Cysteine catabolism and glucosinolate turnover in *Arabidopsis thaliana*

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Abstract

Sulfur is an important chemical element in plants. It is taken up in form of sulfate and assimilation takes place in the cytosol, mitochondria and chloroplasts. The result of this process is, among others, the amino acid cysteine and the secondary metabolite glucosinolate. Under abiotic stress conditions such as low light or extended darkness, the plant has to catabolize amino acids and other metabolites to gain nutrients and energy. Cysteine can be catabolized via three pathways to pyruvate and persulfide. The first reaction step of the mitochondrial pathway, in which glutathione persulfide is oxidized to sulfite by the enzyme ETHE1, was yet unknown. Within this thesis, this reaction step was identified by using isolated mitochondria and recombinantly expressed enzymes (Chapter 4.1). This step requires a mitochondrial aminotransferase, which transaminates L-cysteine to yield 3-mercapto-pyruvate. The pathway continues including three further steps, catalyzed by the enzymes Str1 and ETHE1, to produce pyruvate and thiosulfate as products. An early senescent phenotype and an altered amino acid profile in leaves under light limiting conditions have been shown before in the *ethe1-1* mutant and were confirmed, in the course of this thesis, for the *ethe1-1/str1-1* double mutant. Furthermore, the delay in seed development was increased under extended darkness and mitochondrial structure as well as the amino acid metabolism were altered in the single mutant (Chapter 4.2). Due to these alterations, the breakdown of cysteine via ETHE1 is proposed to play an important role during light- and carbon limiting conditions to gain energy. Extended darkness conditions also influence glucosinolate metabolism in *Arabidopsis thaliana*: The leaf glucosinolate content is decreased and the activities and abundances of the enzymes myrosinase and nitrilase are increased (Chapter 4.3). These findings show that glucosinolates are turned over under carbon starvation conditions and can possibly act as alternative respiratory substrates, like shown for cysteine. This is remarkable, because the breakdown products of glucosinolates are referred to be important active substances. In addition, new insights into the regulation of glucosinolate metabolism in *Arabidopsis thaliana* were gained in the frame of this thesis. This includes developmental changes in leaf myrosinase activity under different growth conditions, developmentally altered protein abundance of the myrosinases TGG1 and TGG2 as well as a circadian rhythm for leaf myrosinase activity in two week old plants.

Keywords: Sulfur-containing compounds, cysteine catabolism, glucosinolate turnover

Zusammenfassung

Schwefel ist ein wichtiges chemisches Element in Pflanzen. Es wird in Form von Sulfat aufgenommen und im Cytosol, in den Mitochondrien und in den Chloroplasten assimiliert. Als Endprodukte entstehen dabei unter anderem die Aminosäure Cystein und das Sekundärmetabolit Glucosinolat. Unter abiotischen Stressbedingungen, wie z.B. Lichtmangel oder verlängerten Dunkelphasen, muss die Pflanze Aminosäuren und andere Metabolite abbauen um Baustoffe und Energie zu gewinnen. Cystein kann über drei Wege zu Pyruvat und Persulfid abgebaut werden. Im mitochondrialen Weg, in dem Glutathion-Persulfid von dem Enzym ETHE1 zu Sulfit oxidiert wird, war der erste Reaktionsschritt bisher unbekannt. Dieser wird in der vorliegenden Arbeit mit Hilfe von isolierten Mitochondrien und rekombinant exprimierten Enzymen nachgewiesen (Kapitel 4.1). Eine mitochondriale Aminotransferase transaminiert L-Cystein zu 3-Mercaptopyruvat. In den folgenden drei Schritten, welche mit Hilfe der Enzyme Str1 und ETHE1 katalysiert werden, entstehen Pyruvat und Thiosulfat. Für die *ethe1-1* Mutante wurden bereits zuvor unter Lichtmangel ein Phänotyp mit früher Seneszenz und ein verändertes Aminosäureprofil in Blättern gezeigt. Dies konnte für die *ethe1-1*/*str1-1* Doppelmutante im Rahmen dieser Arbeit ebenfalls bestätigt werden. Darüber hinaus ist die Samenentwicklung bei verlängerten Dunkelphasen verzögert und die mitochondriale Struktur sowie der Aminosäure-Metabolismus in der Einzelmutante verändert (Kapitel 4.2). Aufgrund dieser Veränderungen besitzt der Abbau von Cystein über ETHE1 vor allem bei limitierenden Licht- und Kohlenstoff-Bedingungen eine wichtige Rolle um Energie zu gewinnen. Weiterhin beeinflussen verlängerte Dunkelphasen auch den Glucosinolat-Metabolismus in *Arabidopsis thaliana*: Der Glucosinolatgehalt in Blättern wird verringert und die Aktivitäten und Abundanzen der Enzyme Myrosinase und Nitrilase, die am Glucosinolat-Katabolismus beteiligt sind, sind erhöht (Kapitel 4.3). Diese Ergebnisse zeigen, dass Glucosinolate unter Kohlenhydratmangel umgesetzt, und, wie für Cystein gezeigt, eventuell als alternative respiratorische Substrate verwendet werden. Dies ist auch deshalb beachtenswert, da die Abbauprodukte der Glucosinolate als wichtige Wirkstoffe angesehen werden. Im Rahmen dieser Arbeit wurden zusätzlich neue Erkenntnisse über die Regulation des Glucosinolat-Metabolismus in *Arabidopsis thaliana* gewonnen. Diese schließen die entwicklungsbedingte Myrosinaseaktivität unter verschiedenen Wachstumsbedingungen, Änderungen der Proteinabundanzen der Myrosinasen TGG1 und TGG2, sowie den circadianen Rhythmus für die Myrosinaseaktivität in zwei Wochen alten Blättern ein.

Schlüsselwörter: Schwefelhaltige Verbindungen, Cystein-Katabolismus, Glucosinolat-Umsatz

Contents

Abbreviations

Acetyl-CoA	Acetyl-Coenzyme A
AOP	2-oxoglutarate-dependent dioxygenase
APK	APS kinase
APR	APS reductase
APS	Adenosine 5`-phosphosulfate
A. thaliana	*Arabidopsis thaliana*
ATP	Adenosintrophosphate
ATPS	ATP sulfurylase
BCAA	Branched-chain amino acid
CBL	Cystathione β-lyase
CGS	Cystathione γ-synthase
CSC	Cysteine synthase complex
DMS	Dimethylsulfide
DMSP	Dimethylsulfoniopropionate
ED	Extended darkness
ESM1	Epithiospecifier-modifier 1
ESP	Epithiospecifier protein
ETHE1	Ethylmalonic encephalopathy protein 1
$FADH_2$	Dihydroflavine-adenine dinucleotide
GLS/GLSs	Glucosinolate/ glucosinolates
GSH	Glutathione
GSSH	Glutathione persulfide
GTR	Glucosinolate transporter
IPMDH	Isopropylmalate dehydrogenase
IPMI LSU/SSU	Isopropylmalate isomerase large subunit/small subunit
ITC/ITCs	Isothiocyanate/ isothiocyanates
MAM	Methylthioalkylmalate synthase
MBP	Myrosinase-binding protein
MVP1	Modified vacuolar phenotype 1

MyAP	Myrosinase-associated protein
NADH	Nicotinamide adenine dinucleotide
NSP	Nitrile-specifier protein
OAS	*O*-acetylserine
OAS-TL	*O*-acetylserine (thiol) lyase
OPH	*O*-phosphohomoserine
OXPHOS	Oxidative phosphorylation
PAPS	3`-phosphoadenosine 5`-phosphosulfate
PEN2	Penetration 2 β-glucosidase
PYK10	Atypical myrosinase
SAM	*S*-adenosylmethionine
SAT	Serine acetyl transferase
SLIM1	Sulfur Limitation1
SiR	Sulfit reductase
Str	Sulfurtransferase
TFP	Thiocyanate forming protein
TGG	Thioglucoside glucohydrolases
QTL	Quantitative trait loci

1 Sulfur in nature and plants

1.1 Occurrence of sulfur in nature and importance in plants

Sulfur is a widespread element, which occurs mainly in the form of sulfate (SO_4^{2-}) in our ecosystem. This element is cycling in the ecosystem of the earth including aqua, air, land and soil (Takahashi et al. 2011; ***Figure 1***). Volcanos as well as hot springs and the heavy industry are producing volatile sulfur in form of sulfide (S^{2-}) and sulfur dioxide (SO_2). These components can be oxidized to sulfate and brought back to the biosphere via rainfalls. Living organisms can convert this sulfate to sulfur containing compounds, e.g. algae are synthesizing dimethylsulfoniopropionate (DMSP) from sulfate and further, dimethylsulfide (DMS) is released as a volatile compound to the air. This contributes to 97% of volatile sulfur released from the oceans into the atmosphere (Cline & Bates, 1983). Plants and microorganisms can assimilate and dissimilate sulfate of the soil coming from the rainfall to generate organic sulfur and inorganic sulfate.

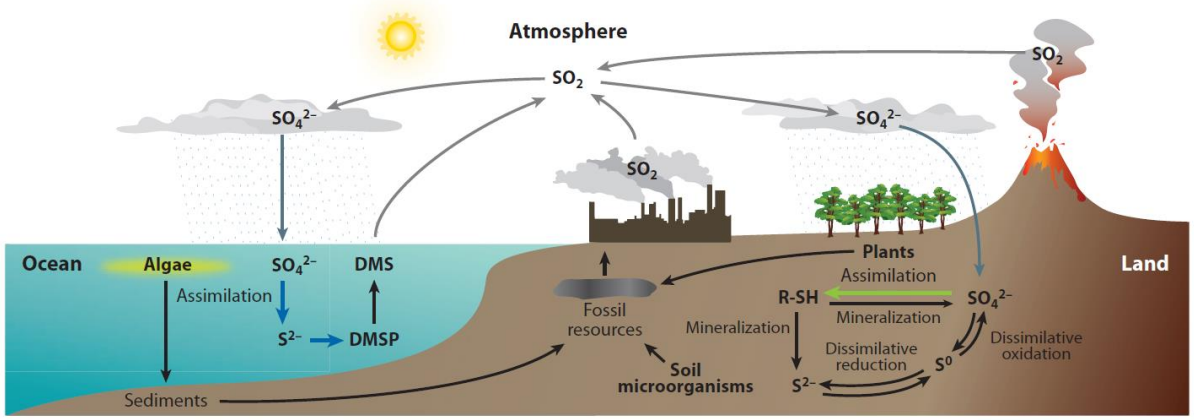

Figure 1: The sulfur cycle in the ecosystem of the earth (Takahashi et al., 2011). Sulfur dioxide (SO_2) is produced by the heavy industry and volcanos and can be oxidized to sulfate (SO_4^{2-}). Sulfate can be assimilated in water by algae and at the landscape by plants. From the ocean, DMS (dimethylsulfide) can evaporate to the air and is cycled back to sulfur dioxide. Fossils of plants, soil microorganism and sediments can be used by the heavy industry, producing sulfur dioxide.

For plants, sulfur is an essential compound, which contributes to various functions. The redox-system within the cells is enabled by thiols of proteins and the reducing agent glutathione (GSH). Moreover, hormones are regulated via sulfation (Gidda et al., 2003; Marsolais et al.,

2007) and hydrogen sulfide itself is discussed to be an important signal molecule (Alvarez et al. 2012; Calderwood and Kopriva 2014). Sulfur-containing metabolites are required in the primary as well as in the secondary metabolism. Cysteine and methionine are the two amino acids being synthesized during sulfur assimilation. Important secondary metabolites containing sulfur and a sulfonated oxime group are glucosinolates (GLSs), acting as defense compounds.

1.2 Transport and assimilation of sulfur in plants

The uptake of sulfur in form of sulfate takes place at the surface cell layers of the roots of plants. Sulfate transporters are driven by a proton/sulfate cotransport, which requires energy. Two high-affinity sulfate transporters in *Arabidopsis thaliana* (*A. thaliana*) are SULTR1;1 (AT4G08620) and SULTR1;2 (AT1G78000) whereas the major function is attributed to SULTR1;2 (Takahashi et al., 2011; Shibagaki et al., 2002). These two transporters are located at the root hairs, epidermis and cortex (Maruyama-Nakashita et al., 2004). When sulfate is transported to the epidermis cells, further transport via plasmodesmata occurs and ends up in the central cylinder. Additional transporters (SULTR4;1, AT5G13550 and SULTR4;2, AT3G12520) are localized in the tonoplast of the root cell vacuoles. These transporters are increasing the symplastic fluxes of sulfate by efflux of sulfate from the vacuoles (Kataoka et al., 2004). Apoplastic sulfate can be transported to the symplast via SULTR2;1 (AT5G10180) which is expressed in the central cylinder. Once sulfate entered the xylem by a yet unknown mechanism, it can be transferred to the shoots. Sulfur is taken up by mesophyll cells and either stored in vacuoles or reduced in the plastids, chloroplasts or cytosol.

The assimilation starts with the activation of sulfate by adenylation. This is catalized by the ATP sulfurylase (ATPS) and adenosine 5`-phosphosulfate (APS) is produced (***Figure 2***). Two further pathways are possible. On the one hand, the APS is phosphorylated via APS kinase (APK) and 3`-phosphoadenosine 5`-phosphosulfate (PAPS) is built. PAPS can be synthesized in the cytosol as well as in the chloroplast/plastid. To serve as sulfur donor for a variety of compounds such as GLSs, PAPS has to be carried to the cytosol by an unknown transport mechanism. Sulfotransferases then transfer the sulfur to the target compound. On the other hand, APS is reduced to sulfite by the enzyme APS reductase (APR) and further reduced to

sulfide via ferredoxin dependent sulfite reductase (SiR). These reduction steps only take place in the chloroplast/plastid. The enzyme *O*-acetylserine (thiol) lyase (OAS-TL) catalyzes the reaction from sulfide to cysteine. Furthermore, glutathione (GSH) and methionine can be produced from cysteine as precursor. In conclusion, sulfate is taken up by sulfate transporters, transferred to the shoots and reduced to different sulfur-containing compounds (for review see Takahashi et al., 2011; Leustek & Saito, 1999).

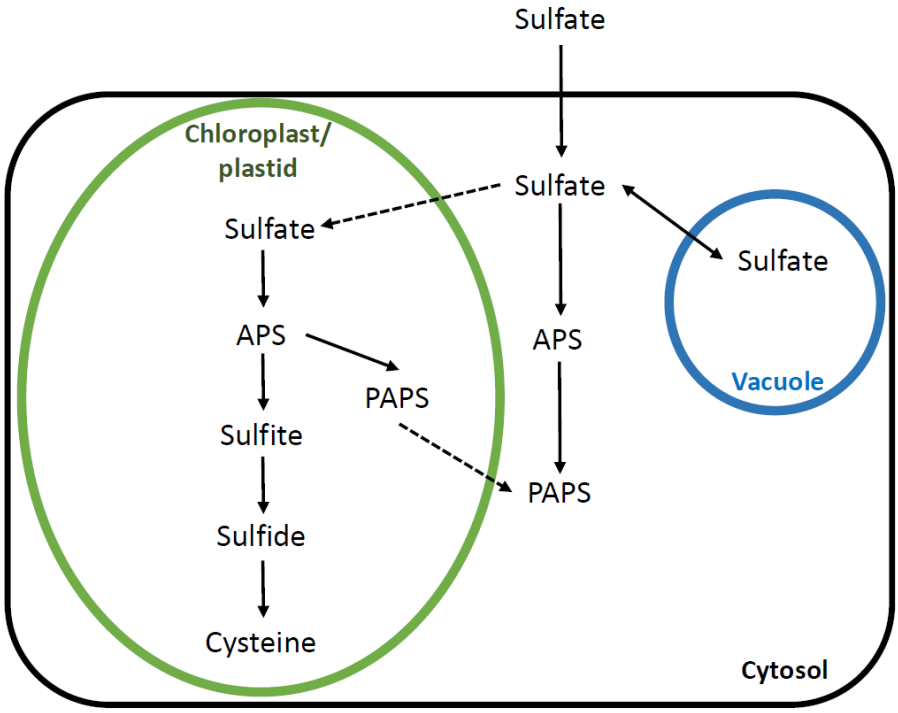

Figure 2: Sulfate assimilation in plants. Sulfate is taken up by the roots and transferred to mesophyll cells. Within these cells, sulfate can be assimilated in the cytosol to APS (adenosine 5`-phosphosulfate) and phosphorylation to PAPS (3`-phosphoadenosine 5`-phosphosulfate) generates a sulfur donor. Within the chloroplast, APS is reduced to sulfite and further on to sulfide. Sulfide and O-acetylserine (OAS) is further needed to produce cysteine.

Sulfur uptake as well as the assimilation are well regulated. Concerning sulfate transporter, SLIM1 (Sulfur Limitation1) acts as transcriptional regulator inducing SULTR1;2 under sulfur deficiency conditions (Takahashi et al., 2011). Development, circadian rhythm, nitrogen nutrition (Koprivova et al., 2000), and hormones like jasmonate and methyljasmonate (Harada et al., 2000; Jost et al., 2005), abscisic acid (Barroso et al., 1999) and salicylic acid (Koprivova et al., 2008; Fodor et al., 1997) are responsible for direct and indirect regulation of assimilation at the transcriptional level. At the post-transcriptional level, cysteine and GSH function as

feedback inhibitors at various steps of the assimilation pathway (Kopriva, 2006; Wirtz & Droux, 2005; Vauclare et al., 2002).

1.3 Primary metabolism: S-containing amino acids

In plants, 20 proteinogenic amino acids are present. The functions of these amino acids vary from incorporation into proteins to being involved in nitrogen assimilation or serving as precursors for phytohormones and secondary compounds. Two amino acids, namely cysteine and methionine, contain sulfur in addition to the overall composition of amino acids with an amino and a carboxyl group. Besides the function of sulfur in proteins, cysteine and methionine have various functions in cell metabolism.

Cysteine is the first organic sulfur compound of the sulfur assimilation pathway and therefore an important intermediate between the reduction of sulfur and the pool for reduced sulfur for compounds of the cell metabolism (Hell et al. 2002). The synthesis of cysteine is divided into two steps (***Figure 3***). First, serine is acetylated with acetyl-coenzyme A (acetyl-CoA) by serine acetyl transferase (SAT) to form *O*-acetylserine (OAS). Second, β-replacement of the acetyl moiety of OAS by sulfide generates cysteine and acetate. This step is catalyzed by OAS-TL. SAT and OAS-TL form a complex named cysteine synthase complex (CSC). This complex is sensing the demand of reduced sulfur in the cell by assembling and disassembling. At high sulfur conditions, SAT and OAS-TL are assembled which leads to an active SAT and an inactive OAS-TL within the complex. The active SAT produces OAS and additional active OAS-TL (homodimers) are generating cysteine with sulfide. At low sulfur supply, this sulfide cannot be provided by the reduction of sulfate and OAS is accumulating consequently. This accumulation leads to the disassembly of the CSC and therefore to an inactive SAT. Furthermore, the higher amount of OAS triggers the induction of genes coding for sulfate transporter and reduction. As far as enough sulfide is provided by the cell, the disassembled and now active OAS-TL can produce cysteine again which lowers the free OAS. Because of the decreased OAS concentration, CSC is built up again and SAT becomes active (Hell and Hillebrand 2001). Cysteine is produced at three sites of the cell: cytosol, chloroplast/plastid and within the mitochondrion. Different isoforms of the two enzymes involved in cysteine biosynthesis, SAT and OAS-TL are localized in these compartments. *A. thaliana* contains five SAT and nine OAS-

TL isoforms (Howarth et al., 2003; Wirtz et al., 2004). Among OAS-TL isoforms, the cytosolic OAS-TL A and the plastid OAS-TL B are responsible for 95 % of the overall activity. Nevertheless, both isoform are compensated for each other, showing no phenotype with T-DNA insertion lines *oastlA* and *oastlB* (Heeg et al., 2008). An insertion line of the mitochondrial OAS-TL C isoform was retarded in growth by 25 %, suggesting an important role for cysteine synthesis in mitochondria for development (Heeg et al., 2008). The reduction of sulfur to sulfide only occurs in the plastids. Sulfide is necessary for cysteine synthesis and it is yet unknown how sulfide is transported across the membrane into the cytosol or the mitochondria. Hydrogen sulfide was suggested to be able to diffuse through membranes (Jacques, 1936; Wirtz & Hell, 2007; Mathai et al., 2009). Heeg et al. 2008 showed that not only hydrogen sulfide but also cysteine have to be transported between the compartments. The occurrence of cysteine synthesis in three compartments, which are all able to synthesize proteins, points out the necessity of cysteine for this process (Hesse et al., 2004). Nevertheless, cysteine synthesis was of more importance in the cytosol and mitochondria compared to the plastid (Heeg et al., 2008). In mitochondria, cysteine is used as substrate to produce Fe-S cluster, thiamin, biotin, lipoate, and detoxification of cyanide (Gueguen et al., 2000; Gerber & Lill, 2002; Picciocchi et al., 2003; Droux, 2004; Birke et al., 2012).
Overall, cysteine is essential for plant metabolism and plays an important role at three different sites of the plant cell. This amino acid contains reduced sulfur from the sulfur assimilation pathway and can therefore serve as precursor for GSH and methionine biosynthesis, but also for phytochelatines, hormones, and cofactors (Höfgen et al., 2001).

Methionine is the second amino acid containing sulfur. The biosynthesis of methionine is localized in the plastids and consists of three enzymatic steps (***Figure 3***). First, cysteine is condensates with *O*-phosphohomoserine (OPH) to form cystathionine. This step is catalyzed by cystathione γ-synthase (CGS). Second, cystathione β-lyase (CBL) produces homocysteine and third, methionine synthase methylates homocysteine and therefore generates methionine. In *A. thaliana*, three isoforms of the methionine synthase are expressed of which one isoform is located to the chloroplast and two isoforms are present in the cytosol. Therefore, methionine is also produced in the cytosol and can further be converted to *S*-adenosylmethionine (SAM), which can serve as methyl donor (Ravanel et al., 2004). The synthesis of methionine is closely coupled with the synthesis of threonine via OPH (Lee et al.,

2005; Hesse & Hoefgen, 2003). OPH derives from homoserine and can be either converted to threonine by threonine synthase or to cystathionine by CGS. Therefore, SAM and methionine regulate this branching point (Bartlem et al., 2000; Hesse & Hoefgen, 2003). High levels of SAM are inducing the activity of threonine synthase, to produce more threonine (Curien et al., 1998). At the same time, plethora methionine is feedback-inhibiting CGS at the mRNA and post-transcriptional level (Chiba et al., 1999; Gakière et al., 2000; Inaba et al., 1994).
Methionine has various functions within the cell metabolism such as initiation of mRNA translation. It is also incorporated into proteins and serves as precursor for the secondary metabolites GLSs (Hesse & Hoefgen, 2003; Fahey et al., 2001).

1.4 Secondary metabolism: Glucosinolates

Products of the primary metabolism are essential for metabolic processes in plants. In contrast, secondary products are internally important and play a role in plant-environment interactions. In Brassicales, including *A. thaliana*, the secondary metabolites GLSs are the most characteristics with up to 40 different glucosinolate (GLS) compounds within one species (D'Auria & Gershenzon, 2005; Halkier & Gershenzon, 2006). The GLS composition is variable and depends mainly on the developmental stage and the environmental conditions (Brown et al., 2003; Petersen et al., 2002; Martínez-Ballesta et al., 2013). GLSs are composed of a glycone group bound to sulfur derived from cysteine or GSH (Sønderby et al., 2010; Falk et al., 2007). This sulfur is further bound to the central carbon atom that has two additional bonds. One is a sulfonated oxime group that is bound via a nitrogen and the other is the side group R (***Figure 3***). The structure of the R group is defined by the amino acid of which the GLS derived from. GLSs have a high variety in structure and up to 120 are described in plants (Halkier & Gershenzon, 2006). The synthesis of these compounds is assumed to be localized along the vasculature in the leaves, while grafting experiments showed that synthesis was also possible in roots (Madsen et al., 2014; Andersen et al., 2013). The amino acids methionine, alanine, valine, leucine, and isoleucine are precursor for aliphatic GLSs, phenylalanine and tyrosine are used to build aromatic GLSs and tryptophan is the precursor of indole GLSs. The synthesis includes three main steps (***Figure 3***). First, the chain elongation of methionine occurs, then the core structure is built, and lastly, GLSs are modified. The chain elongation is catalyzed in five steps and is mainly localized in the chloroplast. The amino acid is deaminated in the cytosol

and then transported to the chloroplast. In the latter named organelle, condensation with acetyl-CoA via methylthio-alkylmalate synthase (MAM1, AT5G23010; MAM3, AT5G23020), isomerization by isopropylmalate isomerase (IPMI LSU1, AT4G13430; IPMI SSU2, AT2G43100; IPMI SSU3, AT3G58990), oxidative decarboxylation through an isopropylmalate dehydrogenase (IPMDH1, AT5G14200), and transamination to form the elongated amino acid occur (Sønderby et al., 2010). This cycle of chain elongation can reach up to nine rounds and generates GLSs with four to nine methylene groups (C4 to C9) in their side chain (Fahey et al., 2001). The elongated amino acid is transported back to the cytosol and undergoes the core structure synthesis.

