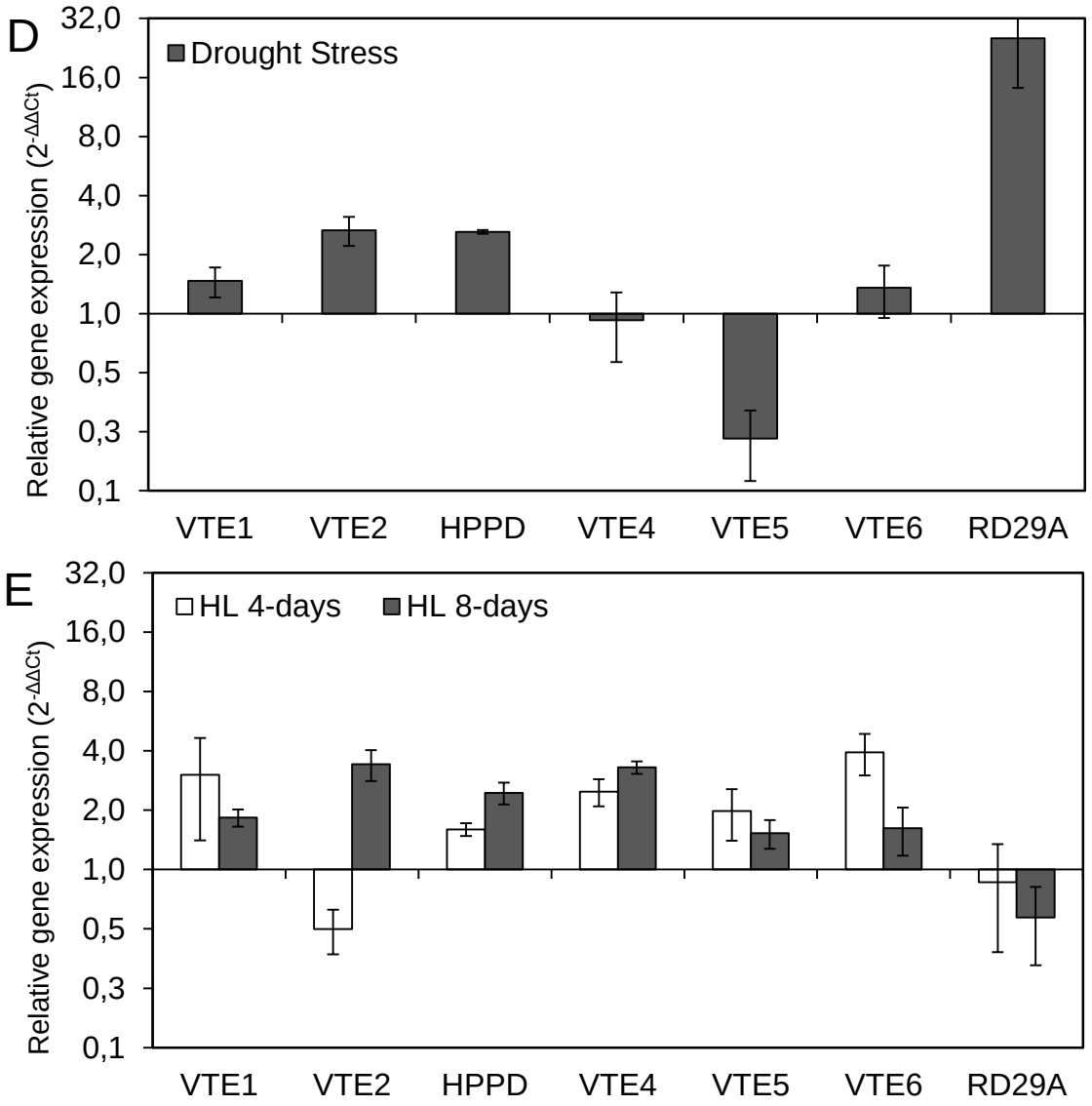


Figure 21: Relative gene expression of Arabidopsis Col-0 in response to abiotic stress

Gene expression of tocopherol biosynthesis genes and *RD29A* in leaves of Arabidopsis Col-0 after treatment with (A) PEG8000, (B), exogenous ABA, (C) nitrogen deficiency, (D) drought stress on soil and (D) high light. Results are presented as differential relative transcript abundance ($2^{-\Delta\Delta Ct}$), normalized to the expression of the reference gene *PP2A* and referred to the gene expression under the respective control conditions. Bars represent mean values ± SD of 3 biological replicates.

4.2.4 ABA Content under Abiotic Stress

The results from tocopherol quantification and qPCR analysis of tocopherol genes suggested that tocopherol biosynthesis is regulated in ABA-dependent

and ABA-independent ways during abiotic stress. To corroborate these findings, the ABA content was measured in the same samples.