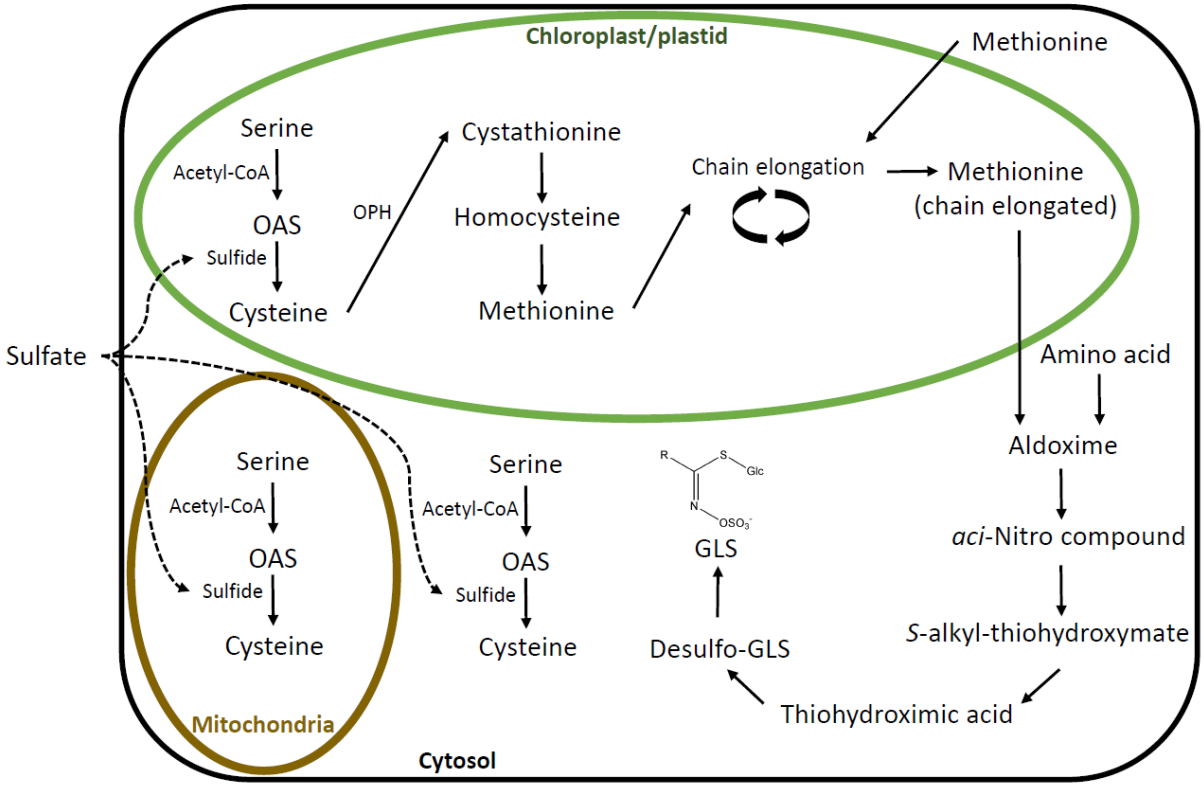

Figure 3 Cysteine, methionine and glucosinolate biosynthesis. Cysteine synthesis is located in the cytosol, the mitochondrion and the chloroplast. In each compartment, the reaction steps are equal and sulfide derived from the sulfate assimilation pathway (dashed arrows). The biosynthesis of methionine is restricted to the chloroplast, but the last step was also shown to take place in the cytosol. Glucosinolates (GLSs) derive from methionine and other amino acids. After chain elongation of methionine in the chloroplast, six enzymatic steps are required to form GLSs. OPH: *O*-phosphohomoserine. OAS: *O*-acetylserine.

Six enzymatic steps are required to form the GLSs and the specific genes are listed in Sønderby et al., 2010. The first two steps are catalyzed by the cytochrome P450 proteins CYP79 and CYP83. CYP79 is forming an aldoxime, which is converted to an *aci*-nitro compound or nitrile

oxide by CYP83. In the next step, *S*-alkyl-thiohydroximate is formed, possibly by a glutathione-S-transferase-like enzyme using cysteine as substrate. A *C-S* lyase converts *S*-alkyl-thiohydroximate into thiohydroximic acid that is further metabolized to desulfo-GLS by *S*-glucosyltransferase. The last step of the biosynthesis is catalyzed by the sulfotransferase to form GLSs. One reason for the high variety of GLSs are the secondary modifications after the core synthesis of GLSs like oxidation, hydroxylation, methoxylation and esterification (Mikkelsen et al. 2002; Tokuhisa et al. 2004; Wittstock and Halkier 2002). Methionine-derived GLSs are the main GLSs being modified at the R group (Halkier & Gershenzon, 2006). The sulfur atom in the methylthioalkyl side chain can be oxidized step-by-step to yield methylsulfonylalkyl moieties in the end. The intermediate of this oxidation step is methylsulfinylalkyl–GLS. This intermediate can either be converted to alkenyl-GLS via AOP2, a 2-oxoacid-dependent dioxygenase, or to hydroxyalkyl-GLS by AOP3. If the alkenyl–GLS contains n=2, 2-hydroxybut-3-enyl-GLS is built at the presence of AOP2 and MAM1 (Sønderby et al., 2010).

Regulation of the GLS biosynthesis and accumulation in Arabidopsis is performed by quantitative trait loci (QTL), among others. Quantitative traits were defined as “phenotypic variation owing to genetic and/or environmental influences” (Abiola et al., 2003). QTL are genetic loci, which affect these variations in e.g. GLS profiles. Total aliphatic GLS accumulation as well as total indole GLSs are controlled each by six loci and aromatic GLSs are under the control of three QTLs. Two of the loci controlling aliphatic GLSs, are the biosynthetic loci *GS-Elong* and *GS-AOP* (Kliebenstein et al., 2001a), whereby *GS-AOP* plays a role in secondary modifications together with *GS-OX* and *GS-OH*. The locus *GS-OX* is controlling the conversion of methylthioalkyl-GLSs to methylsulfinylalkyl-GLSs and *GS-OH* is responsible for the production of 2-hydroxybut-3-enyl-GLSs (Kliebenstein et al. 2001; Magrath et al. 1994; Mithen et al. 1995). Another regulation mechanism for GLS amounts at the gene level are the CYP79 genes, involved in the GLS biosynthesis, that can be induced and as a result of this, respective GLSs are accumulating (Mikkelsen et al., 2003). Furthermore, hormones like jasmonic acid, methyl jasmonate and salicylic acid and mechanical wounding can induce levels of specific GLSs (Brader et al., 2001; Kliebenstein et al., 2002; Mikkelsen et al. 2003; Sasaki-Sekimoto et al. 2005). As described before, the variation of GLSs in composition and amounts in plants

show a regulatory mechanism at the gene level as well as by environmental factors (Halkier & Gershenzon, 2006).

Transport of the synthesized GLSs is performed by the three glucosinolate transporters GTR1 (AT3G47960), GTR2 (AT5G62680), and GTR3 (AT1G18880), that are localized to the plasma-membrane (Nour-Eldin et al., 2012; Jørgensen et al., 2017). GTR1 and GTR2 are high affinity H^+/GLS symporter and transport GLSs from the apoplasm to the cytosol. GTR2 is suggested to have a main role in apoplasmic loading of GLSs to the phloem and therefore, this transporter carries the long-distance transport of GLSs. GTR1 possibly contributes to the distribution of GLSs within the leaves (Chen et al., 2001; Nour-Eldin et al., 2012). GTR3 is highly expressed in the root phloem companion cells and specifically transports indole GLSs (Jørgensen et al., 2017). GLSs can be transported out of the biosynthetic cells to the apoplast by a yet unknown transporter. For further GLS distribution, three pathways are possible: 1. Uptake of the GLSs in phloem companion cells by GTR. 2. GTR driven uptake into S-cells and further storage in vacuoles of these cells. 3. Adjacent mesophyll cells take the GLSs up (GTR) and flux via plasmodesmata allows GLSs to enter leaf edge cells (Madsen et al., 2014). The overall distribution of different GLSs within an Arabidopsis plant was shown by Andersen et al. 2013. Roots and leaves are capable to synthesize aliphatic and indole GLSs. The main storage side of long-chain (C6 to C8) aliphatic GLSs are the roots, whereas the leaves are the main storage side for short-chain (C3 to C5) aliphatic GLSs. Indole GLSs are distributed equally in both plant parts. In the leaf margin and tip, a high concentration of GLSs was found and further more in the leaf abaxial epidermis (Madsen et al., 2014). Depending on the development, the GLSs are also found in the organs of the bolting stem, e.g., cauline leaves, siliques and seeds (Brown et al., 2003). The storage of GLSs takes place in the vacuoles of the S-cells (Koroleva et al., 2000). These specialized cells are up to 1000 µm long and 30 µm in diameter and contain > 100 µM of GLSs. The localization within the plant is on the periphery of the vascular bundles next to so called myrosin cells (Andréasson et al., 2001; Husebye et al., 2002). This is another form of specialized cells, containing large amounts of myrosinase, the first enzyme in the GLS catabolism (see 2.2).

The overall function of GLSs is defense against pathogens and herbivores (Bones and Rossiter 1996; Kliebenstein et al. 2001). This defense strategy, known as the “mustard oil bomb”, occurs after tissue disruption and is followed by a breakdown of GLSs. Volatile products of the

GLS breakdown like isothiocyanates (ITCs) and nitriles are deterring herbivores. Beside the function of defense via GLS breakdown products, GLSs themselves have minor studied functions. Nevertheless, Mewis et al., 2002 showed that benzyl-GLS and to a lower extent allyl-GLS attracted the cabbage webworm *Hellula undalis* for oviposition. In general, GLSs have beneficial functions like feeding stimulants, oviposition and defense against enemies for specialist insects. For generalist insects, GLSs act as defense compound (Rask et al., 2000). The function of the breakdown products are discussed in chapter 2.2.

There are several interconnections between sulfur and the GLS metabolism. GLSs with side chains deriving from methionine comprises the major class of GLSs in Brassicaceae (Brown et al. 2003; Campos de Quiros et al. 2000; Kliebenstein et al. 2001). The synthesis of this amino acid is connected to the sulfur assimilation via cysteine (see 1.3). Furthermore, the last step in GLS biosynthesis is catalyzed by a sulfotransferase to form GLS from a desulfo-GLS. PAPS provide the sulfur needed in this reaction. PAPS is built from APS via APS kinases (APK) and a double knockout mutant *apk1 apk2* showed a reduced GLS content and accumulation of desulfo-GLSs compared to the wild type (Mugford et al., 2009). Sulfur in GLSs derived from GSH or possibly cysteine or PAPS and methionine in the side chain and comprises up to 30 % of total sulfur content in plants (Falk et al., 2007). Thus, GLSs are suggested to be a storage form of sulfur. Under sulfur limiting conditions, biosynthetic genes as well as GLS contents were reduced and genes for sulfur assimilation were increased (Falk et al., 2007).

2 Catabolism of S-containing compounds in plants

The catabolism or turnover of metabolites in plants is important under specific conditions. In situations of abiotic stress, there is an enhanced requirement of carbon skeletons, nutrients, reduction equivalents, and energy in form of ATP to maintain the complete cellular metabolism. Stress situations like limitation of nutrients, drought, high salinity, heat or low light can therefore increase the turnover of metabolites. A special focus was given to carbon starvation conditions with respect to cysteine and GLSs in 2.3.

2.1 Cysteine degradation in various plant organelles

The degradation of cysteine in Arabidopsis is located in the cytosol, plastid and mitochondria. Different pathways leads to pyruvate as product, catalyzed by different enzymes (Hildebrandt et al., 2015). The cytosolic cysteine desulfhydrase deaminates cysteine and the products are pyruvate, ammonium and hydrogen sulfide (H_2S). Alvarez et al. 2010 showed the importance of this protein in cysteine equilibrium within the cells by a 20-25 % higher cysteine content in knockout mutants. Furthermore, this enzyme is suggested to be involved in H_2S signaling. Stomatal opening and autophagosome alterations could be detected in cysteine desulfhydrase knockout mutants and were reversible by addition of H_2S (Alvarez et al., 2012; Jin et al., 2013). An additional D-cysteine desulfhydrase was investigated in mitochondria with several suggestions about the function like regulation of L-amino acid biosynthesis and D-amino acid degradation via “compartmentalization” of both processes using different chiralities (Riemenschneider et al., 2005).

Another pathway of cysteine catabolism is provided by cysteine desulfurases, also named NifS-like proteins, of which isoforms are localized in the mitochondria (NSF1; AT5G65720) and plastid (NFS2; AT1G08490) (Kushnir et al., 2001; Pilon-Smits et al., 2002; Léon et al., 2002). NifS-like proteins catalyze the formation of alanine and elemental sulfur from cysteine and in mitochondria, alanine aminotransferase forms glutamate and pyruvate. The elemental sulfur is exclusively transferred to iron-sulfur scaffold proteins, which are part of the iron-sulfur-cluster biosynthesis (Couturier et al., 2013). Therefore, this pathway aims to provide reduced

sulfur for the synthesis of Fe-S-cluster, biotin and thiamin and do not play a major role in cysteine catabolism (Couturier et al., 2013; Hildebrandt et al., 2015).

A third pathway is localized in the mitochondria of Arabidopsis and comprises the complete oxidation of L-cysteine via pyruvate to thiosulfate (see 4.1: publication 1). The first step is catalyzed by an unknown aminotransferase to yield 3-mercaptopyruvate, where the amino group of cysteine is transferred to 2-oxoglutarate (***Figure 4***). The sulfhydryl group from 3-mercaptopyruvate is then carried by the mitochondrial sulfurtransferase (Str1; AT1G79230) to glutathione (GSH) to produce the substrate glutathione persulfide (GSSH) for the following enzymatic step and pyruvate as side product. The mitochondrial sulfur dioxygenase Ethylmalonic encephalopathy protein 1 (ETHE1; AT1G53580) oxidizes GSSH to sulfite (H_2SO_3) and Str1 adds a second persulfide group to H_2SO_3 to produce thiosulfate ($H_2S_2O_3$).

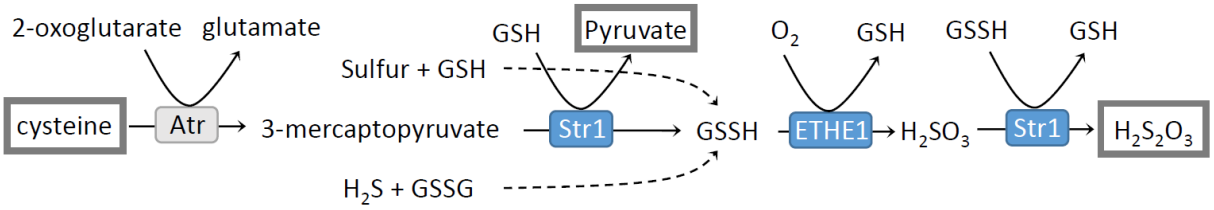

Figure 4: Cysteine catabolism in mitochondria of Arabidopsis. Four enzymatic steps catabolize cysteine to pyruvate and thiosulfate ($H_2S_2O_3$). The first step is catalyzed by a yet unknown aminotransferase (Atr) to yield 3-mercaptopyruvate. Sulfurtransferase (Str) then produces persulfides (GSSH) and later on thiosulfate from sulfite (H_2SO_3). ETHE1 oxidizes the persulfides to sulfite by generating glutathione (GSH).Persulfides can also be generated from sulfur and GSH or hydrogen sulfide (H_2S) and oxidized persulfides (GSSG).

The knockdown of ETHE1 in Arabidopsis showed early senescence under light limiting conditions and severe delay in embryo development (Krüßel et al., 2014). Furthermore, the application of extended darkness (ED) altered the seed composition concerning lipid, free sugar and amino acid content and enhanced the effect of delayed embryo development (see 4.2: manuscript 1). An interaction of ETHE1 with the branched-chain amino acids (BCAA), valine, leucine and isoleucine was suggested due to accumulation of these amino acids under ED conditions in leaves of the *ethe1-1* knockdown mutant (Krüßel et al., 2014). In seeds however, alanine and glycine were suggested to interact with the ETHE1 pathway (see 4.2: manuscript 1).

2.2 Glucosinolate breakdown and turnover

Defense is the main function of GLSs and the corresponding breakdown products. While GLSs were shown to be part of plant-insect interactions, breakdown products seem to have various effects on herbivores as well as on cancer cells and humans (Halkier & Gershenzon, 2006; Rask et al., 2000; Navarro et al., 2011).

GLSs are catabolized by myrosinase to an instable aglucone (***Figure 5***). A spontaneous reaction, called Lossen rearrangement, releases sulfate and the product isothiocyanate (ITC). In the presence of specifier proteins, simple nitriles, epithionitriles, thiocyanates, and oxazolidine-2-thione are formed. Further breakdown of nitriles to carboxylic acids via nitrilases is suggested (Janowitz et al., 2009). In Brassicaceae, the myrosinase gene family is composed of three subfamilies, named MA, MB and MC (Rask et al., 2000). In Arabidopsis four functional typical myrosinases (thioglucoside glucohydrolases; TGG), named TGG1 (AT5G26000), TGG2 (AT5G25980), TGG4 (AT1G47600) and TGG5 (AT1G51470) are present, but are genetically distinct from the MA, MB and MC subfamilies (Rask et al., 2000). TGG3 (AT5G48375) and TGG6 (AT1G51490) are non-functional genes due to deletions, insertions, and premature termination of the translation (Wang et al. 2009; Zhang et al. 2002). The isoforms TGG1 and TGG2 are mainly expressed in leaves, whereas TGG4 and TGG5 are located in the roots (Wittstock & Burow, 2010). TGG1 and TGG2 form dimers with 150 kDa and 126 kDa, repectively (Zhou et al., 2012). Glycosylation by oligomannosidic N-glycans at nine (TGG1) and four (TGG2) sites is suggested to stabilize the proteins and protect them from their hydrolysis products (Liebminger et al., 2012; Halkier & Gershenzon, 2006).

Myrosinases are stored in vacuoles of specialized cells, known as myrosin cells (Carter et al., 2004; Ueda et al., 2006). These cells are located in the phloem parenchyma in close proximity to the S-cells, that contain GLSs (Andréasson et al., 2001; Husebye et al., 2002). TGG1 is, in addition to myrosin cells, located in guard cells (Islam et al., 2009; Husebye et al., 2002; Thangstad et al., 2004).

Besides the typical myrosinases, atypical myrosinases are present in Arabidopsis. PEN2 is a peroxisomal β-glucosidase (AT2G44490) that favors indole GLSs for hydrolysis (Bednarek et al., 2009; Lipka et al., 2005). The accumulation of peroxisomes with PEN2 at the fungal entry site showed the importance of this atypical myrosinase in preinvasion resistance mechanism (Lipka et al., 2005). A member of the subfamily of PEN2 is PYK10 (AT3G09260), first identified

by Nitz et al. 2001 as root and hypocotyl myrosinase in *A. thaliana*. PYK10 is the main component of ER bodies in roots, hypocotyls, and cotyledons (Xu et al., 2004; Matsushima et al., 2003). This enzyme has β-D-glucosidase and β-D-fucosidase activity and forms complexes with myrosinase-binding proteins (MBPs) as well as myrosinase-associated proteins (MyAPs) to alter its activity (Matsushima et al., 2004; Nagano et al., 2008).

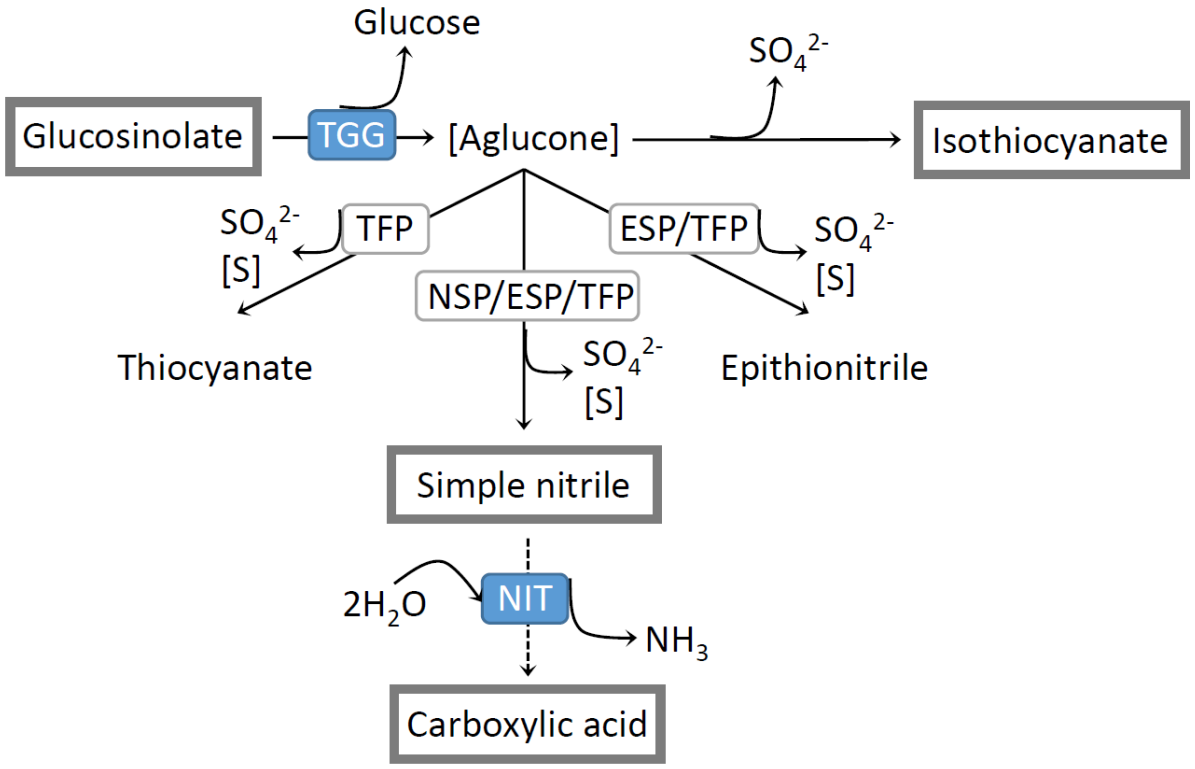

Figure 5: Glucosinolate breakdown into isothiocyanate or carboxylic acid. Thioglucoside glucohydrolases (**TGG**) are catalyzing the first step and produce glucose and an unstable aglucone. Via spontaneous Lossen rearrangement, ITC (isothiocyanate) is built. In presence of specifier proteins (TFP, ESP and NSP), different products like thiocyanate, epithionitriles and simple nitriles were non-enzymatically produced. Nitrilases (**NIT**) can further catabolize nitriles to carboxylic acids. By products are glucose, sulfate (SO_4^{2-}), elemental sulfur ([S]) and ammonium (NH_3). **TFP**: Thiocyanate forming protein, **ESP**: Epithiospecifier protein, **NSP**: Nitrile-specifier protein.

Two additional MyAP-like proteins are present in Arabidopsis, ESM1 and MVP1. Epithiospecifier-modifier 1 (ESM1; AT3G14210) interacts with the ESP (epithiospecifier protein) locus and thus promoted ITC formation and inhibited the production of nitriles (Zhang et al., 2006). The second MyAP is named Modified vacuolar phenotype 1 (MVP1; AT1G54030) and specifically interacts with TGG2. The function of this could be the correct location of TGG2 to prevent toxic products (Agee et al., 2010). Beside the MBPs and MyAPS, ascorbic acid was

shown to alter the activity of myrosinase (Burmeister et al., 2000; Bones & Rossiter, 2006; Andersson et al., 2009).

Specifier proteins influence the variety of breakdown products. These proteins do not show any hydrolytic activity, but upon their presence, lead to different products of GLS breakdown (Tookey, 1973; Halkier & Gershenzon, 2006). Six specifier proteins were found in Arabidopsis, one epithiospecifier protein (ESP) and five nitrile-specifier proteins (NSPs) (Burow et al. 2009; Kissen and Bones 2009; Lambrix et al. 2001). Thiocyanate formation is enabled by TFP (thiocyanate forming protein) although this product has not been reported for Arabidopsis (Burow et al. 2007; Wittstock and Burow 2007, 2010). Epithionitrile is produced in the presence of ESPs and TFPs. The production of simple nitriles either requires NSPs or low pH (<5) or high (>0.01 mM) ferrous ion concentrations (Wittstock & Burow, 2007, 2010). Furthermore, ESP and TFP also conduct NSP activity (Burow et al. 2006, 2007).

The defense system of GLSs is an important mechanism against herbivores and pathogens (Halkier & Gershenzon, 2006; Matile, 1980). The separately stored GLS and myrosinase get in contact to each other after tissue disruption. Depending on the genotype, the organ, the developmental stage and environmental influences, up to three different products could be built from one GLS (Wittstock & Burow, 2010). The toxic compound ITC is well researched and is active against various plant pests (Brown & Morra, 1997; Fahey et al., 2001). Rask et al. 2000 summarized the effect of the defense system on humans, insects, fungi, and natural enemies. Generalist insects and fungi are deterred by GLSs and breakdown products. Specialist insects on the other hand, are adapted to the GLS/myrosinase system by various mechanisms (Winde & Wittstock, 2011; Ratzka et al., 2002; Wittstock et al., 2003) and use it to find their host, for oviposition, and as feeding stimulants. Indirect defense is possible by the attraction of parasitoids of insect pests.

The effects of GLSs and their breakdown products on humans are mostly beneficial (Traka & Mithen, 2009). Many eatable vegetables like cabbage and broccoli (*Brassica oleracea*), radish (*Raphanus sativus*), and horseradish (*Armoracia rusticana*) contain GLSs and are valued for their spice. In addition, the breakdown products have chemopreventive properties, mostly shown for ITCs (Hecht, 1999). The GLS breakdown products can act at various stages of cancer formation (Das et al., 2000). Phase I enzymes, which activate the carcinogens are inhibited and Phase II enzymes, which function in detoxification of activated carcinogens are induced.

The cell cycle of cancer cells can be arrested and furthermore, even apoptosis can be induced (Hayes et al., 2008; Navarro et al., 2011). ITCs were identified to be among the most effective chemopreventive agents (Hecht, 1995).

In the last decade, turnover of GLSs in intact plant tissues was discussed (Burow and Halkier 2017; Grubb and Abel 2006; Neilson et al. 2013; Wittstock and Burow 2010; Yan and Chen 2007). Three main points could be defined that supports the hypothesis of a GLS turnover. 1. The changing GLS content during development of *A. thaliana*. 2. GLS concentration is influenced by sulfur nutrition and therefore, GLSs were suggested to act as sulfur storage compound. 3. Breakdown of GLSs into carboxylic acids releases glucose, elemental sulfur, sulfate and ammonia.

During development, the different plant organs like leaves, roots and at later stages, siliques and seeds change the composition of GLSs (Petersen et al., 2002; Brown et al., 2003). Myrosinase, the enzyme catalyzing the first step of breakdown, showed corresponding changes in activity and content (Petersen et al., 2002). This indicates turnover rather than or additionally to transport of GLSs. Concerning GLSs as sulfur storage, Falk et al., 2007 showed increased level of GLSs under high S conditions and furthermore a decreased content during S-depletion. This could be seen as a hint of the usage of sulfur from GLSs via turnover. The turnover via simple nitriles would firstly avoid the production of toxic ITCs by the rearrangement of the instable aglucone and secondly release nutrients that can serve as energy supply or could be channeled back to primary metabolism for plant development (James and Rossiter 1991; Neilson et al. 2013; Schonhof et al. 2007).

2.3 Turnover under extended darkness conditions

Plants are photoautotrophic organisms, generating their energy via light and simple anorganic molecules. The mechanism is called photosynthesis and generates carbohydrate molecules. When extended darkness (ED) is induced to plants, photosynthesis could not take place and therefore carbon starvation occurs. Nevertheless, Ishizaki et al., 2005 showed, that *A. thaliana* wild type (ecotype Col-0) plants survived up to 15 d of darkness. For plant survival, several catabolic pathways are upregulated and the respective products were used as alternative respiratory substrates (Araújo et al., 2011). Koch 1996 presented various genes of lipid, starch

and protein breakdown, which were upregulated upon carbon starvation. The activity of protein catabolizing enzymes like protease or endopeptidase were increased (James et al. 1996; Moriyasu and Ohsumi 1996; Tassi et al. 1966; Thomas 1978) and compatible to these findings, Brouquisse et al. 1992 showed a decline of proteins by 92 % after eight days of darkness in maize (*Zea mays* L.). The content of amino acids as well as lipids are decreasing under carbohydrate starvation conditions. The complete oxidation of amino acids is generating energy in form of NADH, $FADH_2$ and ATP (Hildebrandt et al., 2015). Aubert et al. 1996 showed a decrease in membrane polar lipids like phospholipids and galactolipids in Sycamore cells (*Acer pseudoplatanus* L.). Taken together, the status of a plant cell under ED is changing at translational and posttranslational levels to gain alternative respiratory substrates. These substrates support the OXPHOS (oxidative phosphorylation) system in mitochondria and the primary metabolism of the plant can therefore be kept in a "stand-by mode" (Keech et al., 2007).

The breakdown of the amino acid cysteine could lead to the generation of alternative substrates like pyruvate and further on acetyl-CoA (see 2.1). Under carbon starvation conditions, ETHE1 is induced and cysteine accumulates in an *ethe1-1* knockdown mutant (Krüßel et al., 2014). This leads to the conclusion that cysteine served on the one hand as an energy source under ED conditions (Hildebrandt et al., 2015). On the other hand, it is important to reduce the reactive cysteine, which could be toxic in high concentrations (Osman et al., 1997; Höfler et al., 2016: publication 1).

GLSs were also shown to be altered during carbon starvation. Bennett et al. 1995 showed a significant decrease in the enzyme, catalyzing the conversion from homophenylalanin to the respective aldoxime, one of the first steps in GLS biosynthesis in oilseed rape (*Brassica napus*) under darkness conditions. In broccoli sprouts, the GLS content was observed to be higher in light grown seedling compared to dark grown seedlings (Pérez-Balibrea et al., 2008). The lower GLS content could also be confirmed by Brandt et al., 2017, in preparation (see 4.3: manuscript 2) for six week old *A. thaliana* plants, transferred to darkness for 7 d. Furthermore, GLS catabolizing enzymes like the myrosinases TGG1 and TGG2 and nitrilase are enhanced in their activity and abundance during ED (Brandt et al., 2017, in preparation; see 4.3: manuscript 2). The conversion of methylsulfinyl- GLSs into alkenyl- GLSs is catalyzed by AOP2 and belongs to

the secondary modifications of GLSs. Transcripts of this enzyme were not detectable under darkness condition in *A. thaliana* ecotype Pitztal (Neal et al., 2010).

Extended or prolonged darkness is used to introduce carbon starvation condition in plants. Furthermore, dark periods were also used to study dark-induced senescence, because many naturally senescent symptoms occur (Buchanan-Wollaston et al., 2005). A comparison between developmental- and dark-induced senescence gene expression data from Arabidopsis showed 53 % accordance of upregulated genes (Buchanan-Wollaston et al., 2005). Although not completely comparable, dark-induced senescence could also be used to study developmental senescence in plants. For example, the question of the decreasing GLS content in Arabidopsis during senescence could possibly be answered by using extended darkness. If transport or turnover of GLSs occur, is still unknown (Burow and Halkier 2017), but first results showed a possible interplay of both mechanisms (see Brandt et al, 2017, in preparation, see 4.3: manuscript 2).

In the frame of this thesis, new insights into mitochondrial cysteine catabolism are presented (publication 1). Additionally, the role of this catabolic pathway during seed germination has been examined and alterations of the embryo development, the amino acid metabolism and cellular structures were observed (manuscript 1). Finally, the regulation of glucosinolate turnover has been investigated (manuscript 2). Since glucosinolate breakdown products have a great potential as drug, specific conditions for induction of GLS catabolism were searched. Evidence is shown that extended darkness has a clearly stimulating effect on GLS turnover.

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4 Publications and Manuscripts

4.1 Publication 1

Dealing with the sulfur part of cysteine: four enzymatic steps degrade L-cysteine to pyruvate and thiosulfate in Arabidopsis mitochondria

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Dealing with the sulfur part of cysteine: four enzymatic steps degrade L-cysteine to pyruvate and thiosulfate in Arabidopsis mitochondria

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Amino acid catabolism is essential for adjusting pool sizes of free amino acids and takes part in energy production as well as nutrient remobilization. The carbon skeletons are generally converted to precursors or intermediates of the tricarboxylic acid cycle. In the case of cysteine, the reduced sulfur derived from the thiol group also has to be oxidized in order to prevent accumulation to toxic concentrations. Here we present a mitochondrial sulfur catabolic pathway catalyzing the complete oxidation of L-cysteine to pyruvate and thiosulfate. After transamination to 3-mercaptopyruvate, the sulfhydryl group from L-cysteine is transferred to glutathione by sulfurtransferase 1 and oxidized to sulfite by the sulfur dioxygenase ETHE1. Sulfite is then converted to thiosulfate by addition of a second persulfide group by sulfurtransferase 1. This pathway is most relevant during early embryo development and for vegetative growth under light-limiting conditions. Characterization of a double mutant produced from *Arabidopsis thaliana* T-DNA insertion lines for ETHE1 and sulfurtransferase 1 revealed that an intermediate of the ETHE1 dependent pathway, most likely a persulfide, interferes with amino acid catabolism and induces early senescence.

Introduction

Amino acid catabolism in plants is involved in the regulation of steady state levels of free amino acids and is particularly important in conditions of increased protein turnover such as germination and senescence. It is critical for nutrient redistribution from senescing leaves to newly formed sink organs such as young leaves and developing seeds. The degradation of amino acids also contributes to energy production in situations of carbohydrate starvation, which may occur during drought or unfavorable light conditions (Araújo et al. 2011). Pool sizes of free amino acids are highly diverse and dynamically change in response to environmental or developmental factors (Hildebrandt et al. 2015). However, the intracellular concentration of cysteine is amongst the lowest known for proteinaceous amino acids. The thiol group of cysteine is highly reactive, it can deplete cells of pyridoxal phosphate by forming thiazolidine derivatives and in addition auto-oxidation in the presence of transition metals generates reactive oxygen species (Osman et al. 1997). Therefore, intercellular concentrations have to be tightly controlled in order to avoid toxic effects. However, conversely cysteine levels must also be sufficiently high to support protein synthesis and the production of other essential molecules

Abbreviations – DAP, days after pollination; GC-MS, gas chromatography-mass spectrometry; GSH, reduced glutathione; GSSH, glutathione persulfide; HPLC, high-performance liquid chromatography; S8, elemental sulfur; SDO, sulfur dioxygenase; TCA cycle, tricarboxylic acid cycle; H_2S, hydrogen sulfide; PCR, polymerase chain reaction.

such as glutathione, coenzyme A and reduced sulfur for the biosynthesis of biotin, thiamin, iron–sulfur clusters and molybdenum cofactors (Van Hoewyk et al. 2008, Balk and Pilon 2011). Thus, synthesis as well as the degradation of cysteine has to be tightly regulated.

During degradation, the carbon skeleton of cysteine is converted to pyruvate via removal of the amino and sulfhydryl groups. These reactions can be catalyzed by different pathways. Cysteine desulfhydrases deaminate cysteine to pyruvate, ammonia and hydrogen sulfide (H_2S). Specific isoforms using L-cysteine (DES1, EC 4.4.1.1) and D-cysteine (D-CDES, EC 4.4.1.15) as a substrate are present in the cytosol and the mitochondrial matrix, respectively (Riemenschneider et al. 2005, Alvarez et al. 2010). Both enzymes are induced during senescence and therefore have been implicated in cysteine catabolism for nutrient remobilization. DES1 deficient Arabidopsis mutants accumulate cysteine and in addition show alterations in autophagosome formation and stomatal opening, which are reversible by supplementation with H_2S (Alvarez et al. 2010, 2012, Jin et al. 2013). Taken together, these results demonstrate that the desulfhydration reaction is relevant for cysteine homeostasis and also regulates the production of the signaling molecule H_2S. However, the further fate of H_2S, which is highly toxic already at low micromolar concentrations, has not yet been analyzed. In contrast, the physiological function of cysteine desulfurases (NifS-like proteins, EC 2.8.1.7) is to provide reduced sulfur for the synthesis of iron–sulfur clusters, biotin and thiamin (Couturier et al. 2013), and therefore this reaction is most likely not relevant for cysteine homeostasis.

We recently demonstrated a role of the sulfur dioxygenase ETHE1 (EC 1.13.11.18) in plant cysteine catabolism (Krüßel et al. 2014). ETHE1 is localized in the mitochondrial matrix and oxidizes glutathione persulfide (GSSH) to sulfite. Knockout of the ETHE1 gene (AT1G53580) in Arabidopsis leads to an arrest of embryo development at the early heart stage (Holdorf et al. 2012). Knockdown mutants are viable but show a delay in embryo development (Krüßel et al. 2014). Interestingly, ETHE1 is highly induced by carbohydrate starvation and mutants develop premature leaf senescence under light limited growth conditions indicating a role in the use of amino acids as alternative energy source.

Here we present the additional steps of the ETHE1-dependent mitochondrial sulfur catabolic pathway catalyzing complete oxidation of L-cysteine to pyruvate and thiosulfate. After transamination to 3-mercaptopyruvate, the sulfhydryl group from L-cysteine is transferred to glutathione by sulfurtransferase 1. Interestingly, this enzyme also converts sulfite to the final product thiosulfate by addition of a second persulfide group. Alternatively, sulfite could be transported out of the mitochondria and either oxidized to sulfate by peroxisomal sulfite oxidase or reduced to sulfide by plastidal sulfite reductase for reassimilation into cysteine (Lang et al. 2007, Khan et al. 2010).

Sulfurtransferases catalyze the transfer of a sulfur atom from a suitable sulfur donor to nucleophilic sulfur acceptors. Twenty putative sulfurtransferase isoforms are annotated in the Arabidopsis genome and some of them have already been characterized on a protein basis (Bartels et al. 2007). They might play a role in plant development and stress response, however, the exact function has not been identified yet. In vitro, the activity is measured using either thiosulfate or 3-mercaptopyruvate as sulfur donor and cyanide as acceptor. Mao et al. (2011) recently demonstrated that the mitochondrial sulfurtransferase Str1 (AT1G79230, EC 2.8.1.2) contributes the main mercaptopyruvate sulfurtransferase activity in Arabidopsis. Knockout plants had no visible phenotype under long-day growth conditions, but seed development was severely compromised. The majority of seeds were shrunken and not able to germinate. Embryo development arrested at the heart stage producing abnormal morphological shapes and eventually aborted.

In order to identify the role of mitochondrial cysteine catabolism and the individual reaction steps in seed development as well as plant energy metabolism, we produced and characterized a double mutant from the ETHE1 knockdown line *ethe1-1* (Krüßel et al. 2014) and the Str1 knockout line *str1-1* (Mao et al. 2011).

Materials and methods

Plant material and growth conditions

All *Arabidopsis thaliana* plants used for this study were of the Columbia ecotype (Col-0). Plants were grown in climate chambers under long-day conditions (16/8 h light/dark) or short-day conditions (8/16 h light/dark) at 22°C, 85 $\mu mol\, s^{-1} m^{-2}$ light and 65% humidity. The T-DNA insertion line SALK_021573 (*ethe1-1*) for the gene AT1G53580 has been characterized in our lab before (Krüßel et al. 2014). Seeds of the line SALK_015593 (*str1-1*) for the gene AT1G79230 were obtained from the Nottingham Arabidopsis Stock Centre (University of Nottingham, UK). This line has been described by Mao et al. (2011). The *ethe1-1* × *str1-1* double mutant was produced by crossing of the two T-DNA insertion lines described above using *str1-1* as the female parent.

Homozygous mutant lines were identified by genomic PCR using gene-specific primers (5′-TGGAATTGG GTTATATGGTGG-3′ and 5′-CGGATCAATCAACTGCTC

ATC-3′ for *ethe1-1* and 5′-AAAGGGGATCTTTAGTGCA GC-3′ and 5′-GTGGGAAGGAAGCAAATTCTC-3′ for *str1-1*) and the T-DNA left border primer LBb1.3 (5′-ATTTTGCCGATTTCGGAAC-3′).

Complete rosettes of wild-type and mutant plants were harvested at dawn and used for metabolite analysis.

Expression and purification of ETHE1 and Str1

Expression and purification of recombinant ETHE1 protein was performed like described before (Krüßel et al. 2014). The plasmid encoding Arabidopsis Str1 was generously provided by Jutta Papenbrock (Institute for Botany, Leibniz University Hannover, Germany). Str1 was expressed in the same way as ETHE1.

Cell suspension cultures and isolation of mitochondria

Arabidopsis cell suspension cultures were established and maintained as described by May and Leaver (1993) and Sunderhaus et al. (2006). Mitochondria were prepared following the procedure outlined by Werhahn et al. (2001).

Sulfur dioxygenase activity test

Sulfur dioxygenase activity was measured at 25°C in a Clarke-type oxygen electrode (Oroboros Oxygraph and Hansatech DW1 Oxygrapy) following the procedure described in Hildebrandt and Grieshaber (2008). The reaction contained 1–2 μg ml^{-1} purified enzyme or 150–300 μg ml^{-1} mitochondrial protein in 0.1 *M* potassium phosphate buffer pH 7.4. For the standard activity test, 1 m*M* GSH (final concentration) was added, followed by 15 μl ml^{-1} of a saturated elemental sulfur solution in acetone. Acetone did not inhibit enzyme activity. Rates were measured during the linear phase of oxygen depletion, which occurred in the first 2–3 min.

Mercaptopyruvate sulfurtransferase activity test

The enzyme assay was performed as described in Papenbrock and Schmidt (2000) with slight modifications. A total of 50 mg of pulverized rosette leaves were incubated with 150 μl 20 m*M* TRIS HCl, pH 8.0 for 20 min on ice. After centrifugation, the crude plant extract was used for enzyme activity tests. The reaction was composed of 0.1 m*M* TRIS HCl, pH 9.0; 5 m*M* β-mercaptopyruvate and 30 μl plant extract and was initiated by addition of 10 m*M* KCN. After incubation at 37°C for 10, 20 and 30 min, the reaction was stopped by adding 60 μl acidic iron reagent (2.5 g 50 ml^{-1} $FeCl_3$, 12 ml 53% HNO_3) to 300 μl of the sample. After centrifugation at 13 000 *g* for 3 min, the absorption was read at 452 nm. Enzyme activity was quantified using a standard curve carried out with sodium thiocyanate in 0.1 m*M* TRIS HCL, pH 9.0. The protein content was measured using the Pierce™ Coomassie Plus (Bradford, UK) Assay Kit.

Leaf respiration measurements

Respiration was measured at 25°C in a Clark-type oxygen electrode (Oroboros Oxygraph). Leaf samples (0.03 g) of 5-week-old plants were taken during the last 4 h of the dark period, cut into pieces and added to 2 ml of air-saturated potassium phosphate buffer (25 m*M*, pH 6.8). After a constant oxygen uptake was reached, respiration was inhibited via 1 m*M* potassium cyanide and 20 m*M* salicylhydroxamic acid.

Phenotypic analysis

For general phenotypic analysis, a modified version of the procedure described in Boyes et al. (2001) was used. Plants were grown under long-day or short-day conditions and growth parameters were measured once per week during the first 60 days after sowing. Plants grown under long-day conditions completed their life cycle within this time frame (Fig. S5). However, when grown under short-day conditions plants started flowering much later (after 10–11 weeks), and further development was monitored by taking pictures.

Embryo morphology was analyzed with a microscope equipped with Nomarski optics. Seeds were dissected from the siliques and cleared in Hoyer's solution (15 ml distilled water, 3.75 g gum arabic, 2.5 ml glycerol, 50 g chloral hydrate) overnight before analysis.

Germination rates were determined for seeds of wild type, *ethe1-1* and three different morphological types of *str1-1* and *ethe1-1* × *str1-1*. The seeds were surface sterilized with 6% sodium hypochlorite and 100% ethanol followed by five washing steps with sterilized water. For vernalization, seeds were incubated for 2 days at 4°C in the dark. Approximately 20 seeds were sown per plate (three replicates per sample) on MS-medium without a carbon source and incubated for another 2 days at 4°C in the dark. Afterwards, the plates were placed into a growth chamber (24°C, 16/8 h light/ dark). After 24, 48 and 72 h, germinated seeds were counted. A seed was considered to have germinated when the radicle raptures the endosperm and the testa.

Metabolite analysis

For extensive metabolite profiling, complete rosettes of wild-type and mutant plants grown under short-day

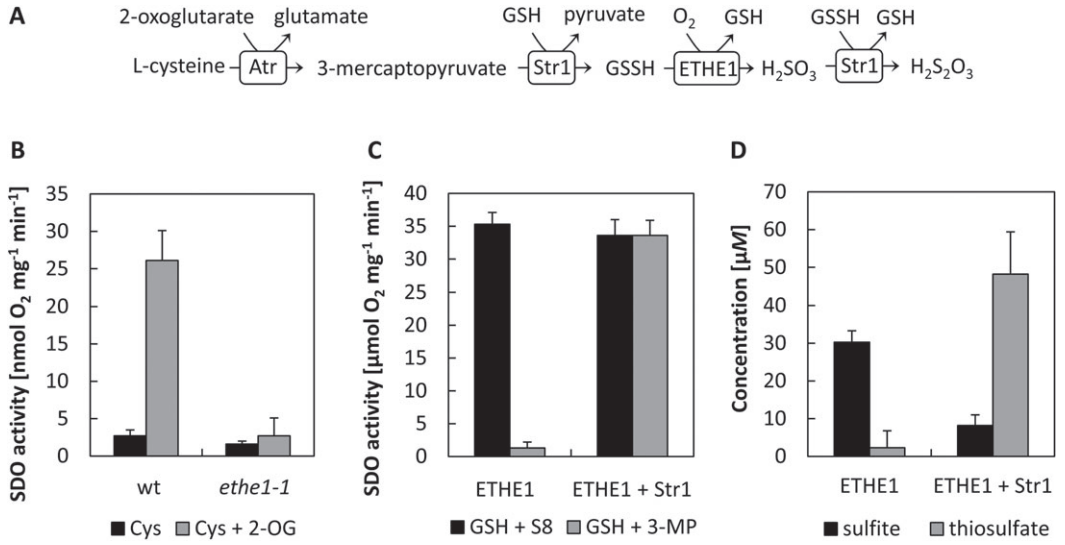

Fig. 1. Reaction steps of the mitochondrial L-cysteine catabolic pathway. (A) Reaction scheme of the oxidation of L-cysteine to pyruvate and thiosulfate catalyzed by a cysteine transaminase (Atr), sulfurtransferase 1 (Str1), and the sulfur dioxygenase ETHE1. (B) Sulfur dioxygenase (SDO) activity (nmol O_2 mg protein^{-1} min^{-1}) of mitochondria isolated from wild type and *ethe1-1* cell culture after the addition of either L-cysteine (5 mM, black bars) or L-cysteine (5 mM) plus 2-oxoglutarate (5 mM, gray bars) as a substrate. (C) SDO activity (µmol O_2 mg protein^{-1} min^{-1}) of isolated recombinant ETHE1 enzyme in the absence and presence of isolated recombinant Str1 after addition of reduced glutathione (1 mM) plus elemental sulfur (15 µl ml^{-1}) (GSH + S8), which non-enzymatically produce glutathione persulfide (black bars), or reduced glutathione (1 mM) plus 3-mercaptopyruvate (5 mM) (GSH + 3-MP, gray bars). (D) Concentrations of the persulfide oxidation products sulfite (black bars) and thiosulfate (gray bars) in the reaction mixture containing 2 µg ml^{-1} purified recombinant ETHE1 enzyme in the absence or presence of 20 µg ml^{-1} purified recombinant Str1 10 min after the addition of GSH plus sulfur as a substrate.

conditions were harvested at the end of the dark period at the age of 42 and 100 days. Metabolite analysis by gas chromatography-mass spectrometry (GC-MS) was performed essentially as described by Lisec et al. (2006) and by extracting of metabolites for injection of extracts from 1 mg fresh weight of plant material into the GC/time-of-flight mass spectrometer. Chromatograms and mass spectra were evaluated using TagFinder 4.0 software (Luedemann et al. 2008) and Xcalibur 2.1 software (Thermo Fisher Scientific, Waltham, MA). Metabolites were identified in comparison with database entries of authentic standards (Kopka et al. 2005, Schauer et al. 2005). Peak areas of the mass (*m/z*) fragments were normalized to the internal standard (ribitol) and fresh weight of the samples. Identification and annotation of detected peaks are shown in Table S1 following recent recommendations for reporting metabolite data (Fernie et al. 2011).