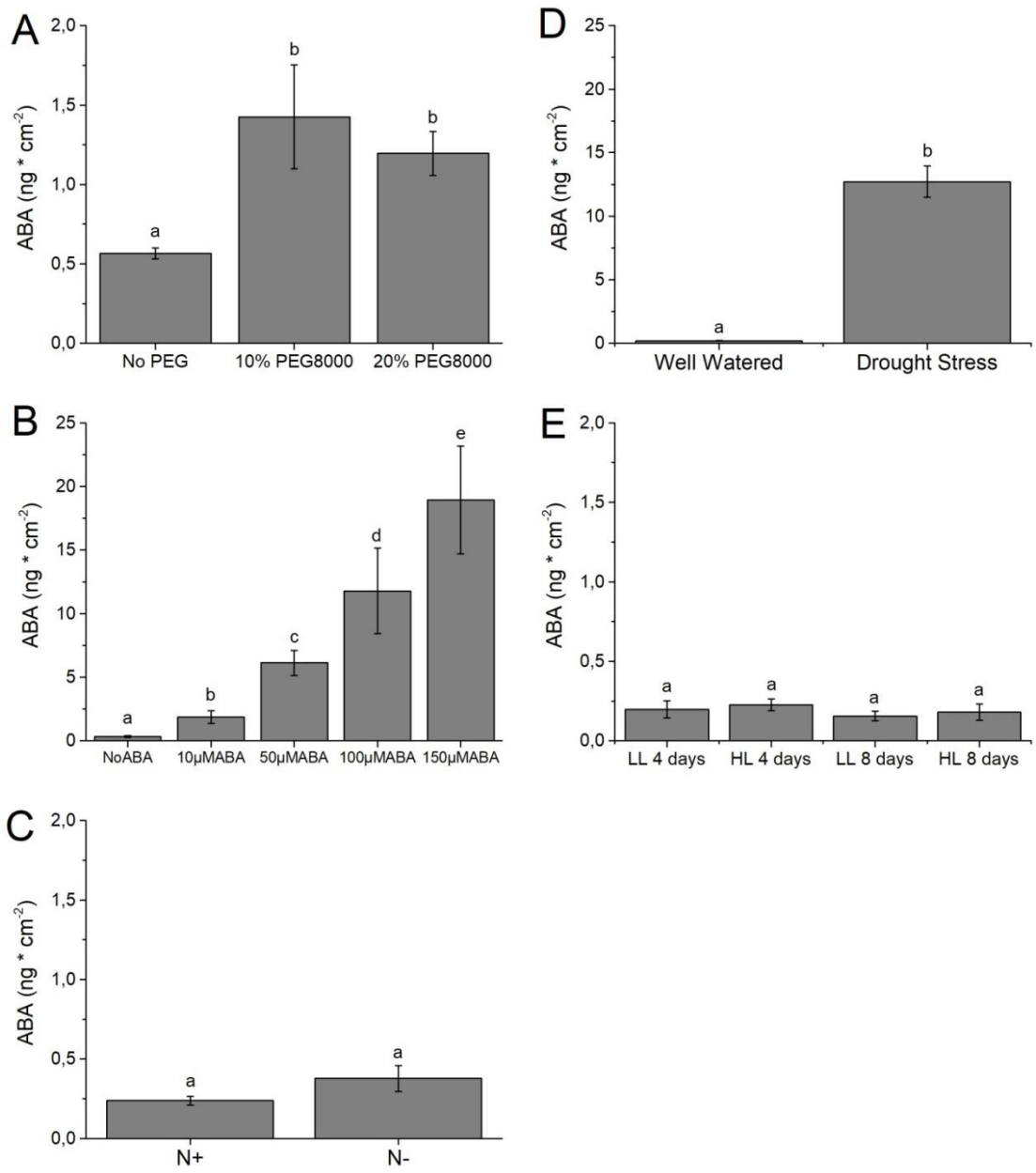


Figure 22: ABA content of Arabidopsis Col-0 in response to abiotic stress

Amount of ABA in leaves of Arabidopsis Col-0 after treatment with (A) PEG8000, (B) exogenous ABA, (C) nitrogen deficiency, (D) drought stress on soil and (E) high light. Bars represent mean values ± SD of 5 biological replicates. Different letters indicate significant differences between means at a significance level of 0.05 in One-Way-ANOVA (Fisher LSD).

Phytohormone measurements using LC-MS show that the ABA content increased after treatment with 10% or 20% PEG8000. When treated with 10%

PEG8000, the amount of ABA almost tripled in comparison to the control conditions. The ABA content was similarly increased with 20% PEG8000 (Figure 22 A). The ABA content increased in Col-0 leaves dependent on ABA concentration. The ABA content increased about 20-fold after exposure to 150 µM ABA (Figure 22 B). In contrast to the relative gene expression data during nitrogen deficiency (where the *RD29A* gene was slightly induced), the ABA content was not changed during nitrogen deprivation (Figure 22 C). Drought stress on soil after withholding water led to the strongest accumulation of ABA. After withholding water for 10 days, the ABA content was about 15-fold higher than in well-watered plants (Figure 22 D). In contrast, the high light treatment did not change the ABA content in Col-0 plants, in accordance with the transcript data of RD29A (Figure 22 E).

4.2.5 ABA Treatment of ABA-insensitive mutants

Tocopherol biosynthesis can presumably be induced in an ABA-dependent and -independent way, since the abiotic stress treatments tested so far induce a mixture of ABA-dependent and -independent pathways. Therefore, ABA-insensitive mutants were measured under the application of exogenous ABA.

4.2.5.1 Tocopherol Content in *abi* Mutants after ABA-Treatment

The ABA-insensitive mutants were grown for 9 days on medium containing no, 50 µM or 150 µM ABA. Wild type Col-0 accumulated tocopherol dependent on the ABA concentration in the medium. Also the other ecotypes Ler and Ws accumulated tocopherol after ABA application. In all *abi* mutants, tocopherol increased after exposure to ABA. But tocopherol accumulation was indeed compromised in two mutants, in *abi4-102* and *pyr1pyl2pyl4pyl5pyl8*. In the absence of ABA, plants of the two mutants and the Col-0 accumulated similar tocopherol amounts. But, especially in the *abi4-102* mutant, tocopherol increased to a much lower extent compared with wild type under the two ABA concentrations. The *pyr1pyl2pyl4pyl5pyl8* mutant still accumulated tocopherol under the two ABA concentrations, but the tocopherol amount was also compromised compared to the wild type (Figure 23). The other tested ABA-

insensitive mutants had total tocochromanol levels comparable to the ones of their corresponding wild types.

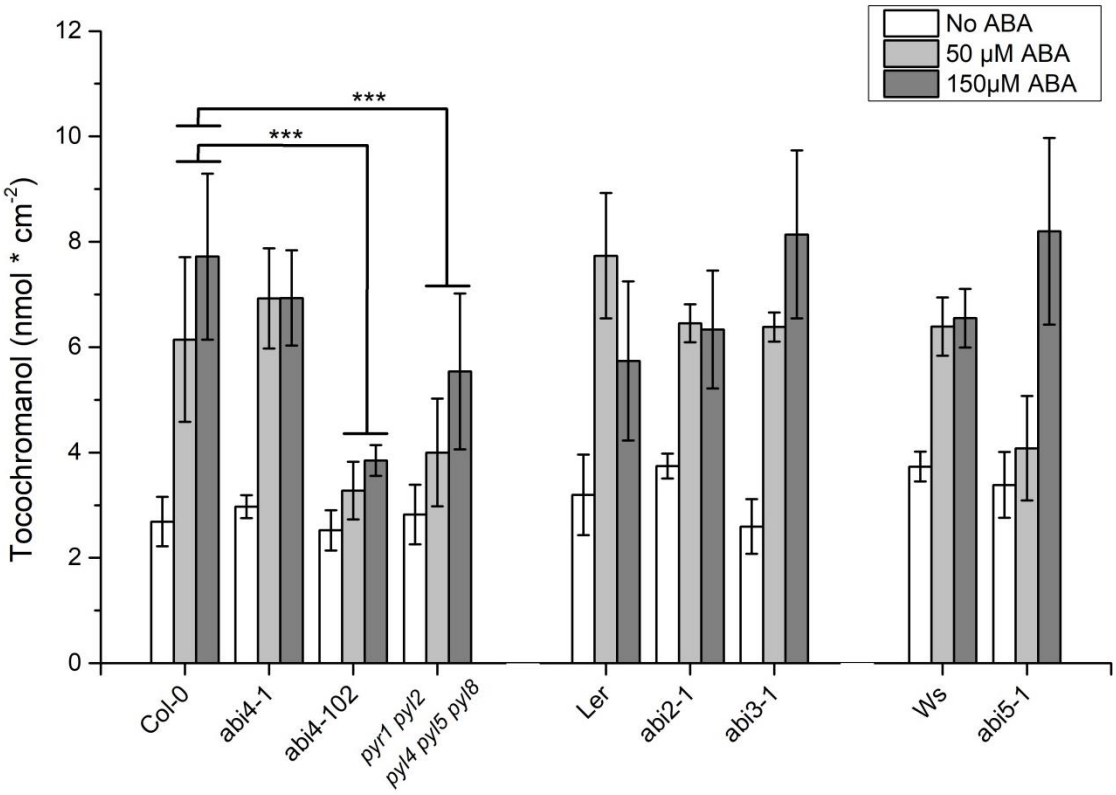


Figure 23: Tocochromanol accumulation of ABA-insensitive mutants after ABA treatment
Tocochromanol content in leaves of Arabidopsis ABA-insensitive mutants, with their corresponding wild types, grown on MS medium containing no, 50 µM or 150 µM ABA for 9 days. Bars represent mean values ± SD of at least 5 biological replicates (n=5-15). Asterisks indicate significant differences between means of the corresponding WT and the mutant under various ABA concentrations in One-Way-ANOVA (Fishers LSD; ***P<0.001).