Sulfur-containing metabolites as well as products of the sulfur dioxygenase reaction were analyzed by High-performance liquid chromatography (HPLC) (Hildebrandt and Grieshaber 2008). Shortly, sulfur compounds were labeled with the fluorescent dye monobromobimane (2.9 m*M*) in a buffer containing 50 m*M* Hepes (pH 8.0), 5 m*M* EDTA and 37.5% (v/v) acetonitrile for 30 min in the dark and quantified after separation by reversed-phase HPLC using external standards.

Results

Enzymatic steps of the mitochondrial cysteine catabolic pathway

In a previous study, we demonstrated that the mitochondrial sulfur dioxygenase ETHE1 oxidizes persulfide groups derived from 3-mercaptopyruvate to sulfite and postulated a role for this reaction sequence in L-cysteine catabolism (Krüßel et al. 2014). In order to identify the complete pathway and provide evidence for the individual reaction steps (Fig. 1A), we measured sulfur dioxygenase activity in mitochondria after the addition of L-cysteine as a substrate (Fig. 1B) and in addition reconstituted part of the pathway using isolated recombinant enzymes (Fig. 1C, D). Our results indicate that L-cysteine is oxidized by the ETHE1 pathway after transamination to 3-mercaptopyruvate. Mitochondrial oxygen consumption with L-cysteine as a substrate was detectable only if 2-oxoglutarate was included in the reaction mixture as an amino group acceptor for transamination. The activity was very low in the *ethe1-1* knockdown mutant showing that the oxygen consumption rate in wild-type mitochondria was indeed specific for the ETHE1-dependent pathway (Fig. 1B).

Next, we tested whether ETHE1 in combination with the main mitochondrial sulfurtransferase Str1 is

able to catalyze the remaining part of the postulated pathway, i.e. transfer of the sulfhydryl group from 3-mercaptopyruvate to glutathione, oxidation to sulfite and addition of a second persulfide group to produce thiosulfate (Fig. 1A). Isolated recombinant ETHE1 enzyme oxidizes glutathione persulfide (GSSH), which is non-enzymatically produced from reduced glutathione (GSH) plus elemental sulfur (S8), to sulfite but cannot use the sulfhydryl group of 3-mercaptopyruvate as a substrate (left side of Fig. 1C, D). However, in the presence of isolated recombinant sulfurtransferase 1 protein, the sulfur dioxygenase activity is comparable with elemental sulfur or 3-mercaptopyruvate added as a substrate (right side of Fig. 1C). Thus, the sulfurtransferase is able to produce GSSH as a substrate for ETHE1 by transferring the sulfhydryl group of 3-mercaptopyruvate to GSH. Thiosulfate did not serve as a substrate for this reaction sequence (data not shown). Our results also provide evidence for a second function of Str1 in the cysteine catabolic pathway. The main product of persulfide oxidation by ETHE1 in the presence of Str1 is thiosulfate, indicating that the sulfurtransferase adds an additional persulfide group to the sulfite produced by the sulfur dioxygenase reaction (left side of Fig. 1D).

Physiological roles of mitochondrial cysteine catabolism

Having identified the individual steps of mitochondrial cysteine catabolism, we next addressed the physiological role of this reaction sequence during the Arabidopsis life cycle. T-DNA insertion lines for both, ETHE1 and Str1 have already been characterized (Mao et al. 2011, Holdorf et al. 2012, Krüßel et al. 2014). Knockout of Str1 produced consistent effects in the two independent T-DNA lines analyzed, and complete knockout of ETHE1 is embryo lethal. Therefore, we selected the strong knockdown line *ethe1-1* (Krüßel et al. 2014) and the knockout line *str1-1* (Mao et al. 2011) as representative parent lines to produce a double mutant.

Seed and embryo development is severely impaired in the *ethe1-1* × *str1-1* double mutant

Because embryo development is effected in ETHE1 as well as Str1 deficient mutants, we first analyzed the seeds of the *ethe1-1* × *str1-1* double mutant to see whether downregulation of both enzymes had additive effects. In summary, embryo development of the double mutant was comparable with *str1-1* (Fig. 2). Wild-type embryos developed very uniformly reaching the mature stage at 7 days after pollination (DAP), whereas several growth stages were simultaneously present in *ethe1-1* siliques, and not until 10 DAP all of them were mature (Fig. 2B). In contrast, embryogenesis was severely delayed in *str1-1* and the *ethe1-1* × *str1-1* double mutant. Most of the mutant embryos were still in the pre-globular or globular stage at 5 DAP when the wild type had already reached cotyledon stage (Fig. 2B). At 7 DAP, wild-type seeds were already dark green indicating that the embryos had developed their full photosynthetic capacity (Fig. 2A). In contrast, seeds of *str1-1* plants and the double mutant were smaller, of a white, light green or even light brown color, and contained embryos in the globular to cotyledon stage. Starting from about 10 DAP different degrees of a brown, shriveled seed phenotype became apparent (Fig. 2A, Figs S1 and S2). Embryos developed morphological abnormalities such as giant heart stage or asymmetric cotyledons (Fig. 2A, Figs S3 and S4). Less than 50% of the *str1-1* and *ethe1-1* × *str1-1* embryos finally reached the mature stage whereas the rest mainly aborted at the heart stage.

While mature *ethe1-1* seeds were morphologically indistinguishable from the wild type, only about one fifth of the seeds from *str1-1* plants and the double mutant appeared normal. An equal proportion of the remaining seeds were of a rectangular shape or severely shrunken (Fig. 3). Germination was slightly delayed in *ethe1-1* with 82 ± 3% germinated seeds after 24 h compared with 93 ± 8% in the wild type (Fig. 3). However, after a mere 48 h, there was no significant difference between germination rates of *ethe1-1* and wild-type seeds. In contrast, the germination capacity of *str1-1* seeds was severely compromised. Even normal looking seeds were not completely germinated after 72 h, and in rectangular and shriveled seeds the germination rates dropped to 43 ± 8 and 20 ± 5%, respectively. Interestingly, seeds of the double mutant germinated significantly better than those of the *str1-1* mutant (marked by crosses in Fig. 3). The normal looking seeds behaved like *ethe1-1* seeds and also higher percentages of the misshaped seeds compared with the *str1-1* mutant were able to germinate (63 ± 10% of rectangular and 35 ± 5% of shriveled seeds).

Patterns of early leaf senescence under light limitation

When grown under long-day conditions (16/8 h light/dark) the phenotype of the three analyzed mutant lines was comparable with the wild type (Fig. S5). Shortening of the light period to 8 h per day led to reduced growth and early leaf senescence in *ethe1-1* plants as described before (Krüßel et al. 2014), whereas the *str1-1* plants were indistinguishable from the wild type (Fig. 4). While development proceeded very uniformly in the wild type as well as in both individual mutant lines,

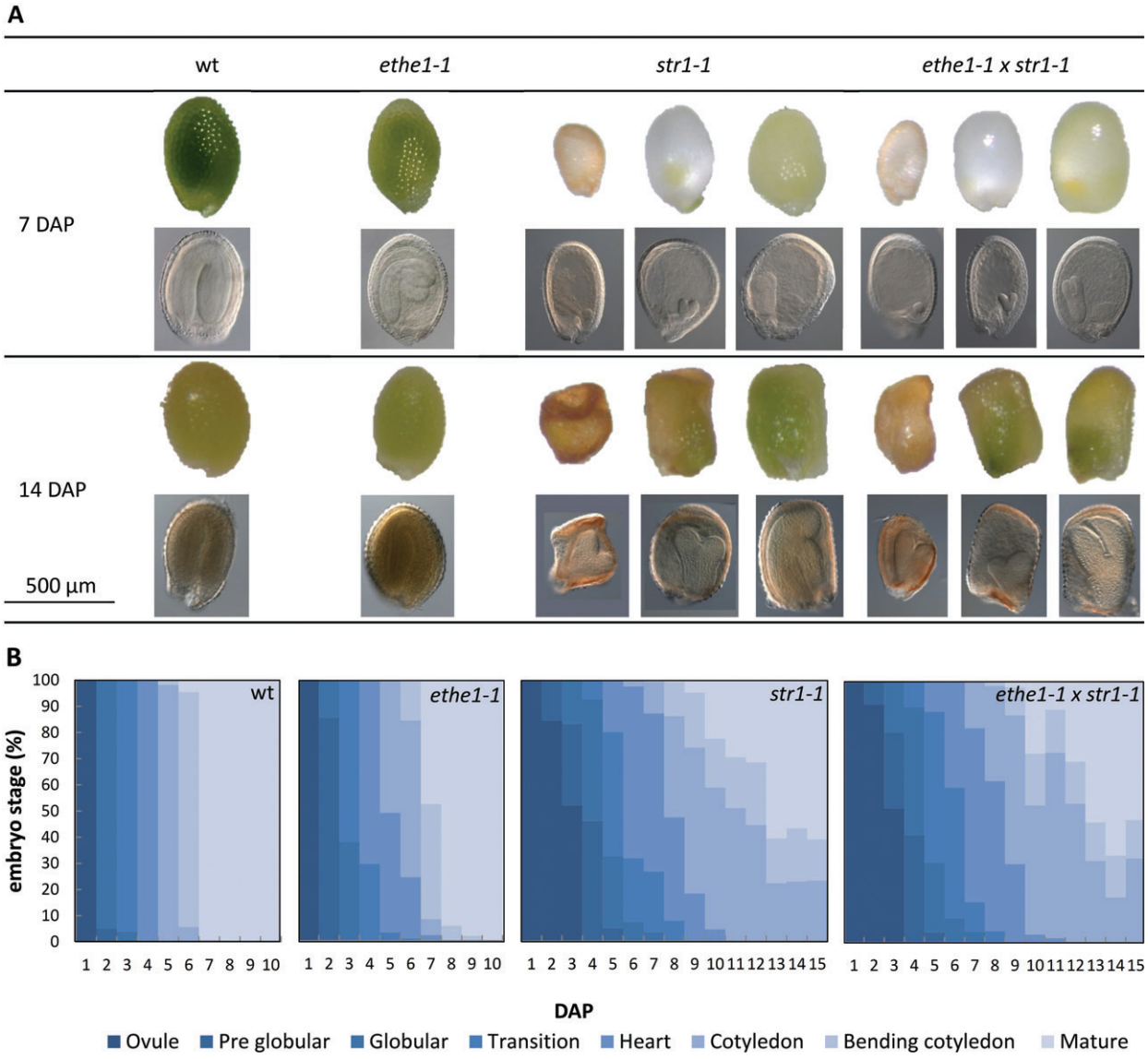

Fig. 2. Embryo development in the *ethe1-1* × *str1-1* double mutant compared with *ethe1-1* and *str1-1* single mutants and the wild type. (A) Representative images of seeds and embryos at 7 and 14 days after pollination (DAP). (B) Progression of embryo development from ovule to mature stage. Approximately 220 seeds were analyzed for each day and genotype, and bars represent the percentage of embryos in the developmental stage indicated.

the phenotype of the *ethe1-1* × *str1-1* double mutant was diverse. Growth rates of the nine double mutant plants analyzed differed fivefold between 0.6 and 3 mm per day (Fig. 4A inserted graph). Plant number 3, 4, 6, 7 and 8 grew rapidly and were indistinguishable from the wild type until 10 weeks after sowing. Then, leafs progressively developed chlorotic patches in two of the large plants (numbers 4 and 6). In contrast, growth rates of four double mutant plants (numbers 1, 2, 5 and 9) were reduced in a similar manner as in *ethe1-1*. However, only two of these small plants (numbers 2 and 5) started to become chlorotic after 7 weeks of growth while the other two (numbers 1 and 9) remained dark green until leaves were harvested at the age of 100 days.

In order to identify the metabolic reason for these phenotype variations, we analyzed metabolite profiles of wild-type and mutant plants grown under short-day conditions (Table S2). Samples were harvested immediately after the dark period at two different developmental stages: After 42 days, neither wild-type nor mutant plants showed any visible signs of senescence ('young plants' Fig. S6). However, *ethe1-1* rosettes were significantly

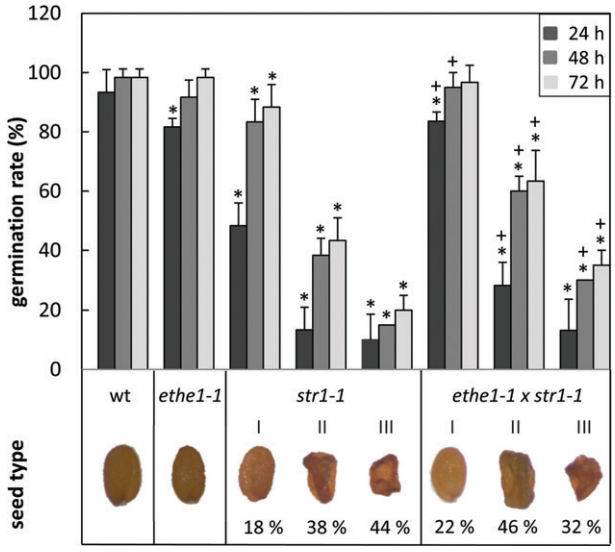

Fig. 3. Phenotype and germination rates of mature seeds in the *ethe1-1* × *str1-1* double mutant compared with *ethe1-1* and *str1-1* single mutants and the wild type. Whereas wild type and *ethe1-1* plants consistently produced intact seeds, three different phenotypes (I, intact; II, rectangular, III, shriveled) were present in *str1-1* plants and the double mutant in a ratio indicated below the respective seed type. Bars represent the germination rates (%) of the different seed types after 24 h (dark gray), 48 h (medium gray) and 71 h (light gray). Asterisks indicate values significantly different from the wild type, and values that are significantly different in the double mutant from the corresponding data points in the str1 mutant are marked with a plus ($P < 0.05$, Student's *t*-test).

smaller than the wild type (6.0 ± 0.8 compared with 9.3 ± 0.9 cm) and contained less leaves (18.7 ± 1.2 vs. 23.9 ± 2.5). The rosette diameter of *ethe1-1* × *str1-1* double mutants varied between 1.6 and 8.5 cm, and small as well as big plants were used for the metabolite analysis. The second set of leaf samples was harvested after 100 days ('old plants'; Fig. 4B), when all plants had already started flowering. *Ethe1-1* leaves were about 50% chlorotic and the double mutant displayed the four different phenotypes described above (see Fig. 4B), with an *ethe1-1*-like phenotype (plants 2 and 5), a wild-type-like phenotype (plants 3, 7 and 8), and two intermediate forms, i.e. small green plants (plants 1 and 9), and large plants with yellowing leaves (plants 4 and 6).

Already in the young plants, harvested at 42 days after sowing, a number of specific changes in the metabolite profiles of the mutants compared with the wild type became apparent (Figs 5 and 6, Table S2). Nitrogen-rich amino acids accumulated in all mutants, and the levels of secondary metabolites involved in nitrogen turnover such as putrescine and spermidine were also significantly increased (Fig. 5A; upper panel). In addition, we detected significantly higher levels of the amino acids taking part in photorespiration, glycine and serine, in *str1-1* and the double mutant (Fig. 5C). Interestingly, serine was also elevated in *ethe1-1* plants without a concomitant increase in glycine leading to a significant decrease in the glycine/serine ratio, which is often used as an indicator of photorespiratory activity. Specific effects on the tricarboxylic acid cycle (TCA) cycle also became apparent. In all three mutants, succinate and fumarate levels were significantly lower than in the wild type (1.9 to 6.5-fold), and malate was additionally decreased in *str1-1* and the double mutant (Fig. 6).

The metabolite profile of young *ethe1-1* plants revealed two characteristic changes that were not present in *str1-1* or the double mutant. There was a significant increase in the amino acids associated with energy metabolism, i.e. branched-chain amino acids, lysine and aromatic amino acids (Fig. 5B, upper panel). In addition, carbohydrate metabolism was effected with a strong accumulation of galactinol (5.3-fold) and raffinose (8-fold) and lower but still significant increases in sucrose and fructose concentrations while glucose was slightly decreased (Fig. 6). Respiration rates of leafs measured at the end of the dark period were significantly increased by 25% in young *ethe1-1* plants but comparable to the wild type in *str1-1* and the double mutant (Fig. S7).

Metabolite profiles of the old plants harvested 100 days after sowing reflected patterns in comparison to the young plants that are typical for leaf senescence. In particular, a general increase in levels of free amino acids most pronounced in those involved in nitrogen remobilization, a strong decrease in the glycine/serine ratio, and an accumulation of sugars were detected in wild type as well as mutant plants (Figs 5 and 6).

Next, we analyzed the metabolite profiles of the individual plants using the hierarchical clustering tool available in MeV (Fig. 7). The metabolite profile of *str1-1* was very similar to the wild type in the old plants so that the clustering algorithm was not able to distinguish between these two groups. However, all five *ethe1-1* plants were clearly grouped together on the basis of their metabolite profiles and, most interestingly, eight of the nine *ethe1-1* × *str1-1* plants clustered correctly according to their phenotype. The small and chlorotic double mutant plants (numbers 2 and 5), which looked like *ethe1-1* plants of the same age, also had the most similar metabolite profiles followed by the big plants with chlorotic leaves (numbers 6 and 7). Four of the five double mutant plants that were still entirely green at the time of harvest clustered together, and again profiles of the small rosettes (numbers 1 and 9) were clearly separated from those of the big rosettes (numbers 7 and 8). Only one of the double mutant plants with no visible

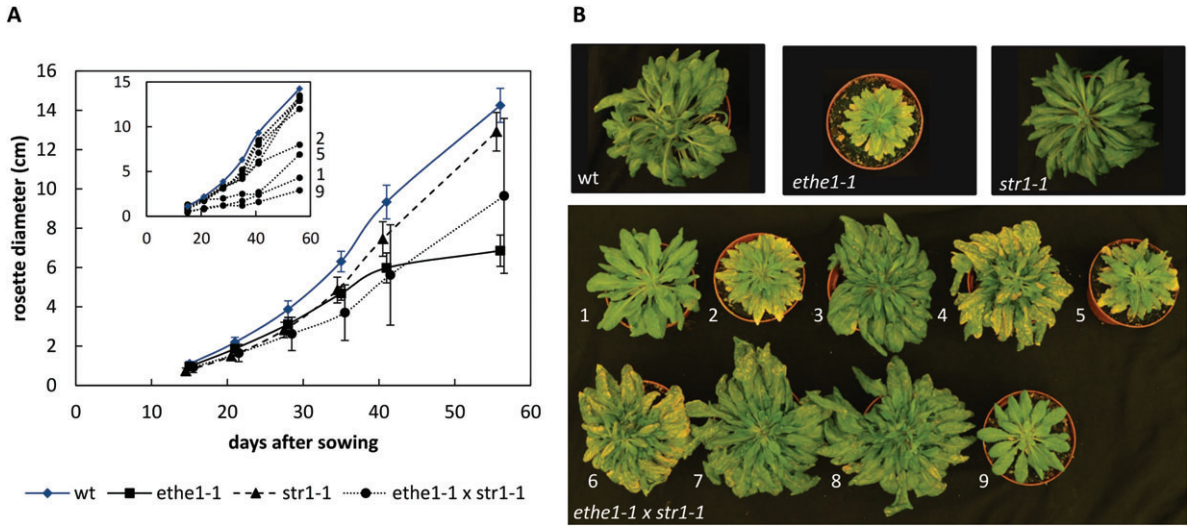

Fig. 4. Phenotype of the *ethe1-1* × *str1-1* double mutant compared with *ethe1-1* and *str1-1* single mutants and the wild type under short-day growth conditions (8/16 h light/dark). (A) Means ± standard deviations of maximal rosette diameters from 9 to 10 plants (cm). Because double mutant plants had a very heterogeneous phenotype, growth curves of the individual plants are shown in the inserted graph. (B) Rosettes of the nine double mutant plants analyzed and representative plants of the *ethe1-1* and *str1-1* single mutants and the wild type after 100 days of growth under short-day conditions.

phenotype (numbers 3) was clustering with the wild type and *str1-1* plants. Because not only the plants but also the metabolites were clustered, it was possible to derive patterns typical for the individual phenotypes (marked by the black boxes in Fig. 7).

In order to estimate, whether the variable phenotype of the double mutant plants might be associated with different activities of the remaining sulfurtransferases, we measured the mercaptopyruvate sulfurtransferase activity in leaf homogenate of the plants harvested after 100 days of growth under short-day conditions (Fig. 8A). As expected, 3-mercaptopyruvate sulfurtransferase activity was significantly decreased (to about 50%) in *str1-1* plants compared with the wild type. Interestingly, ETHE1 deficiency also led to a significant reduction in sulfurtransferase activity. A combination of both effects in the *ethe1-1* × *str1-1* line resulted in a high variation of total mercaptopyruvate sulfurtransferase activity in the nine individual plants analyzed between 72 and 137 nmol thiocyanate mg^{-1} min^{-1} (Fig. 8A, light gray bars). Analysis of sulfur metabolites revealed a general accumulation in *ethe1-1* leaves (Fig. 8B). In contrast, thiosulfate was strongly reduced in both Str1 deficient mutant lines (*str1-1* and the double mutant). Contents of sulfite, cysteine, and GSH were more variable in the nine double mutant plants analyzed (Table S3). Interestingly, the small plants (numbers 1, 2, 5 and 9) had consistently higher GSH contents than the large plants, and cysteine was increased in the double mutants with an *ethe1-1*-like phenotype (numbers 2 and 5).

Discussion

We identified a mitochondrial cysteine catabolic pathway and revealed the physiological relevance of individual reaction steps for seed development and plant growth under limited light conditions.

Three mitochondrial enzymes oxidize L-cysteine to pyruvate and thiosulfate

We demonstrated before that the mitochondrial sulfurdioxygenase ETHE1 oxidizes glutathione-bound persulfide groups to sulfite (Krüßel et al. 2014), and this reaction can easily be monitored via oxygen consumption. Here we show that (1) oxidation of the thiol group from L-cysteine by ETHE1 requires transamination to 3-mercaptopyruvate and this activity is present in Arabidopsis mitochondria, (2) 3-mercaptopyruvate can be oxidized by ETHE1 in the presence of sulfurtransferase 1 and (3) sulfurtransferase 1 converts the sulfite produced by ETHE1 to thiosulfate. Thus, we provide evidence for the complete pathway of L-cysteine catabolism to thiosulfate and pyruvate via 3-mercaptopyruvate, glutathione persulfide and sulfite (Fig. 1A).

A cysteine aminotransferase is present in Arabidopsis mitochondria

L-Cysteine can be oxidized in Arabidopsis mitochondria, but only in the presence of 2-oxoglutarate indicating that a transamination step is involved. Thus, a role

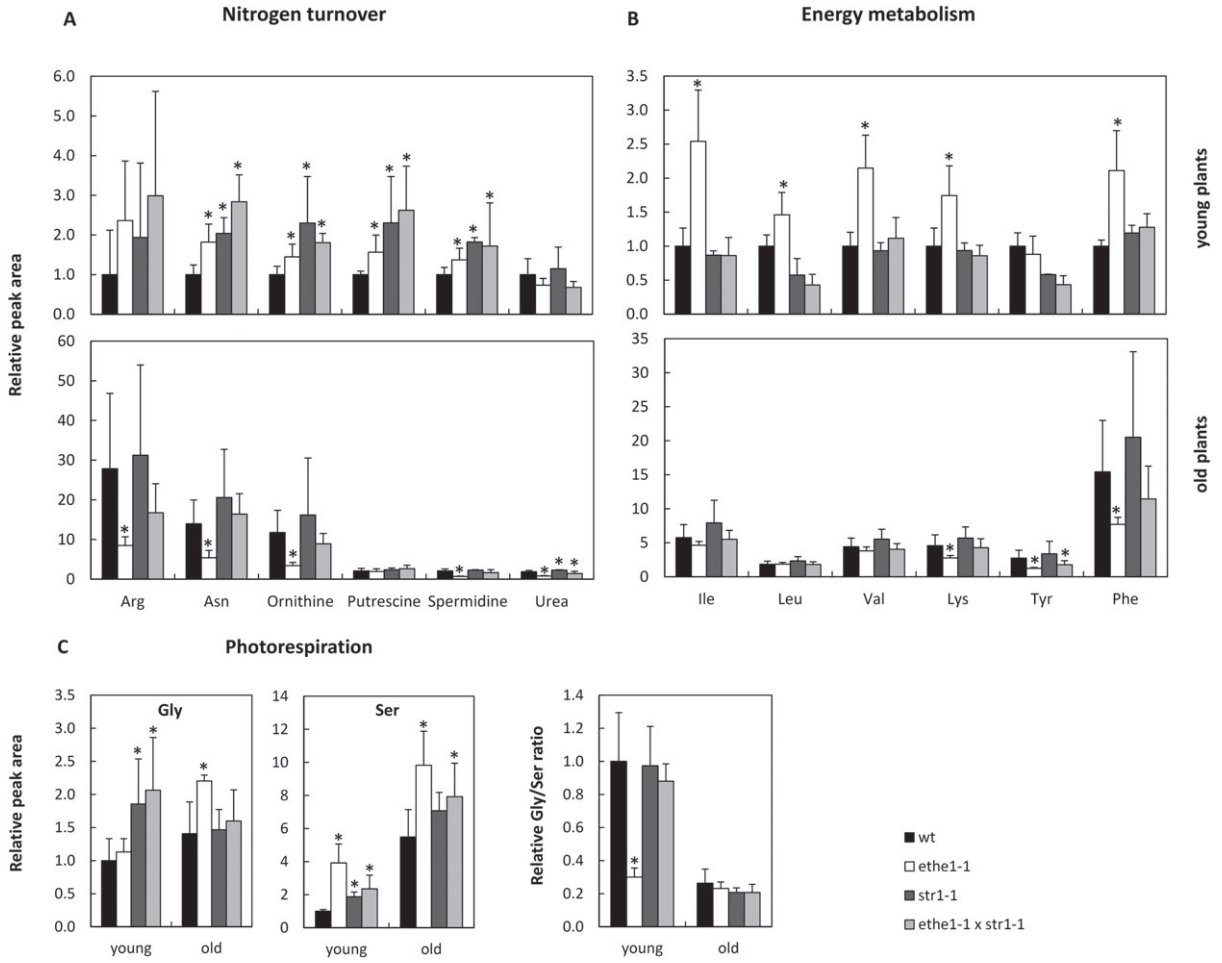

Fig. 5. Amino acid profile of the *ethe1-1* × *str1-1* double mutant compared with *ethe1-1* and *str1-1* single mutants and the wild type under short-day growth conditions (8/16 h light dark). Complete rosettes of 3–5 plants were harvested at dawn at the age of 42 days (young plants) and 100 days (old plants) and analyzed by GC/MS. Amino acid contents are given as relative peak areas normalized to young wild-type plants. *Significantly different from corresponding wild-type samples ($P < 0.05$, Student's *t*-test).

of cysteine desulfurases and desulfhydrases can be excluded. Because cyanide was not present in the reaction mixture, the mitochondrial matrix enzyme β-cyanoalanine synthase is also not involved in the cysteine catabolic pathway described here. The protein catalyzing transamination of cysteine and 2-oxoglutarate to 3-mercaptopyruvate and glutamate has not been identified so far, and no cysteine aminotransferase is annotated in the Arabidopsis genome. However, the same activity has been found in animal mitochondria and postulated to be an additional function of an aspartate aminotransferase (Ubuka et al. 1978). Thirty-one aminotransferases with different substrate specificities have been described in Arabidopsis so far, and 10 of them are probably localized in the mitochondrial matrix (Hildebrandt et al. 2015). These mitochondrial aminotransferases are annotated to be specific for ornithine, aspartate, branched-chain amino acids, alanine and glyoxylate, and it will be the subject of future studies to find out whether one of them or an additional enzyme, as yet not identified as an aminotransferase, catalyzes the transamination of L-cysteine.

Two physiological roles for plant mitochondrial sulfurtransferase

Sulfurtransferases are ubiquitous enzymes present in all the different compartments of plant, animal and also prokaryotic cells. They catalyze the transfer of sulfane sulfur from persulfides, polythionates, thiosulfate and 3-mercaptopyruvate to thiophilic acceptors such as cyanide, sulfite or thiols (GSH, dihydrolipoate, thioredoxin and even protein sulfhydryl groups) (Westley 1973, 1980, Nandi and Westley 1998). However, their

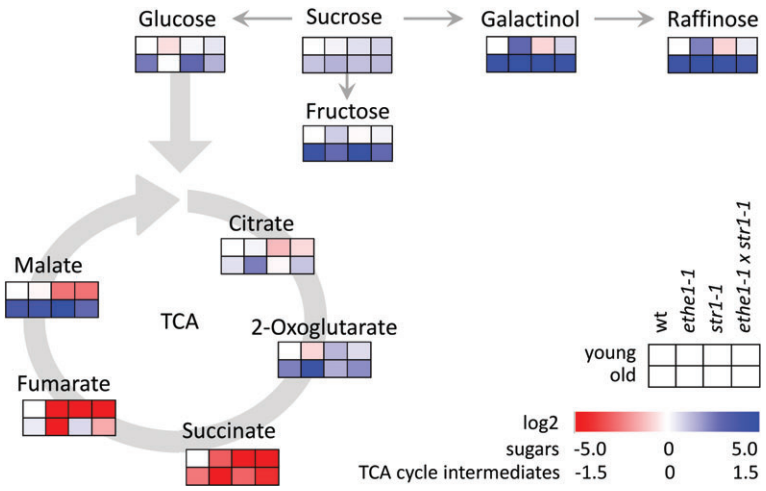

Fig. 6. Heatmap of metabolite changes related to sugar metabolism and the TCA cycle in young and old wild-type and mutant plants. Log_2 ratios of fold changes in relation to young wild-type samples are indicated by shades of blue or red color according to the scale bar. Data represent mean values of three to nine biological replicates for each data point. Rosettes were harvested at dawn after 42 days (young) and 100 days (old) of growth under short-day conditions (8/16 h light/dark). The complete dataset (means ± standard deviations, statistical analysis) is presented in Table S2.

metabolic functions have yet to be completely understood. The detoxification of cyanide has initially been considered to be the main role of sulfurtransferases because cyanide is a very good acceptor for persulfide groups in vitro and the addition of sulfur converts cyanide to the less toxic thiocyanate (Westley 1973). Additional physiologically relevant functions of sulfurtransferases might be control of the sulfane sulfur pool, maintenance of iron–sulfur clusters and molybdenum cofactor synthesis (Westley et al. 1983, Ogata and Volini 1990, Matthies et al. 2004).

Here we demonstrate that the mitochondrial sulfurtransferase Str1 is part of a cysteine catabolic pathway in Arabidopsis and even catalyzes two of the four reaction steps. Isolated recombinant Str1 protein was able to produce a substrate for ETHE1 from 3-mercaptopyruvate (Fig. 1C). Because this activity was only detectable in the presence of GSH, the substrate produced was most likely GSSH, which has been described as a product of sulfurtransferases before (Westley 1973, 1980). In the in vitro assay using cyanide as a sulfur acceptor, Str1 has a higher activity with 3-mercaptopyruvate than with thiosulfate as a substrate and is therefore called mercaptopyruvate sulfurtransferase (Papenbrock and Schmidt 2000). In accordance with this result, we found that Str1 readily transfers the thiol group from 3-mercaptopyruvate but not from thiosulfate to GSH to produce GSSH as a substrate for the sulfur dioxygenase reaction. As already described in animals, sulfurtransferases can also produce thiosulfate by transferring a persulfide group from a suitable donor such as GSSH to sulfite (Westley 1973, Hildebrandt and Grieshaber 2008). Isolated recombinant Arabidopsis ETHE1 enzyme oxidizes persulfides to sulfite, whereas the product of persulfide oxidation in mitochondria is mainly thiosulfate (Fig. 1D, Krüßel et al. 2014). Here we demonstrate that addition of Str1 protein to the reaction mixture is sufficient to produce this product shift from sulfite to thiosulfate (Fig. 1D). Also, the thiosulfate content of rosette leaves was strongly decreased in the Str1 deficient mutant lines compared with the wild type. Thus, in addition to providing GSSH from 3-mercaptopyruvate as a substrate for ETHE1, Str1 can indeed catalyze a second reaction step in the mitochondrial cysteine catabolic pathway and transfer a persulfide group to sulfite producing thiosulfate.

Cysteine degradation and persulfide transfer reactions are essential during embryo development

Complete knockout of either sulfurtransferase 1 or ETHE1 in Arabidopsis severely affects embryo development indicating that cysteine catabolism is highly important during this developmental stage. The reason for the seed defects could either be accumulation of a toxic intermediate or nutrient deficiency. Because both mutant lines are affected, the potential toxic intermediate is most likely produced prior to the first transsulfuration step, i.e. it might be 3-mercaptopyruvate, L-cysteine or the dimer L-cystine, which forms non-enzymatically from oxidized cysteine but, at least in animals, is supposedly less toxic (Baker 2006). An alternative explanation would be the presence of two independent toxic intermediates in the individual mutant lines, because the sulfurtransferase

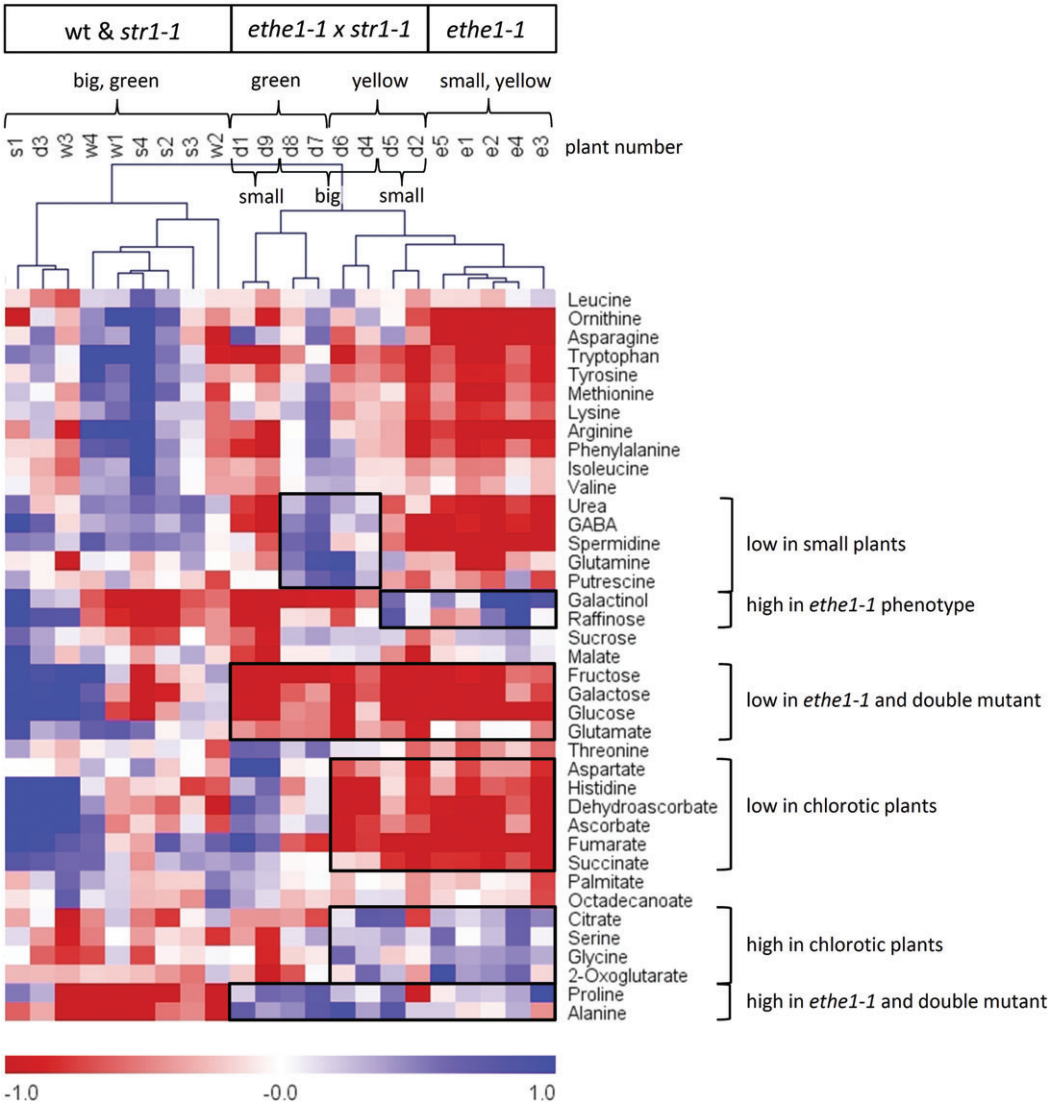

Fig. 7. Clustering of individual mutant and wild-type plants harvested after 100 days of growth under short-day conditions according to their metabolite profiles. Log_2 ratios of fold changes in relation to the mean peak area of each metabolite are indicated by shades of blue or red color according to the scale bar. Rosettes of four wild-type plants (w1-4), four *str1-1* plants (s1-4), five *ethe1-1* plants (e1-5) and nine *ethe1-1* × *str1-1* plants shown in Fig. 4B (d1-9) were harvested at dawn after 100 days of growth under short-day conditions (8/16 h light/dark). Hierarchical clustering was performed using MeV software. Plant numbers are given in the upper part of the figure together with some phenotype information. Black boxes mark metabolite patterns characteristic for individual phenotypes. The complete dataset (means ± standard deviations, statistical analysis) is presented in Table S2.

probably has additional functions to its role in cysteine degradation such as cyanide detoxification. The seed phenotype of *ethe1-1* × *str1-1* plants resembled the *str1-1* single mutant and was not worsened but in contrast even slightly improved by the additional downregulation of sulfur dioxygenase activity. This result would be in agreement with the postulated accumulation of toxic cyanide because of Str1 knockout and toxic persulfide because of ETHE1 knockdown. Persulfide accumulation is most likely reduced in the double mutant because the sulfurtransferase producing it is missing. Residual persulfides potentially resulting from other reactions would readily react with cyanide and thus lessen its toxic effect. Knockdown of ETHE1 leads to an accumulation of most amino acids in the seeds (Krüßel et al. 2014). Thus, defects in cysteine degradation probably interfere with protein catabolism in general and might create a shortage of nitrogen supply. However, this option has to be further investigated in order to gain a deeper understanding of the underlying molecular mechanisms.

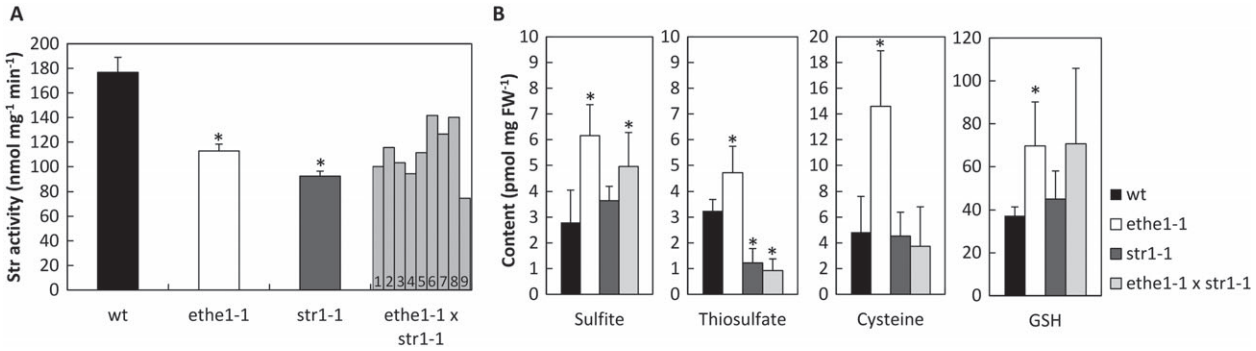

Fig. 8. Sulfurtransferase activities and contents of sulfur metabolites in mutant and wild-type plants harvested after 100-days of growth under short-day conditions. (A) Mercaptopyruvate sulfurtransferase activity (nmol thiocyanate produced mg protein^{-1} min^{-1}). For *ethe1-1* × *str1-1* activities of the nine plants analyzed are shown individually (light gray bars, plant numbers are indicated). (B) Sulfur metabolite content (pmol mg fresh weight^{-1}). Metabolite contents of the individual double mutant plants are given in Table S3. Data represent mean values of three to nine biological replicates for each data point. *Significantly different from corresponding wild-type samples ($P < 0.05$, Student's *t*-test).

In contrast to the situation in seeds, the function of sulfurtransferase 1 appears to be non-essential for vegetative growth in Arabidopsis, at least under non-stressed growth conditions. All necessary physiological functions can probably be compensated by one of the additional 19 sulfurtransferase isoforms (Bartels et al. 2007). Because cysteine and GSH levels were normal in the *str1-1* plants, the residual mercaptopyruvate sulfurtransferase activity of about 50% was obviously sufficient to efficiently catalyze the second step in the cysteine degradation pathway. Interestingly, thiosulfate contents were significantly reduced in all Str1 deficient plants indicating that thiosulfate production might be a more specific function of this sulfurtransferase isoform.

The early senescence phenotype characteristic for *ethe1-1* plants grown under short-day conditions was only present in some of the *ethe1-1* × *str1-1* double mutants and correlated quite well with an increased cysteine content. Thus premature senescence as well as cysteine accumulation is most likely caused by reactive sulfur intermediates, which are produced in different amounts in the individual double mutant plants. This would be consistent with the high variability in seed development and different levels of residual sulfurtransferase activity present in the *ethe1-1* × *str1-1* plants.

Defects in cysteine catabolism induce metabolic shifts characteristic for developmental senescence

Plant senescence is a highly coordinated physiological process essential for nutrient remobilization to the growing seeds (Lim et al. 2007). Recently, comprehensive metabolite profiles of the senescence process in Arabidopsis have been published, providing a catalog of metabolite patterns characteristic for the onset of developmental senescence that can be used as markers (Watanabe et al. 2013). Interestingly, specific shifts in the metabolite profile already occur in pre-senescent tissues before chlorosis becomes apparent.

We detected several senescence-specific metabolite changes in young rosettes of all mutant lines analyzed. The concentrations of amino acids as well as secondary metabolites associated with nitrogen turnover were elevated in the mutants compared with the wild type. High asparagine/aspartate and glutamine/glutamate ratios are typical metabolite markers for the onset of senescence in Arabidopsis, and nitrogen-rich amino acids are closely associated with nitrogen remobilization and transport (Watanabe et al. 2013). Concentrations of succinate and fumarate were consistently reduced in all three mutants related to cysteine catabolism indicating a change in the flux mode of the TCA cycle toward the production of precursors for other pathways that may be connected to a higher nitrogen turnover (Sweetlove et al. 2010). In addition, we detected an increase of serine and glycine levels, which is also characteristic for senescence under short-day conditions (Watanabe et al. 2013). According to these results, defects in the mitochondrial cysteine degradation pathway lead to several metabolic shifts that normally occur during senescence. However, because *str1-1* plants developed normally additional effects were obviously necessary to actually induce premature leaf senescence.

An intermediate from cysteine degradation interferes with amino acid catabolism and affects the C/N status

A shift in the C/N-status reflected by an accumulation of hexoses in combination with increased concentrations

of amino acids and other metabolites related to nitrogen remobilization is often discussed to be an internal signal for the induction of leaf senescence (Wingler et al. 2009, Agüera et al. 2010). While metabolites associated with nitrogen remobilization were high in all mutant plants analyzed, hexoses as well as the stress-related sugars galactinol and raffinose accumulated considerably in the *ethe1-1* plants. Thus, the increase in free sugar levels in combination with a low nitrogen status might act as a metabolic signal for the onset of senescence in young ETHE1 deficient plants. In addition, branched-chain amino acids, aromatic amino acids and lysine accumulated in young *ethe1-1* plants at the end of the dark period. Energy yield from the oxidation of these amino acids is particularly high, and they typically increase during carbohydrate starvation probably to serve as an alternative substrate for ATP production (Araújo et al. 2011, Hildebrandt et al. 2015). Dark respiration was also significantly increased in *ethe1-1* leaves indicating a high metabolic rate. Interestingly, these effects were specific for *ethe1-1* and not present in the *ethe1-1*×*str1-1* double mutant. Therefore, they might be caused by a persulfide intermediate that is not produced in the absence of the sulfurtransferase. Persulfides are highly reactive (nucleophilic and reducing) and have been identified as critical components of redox cell signaling in mammals (Ida et al. 2014). GSSH as well as protein bound cysteine persulfides are endogenously produced and maintained in animal cells, and our results indicate that these reactive sulfur species might have a similar function in plant tissues.

After 100 days of growth under short-day conditions, metabolite profiles clearly indicated that the senescence process had also started in wild type and *str1-1* plants although only minor chlorotic patches were visible in the leaves. Some of the characteristic differences detected in *ethe1-1* and *ethe1-1*×*str1-1* plants such as increased concentrations of the stress-related sugars raffinose and galactinol, reduced levels of antioxidants, and changes in the TCA cycle mode can be attributed to the advanced senescence state of these mutants compared with the wild type. Interestingly, there were also some metabolite patterns apparent in *ethe1-1* and double mutant plants at this late developmental stage that were contrary to the characteristic senescence profile of Arabidopsis leaves and could therefore reflect specific effects of persulfide accumulation. Hexose levels were low in ETHE1 deficient plants indicating that the increased metabolic rate might lead to carbohydrate starvation during senescence. In addition, growth repression could be connected to changes in the nitrogen status, because the contents of amino acids associated with nitrogen remobilization correlated with the size of the double mutant plants.

Conclusions

None of the previously published pathways of cysteine catabolism consider the ultimate fate of the thiol group (Riemenschneider et al. 2005, Alvarez et al. 2010, 2012). However, our results clearly demonstrate that a tight regulation of the concentrations of reactive persulfide intermediates is highly important. An exciting question for further research will be whether persulfides also have a defined signaling function in plants similar to that described in animals. By now, it is already obvious that either toxic or regulatory effects of reactive sulfur intermediates interfere with central aspects of plant metabolism during seed development as well as vegetative growth under light-limiting conditions. It is highly conceivable that a better understanding of the mechanisms underlying these phenomena will provide a basis for improving plant yield and fitness.

Author contributions

S. H. and M. B. performed and evaluated experiments addressing the individual steps of the cysteine catabolic pathway, C. L. and T. B. characterized the *ethe1-1*×*str1-1* double mutant, T. T. and A. R. F. performed metabolite profiling, H. P. B. contributed to the scientific context, T. M. H. designed the experiments, evaluated data and wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Overview of the metabolite reporting list.

Table S2. Metabolite profiles of wild-type and mutant plants grown under short-day conditions (8 h light/ 16 h dark).

Table S3. Contents of sulfur metabolites in wild-type and mutant plants grown under short-day conditions (8/16 h light/dark).

Fig. S1. Seed development in the sulfurtransferase 1 knockout line *str1-1*.

Fig. S2. Seed development in the *ethe1-1* × *str1-1* double mutant.

Fig. S3. Embryo development in the sulfurtransferase 1 knockout line *str1-1*.

Fig. S4. Embryo development in the *ethe1-1* × *str1-1* double mutant.

Fig. S5. Phenotype of the *ethe1-1* × *str1-1* double mutant compared with *ethe1-1* and *str1-1* single mutants and the wild type under long-day growth conditions (16 h light/8 h dark).

Fig. S6. Phenotype of the 'young' plants harvested for metabolite analysis.

Fig. S7. Leaf respiration of wild-type and mutant plants at the end of the dark period.

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4.2 Manuscript 1

Amino acid metabolism and the role of ETHE1 in Arabidopsis thaliana seeds

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Amino acid metabolism and the role of ETHE1 in *Arabidopsis thaliana* seeds

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Abstract

The sulfur dioxygenase ETHE1 oxidizes persulfides in the mitochondrial matrix and is involved in the degradation of L-cysteine. ETHE1 has an essential but yet undefined function in early embryo development of *Arabidopsis thaliana*. In leaves, ETHE1 is strongly induced by extended darkness and takes part in the use of amino acids as alternative respiratory substrates during carbohydrate starvation. Thus, we tested the effect of darkness on seed development in an ETHE1 deficient mutant compared to the wild type. Since ETHE1 knock out is embryo lethal, the knock down line *ethe1-1* with about 1 % residual sulfur dioxygenase activity was used for this study. We performed phenotype analysis, metabolite profiling and comparative proteomics to investigate the general effect of extended darkness on seed metabolism and further define the particular function of the mitochondrial sulfur dioxygenase ETHE1 in seeds. Shading of the siliques had no morphological effect on embryogenesis in wild type plants. However, the developmental delay that was already visible in *ethe1-1* seeds under control conditions was further enhanced in the darkness. Interestingly, dark conditions strongly affected seed quality parameters of wild type as well as mutant plants. The lipid content of the seeds was severely decreased to about 20 % of light conditions, and there was also a clear reduction in free sugar concentrations. In contrast, amino acids accumulated with the strongest effect on the nitrogen-rich amino acids asparagine and glutamine. Proteome as well as metabolite data revealed a major regulatory effect of light on nitrogen turnover in the developing seeds. In addition, first evidence for a function of branched-chain amino acid catabolism in seed energy production was provided. Knockdown of ETHE1 lead to alterations in endosperm cellularization as well as mitochondrial structure. Early seedling establishment was less sensitive to absicic acid inhibition in *ethe1-1* than

in the wild type. Thus, the sulfur dioxygenase ETHE1 might have a function in ABA signal transduction. Interestingly, the effect on amino acid profiles was clearly different from that found in leaves indicating that in seeds the ETHE1 dependent pathway interacts with alanine and glycine rather than branched-chain amino acid metabolism.