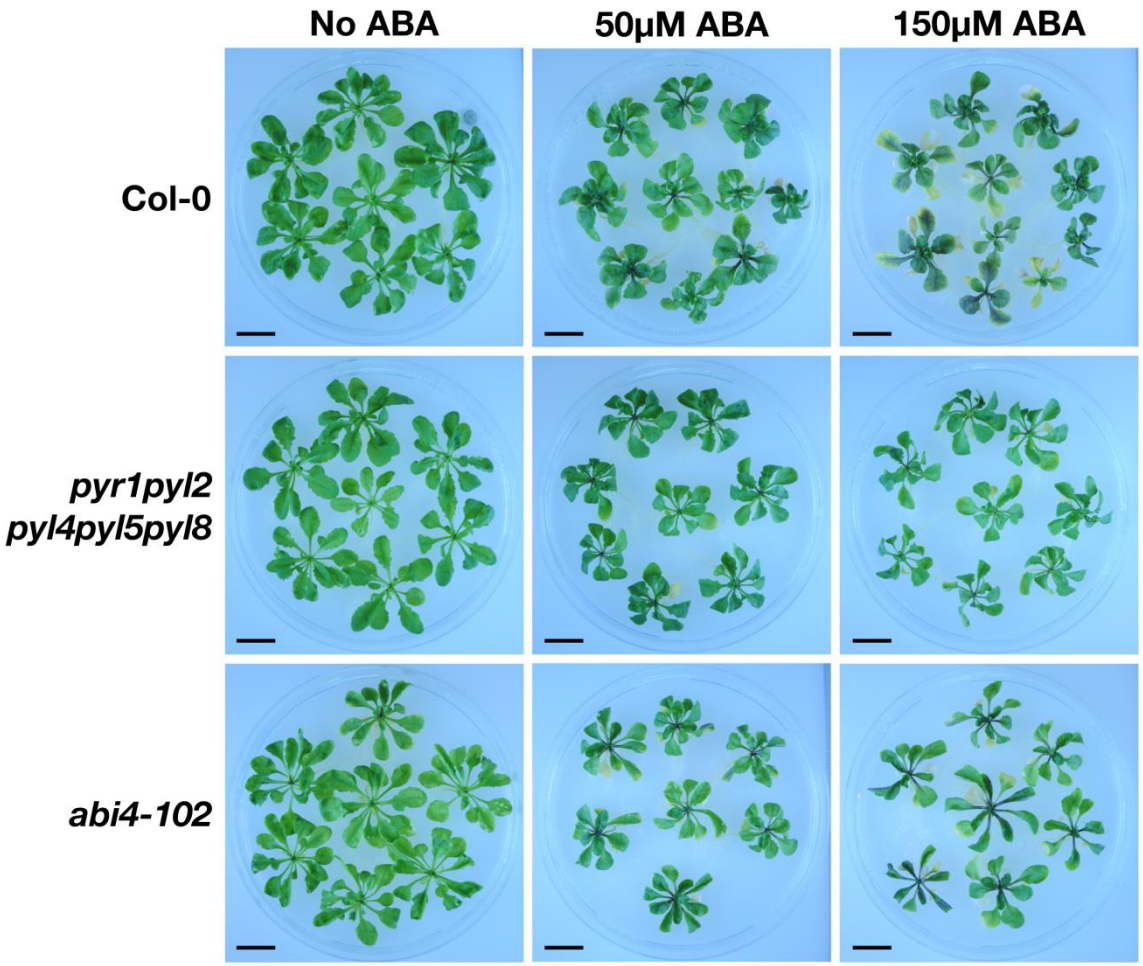


Figure 24: Arabidopsis Col-0 and ABA-insensitive mutants grown on synthetic medium with different concentrations of ABA

Photos of Arabidopsis Col-0, *pyr1pyl2pyl4pyl5pyl8* and *abi4-102* plants, grown on synthetic medium containing no, 50 µM or 150 µM ABA. Bars = 2 cm.

The two mutants, *abi4-102* and *pyr1pyl2pyl4pyl5pyl8*, also showed different morphological changes after ABA treatment. While Col-0 plants suffered strongly on medium containing 50 µM and 150 µM ABA, the plants of the *abi4-102* and *pyr1pyl2pyl4pyl5pyl8* mutants showed an altered response to exogenous ABA. Growth of Col-0 plants was retarded when grown under the two concentrations of ABA. Under 150 µM ABA, some leaves were yellow while others were dark green and purple, probably due to anthocyanin accumulation. The plants of the pentuple mutant *pyr1pyl2pyl4pyl5pyl8* also showed retarded growth in response to the two ABA concentrations. But leaves did not turn yellow or dark green. A similar morphology was observed for the *abi4-102* mutants. They also were inhibited in growth after ABA application, but did not

show such an obvious stress response compared to the wild type. But some of the leaves of the *abi4-102* mutants were curled after ABA treatment (Figure 24).

4.2.5.2 Relative Gene Expression in *abi* Mutants after ABA Treatment

After the tocopherol analyses revealed that the two mutants, *abi4-102* and *pyr1pyl2pyl4pyl5pyl8*, indeed have reduced amounts of tocopherol after ABA application, the gene expression of tocopherol genes was analyzed. To this end, samples were again harvested at the same time point as for tocopherol measurements to investigate the gene expression at the moment when plants are stressed. After 9 days of ABA application, the relative gene expression of selected tocopherol biosynthesis genes was measured by qPCR in the two mutants, *abi4-102* and *pyr1pyl2pyl4pyl5pyl8*.

In wild type Col-0, the expression of *VTE2* and *VTE6* was activated by ABA application dependent on the ABA concentration. Also the *RD29A* gene expression was strongly induced by ABA. The expression of *HPPD* and *VTE4* was only induced under the high concentration of 150 µM ABA, while the expression of *VTE1* and *VTE5* was decreased under the two concentrations of ABA (Figure 25 A).

In the *pyr1pyl2pyl4pyl5pyl8* mutant, the expression of *VTE2* and *VTE6* was induced by ABA as well. But while in the wild type the expression of VTE2 and VTE6 increased in an ABA-dependent manner, their expression in the pentuple mutant was lower under both ABA concentrations. The ABA marker gene *RD29A* was also much less induced and showed wider variation. On the other hand *VTE4* gene expression is strongly increased under the two ABA concentrations in the pentuple mutant, while in the wild type, VTE4 gene expression was only slightly induced by 150 µM ABA. Remarkably, the *VTE1* gene expression was also slightly increased in the *pyr1pyl2pyl4pyl5pyl8* mutant, while not induced in the wild type. Similar to the wild type the gene expression of *HPPD* and *VTE5* was also decreased in the pentuple mutant (Figure 25 B).