Introduction

Seed development of plants starts with the unique event of double fertilization. The resulting seed compartments endosperm and embryo undergo specific developmental sequences with dramatic changes on cellular and molecular level from tissue differentiation and growth to storage deposition during seed filling. The endosperm is essential for embryo development. It embeds the embryo and is surrounded by the seed coat. In *A. thaliana* the endosperm is of transient nature and only the aleurone layer remains until seed maturity (Novack et al. 2010). Its development is characterized by a stepwise differentiation including transition from a syncytial to a cellular phase (Olsen 2004, Berger 2003, Brown et al. 2003, Brown et al. 1999). The process of cellularization has a great impact on the accurate embryo development (Hehenberger et al. 2012) and conducts the initiation of embryo growth (Brown et al. 1999). During seed maturation the embryo massively accumulates storage compounds within the cotyledons and becomes a highly specialized storage tissue. At early stages starch and hexoses accumulate transiently, but their amount gradually decreases accompanied by a rapid increase of protein and lipid storage (Borisjuk et al. 2005, Baud et al. 2002). The synthesis of storage compounds requires provision of sufficient amounts of precursor molecules, reductants and energy (Gallardo et al. 2008, Fait et al. 2006). Nutrients such as sugars and amino acids delivered from the mother plant are precursors for storage product biosynthesis in seeds (Melkus et al. 2009). An adaption of energy metabolism including photosynthesis and respiration is essential to enable efficient storage product accumulation. Seed plastids hold special structures and have adapted their metabolism to cope with reduced light levels available for photosynthetic reactions (Borisjuk et al. 2013, Borisjuk et al. 2004). The permeability of gases into seeds is low, and plastidial activity during the maturation phase contributes to oxygen allocation and reassimilation of CO_2 (Borisjuk and Rolletschek 2009, Rolletschek et al. 2005, Borisjuk et al. 2005, Ruuska et al. 2004). Highest photosynthetic activity is observed during storage product synthesis, suggesting a correlation of metabolic processes and photosynthesis (Fait et al. 2006, Ruuska et al. 2002). Recently it was

postulated that amino acids are not only precursors for storage proteins within seeds, but might also serve as alternative substrates for mitochondrial metabolism during situations of high energy demand (Galili et al. 2014). In vegetative tissues amino acid catabolism is induced by carbon starvation situations e.g. periods of extended darkness (Hildebrandt et al. 2015, Araujo et al. 2011 and 2010) First studies denote a potential effect of amino acid degradation also on the energy status of seeds (Credali et al. 2013, Angelovici et al. 2011, Gu et al. 2010, Angelovici et al. 2009, Weigelt et al. 2008, Zhu and Galili 2003). However, biosynthesis as well as catabolism of amino acids in seeds is still largely unknown. The mitochondrial sulfur dioxygenase ETHE1 (AT1G53580) is part of a sulfur catabolic pathway that catalyzes the oxidation of sulfide or persulfides derived from amino acids to thiosulfate and sulfate. In *Arabidopsis* leaves ETHE1 has a key function in amino acid catabolism in situations of carbohydrate starvation such as extended darkness (Krüßel et al. 2014). In seeds, ETHE1 knock out leads to alterations in endosperm formation and finally causes seed abortion (Holdorf et al. 2012). It has been shown that a sulfur dioxygenase activity of 1% present in an ETHE1 knock down mutant (*ethe1-1*) is sufficient for embryo survival, but development is severely delayed (Krüßel et al. 2014), which underlines the importance of this enzyme for seed metabolism.

This study aimed to investigate the physiological role of the mitochondrial sulfur dioxygenase ETHE1 in seeds. One major aspect was to establish whether the functional context of ETHE1 in leaves, the use of amino acids as alternative respiratory substrates, is relevant during embryo development. Therefore, our experimental approach included shading of the siliques combined with metabolite and proteome analysis. Our results show a strong effect of extended darkness on amino acid metabolism in seeds and indicate an additional role of ETHE1 in ABA signaling and cell structure formation.

Results

A combination of phenotype analysis, metabolite profiling and comparative proteomics was used to investigate amino acid metabolism in general and the particular function of the mitochondrial sulfur dioxygenase ETHE1 in seeds. Since ETHE1 knock out is embryo lethal (Holdorf et al. 2012), the knock down line *ethe1-1* described in Krüßel et al. (2014) was used for all experiments.

ETHE1 knock down causes a delay in embryogenesis which is further enhanced in the darkness

In leaves ETHE1 is strongly induced by extended darkness and has a key function in the use of amino acids as alternative respiratory substrates during carbohydrate starvation (Krüßel et al. 2014). Thus, we tested the effect of darkness on seed development in the *ethe1-1* mutant by covering the young siliques with aluminium foil one day after pollination (DAP, Supp. Fig. 1). The role of photosynthesis in seeds is still largely unknown. Since carbohydrates are mainly provided by the mother plant, additional functions compared to leaves can be expected. Therefore, we first tested the effect of darkness on seed development in wild type plants. Overall development of the siliques and the number of seeds produced was not influenced by the shading procedure (data not shown). However, the seeds did not turn green at 4-5 DAP like under light conditions but appeared yellowish (Figure 1A). Seeds grown under light and dark conditions were harvested over a period of 1 to 9 DAP. For each time point (1-9 DAP) and light condition (light, dark) embryo development in seeds from 5 siliques was analyzed using a seed clearing method. Interestingly, there were no morphological differences between wild type embryos growing under light or dark conditions. Development from globular to mature stage proceeded very uniformly, and all seeds in a silique were in the same developmental stage (Figure 1B). In contrast, we observed a delay of embryogenesis in *ethe1-1* seeds with several embryo stages present within one silique. Dark conditions induced a stronger phenotype in *ethe1-1* seed, which became apparent starting from 3 DAP were most of the embryos were still in the globular stage, while light grown *ethe1-1* embryos were in the transition stage. At 9 DAP most of the embryos were fully matured, but some were still in the cotyledon and bending cotyledon stage. The

developmental delay of *ethe1-1* seeds compared to the wild type observed under control conditions is further increased by about two days in darkness.

Darkness leads to reduced seed weight and a changed biomass composition

Mature seeds of wild type as well as *ethe1-1* plants grown under light or dark conditions were of the same size and morphologically indistinguishable. However, the final seed weight was drastically reduced in darkness for both genotypes (Figure 2A). To investigate whether the lower weight is caused by a shift in storage product accumulation, we analyzed the final biomass composition of the dormant seeds (Figure 2B). For both, wild type and *ethe1-1* seeds, lipid contents were decreased to about 20% of light grown seeds. In addition shading of the siliques induced an accumulation of free amino acids. Most notably contents of the nitrogen-rich amino acids Asn and Gln increased more than 20-fold in the darkness compared to control conditions (Figure 2C) In contrast, the protein content was slightly reduced in dark grown seeds, whereas final starch levels remained unaffected. However, concentrations of hexoses and sucrose were strongly decreased after the dark treatment in wild type as well as *ethe1-1* seeds. In agreement with the postulated impact of photosynthesis on germination timing (Allorent et al. 2015), we observed a reduced germination capacity of dark grown seeds (Supp. Fig. 2). Only minor differences were observed regarding biomass composition and germination rate between wild type and *ethe1-1* seeds.

Proteomics and metabolite profiles

To further investigate the role of ETHE1 in seed metabolism as well as the influence of light on storage product accumulation we performed comparative proteomics and analyzed metabolite profiles of wild type and *ethe1-1* seeds grown under light and dark conditions. Samples were taken at two different time points (Figure 1A, indicated by blue arrows). We selected 5 DAP, since the developmental delay in the mutant seeds was most pronounced at this stage. The endosperm still has an important function and covers a large part of the seed. A second set of samples was collected at 9 DAP, when the seed was completely filled by the embryo and there was no morphological difference between the genotypes or light conditions. Seeds from 8 batches including 10 plants each were pooled and used for shotgun proteomics as well as metabolite

analysis via GC/MS. In total 1647 and 1485 unique proteins were identified in the shotgun proteomics approach for seed samples harvested at 5 and 9 DAP, respectively. Technical replicates of the samples clustered very well in a principal component analysis and the different groups were clearly separated so that it was possible to identify sets of differentially abundant proteins (Figure 3). For both harvest time points we compared protein abundances in dark vs. light grown seeds of both genotypes, and in addition we analyzed the effect of ETHE1 deficiency on seed metabolism by comparing *ethe1-1* and wild type seeds grown under control conditions. The complete dataset is available in Supplemental Table S1.

Effects of darkness on seed primary metabolism and storage product accumulation

The metabolite profiles of wild type as well as *ethe1-1* seeds revealed only a mild shortage of carbohydrates in the absence of photosynthesis. The level of sucrose, which is the main sugar present at 9 DAP, was nearly identical, and also glucose and fructose which are dominant earlier in development were only moderately reduced to 65-95 % of the level present in seeds grown under light conditions. The TCA cycle intermediates citrate, 2-oxoglutarate, succinate, fumarate, and malate were even increased up to 4-fold (Figure 4). In contrast, we detected strong changes in the amino acid composition of seeds grown in shaded siliques (Figure 4). Like already detected in the mature seeds, the most pronounced effect was on amino acids used for nitrogen storage and transport with a 67 fold increase in asparagine concentrations in dark-grown seeds compared to control conditions at 9 DAP. At 5 DAP there was also a clear accumulation of branched-chain and aromatic amino acids detectable in the darkness, which became less pronounced later in development.

The proteome comparison indicated a large impact of the availability of light on various metabolic pathways. To get a global overview of pathways potentially affecting seed composition, proteins were annotated to functional categories, and the mean of $\log_2$-fold changes in protein abundances was calculated for each category (Figure 5A). While the total number of proteins with significantly changed abundance was similar in seeds harvested at 5 and 9 DAP and also in mutant and wild type samples (Figure 3), there were clear differences in the level of regulation (Figure 5A). In general, the response was much stronger in older seeds, and $\log_2$-ratios between dark and light conditions were also higher for *ethe1-1* samples than for the wild type. As expected, darkness led to a clear decrease in proteins related to the light reaction of

photosynthesis in all comparisons analyzed. In seeds harvested at 5 DAP the calvin cycle, starch metabolism, and fatty acid synthesis were also reduced. However, at 9 DAP most metabolic pathways were up-regulated in dark vs. light grown seeds including carbohydrate catabolism, protein metabolism, and amino acid metabolism. In contrast, there was a strong decrease in proteins related to the synthesis of triacylglycerides, and starch synthesis was also slightly down-regulated. A more detailed analysis of amino acid synthesis and degradation pathways identified a few exceptions to the general trend of increased protein abundances in the darkness (Figure 5B). The synthesis of Gln, Thr, and Met was down-regulated in shaded wild type seeds, and Cys catabolism was decreased in the dark in *ethe1-1*.

Effects of ETHE1 deficiency on seed metabolism and development

While the composition of mature seeds was very similar in the ETHE1 knockdown mutant and in the wild type, specific differences in the metabolite profile became apparent at earlier developmental stages (Figure 4). At 5 DAP alanine and glycine concentrations were 4 to 5 fold increased compared to the wild type under light as well as dark growth conditions. Comparison to the metabolite profile of wild type seeds harvested at 4 DAP confirmed, that this effect was not due to the earlier developmental stage present in the mutant. There was a trend towards a general increase in free amino acids in *ethe1-1* seeds compared to the wild type (on average 1.7 fold in the light and 1.9 fold in the darkness) (Figure 4C). Hexose levels in the mutant were also elevated at 5 DAP, which could however be linked to the earlier developmental stage of the *ethe1-1* embryo, since in the wild type hexose concentrations decreased between 4 and 5 DAP (Figure 4A). Sucrose as well as TCA cycle intermediates were unaffected (Figure 4C). At 9 DAP the metabolite profiles of the ETHE1 deficient seeds were more similar to the wild type than at the earlier stage. Amino acid concentrations were only slightly increased (on average 1.3 fold), hexose levels were 1.5-2.5 fold higher, and sucrose was decreased to 70 % of wild type level in the light and to 60 % in shaded siliques.

The proteome analyses of *ethe1-1* seeds compared to wild type revealed 215 (5 DAP) and 115 (9 DAP) proteins significantly higher in abundance whereas 206 (5 DAP) and 155 (9 DAP) proteins showed a reduced abundance (Figure 3; a full list of proteins with significantly changed abundance is given in the supplementary material). To get a global overview of pathways

potentially influenced by ETHE1, proteins were annotated to functional categories and the mean of log_2-fold changes in protein abundances was calculated for each category (Figure 6A). Since there is a delay in early seed development in *ethe1-1* compared to the wild type, *ethe1-1* seed samples harvested at 5 DAP were compared to wild type samples of the same age (5 DAP) and in addition to wild type samples in the same developmental stage (4 DAP). The variable color pattern in the first two columns of Figure 6A indicates that several differences between the proteomes of mutant and wild type seeds might be due to the developmental delay. For example, in *ethe1-1* seeds harvested at 5 DAP proteins related to photosynthesis were of slighltly higher abundance than in wild type seeds harvested at 4 DAP but strongly reduced compared to 5 DAP wild type seeds, which can be easily explained by the increasing photosynthetic activity during early seed development. To further elucidate the level of consistency in the proteomic characterization of *ethe1-1* seeds at 5 DAP we produced a vulcano plot to compare the protein abundances in m5L/wt 4L using only the proteins that are of significantly changed abundance in m5L/wt5L (Figure 6B). In this diagram red squares located in the upper right part are consistenly up-reguated in *ethe1-1* compared to wild type seeds of the same age and of the same developmental stage, and blue squares located in the upper left part are consistenly down-regulated. Consistent changes include the induction of several enzymes involved in sugar metabolism such as raffinose synthase, myo-inositol-1-phosphate synthase, sucrose synthase, and xylose isomerase. At 9 DAP photosynthesis is clearly up-regulated in *ethe1-1* compared to the wild type, whereas lipid and amino acid catabolism are most stronly reduced (Figure 6A).

ETHE1 knock down leads to impaired endosperm cellularization

Endosperm cellularization has been shown to be affected by ETHE1 knockout (Holdorf et al. 2012). Therefore, we measured the size of central endosperm cells obtained at 7 DAP, in microscopic sections of wild type as well as mutant seeds stained with Toluidine Blue O (Figure 8A and B). The area of about 50 cells per sample type was calculated. The average cell size in wild type endosperm was 156±40 µm². In contrast *ethe1-1* seeds showed characteristically enlarged endosperm cells with a size of 305±96 μm^2. Darkness further increased the endosperm cell size in the mutant, while endosperm of wild type seeds was not affected. Analysis of the proteomics dataset revealed specific changes in the abundance of proteins related to cell

organization (Figure 7C). Several isoforms of actin and tubulin were increased in *ethe1-1* seeds compared to the wild type at 9 DAP, whereas proteins involved in vesicle transport were reduced.

Aberrant mitochondria in ethe1-1seeds

Previous results obtained with the ETHE1 knockout mutant (Holdorf et al. 2012) indicate potential effects of ETHE1 deficiency on seed mitochondria. Therefore, we next investigated the ultracellular structure of embryo and endosperm cells in *ethe1-1* by using transmission electron microscopy (TEM). The overall organellar structure was relatively normal compared to wild type, with the exception of the appearance of mitochondria (Figure 8C). The main population of mitochondria in both wild type and *ethe1-1* were similar in structure (e.g. well distinguished double membrane, lamellar cristae, and relative small area of electron-transparent matrix). The maximal differences in size of mitochondria in embryo of wild type cells (0.08-0.28 µm²) and of *ethe1-1* (0.04-0.38 µm²) displayed high heterogeneity of population in both genotypes. Similar results were achieved by comparison of mitochondria in endosperm, namely mitochondria size in wild type (0.05-0.42 µm²) and *ethe1-1* (0.04-0.34 µm²). Extremely high variability of individual organelles did not allow defining any statistically significant differences. However, embryo cells of *ethe1-1* showed aberrant mitochondria, which differed from wild type at the ultrastructural level in means of strongly enlarged size (up to 5-fold in length), less pronounced lamellar cristae, larger electron transparent areas in the matrix and some electron-dense insertions (Figure 8C).

ETHE1 knock down affects ABA signal transduction

Defects in endosperm cellularization have also been described for mutants with altered levels of the plant hormone abscisic acid (Cheng et al. 2014, Sreenivasulu et al. 2010). Since ETHE1 expression is strongly induced by ABA (eFP Browser, Winter et al. 2007), we decided to investigate, whether ABA signaling might be affected in *ethe1-1* seeds. ABA inducible proteins were mostly down-regulated in *ethe1-1* compared to wild type seeds at 9 DAP (Figure 8A), which might be explained by either lower levels of the signaling molecule or a defect in ABA signal transduction. ABA insensitive mutants can be identified by their ability to grow on ABA containing agar plates, since in the wild type ABA delays seed germination and inhibits early seedling development (Chen et al. 2008). Germination was equally delayed in the presence of 1 µM ABA in wild type and *ethe1-1* seeds, but after 8 days the number of germinated seeds was

comparable to the control without ABA for both genotypes (data not shown). However, while most of the wild type plants arrested growth immediately after emergence of the radicle, several *ethe1-1* seedlings continued growing and developed leaves (Figure 8B). The relative growth rate in the presence of exogenous ABA was significantly (about 2 fold) higher in *ethe1-1* seedlings than in the wild type (Figure 8C).

Discussion

The sulfur dioxygenase ETHE1, which is involved in cysteine catabolism, has an essential but yet undefined function in *Arabidopsis* seed development. In this study, metabolomics and shotgun mass spectrometry based proteomics were implemented to (i) investigate the general role of amino acid metabolism in seeds and the specific impact of changing light conditions on seed filling and (ii) the characterization of ETHE1 function in developing seeds.

Impact of changing light conditions on seed metabolism

Darkness reduces the seed filling capacity

In serveral dicotyledonous species such as *Arabidopsis* embryos become green during development. Seed plastids differ in their structure from those present in leaves, and since carbohydrates are mainly supplied by the mother plant, one can assume that plastidial metabolism is changed as well (Ruuska et al. 2002). Nevertheless, it has been proposed that seed photosynthesis has a great impact on seed metabolism and supports efficient storage product accumulation (Andriotis et al. 2010, Fait et al. 2006, Goffman et al. 2005). Our results confirm that shading of siliques, which obviously prevents photosynthesis, has a great impact on storage metabolism characterized by reduced lipid and sugar levels, slightly reduced protein content and an accumulation of free amino acids in the seeds. It is very likely that the reduced storage capacity in darkness leads to the lower seed weight we observed, since under light conditions storage product accumulation coincides with increasing seed weight (Baud and Lepiniec 2009). Similar results were obtained in a previous study on *B. napus* seeds grown in darkness (Borisjuk et al. 2013). Since gas diffusion into seeds is low, a major function of seed photosynthesis in fatty acid synthesis might be to supply oxygen required for mitochondrial ATP production (Borisjuk

and Rolletschek 2009, Borisjuk et al. 2005). Interestingly, we found an accumulation of TCA cycle intermediates in dark grown seeds, which could be an indication for decreased respiration rates. The role of photosynthesis in seed development can also be studied by using inhibitors (Allorent et al. 2015). Brushing of *Arabidopsis* siliques with DCMU to block photosystem II activity had no effect on storage protein or lipid content. Also, only minor differences in primary metabolites were detected, with the exception of an increase in GABA, proline and galactinol. Therefore, it can be assumed that darkness applied to the growing seeds might cause additional effects on development and metabolism.

Darkness induces an accumulation of nitrogen-rich amino acids

Nitrogen loading of the seeds is achieved via the import of amino acids (asparagine, glutamine, and alanine), which during seed filling are mainly used for storage protein synthesis (Higashi et al. 2006). We observed that the nitrogen-rich amino acids asparagine (Asn) and glutamine (Gln) were most dramatically increased under dark conditions. One reason for this effect could be an induction of their synthesis in the absence of light. In leaves asparagine synthetase shows a diurnal pattern of activity and is inhibited by light as well as high levels of reduced carbon (Nozawa et al. 1999). So far no information is available on the relevance of Asn synthesis vs. import in seeds, but it is postulated that Asn has a role in storage product accumulation and might act as signal of the internal nitrogen status (Hernandez-Sebastia et al. 2005). We detected a general increase in proteins related to amino acid synthesis in dark grown seeds. However, asparagine synthase was not detected, and the glutamine synthetase isoforms 1-2 and 1-3 that were detected in our samples were consistently down-regulated in the darkness indicating that the increase in free amino acids might be primarily achieved by an induction of import.

Darkness induces accumulation of branched chain and aromatic amino acids at early stage of seed development

In vegetative tissues, amino acids serve as alternative substrate for mitochondrial respiration in situations of carbon starvation induced by extended darkness (Araujo et al. 2011). The branched-chain amino acids valine, leucine, and isoleucine as well as lysine are particularly relevant in this

functional context since their catabolic pathways produce high amounts of ATP and directly transfer electrons into the respiratory chain. Today only limited information on amino acid metabolism in seeds is available. Recently, results related to amino acid catabolism obtained from leaves were hypothetically transferred to seeds. It is proposed that in response to specific developmental processes and stress situations, amino acid catabolism together with photosynthesis contributes to energy supply in seeds (Gallili et al. 2014). The metabolite profiles of dark grown seeds revealed only a mild shortage of carbohydrates in the absence of photosynthesis, which was expected since sugars are delivered from the mother plant and further metabolized (Melkus et al. 2009). Thus, shading of the silique does not necessarily induce carbon starvation conditions, but we could show that it nevertheless induces amino acid accumulation. Interestingly, at least in young seeds (at 5 DAP) levels of branched-chain and aromatic amino acids were clearly elevated. This first experimental evidence highlights that amino acids are presumably not only used for protein biosynthesis but might also contribute to the energy status in developing seeds by alternative respiration.

Physiological function of ETHE1 in seeds

ETHE1 affects seed amino acid homeostasis

Amino acid concentrations in *ethe1-1* seeds show a general trend towards accumulation compared to the wild type. In particular, *ethe1-1* seeds accumulated high amounts of alanine and glycine. The endosperm is the first sink within the seed, and amino acids concentration is high during early stages. Melkus et al. (2009) demonstrated that a decrease of amino acid levels in the endosperm coincides with storage activity in the embryo. However, the reason for the specific accumulation of alanine and glycine is presently unknown. The proteome dataset does not provide any indication for either induced synthesis or decreased degradation of these amino acids. Alanine has been shown to accumulate under hypoxic conditions and might be involved in low-oxygen tolerance (van Dongen et al. 2013, Miyashita et al. 2007). Further studies will be required to clarify whether ETHE1 deficient seeds suffer from hypoxia at any stage during development.

ETHE1 is required for cell organisation and cell wall establishment

Phenotype analysis revealed that *ethe1-1* mutants display a higher sensitivity to changing light conditions, indicated by an enhanced delay of embryogenesis during darkness. However, the final biomass composition of the dormant seeds was not affected by a knock down of ETHE1. In *ethe1-1* seeds, the endosperm is composed of enlarged irregularly shaped cells during cellularization. Our proteomic results confirm that ETHE1 is involved in the establishment of cell walls during development. Proteins related to cell wall degradation such as glucanase and glucosidase were increased in abundance in the mutant compared to the wild type and cell wall proteins were reduced. Overall, we found high amounts of actin and tubulin in *ethe1-1* seeds. In plants, tubulins accumulate during high activity of cell division, cell expansion processes, and also during development (Wasteneys 2002). A high turnover of cell wall elements and defective cell structure establishment might not only affect endosperm cellularization but also nutrient transport to the embryo during development of *ethe1-1* seeds. It has been shown that a mutant blocking transfer cell formation (pea E2748) is defective in normal establishment of embryonic epidermis, which induces enlarged cell wall layers and impaired embryo growth (Borisjuk et al. 2002). Therefore, proper tissue formation is essential for nutrient transport. Our data might indicate that *ethe1-1* mutant embryos suffer from nutrient starvation due to impaired tissue formation. It is proposed that a switch from high hexose to high sucrose level is essential to ensure proper differentiation and cell expansion that elevates storage activity of the embryo (Rolland et al. 2006, Weber et al. 2005). At 5 DAP high concentrations of glucose and fructose and comparatively low levels of sucrose were detected. Enhanced abundance of sucrose synthase activity might induce an increase of relative levels of hexoses observed in *ethe1-1* seeds. However, analyzing wild type seeds with an equal embryo stage (4 DAP) indicates that the difference observed are rather linked to the general delay of seed development than to a specific ETHE1 function. The switch to high sucrose levels required for proper embryo development is clearly visible at 9 DAP, though sucrose levels are decreased in *ethe1-1* compared to wild type seeds.