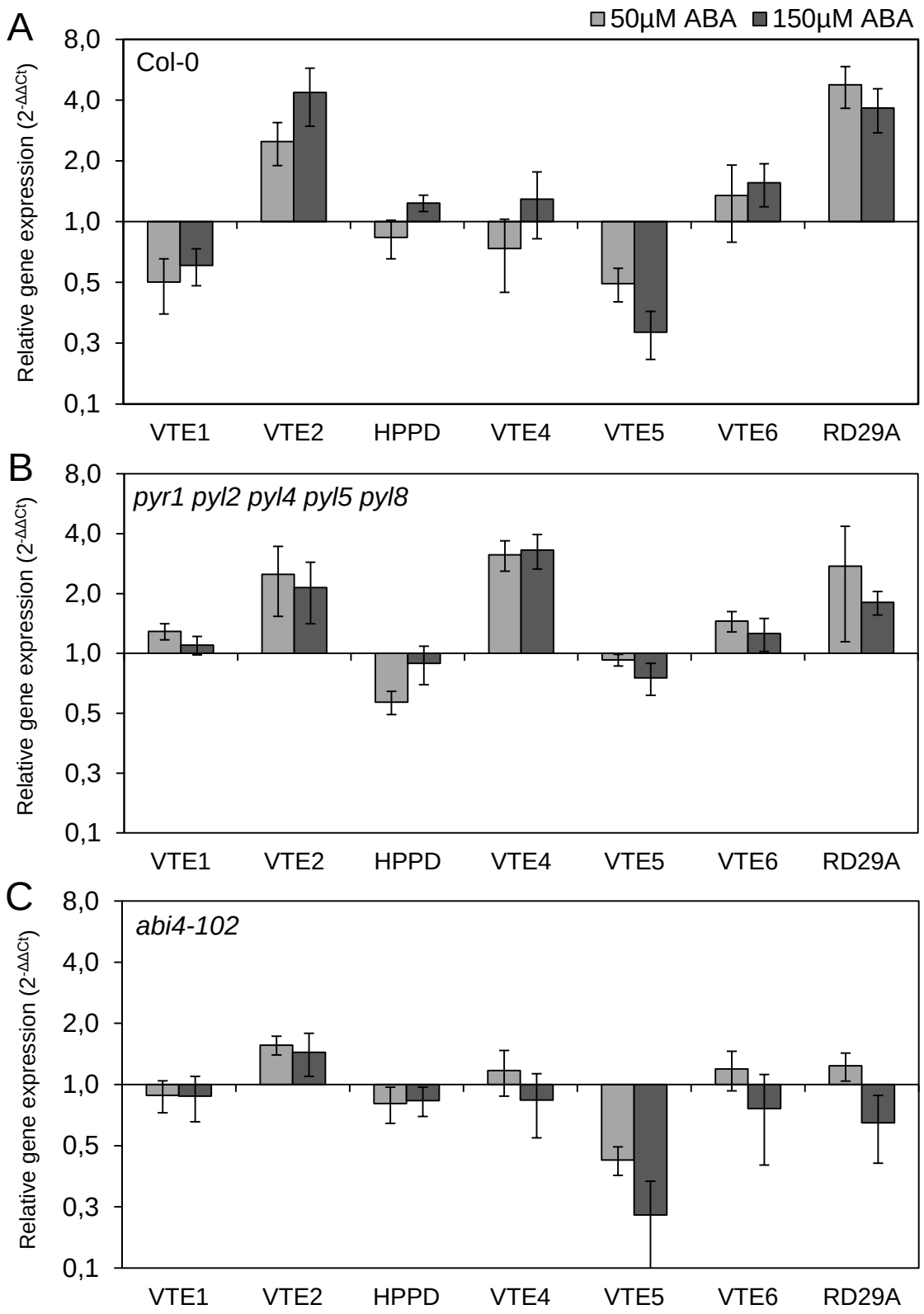


Figure 25: Relative gene expression of ABA-insensitive mutants after ABA Treatment

Gene expression of selected tocopherol biosynthesis genes and *RD29A* in leaves of the Arabidopsis Col-0 and two ABA-insensitive mutants, *abi4-102* and *pyr1pyl2pyl4pyl5pyl8*. The gene expression levels were determined by qPCR analysis. Results are presented as

differential relative transcript abundance ($2^{-\Delta\Delta Ct}$), normalized to the expression of the reference gene *PP2A* and referred to the gene expression under the respective control conditions. Bars represent mean values ± SD of 3 biological replicates.

The *abi4-102* mutant showed a completely different gene expression pattern than the wild type. None of the tested tocopherol biosynthesis genes was 2-fold higher or lower expressed in *abi4-102* after ABA treatment. The ratio of 2 fold is generally seen as a threshold for true differential expression. Expression of *VTE2*, *VTE6, VTE4* and RD29A was only marginally increased under 50 µM ABA. But with higher concentrations of 150 µM ABA, *RD29A* was rather down regulated. *VTE1*, *HPPD* and *VTE5* were downregulated by 50 µM or 150 µM ABA treatment (Figure 25 C).

5. DISCUSSION

Water deficit or other environmental stresses in plants lead to elevated production of reactive oxygen species (ROS) that can have injurious effects on photosystems and thylakoid membranes (Farooq *et al.*, 2009; Ahmad *et al.*, 2010; Lisar *et al.*, 2012). To limit damage, plants increase the production of antioxidants such as tocopherol (Miret & Munné-Bosch, 2015; Boubakri *et al.*, 2016). Additionally, abiotic stress also causes elevated levels of abscisic acid (ABA) (Shinozaki & Yamaguchi-Shinozaki, 2007). The decipherment of the regulatory network between ABA and tocopherol synthesis during stress in *Arabidopsis* may help to understand the role of tocopherol during environmental stresses. Therefore, abiotic stress treatments were used to increase tocopherol synthesis in Arabidopsis wild type and in ABA-deficient (*aba*) and ABA-insensitive (*abi*) mutant plants to address the basic questions whether ABA regulates this pathway in response to stress.

5.1 *abi* Mutants have Reduced Tocopherol levels under Osmotic Stress

In the majority of the investigated ABA-deficient and ABA-insensitive mutants, absolute tocochromanol contents and relative distributions of the tocopherol components were similar as in their corresponding wild types, when exposed to drought and osmotic stress (Figure 11 and Figure 12). Only the three mutant lines *abi4-1*, *abi4-102,* and *abi2-1* showed less accumulation of total tocochromanol under osmotic stress (PEG8000). In principle, one would have expected that tocopherol accumulation is compromised, if the signalling pathway between ABA and tocopherol biosynthesis is interrupted due to a mutation in the ABA biosynthesis or ABA signaling cascade, i.e. in all *aba* and *abi* mutant lines.

Different scenarios might explain why the ABA-deficient mutants do not show a decrease in total tocochromanol amount. One explanation could be that ABA itself, ABA precursors or conjugates in fact are not involved in tocopherol biosynthesis pathway. Alternatively, the remaining ABA amounts, which were measured in the *aba* mutants, are sufficient to induce tocopherol synthesis. Furthermore, other regulatory pathways might be involved in stimulating

tocopherol synthesis under drought. Since this is the first study on the regulation of tocopherol biosynthesis through ABA, there is no information about the amount of ABA that is necessary or sufficient to induce tocopherol synthesis. Even if the ABA levels are drastically reduced as in the *aba1-6* and *aba2-1* mutants (Figure 15), minor amounts of ABA which are still present may be sufficient to stimulate the ABA signaling cascade which in turn positively regulates tocopherol biosynthesis.

Only the *aba1-6* (N3772, Gly160Ser mutation) (Niyogi *et al.*, 1998; Barrero *et al.*, 2005) and *aba2-1* (N156, Ser264Asn) (Léon-Kloosterziel *et al.*, 1996; Cheng *et al.*, 2002) mutants showed a strong ABA reduction, while *aba3-2* (N158, Leu387Stop, Mo-cofactor sulfurylase with several paralogs) (Xiong *et al.*, 2001) showed a ~40% decrease, and the ABA content in *aba2-4* (N3835, Ala45Val, conserved mutation) (Laby *et al.*, 2000; Cheng *et al.*, 2002) and *aba3-1* (N157, Gly469Glu, ABA3 contains 819 aa) (Léon-Kloosterziel *et al.*, 1996; Xiong *et al.*, 2001) were not different from WT. The remaining ABA content in *aba* mutants could be derived from leaky mutations or paralogous enzymes catalyzing the same reactions. Alternatively, the residual amount of ABA in these lines might be explained by the existence of an independent pathway in Arabidopsis which is derived from farnesyldiphosphate instead of violaxanthin, and which has already been described in fungi (Inomata *et al.*, 2004; Barrero *et al.*, 2005; Hartung, 2010).

Three ABA-insensitive mutant plants, *abi4-1* (N8104, frameshift at aa157 resulting in early stop) (Finkelstein, 1994; Finkelstein *et al.*, 1998), *abi4-102* (N3837, Trp80Stop) and *abi2-1* (N23, Gly168Asp) (Koornneef *et al.*, 1984) have reduced amounts of tocopherol after PEG8000 treatment, indicating that there is a link between the ABA signaling and tocopherol biosynthesis. The fact that two *abi4* mutant alleles are among the tocopherol deficient plants represents strong evidence that the *ABI4* gene is involved in regulating tocopherol synthesis under PEG8000 treatment. *ABI4* encodes a transcription factor with an AP2 (APETALA2) domain (Finkelstein *et al.*, 1998). Whereas ABI4 is a unique protein, the AP2 domain is a DNA-binding domain which is highly conserved in 145 proteins of the AP2/ERF family in Arabidopsis. Members of

this family specifically bind to ABRE elements of the promoter of abiotic stress-responsive genes and regulate their expression (Sakuma *et al.*, 2002; Wind *et al.*, 2013). It is highly expressed in seeds and plays a role especially in seed maturation and seed germination (Finkelstein, 1994; Penfield *et al.*, 2006). Different alleles of the *ABI4* locus were investigated independently by several groups because of the *abi4* sugar- and glucose-insensitive phenotype (Finkelstein et al., 2002). Originally, the *sis5* (sugar insensitive) mutant alleles were identified because of their ability to develop relatively normal shoot systems on high concentrations of exogenous sugar, and they were then renamed *abi4-101*, *abi4-102*, *abi4-103* and *abi4-104* because they harbor mutations in the ABI4 locus (Laby *et al.*, 2000). Later it was shown that ABI4 also plays a role as a repressor of lipid breakdown in the embryo, and thereby, it regulates how endosperm oil reserves are used to fuel seedling establishment (Penfield *et al.*, 2006). But ABI4 was also found to act in the regulation of abiotic stress responses (Reeves *et al.*, 2011). In summary, ABI4 functions in several plant processes but was mainly investigated in seeds or developing seeds. Data on vegetative tissue is hardly available. Whether tocopherol biosynthesis genes are direct or indirect targets of ABI4 remains to be investigated.

The other allele that showed reduced tocopherol levels in response to PEG8000 treatment was *abi2-1*. This is a mutant of the *ABI2* gene in Arabidopsis, encoding a protein serine/threonine phosphatase 2C (PP2C). The *ABI2* gene was identified by the dominant mutation *abi2-1*, which led to pleiotropic changes including reduced responsiveness to ABA during seed germination and seedling growth, reduced seed dormancy and impaired stomatal regulation. Contrary to other ABA-insensitive mutants, *abi2* mutants do not show altered seed storage reserves, but mostly show vegetative growth alterations during ABA- or stress-regulated responses (Koornneef *et al.*, 1984; Finkelstein & Somerville, 1990; Assmann *et al.*, 2000) ABI2, and the homologous PP2C phosphatase ABI1, play partly redundant roles in controlling ABA responses, acting in a negative regulatory feedback loop (Merlot *et al.*, 2001).

Other than ABI4, which might have a direct or indirect effect on tocopherol biosynthesis, ABI2 can only have an indirect influence. ABI4 is a transcription factor that might activate other factors or build heterodimers, which then could bind to promoter sides of tocopherol biosynthesis genes. Or ABI4 directly binds to possible motifs of tocopherol biosynthetic genes for transcriptional activation. In 2013, Wind and coworkers reported specific DNA sequences to which the AP2 domain of ABI4 binds, either as a repressor or activator (Wind *et al.*, 2013). These promoter sequences to which ABI4 binds include the CE1 element CACC(G) and the partially overlapping G-box seuquence CACGT. It will be interesting to test whether these specific elements are found in the promoter regions of tocopherol biosynthesis genes (VTE1, VTE2, etc.), if they are actual targets of ABI4.

The effect of suppression of tocopherol increase due to the mutation in the *ABI2* gene can only be explained by a downstream signal. It is possible that the impairment of ABI2 activity results in the inactivation of other transcriptional factors, such as ABI4, thereby leading to reduced tocopherol synthesis. In the course of the tocopherol measurements, no differences for *abi3* or *abi5* mutants were detected. Therefore, even though ABI3 and ABI5 act together with ABI4 in the regulation of abiotic responses (Skubacz *et al.*, 2016), these two transcription factors seem to have no impact on tocopherol synthesis under the tested conditions.