Possible role of ETHE1 in ABA signaling

ABA has critical functions during several steps in seed development, germination, and early seedling growth. ABA levels in the siliques peak during early seed maturation and are maximal at 9 DAP (Kanno et al. 2010). Our proteome data show that at this developmental stage ETHE1 deficiency leads to a specific decrease in the abundance of ABA inducible proteins indicating that the sulfur dioxygenase might have a presently unknown function in ABA signal transduction. Another indication for a functional connection is the strong induction of ETHE1 expression by ABA (eFP Browser, Winter et al. 2007). ABA signal transduction involves a number of transcription factors that regulate the expression of ABA-dependent downstream genes by binding to the ABA response elements in the promotor region (Hubbard et al. 2010). ABA insensitive mutants deficient in individual transcription factors are able to germinate and grow in the presence of higher concentrations of exogenous ABA than the wild type. ABA leads to an arrest of seedling growth at a checkpoint immediately after the emergence of the radicle from the seed coat probably to keep the germinated seed in a quiescent state if environmental conditions are unfavorable for the survival of the young seedling (Lopez-Molina et al. 2001). In fact, at the low concentration used for this study (1 µM), ABA is rather an inhibitor of early seedling growth than of germination. We detected a strong decrease in ABA sensitivity in the *ethe1-1* mutant, which is comparable to the results obtained with mutants defective in ABA signaling (Chen et al. 2008). Interestingly, the transcription factor HY5 responsible for the activation of many light regulated genes is also a transcriptional activator of the ABA-responsive transcription factor ABI5 so that light positively regulates ABA signaling (Chen et al. 2008). Thus, the stronger effect of shading on the development and the proteome of *ethe1-1* compared to wild type seeds we detected in this study might be connected to defects in ABA signaling. Mutations in ABA2, an enzyme involved in the biosynthesis of ABA, lead to a delay in endosperm cellularization and an increased embryo cell number (Cheng et al. 2014). In barley, ploidy levels of the developing endosperm are inversely correlated to the ABA content suggesting an influence of ABA on cell-cycle regulation (Sreenivasulu et al. 2010). Thus, the defects in endosperm cellularization detected in the *ethe1-1* seeds might also be a consequence of the postulated disturbance in the ABA signaling process. The primary reason for the different effects detected in *ethe1-1* seeds could be an accumulation of persulfides, which are the substrate of the sulfur dioxygenase ETHE1. Deficiency of the cysteine desulfhydrase DES1 has been shown to interfere with several

metabolic pathways due to decreased production of the signaling molecule hydrogen sulfide (Romero et al. 2013, Gotor et al. 2015). Interestingly, one of the effects in the des1 mutant is a block in ABA induced stomatal closure indicating a function of sulfide in ABA signal transduction (Garcia-Mata and Lamattina 2010). Hydrogen sulfide and also persulfide signaling might be mediated by persulfidation of specific cysteine residues in proteins. More than 2000 Arabidopsis proteins have been shown to be persulfidated under control conditions, and among them were ABA receptors as well as cytoskeleton proteins (Aroca et al. 2017). Further research will be required to elucidate the potential role of the mitochondrial sulfur dioxygenase ETHE1 in sulfur signaling and protein persulfidation.

Material and Methods

Plant growth and seed harvesting

A. thaliana wild type (ecotype Columbia) and *ethe1-1 (SALK_021573*, Nottingham Arabidopsis Stock Centre, University of Nottingham) plants were grown in a climate chamber under longday conditions (16h light/8h dark, 22°C, 85 µmol s^{-1} m^{-2} light intensity and 65% humidity). Flowers were labeled at the day of pollination. Subsequently siliques were harvested from 1 to 9 DAP. For dark treatment, siliques were shaded with aluminium foil 24 hours after flower tagging while the rest of the plant and control siliques were grown under normal light conditions.

Phenotypic analysis of seed samples

Whole-mount preparations were used for microscopic analysis of *A. thaliana* wild type and *ethe1-1* embryo development grown under light and dark conditions. Seeds from at least 5 siliques each were cleared by incubation with Hoyer´s solution [15 mL distilled water, 3.75 g gum Arabic, 2.5 mL glycerine, 50 g chloral hydrate] overnight. Embryo development was analysed by using a Normarski optics and light microscopy. Observed embryo stages were counted. For analyzing seed tissue differentiation histological sections were prepared form 7 DAP *ethe1-1* and wild type seeds grown under light and dark conditions. Seed samples were fixed with 1.25% formaldehyde and 0.1 M phosphate buffer overnight followed by dehydration

of the samples by a series of ethanol aqueous solutions (30-99%). After dehydration samples were infiltrated with Historesin (Leica). Sections of 3.5 µm were prepared using a microtome (HYRAX M55, Zeiss) and Sec55 low profile blades (MICROM). Sections were stained with 0.5% Toluidine Blue O in 200mM phosphate buffer. Photographs of cleared seeds and sections were taken with a Zeiss microscope (Axioskop2) and AxioCam MRc5 camera. Seed size and weight were measured for dormant seeds of *ethe1-1* and wild type grown under light and dark conditions for 100 seeds each. Significant differences were calculated using a Student´s t-test (p-value ≤0.05). For electron microscopy of mitochondria seeds of wild type and *ethe1-1* (5 DAP) were fixed and embedded in accordance to Tschiersch et al. (2011). Digital records of seed mitochondria were made on a Zeiss 902 electron microscope at 80 kV.

Germination rates

Arabidopsis seeds of wild type (ecotype Columbia, Col0) and *ethe1-1* grown under light and dark conditions were surface sterilized with 6% sodium hypochloride and 100% ethanol followed by five washing steps with sterilized water . For vernalization seeds were incubated for 2 days at 4°C in the dark. Approximately 20 seeds were sown per plate (3 replicates per sample) on MS-medium [60mM sucrose, 1% Agar, 0.5% MS-medium (Duchefa), pH 5.7-5.8 with KOH] and MS medium without a carbon source and incubated for another 2 days at 4°C in the dark. Afterwards the plates were placed to a growth chamber (24°C, 16 h light/8 h dark). After 72h germinated seeds were counted. A seed is considered to be germinated when the radicle raptures the endosperm and the testa.

Silique culture

Siliques were harvested from wild type and *ethe1-1* plants at 2 DAP and cultured in a medium [1.5% MS and 2mM MES, pH 5.6] (control). For amino acid treatments 0.1mM, 0.5mM and 1 mM cysteine and leucine were added to the silique culture. A concentration of 10 mM resulted in seed abortion for both genotypes (data not shown). Siliques were cultured for 3 days in a climate chamber under longday conditions (seed stage equivalent to 5 DAP). Subsequently, seeds were harvested and embryo stages investigated by seed clearing and light microscopy as described above.

Metabolite profiling

Metabolites of *ethe1-1* and wild type seeds (4, 5 and 9 DAP) grown under light and dark conditions were extracted and subsequently analysed by GC-TOF MS as described by Lisec et al. (2006). Chromatograms and mass spectra were evaluated by using TagFinder 4.0 software (Luedemann et al., 2008) and Xcalibur 2.1 software (Thermo Fisher Scientific, Waltham, USA). Metabolites were identified in comparison to database entries of authentic standards (Kopka et al., 2005; Schauer et al., 2005). Peak areas of the mass (m/z) fragments were normalized to the internal standard (ribitol) and fresh weight of the seed samples. Identification and annotation of detected peaks followed recent recommendations for reporting metabolite data (Fernie et al., 2011). Metabolites (starch, total proteins, total lipids and amino acids) of dormant wild type and *ethe1-1* seeds grown under light and dark conditions were extracted and analyzed as described in Schwender et al. (2015).

Sample preparation for mass spectrometry

Proteins of wild type and *ethe1-1* (5DAP, 9 DAP, light, dark) were extracted from 30 mg of pulverized seeds (pooled sample) by adding 150 µL extraction buffer [4% SDS (w/v), 125mM TRIS, 20% glycerol (v/v)] and incubating at 60°C for 5 min. Another 150 µL of ddH2O were added to the sample followed by centrifugation at 18000xg for 10 min. The protein concentration of the supernatant was measured by using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Dreieich, Germany). To each sample (total protein 50 µg) 2-mercapto ethanol with a trace of bromophenol blue was added to a final concentration of 5%. SDS gel electrophoresis using a 1D glycine gel (stacking gel 4% acrylamide, separating gel 14% acrylamide) was performed according to Laemmli (1970). The gel run was stopped before proteins entered the separation gel. The gel was fixed with 10% (v/v) acetate in 40% (v/v) methanol for 45 min and stained with Coomassie blue CBB G-250 (Merck, Darmstadt, Germany) for 30 min as described by Neuhoff et al. (1985 and 1990). Gel bands were cut using a scalpel and diced into 1.0-1.5 mm cubes. Carbamidomethylated followed by tryptic digestion and extraction of proteins was performed according to Klodmann et al. (2010). Resulting peptides were resolved in 20 µL of 2% [v/v] ACN, 0.1% [v/v] formic acid (FA) prior to MS analysis.

Shot gun mass spectrometry and relative protein quantification

Shot gun mass spectrometry was performed by using a Q-Exactive (Thermo Fisher Scientific, Dreieich, Germany) mass spectrometer coupled to an Ultimate 3000 (Thermo Fisher Scientific, Dreieich, Germany) UPLC. Three times (technical replicates) four microliter of peptide solution per seed sample were injected into a 2 cm, C18, 5 µm, 100 Å reverse phase trapping column (Acclaim PepMap100, Thermo Fisher Scientific, Dreieich, Germany). Peptide separation was done on a 50 cm, C18, 3 µm, 100 Å reverse phase analytical column (Acclaim PepMap100, Thermo Fisher Scientific, Dreieich, Germany). Peptides were eluted by using a non-linear 2% [v/v] to 34% [v/v] acetonitrile gradient in 0.1% [v/v] formic acid of 60 minutes. For MS analysis a spray voltage of 2.2 kV, capillary temperature to 275°C and S-lens RF level to 50% was tuned. For full MS scans, the number of microscans was adjusted to 1, resolution to 70,000, AGC target to 1e6, maximum injection time to 400 ms, number of scan ranges to 1 and scan range to 400 to 1600m/z. For dd-MS2, the number of microscans was adjusted to 1, resolution to 17,500, AGC target to 1e5, maximum injection time to 120 ms, Loop count to 10, MSX count to 1, isolation window to 3.0 m/z, fixed first mass to 100.0 m/z and NCE to 27.0. Data dependent (dd) settings were adjusted to underfill ratio of 0.5%, intensity threshold to 2.0e3; apex trigger to 10 to 60 s, charge exclusion to unassigned 1, 5, 5 – 8, >8, peptide match to preferred, exclude isotopes to on and dynamic exclusion to 45.0 s. MS/MS spectra were queried against an in-house Arabidopsis protein database also containing frequent contaminations and RNA-editing sites using the Andromeda search engine as part of the MaxQuant (Cox and Mann, 2008) software package. Frequent contaminants (BSA, keratin, trypsin) were removed from the final output lists. MaxQuant analysis was performed using version 1.5.2.8. with the same parameter settings as described previously (Fromm et al. 2016). Label free quantification (LFQ) intensities from the corresponding MaxQuant file were uploaded into the Perseus software and quantitation matrix was built based on log2-transformed LFQ intensity values. Only proteins with a P value <0.05 were considered statistically significant.

In-vitro cultures with ABA

In vitro cultures were used for germination and growth experiments with 1 µM abscisic acid (ABA). The seeds of the wild type (WT) and mutant (*ethe1-1*) were sterilized and planted

together on one plate. As control, the plates contained ½ Murashige and Skoog medium and for the treatment with ABA, this medium was supplemented with 1 µM ABA. The plates were transferred to long day conditions after 2 d of stratification at 4 °C in darkness. From 2 d to 9 d, the germinated (radicula minimal 1 mm) seeds as well as the seeds, which built at least the first two leaves were counted daily.

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Figures

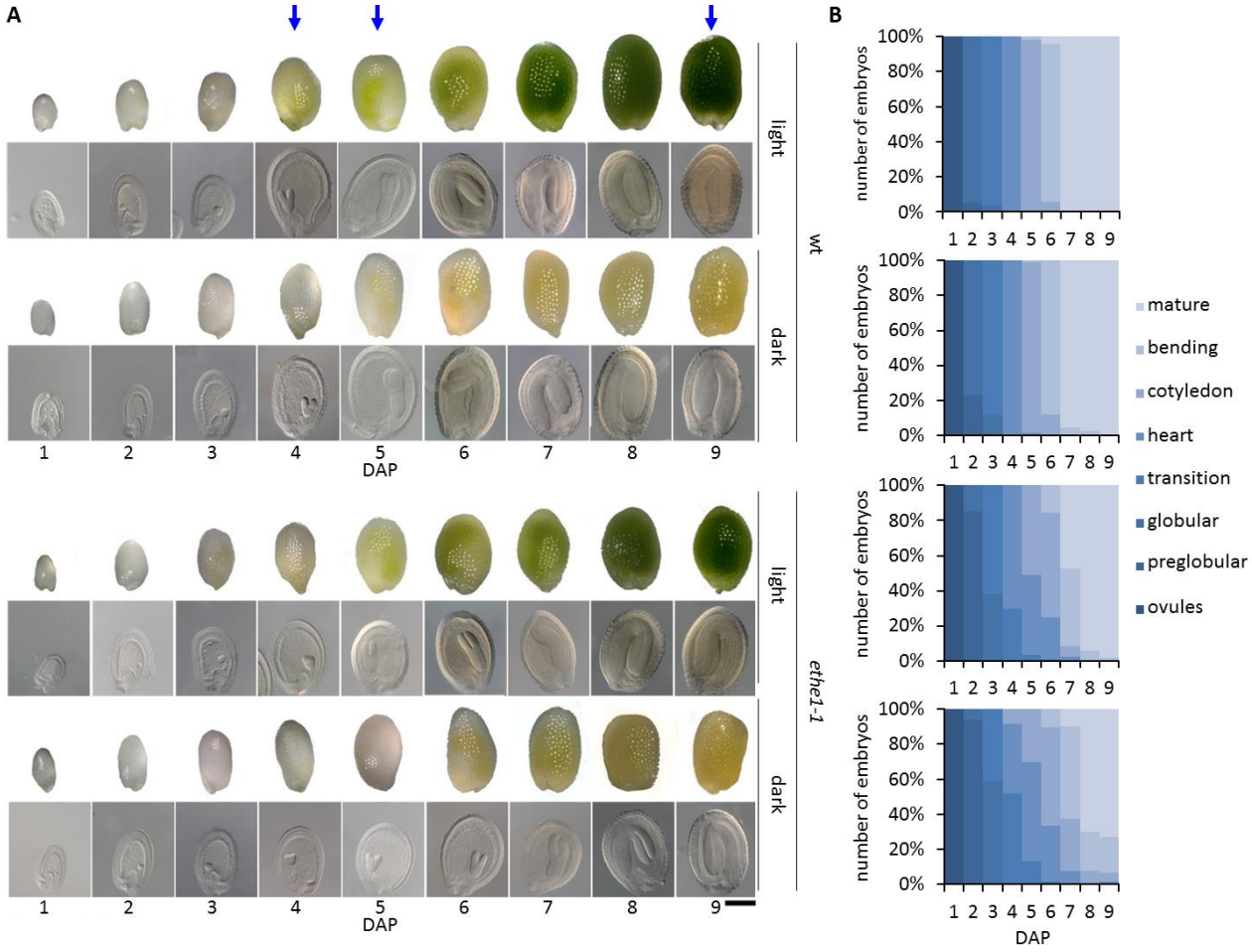


Figure 1: Seed development of *A. thaliana* wild type and *ethe1-1* seeds under light and dark conditions. A: Representative seeds and embryos of wild type (wt) and *ethe1-1* from 1 to 9 days after pollination (DAP) grown under light and dark conditions, bars = 100 µm. **B:** Progression of embryo development in wt and *ethe1-1* seeds. A total of 180 siliques and 7781 seeds between 1 and 9 DAP were analyzed of wild type and *ethe1-1* grown under light and dark conditions. Blue arrows indicates stages investigated by metabolomics and proteomics.

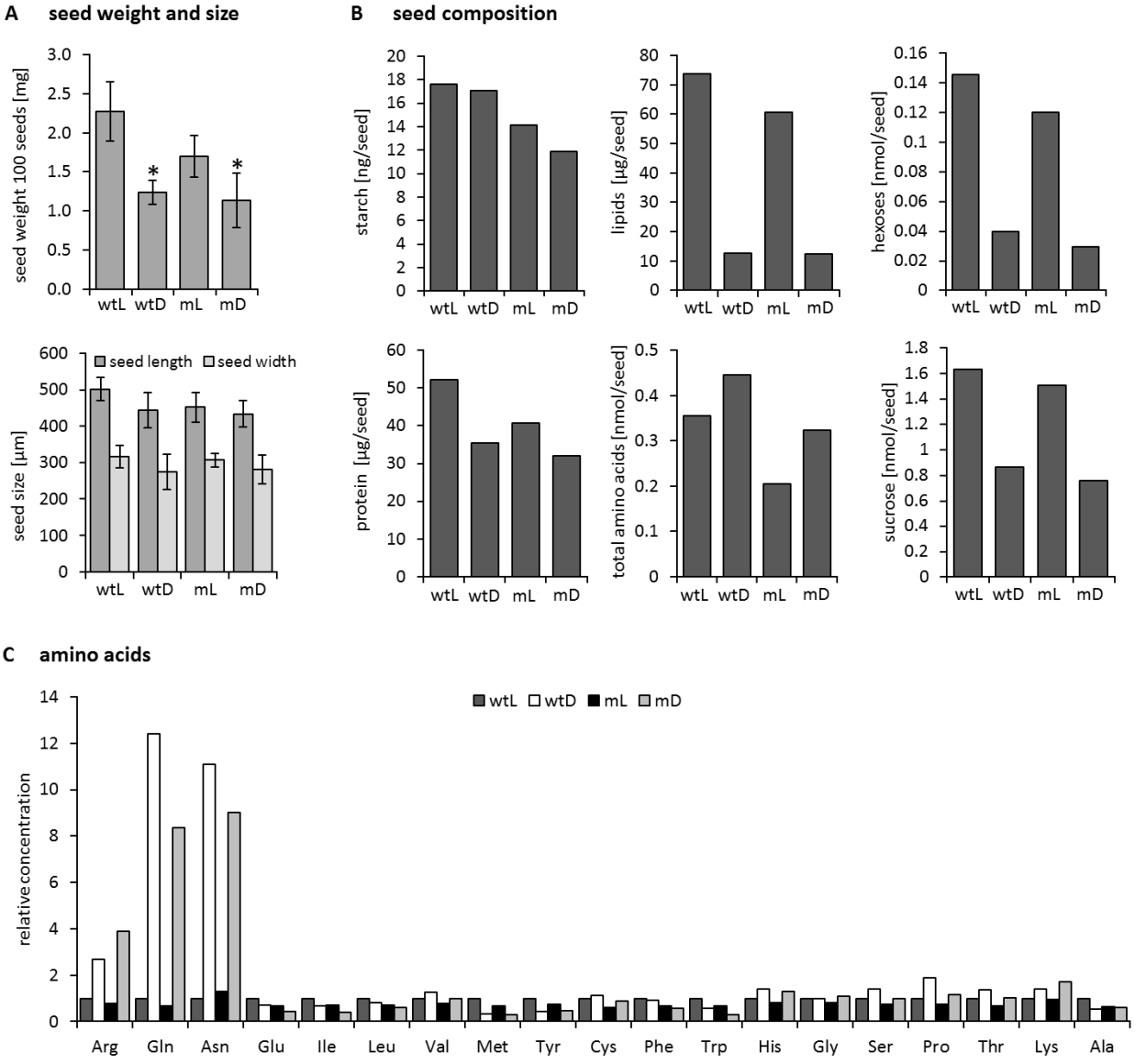


Figure 2: Seed weight, seed size and biomass composition of dormant seeds. A: Seed size and weight of 100 dormant seeds from wild type and *ethe1-1* plants grown under light and dark conditions. Asterisks indicate significant changes based on a Student´s t-test (p-value ≤0.05) **B:** Storage compounds and amino acids were extracted from dormant seeds from wild type and *ethe1-1* grown under light and dark conditions and quantified. **C:** amino acid profiles of dormant seeds from wild type and *ethe1-1*. **wtL** wild type seeds grown under light conditions, **wtD** wild type seeds grown under conditions dark conditions, **mL** *ethe1-1* seeds grown under light conditions, **mD** *ethe1-1* seeds grown under dark conditions.

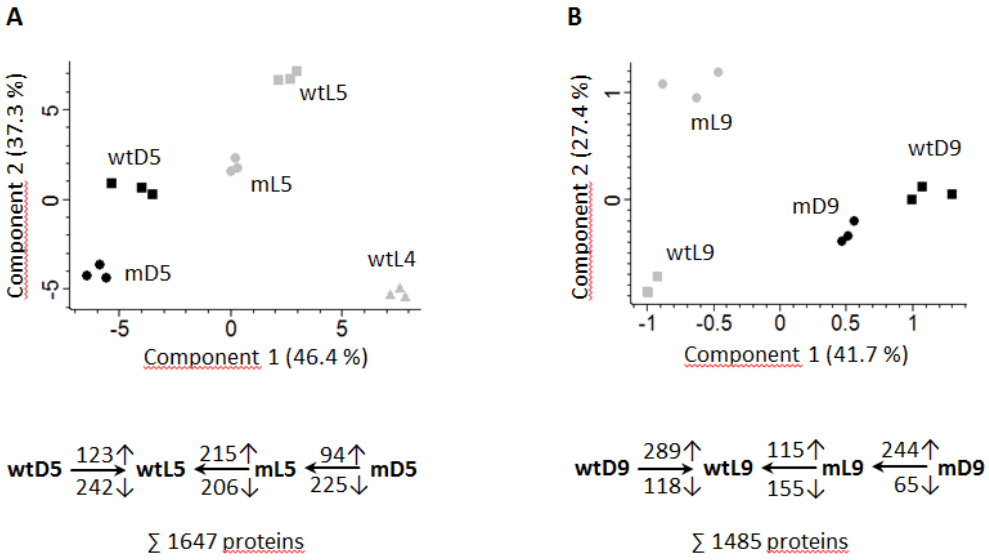


Figure 3: Priciple component analysis and overview of proteomic comparisons. For quality control of the shotgun MS datasets principle component analysis (PCA) was performed for the two harvest timepoints (**A:** 4 and 5 DAP; **B:** 9 DAP) using Perseus software. The numbers of significantly increased and decreased proteins for relevant comparisons are indicated in the lower part of the figure. Comparisons were wtD/wtL, mL/wtL, mD/mL. The total numers of detected proteins in the two datasets are listed in the bottom. **wtL** wild type seeds grown under light conditions, **wtD** wild type seeds grown under dark conditions, **mL** *ethe1-1* seeds grown under light conditions, **mD** *ethe1-1* seeds grown under dark conditions, numbers indicate the age of the seed in days after pollination.