The parallel impairment of five PYR/PYL/RCAR receptors in the *pyr1pyl2pyl4pyl5pyl8* mutant does not show an effect on tocopherol accumulation under PEG8000 stress, possibly because there are 14 homologues of these receptors in Arabidopsis (Gonzalez-Guzman *et al.*, 2012). Therefore, they are highly redundant and the combination of null mutations of “just” five genes is not sufficient to interrupt the signal transduction through ABA (Zhao *et al.*, 2018). Additionally, the ABA signaling cascade is a complex network with several backups and feedback loops. So there might also be other transcription factors or enzymes that play a role in forwarding the ABA signal to the tocopherol biosynthesis pathway, which have not been included in this work (Fujii & Zhu, 2009; Park *et al.*, 2009; Fujii *et al.*, 2011; Zhao *et al.*, 2016).

In response to nitrogen deficiency, all *aba* and *abi* mutants analyzed accumulated strongly elevated levels of total tocochromanol relative to their respective unstressed controls, similar as their corresponding wild types. The two mutants, *aba2-4* and *pyr1pyl2pyl4pyl5pyl8*, had even more tocochromanol after growth on nitrogen-depleted medium (Figure 13). Unlike during PEG8000 treatment, the chlorophyll breakdown and the subsequent synthesis of tocopherol during nitrogen deficiency is probably ABA-independent, because the ABA content did not change very much (Figure 22). None of the analyzed mutants showed a reduction of tocopherol in response to nitrogen depletion. It was previously shown that phytol is a byproduct of chlorophyll degradation and is incorporated into tocopherol (Ischebeck et al., 2006; vom Dorp et al., 2015). This response seems to be independent from the regulation by ABA.

Similarly, the tocopherol content in the seeds of all mutant lines was as high as in the wild type controls (Figure 14), leading to the conclusion that tocopherol synthesis during seed maturation is also probably ABA-independent.

5.2 Abiotic Stress Increases Tocopherol Levels in Wild Type Plants

Arabidopsis Col-0 plants were grown under five different abiotic stresses: (i) PEG8000-induced osmotic stress, (ii) exogenous application of ABA, (iii) nitrogen-depleted medium, (iv) drought stress on soil, and (v) high light treatment. All abiotic stress treatments caused the accumulation of total tocochromanol (Figure 18), while the percent distribution of α-, β-, γ-, δ-tocopherol and plastochromanol (PC-8), showed only minor changes (Figure 19). These results are consistent with prior studies showing that tocopherol levels are elevated in response to drought (Munné-Bosch & Alegre, 2003; Liu *et al.*, 2008; Fleta-Soriano & Munné-Bosch, 2017), high light (Collakova & DellaPenna, 2003a; Kobayashi & DellaPenna, 2008) and even low-temperature (Maeda *et al.*, 2006) or salt stress (Ellouzi *et al.*, 2013) in several plant species at various developmental stages.

Plants grown under control conditions in tissue culture have higher tocopherol contents than plants grown on soil, because the medium for control conditions already has a water potential of -0.4 MPa (Table 2). This is because the medium is solid due to the presence of agarose, and water availability is

reduced. Nevertheless, the elevated levels of tocochromanol produced during abiotic stress in plants growing in tissue culture are due to the upregulation of tocopherol pathway-related enzymes (Lushchak & Semchuk, 2012; Mène-Saffrané, 2017). The increase in total tocochromanol levels was paralleled by an increase in α-tocopherol levels in response to all abiotic stresses. The α-tocopherol levels in plants grown on synthetic medium showed little change during stress treatment (PEG8000, ABA and nitrogen deprivation), but under drought and high light stress the proportions of α-tocopherol declined. However, the levels of other tocopherols increased in stressed Col-0 plants after all conducted abiotic stresses, suggesting that steps of the tocopherol biosynthesis might limit α-tocopherol accumulation during stress. The presence of β- and δ-tocopherol in stressed wild type leaves suggests that the methyltransferase VTE3, which methylates MPBQ to the α-tocopherol precursor DMPBQ (Cheng *et al.*, 2003) might limit α-tocopherol synthesis (Figure 2). High levels of γ-tocopherol were also detected in stressed Col-0 plants, suggesting that VTE4 is also limiting for α-tocopherol synthesis. VTE4 catalyzes the last methylation step of γ- and δ- tocopherol to α- and β-tocopherol, respectively (Shintani & DellaPenna, 1998; Bergmüller *et al.*, 2003) (Figure 2).

Furthermore, a decrease in chlorophyll levels was observed in wild type leaves during drought stress and nitrogen deficiency, while tocochromanol levels increased (Figure 18 and Figure 20). A correlation between chlorophyll degradation and tocopherol accumulation during leaf senescence has been reported previously (Rise *et al.*, 1989). Chlorophyll and phytol are synthesized within the chloroplasts, and phytol released from chlorophyll is used for tocopherol synthesis. Later it was shown that phytol, released during chlorophyll breakdown, is phosphorylated by a phytol kinase to phytylmonophosphate (PMP) in Arabidopsis. This phytol kinase was named VTE5 after the characterization of the *vte5* mutant, which shows an 80% reduction in total seed tocopherols and 65% reduction in leaf tocopherols (Valentin *et al.*, 2006). The PMP produced by VTE5 is subsequently again phosphorylated to phythyldiphosphate (PDP) by a phytylphosphate kinase, designated VTE6. Mutations of the *VTE6* gene caused a severe tocopherol deficiency in leaves

(by 98-100% compared to the wild type) coupled with dramatic growth retardation (vom Dorp *et al.*, 2015). Together VTE5 and VTE6 provide an alternative pathway for the production of PDP (Figure 2), the precursor for the phytyl moiety in tocopherol synthesis.

Contrary to drought stress and nitrogen deficiency, high light treatment only caused a minor decrease in chlorophyll content after 8 days, when tocopherol levels even exceeded the ones from drought stress. Application of exogenous ABA does not cause any changes in chlorophyll level, but tocopherols accumulated. Surprisingly the amount of chlorophyll slightly increased under PEG8000 treatment, incoherent with an increase in tocopherols (Figure 20).

5.3 Tocopherol Synthesis is Induced in ABA-Dependent and -Independent Ways

Biochemical reactions leading to tocopherol biosynthesis and the genes encoding the corresponding enzymes have been identified in several studies, whilst much less is known about regulatory genes and mechanisms controlling tocopherol synthesis. Several times it has been suggested that ABA may stimulate tocopherol synthesis. One study on vitamin E biosynthesis genes in rice reported the presence of ABA-responsive elements (ABREs) in the promoter regions of *OsHPPD*, *OsγTMT* and *OsMPBQMT1.* Increased transcript abundances of these genes were found during dehydration stress (Chaudhary & Khurana, 2009). Moreover, a positive correlation between ABA and tocochromanol contents were shown, at least in part, during drought exposure (Fleta-Soriano & Munné-Bosch, 2017). The only molecular evidence of such a relationship so far has been provided by Ghassemian and colleagues using microarray and GC/MS analyses. ABA-treated Arabidopsis seedlings revealed slightly increased α-tocopherol contents when compared to untreated controls. Furthermore the transcriptional abundances of the tocopherol biosynthesis genes *HPPD*, *VTE2*, *VTE1* and *VTE4* were increased after 3, 6, 12 and 24 h of moderate ABA treatment (Ghassemian *et al.*, 2008).

Based on this knowledge, the relative gene expression of selected tocopherol biosynthesis genes, along with the ABA-response marker gene

RD29A (Yamaguchi-Shinozaki & Shinozaki, 1994) were analyzed, under various abiotic stresses. When applying only exogenous ABA, the expression of *VTE2* and *VTE6* was increased relative to the non-treated control (Figure 21 B). Therefore, *VTE2* and *VTE6* are definitely ABA-dependent among all analyzed tocopherol genes. This fits only partly to the results from the previous transcript study of Ghassemian and coworkers. In both studies transcript levels of *VTE2* are ABA-dependently induced. *VTE2* encodes homogentisate phytyltransferase which catalyzes the committed condensation of HGA and PDP (Collakova & DellaPenna, 2003a). Increased availability of these substrates as well as up-regulation of *VTE2* transcription are likely to contribute to the elevated tocopherol levels under stress. *VTE6* encodes phytylphosphate kinase, probably contributing to enhanced production of the substrate PDP for *VTE2* activity. Ghassemian et al. measured the short term response (several hours) of tocopherol gene expression after ABA treatment. In their study, the genes *VTE1*, *HPPD* and *VTE4* were induced. *VTE6* was not measured. In the present study, gene expression was measured after several days of ABA treatment. Under these conditions, the transcription of *VTE1* was not increased, and that of *HPPD* and *VTE4* were weakly induced and only when very high amounts of ABA are applied. These discrepancies in transcriptional response, obtained by Ghassemian et al. and the present study, are explainable by the alterations in the experimental set-ups, because the analyses were done at different plant ages and with different ABA concentrations.

A similar pattern of gene expression was observed after PEG8000 treatment. Additionally to the increased expression of *VTE2* and *VTE6*, the *HPPD* gene was slightly induced when plants were treated with PEG8000. Also the transcript levels of *RD29A* were strongly raised (Figure 21 A). While under ABA treatment the induction of *RD29A* was observed as expected, the RD29A induction by PEG8000 treatment indicates that ABA increased and is involved in osmotic stress response, or that RD29A expression can also be partially induced in an ABA-independent manner.

Even though the *VTE2* and *VTE4* gene expression was slightly increased under nitrogen deprived conditions, the overall expression showed that most

tocopherol genes were not upregulated (Figure 21 C). These findings are in agreement with the scenario that free phytol from the breakdown of chlorophyll is integrated into tocopherol, but this process is not necessarily regulated by ABA during nitrogen deprivation. The low induction of the *RD29A* gene expression might be caused by the fact that the promoter of *RD29A* contains also *cis*-acting dehydration-responsive elements involved in ABA-independent response to abiotic stress such as chlorotic stress/nitrogen deprivation (Yamaguchi-Shinozaki & Shinozaki, 1994).

Drought stress on soil on the other hand activates tocopherol biosynthesis by increased expression of *VTE1*, *VTE2*, *HPPD* and *VTE6* (Figure 21 D). In previous studies, it was already quoted that ABA is produced under water deficit conditions and plays a pivotal role in response to drought stress (Shinozaki & Yamaguchi-Shinozaki, 2007). As described above, *VTE2* and *VTE6* are activated by ABA treatment and therefore, the induction of *VTE2* and *VTE6* expression might be mostly mediated by the ABA increase during drought stress. On the other hand, *VTE1* and *HPPD* are stress-inducible genes that can be activated without ABA.

A completely different gene expression pattern was detected for plants treated with high light. Here, all selected tocopherol synthesis genes, *VTE1*, *VTE2, HPPD, VTE4, VTE5* and *VTE6*, are activated while the *RD29A* gene expression is decreased (Figure 21 E). This leads to the assumption that the transcription of the tocopherol biosynthesis genes, *VTE2* and *VTE6,* can also be activated without ABA. These results are the first indication that tocopherol synthesis is regulated by ABA-dependent and -independent pathways.

These results could be confirmed with the measurement of ABA levels in the five abiotic stress treatment of Arabidopsis Col-0 (Figure 22). The ABA content of Col-0 plants in response to exogenous ABA application increased with ascending concentrations. ABA is absorbed by the roots from the medium and distributed throughout the plant. Exogenous ABA probably induced the expression of ABA biosynthetic enzymes through the regulatory feedforward pathway (Merlot *et al.*, 2001; Xiong *et al.*, 2002a; Xiong & Zhu, 2003), leading to additional production of endogenous ABA. Drought stress strongly induced ABA

biosynthesis in Arabidopsis Col-0 plants, resulting in a strong increase in ABA level after 10 days of withholding water. These results are consistent with previous studies, showing the activation of ABA biosynthesis through environmental conditions, such as drought (Xiong *et al.*, 2001, 2002a; Iuchi *et al.*, 2001). On the other hand osmotic stress imposed by two concentrations of PEG8000 induced only a slight increase of ABA amounts, indicating that also other ABA-independent pathways induce stress response in these conditions.

Together the ABA levels and qPCR results obtained from PEG8000-induced stress and drought stress on soil additionally explain why the treatment of the *aba* and *abi* mutants led to strong tocochromanol accumulations. Drought stress stimulates a complex pattern of ABA-dependent and -independent responses. The results of the high light experiment clearly show that tocopherol synthesis under stress can also be upregulated in an ABA-independent way. In the ABA-deficient mutants, tocochromanol can still increase during PEG8000 and drought stress, because even if ABA is not involved in mediating the tocopherol increase in *aba* mutants, other pathways can lead to the stimulation of tocopherol synthesis. ABA-insensitive mutants are likewise complemented by ABA-independent pathways, which can explain the increase in total tocochromanol in the *abi4-1*, *abi4-102* and *abi2-1* mutants under PEG8000 treatment. Stress application by PEG8000 showed a similar gene expression as exposure to exogenous ABA. Osmotic stress imposed by PEG8000 also leads to ABA-dependent and –independent responses. In response to nitrogen deficiency and high light treatment the ABA content in Col-0 plants was unchanged, which is in agreement with the gene expression patterns observed. These findings indicate that the regulation of tocopherol biosynthesis is probably mediated in ABA-dependent or -independent pathways. Multifaceted possibilities might explain small residual amounts of ABA content in *aba* or *abi* mutants for an ABA-dependent tocopherol regulation such as leaky mutations, parallel biosynthetic pathways or other non-mutated genes within a gene family, especially in *abi* mutants. During abiotic stresses in which ABA content does not increase or increases only slightly such as PEG8000, N- or high light, an ABA-independent regulatory pathway of tocopherol seems to play an important role.

On the other side when ABA content increases significantly such as during exogenous ABA application or drought stress on soil, ABA should play a predominant role in tocopherol regulation.

A former study demonstrated that the treatment of cannabis plants with gibberellic acid led to increased amounts of α-tocopherol in leaves (Mansouri *et al.*, 2009). Moreover, the seeds of the two Arabidopsis salicylic acid-deficient mutants *NahG* and *sid2* contained more α- and γ-tocopherol than the wild type (Abreu & Munne-Bosch, 2009). Jasmonic acid has been shown to be involved in the regulation of tyrosine aminotransferase (TAT), which catalyzes the transamination from tyrosine to p-hydroxyphenylpyruvate (HPP), a precursor for tocopherol biosynthesis (Figure 2) (Sandorf & Holländer-Czytko, 2002). Additionally it has been suggested that because of the antioxidant activity of α-tocopherol during lipid peroxidation, the formation of secondary oxidation products including jasmonic acid is altered. Consequently, α-tocopherol synthesis could be regulated by the interplay of lipid peroxidation and jasmonic acid contents (Munné-Bosch & Alegre, 2002; Munné-Bosch *et al.*, 2007). In principal it is possible that multiple phytohormones might mediate tocopherol biosynthesis by additive, synergistic or antagonistic activities.

5.4 Two *abi* Mutants are Defect in ABA Signal Transmission

Application of exogenous ABA triggers only the ABA-dependent regulation mechanisms in Arabidopsis that may regulate tocopherol biosynthesis. Thus, mutants containing less tocopherol can be expected to be still capable of ABA signal transduction. However, *abi* mutants deficient in ABA signaling might show alterations in ABA-dependent pathways upon ABA treatment. After the treatment of *abi* mutants with no, 50 µM or 150 µM ABA, two mutants showed less tocochromanol accumulation, compared with the corresponding wild type (Figure 23).

One was the pentuple *pyr1pyl2pyl4pyl5pyl8* mutant, which showed reduced tocochromanol accumulation under the two ABA concentrations. The five mutations are in receptor genes from the PYR/PYL/RCAR family, whose proteins can directly bind ABA (Figure 5) (Gonzalez-Guzman *et al.*, 2012). With 14 members in Arabidopsis, these receptors are highly redundant. Even though

only 5 out of 14 receptors are impaired in the *pyr1pyl2pyl4pyl5pyl8* mutant, the morphological response to the ABA treatment is inhibited, apparently due to the lack in the transmission of the ABA signal (Figure 24). The gene expression analyzed with qPCR underpins these results (Figure 25 B). In the *pyr1pyl2pyl4pyl5pyl8* mutant, *VTE2* and *VTE6* are less strongly expressed than in the wild type. Also the expression of *RD29A* is compromised. Thus the ABA signal transduction is impaired in these mutants. That *VTE2* and *VTE6* are still induced to a certain level might be because of the redundancy of the PYR/PYL/RCAR receptors. Furthermore, *VTE4* is strongly induced in the pentuple mutant, contrary to the wild type. This phenomenon is difficult to explain. Enhanced *VTE4* transcription does only partially affect tocochromanol composition because the proportion of α-tocopherol was increased, but not the proportion of β-tocopherol. The *VTE1* transcription was also induced in the pentuple mutant, while it was not in the Col-0 plants, reflected by an increase in γ-tocopherol in the mutants. An impairment of the PYR/PYL/RCAR receptors might lead to the activation of other regulatory mechanisms, which subsequently start the induction of tocopherol genes, such as *VTE4* or *VTE1*. Even though *VTE1* and *VTE4* are important for tocopherol synthesis, their activities are not limiting in leaves, because it was shown before that *VTE2* performs the committed step in tocopherol synthesis, which is also the (rate-limiting) bottleneck of the pathway (Collakova & DellaPenna, 2003a). The transcription of *RD29A* was still slightly increased in the *pyr1pyl2pyl4pyl5pyl8* mutant. RD29A is induced via its ABRE promoter motifs from AREB/ABF transcription factors, which are activated through SnRK2s (Figure 5). This means that the channel for activation is still open in the pentuple mutant for some remaining activity (Figure 25 B).

The second mutant, *abi4-102*, that accumulated less tocopherol under ABA treatment was also one of the mutants that showed decreased tocopherol levels under PEG8000 treatment. *abi4-102* had less tocopherol under both applied ABA concentrations (Figure 23) and showed also an altered morphological response when compared to the Col-0 wild type (Figure 24). The results obtained by qPCR are consistent with the reduced tocopherol levels.

Most striking was that the *RD29A* gene in *abi4-102* was only marginally activated under 50 µM ABA but not under 150 µM ABA. RD29A expression is not directly affected by ABI4 (Figure 5), but is maybe interrupted by a negative feedback provoked by the defect of *ABI4*. Also the transcription level of *VTE2*, *VTE4* and *VTE6* was not above the level in the control conditions. This is in accordance with the finding that *abi4-102* mutants contain the same amounts of tocopherol under non-stressed conditions as the wild type and almost the same amounts as after ABA application (Figure 25 C). The *abi4-102* mutant appears to be a strong *abi4* mutant allele making it an interesting candidate for further investigations of the regulatory relation between ABA signaling and tocopherol biosynthesis.

Collectively, results from molecular and biochemical analyses indicate that there might be two mechanisms in Arabidopsis that contribute to the regulation of tocopherol biosynthesis, one ABA-dependent and the other ABA-independent.

6. SUMMARY

Abiotic stresses in plants lead to elevated levels of reactive oxygen species that can have detrimental effects on photosystems and thylakoid lipids. To limit damage, plants increase the production of antioxidants such as tocopherol (vitamin E). While tocopherol biosynthesis has been investigated in detail, only little is known about the regulation of this pathway. Previously, it has been suggested that the phytohormone abscisic acid (ABA), which is involved in abiotic stress responses, may be a key regulator of tocopherol biosynthesis.

To decipher the regulatory network between ABA and tocopherol biosynthesis in Arabidopsis, several ABA-deficient (*aba*) and ABA-insensitive (*abi*) mutants were exposed to (i) osmotic stress simulated by PEG8000 containing medium, (ii) drought stress on soil, and (iii) nitrogen deprived medium. Under all stress conditions the tocopherol content in the mutants increased similarly as in the wild type, except for *abi4-1*, *abi4-102* and *abi2-1* under osmotic stress. However, tocopherol synthesis was still upregulated in several *aba* or *abi* mutants. Therefore, Arabidopsis wild type plants were exposed to two additional abiotic stresses, i.e. (iv) ABA treatment and (v) high light exposure. Under all five stresses, tocopherol was increased. Transcript analyses via qPCR supported these findings showing an up-regulation of tocopherol biosynthesis genes under all conditions. LC-MS measurements revealed that ABA content increased in response to ABA treatment, drought stress and osmotic stress, but not during high light and nitrogen deprivation. This indicates that an ABA-independent pathway for regulating tocopherol biosynthesis must exist. Treatment of *abi* mutants with exogenous ABA showed a less pronounced increase in tocopherol content for the *abi4-102* and *pyl1pyr2pyr4pyr5pyr8* mutants. The expression of tocopherol biosynthesis genes is also not altered in *pyl1pyr2pyr4pyr5pyr8* and especially in *abi4-102*. These results support the scenario that tocopherol synthesis is regulated via an ABA-dependent and an ABA-independent pathway, and that the ABA receptor proteins PYL1PYR2PYR4PYR5PYR8 and the AP2 domain transcription factor ABI4 play important roles during ABA dependent tocopherol synthesis.

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