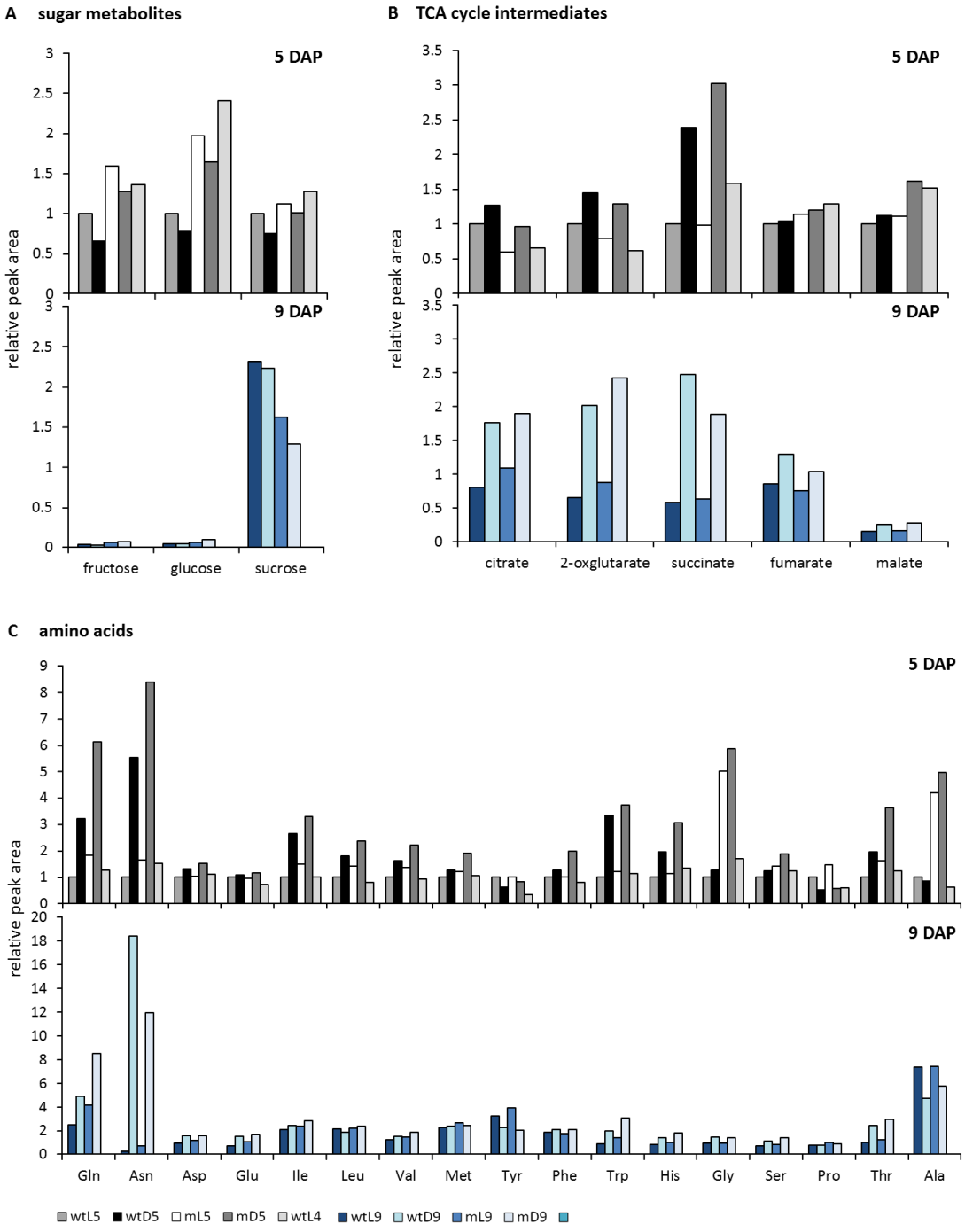


Figure 4: Metabolite profiles. Metabolites (sugar metabolites, TCA cycle intermediates, amino acids) of *ethe1-1* and wild type seeds (4, 5 and 9 DAP) grown under light and dark conditions were extracted and subsequently analysed by GC-TOF MS. Peak areas of the mass (m/z) fragments were normalized to the internal standard (ribitol) and fresh weight of the seed samples. **wtL** wild type seeds grown under light conditions, **wtD** wild type seeds grown under dark conditions, **mL** *ethe1-1* seeds grown under light conditions, **mD** *ethe1-1* seeds grown under dark conditions, numbers indicate the age of the seed in days after pollination (DAP).

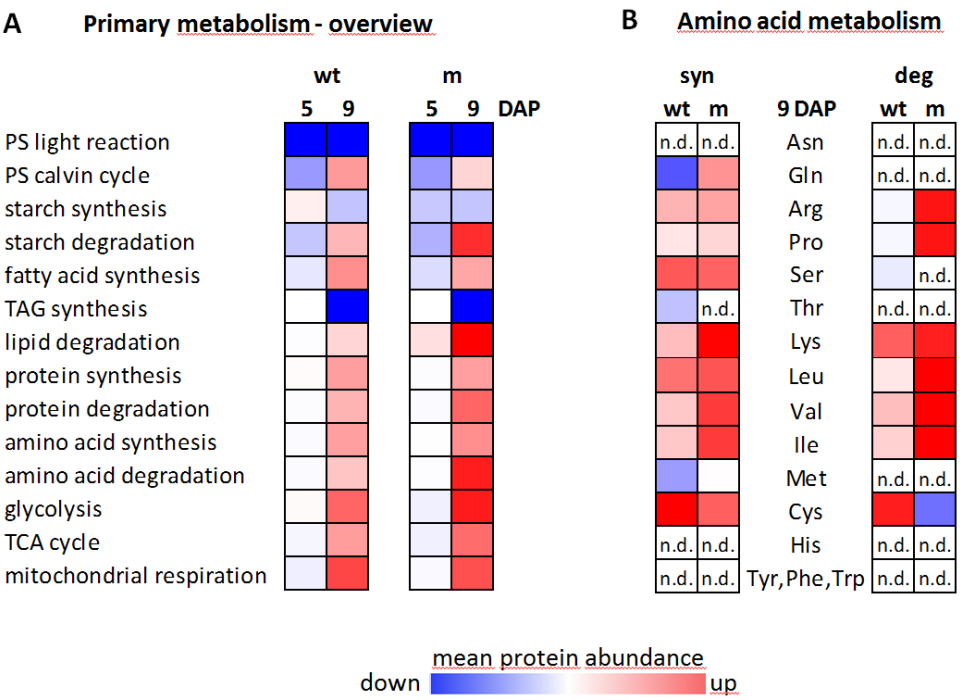


Figure 5: The effect of darkness on the proteome of developing wild type and *ethe1-1* seeds. **A.** Log_2-fold changes in protein abundances were calculated for four different comparisons (dark vs. light grown wild type seeds harvested at 5 and 9 DAP respectively (wt, left side) and dark vs. light grown *ethe1-1* seeds harvested at 5 and 9 DAP respectively (m, right side). The color gradient represents the means of all log_2-ratios (scaling: -1 to +1) for proteins included in the functional categories listed according to the MapMan annotation file (version Ath_AGI_LOCUS_TAIR10_Aug2012). **B.** The effect of darkness on amino acid metabolism at 9 DAP represented by the means of all log_2-ratios (scaling: -1 to +1) for proteins involved in the synthesis (syn) or degradation (deg) of the individual amino acids listed. n.d., not detected

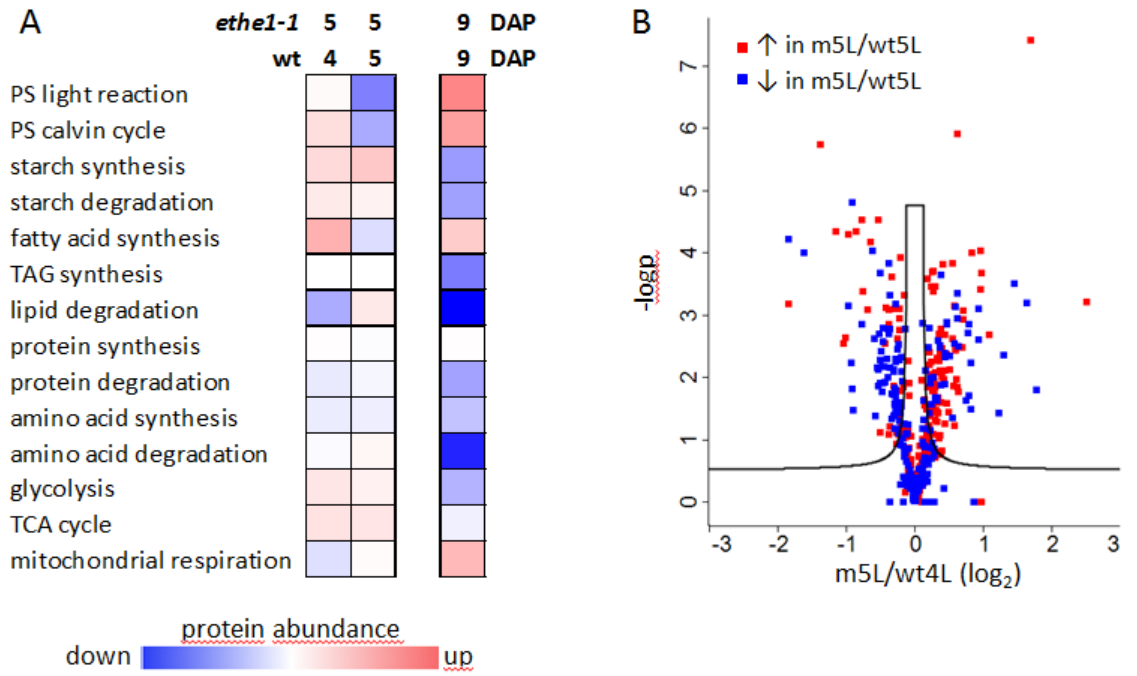


Figure 6: The proteome of *ethe1-1* compared to wild type seeds during development. Since there is a delay in early seed development in *ethe1-1* compared to the wt, *ethe1-1* seed samples harvested at 5 DAP were compared to wt samples of the same age (5 DAP) and in addition to wt samples in the same developmental stage (4 DAP). **A:** Log_2-fold changes in protein abundances were calculated for three different comparisons indicated on top of the columns (m5L/wt4L, m5L/wt5L, m9L/wt9L). The color gradient represents the means of all log_2-ratios (scaling: -1 to +1) for proteins included in the functional categories listed according to the MapMan annotation file (version Ath_AGI_LOCUS_TAIR10_Aug2012). **B:** Vulcano plot illustrating consistent changes in the proteome of young *ethe1* seeds compared to the wild type. Solid lines represent the threshold for significance (FDR: 0.05, s0: 0.1) in the comparison m5L/wt4L. Proteins that are significanly increased or decreased in the comparison m5L/wt5L are shown in red and blue, respectively. Thus, red squares located in the upper right part of the diagramm are consistenly upregulated in *ethe1-1* compared to wild type seeds of the same age (5 DAP) and of the same developmental stage (4 DAP), and blue squares located in the upper left part are consistenly downregulated. **wtL** wild type seeds grown under light conditions, **mL** *ethe1-1* seeds grown under light conditions, numbers indicate the age of the seed in days after pollination (DAP).

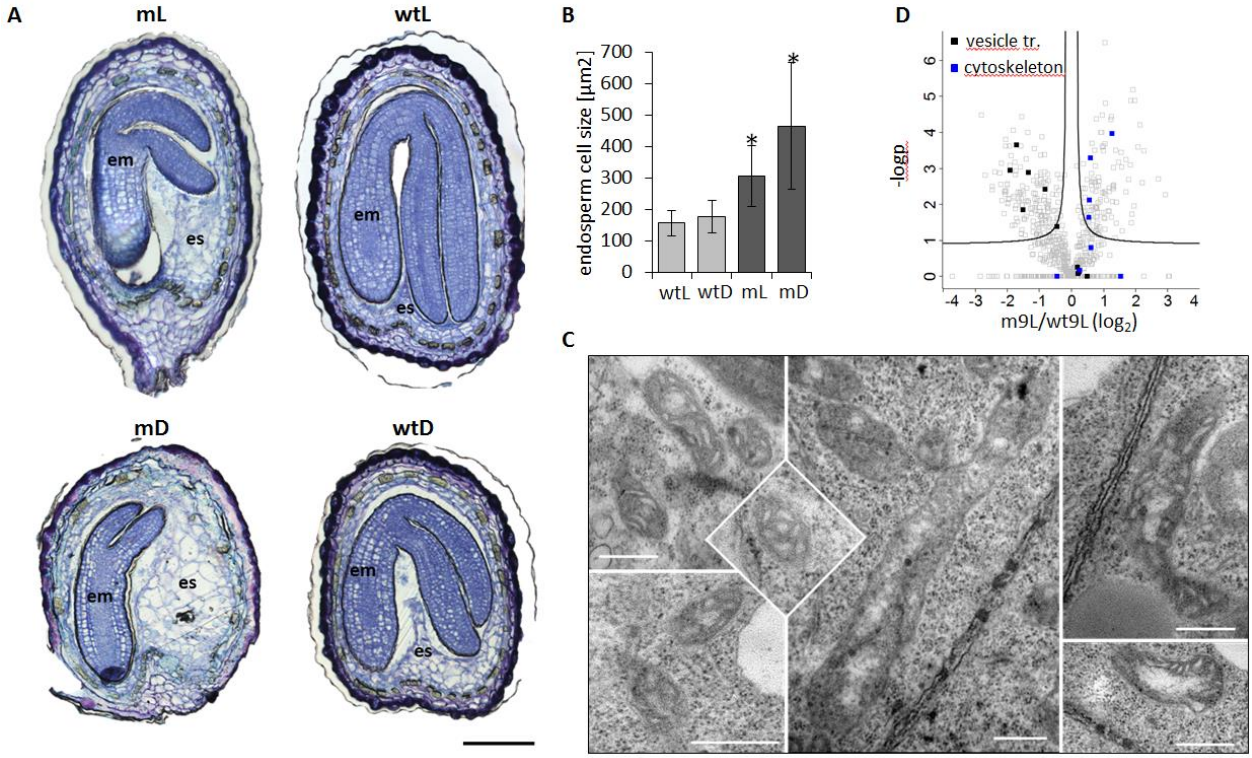


Figure 7: Endosperm cellularization and mitochondrial structure. A: Seed sections (7 DAP) of wild type and *ethe1-1* seeds grown under light and dark conditions. Sections were stained with Toluidine Blue O to visualize endospserm cellularization, bars = 100 µm **B:** Endosperm cell size of ca. 50 cells is given as area [μm^2] obtained from microscopy images using AxioVision software (Version 4.8.1). Asterisks indicate significant changes to wild type light (wtL) based on a Student´s t-test (p-value ≤0.05) **C:** Electron microsopic analysis of mitochondrial structure from *ethe1-1* seeds compared to wild type, bars = 500 nm **D:** Vulcano plot illustrating differences in protein abundance between *ethe1-1* and wt seeds at 9 DAP. Solid lines represent the threshold for significance (FDR: 0.05, s0: 0.1). Proteins included in functional categories that might be relevant for endosperm cellularization (vesicle transport and cytoskeleton) in the MapMan annotation file (version Ath_AGI_LOCUS_TAIR10_Aug2012) are highlighted in black and blue, respectively. **wtL** wild type seeds grown under light conditions, **wtD** wild type seeds grown under conditions dark conditions, **mL** *ethe1-1* seeds grown under light conditions, **mD** *ethe1-1* seeds grown under dark conditions.

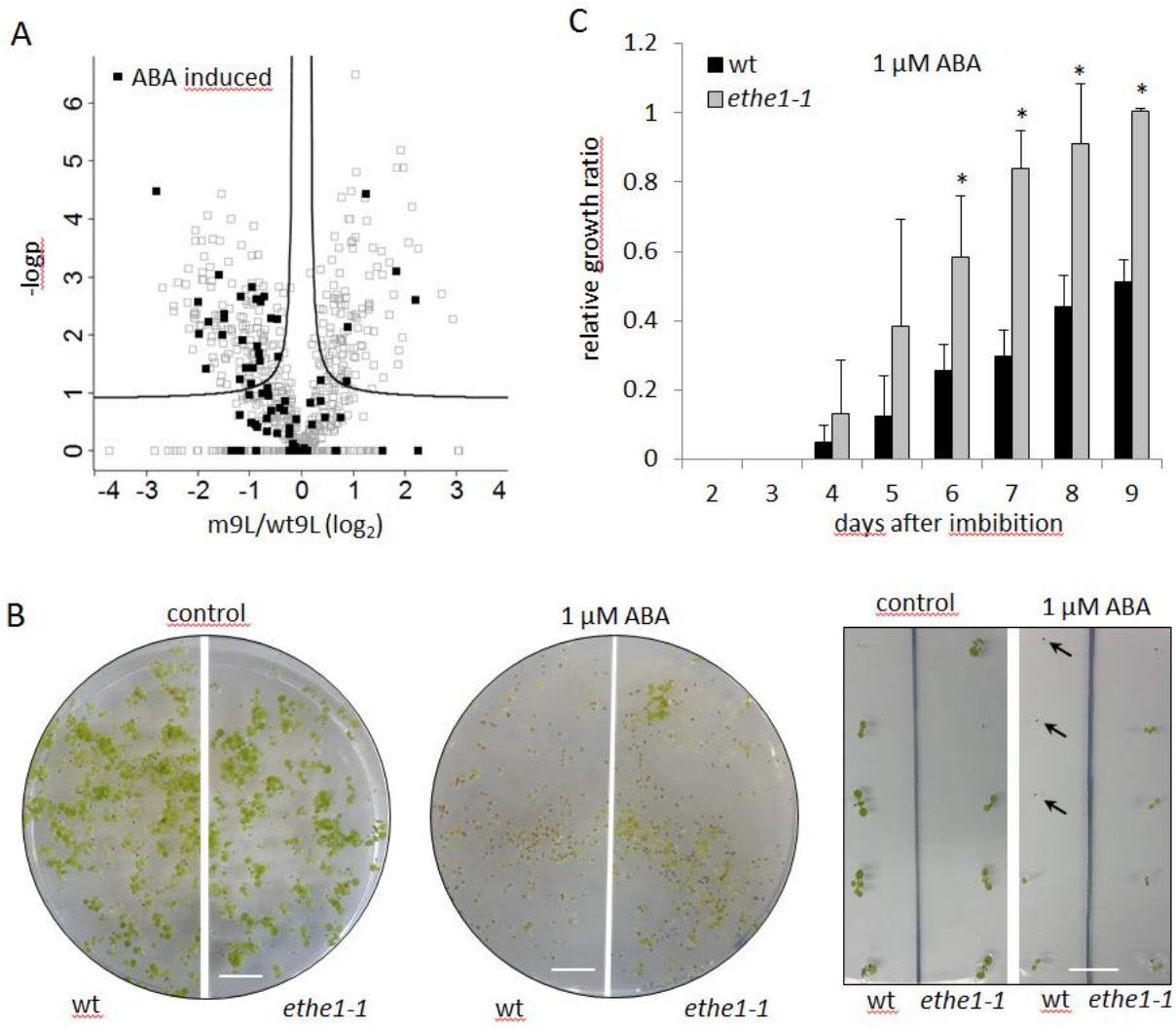
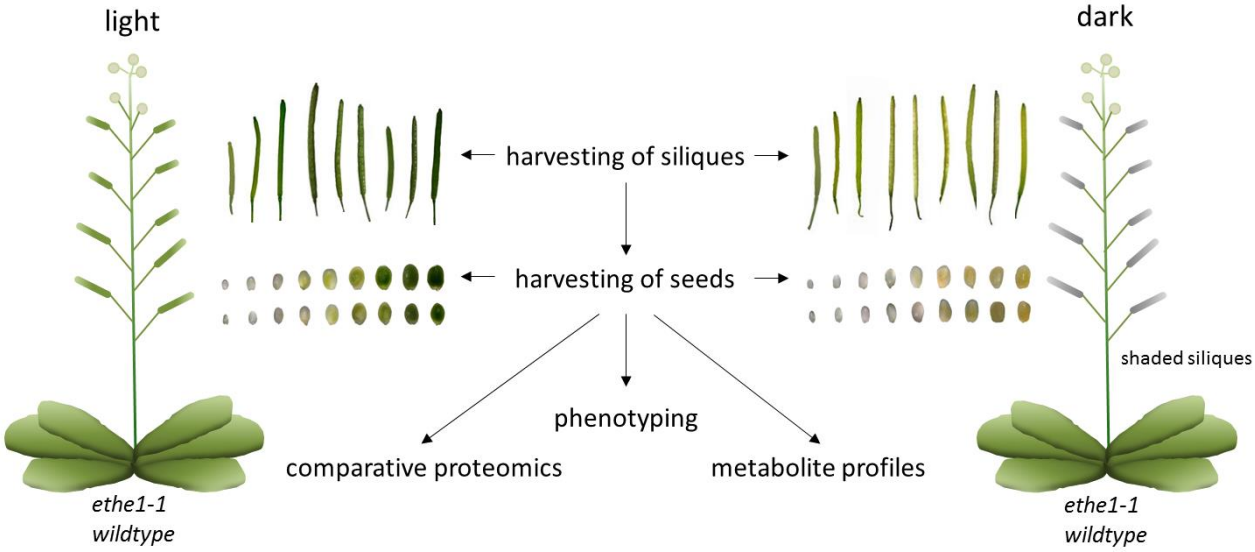
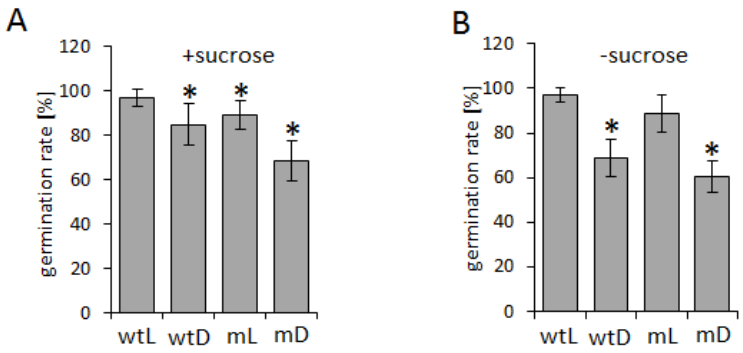


Figure 8: ETHE1 deficiency affects abscisic acid (ABA) signal transduction. A. Vulcano plot illustrating differences in protein abundance between light grown *ethe1-1* and wt seeds at 9 DAP. Solid lines represent the threshold for significance (FDR: 0.05, s0: 0.1). Proteins induced by ABA (Gruner et al. 2013) are marked in black. **B.** Phenotype of wild type and *ethe1-1* seedlings grown on agar plates containing 0 µM (control) or 1 µM ABA 10 days after transfer to light conditions. Arrows indicate seeds that had germinated but arrested growth after emergence of the radicle. scale bars = 1 cm. **C.** Relative growth ratio of wt compared to *ethe1-1* seedlings on agar plates containing 1 µM ABA calculated as the percentage of germinated plants that had also developed leaves. All data points were normalized to the growth ratio of *ethe1-1* seedlings at 9 days after imbibition. Asterisks indicate significant differences to the wild type based on a Student´s t-test (p-value ≤0.05)

Supplemental Figures



Supplementary figure 1: Experimental design. *A. thaliana* wild type (ecotype Columbia) and *ethe1-1* plants were grown in a climate chamber under longday conditions (16h light/8h dark, 22°C, 85 µmol s^{-1} m^{-2} light intensity and 65% humidity). Flowers were labeled at the day of pollination. Subsequently siliques were harvested from 1 to 9 DAP. For dark treatment, siliques were shaded with aluminium foil 24 hours after flower tagging while the rest of the plant and control siliques were grown under normal light conditions. Seeds were investigated by phenotyping, comparative proteomics and measuring metabolite profiles.



Supplementary figure 2: Germination rates. Sterilized seeds of wild type and *ethe1-1* grown under light and dark conditions were were sown per plate (3 replicates per sample) on MS-medium [60mM sucrose, 1% Agar, 0.5% MS-medium (Duchefa), pH 5.7-5.8 with KOH] and MS medium without sucrose and incubated for another 2 days at 4°C in the dark. Afterwards the plates were placed to a growth chamber (24°C, 16 h light/8 h dark). After 72h germinated seeds were counted. A seed is considered to be germinated when the radicle raptures the endosperm and the testa.

4.3 Manuscript 2

Extended darkness induces internal turnover of glucosinolates in Arabidopsis leaves

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Abstract

Prolonged darkness leads to carbohydrate starvation, and as a consequence plants degrade proteins and lipids to oxidize amino acids and fatty acids as alternative substrates for mitochondrial ATP production. We investigated, whether the internal breakdown of glucosinolates, a major class of sulfur-containing secondary metabolites, might be an additional component of the carbohydrate starvation response in Arabidopsis. The glucosinolate content of Arabidopsis leaves was strongly reduced after seven days of darkness. We also detected a significant increase in the activity of myrosinase, the enzyme catalyzing the initial step in glucosinolate breakdown, coinciding with a strong induction of the main leaf myrosinase isoforms TGG1 and TGG2. In addition, nitrilase activity was increased suggesting a turnover via nitriles and carboxylic acids. Internal degradation of glucosinolates might also be involved in diurnal or developmental adaptations of the glucosinolate profile. We observed a circadian rhythm for myrosinase activity in two-week-old plants. Furthermore, leaf myrosinase activity and protein abundance of TGG2 varied during plant development, whereas leaf protein abundance of TGG1 remained stable indicating regulation at the transcriptional as well as post-translational level.

Keywords: Glucosinolate turnover, myrosinase, nitrilase, development

Abbreviations

ED	Extended darkness
GLS	Glucosinolates
I3M	Indol-3-ylmethyl-GLS
ITC	Isothiocyanate
LD	Long day
MVP1	Modified vacuolar phenotype 1
SD	Short day
TGG	Thioglucoside glucohydrolase
1MI3M	N-Methoxy-indol-3-ylmethyl-GLS
4MI3M	4-Methoxy-indol-3-ylmethyl-GLS
4MSOB	4-Methylsulfinylbutyl-GLS
4MTB	4-Methylthiobutyl-GLS

5MTP	5-Methylthiopentyl-GLS
5MSOP	5-Methylsulfinylpentyl-GLS
6MSOH	6-Methylsulfinylhexyl-GLS
7MSOH	7-Methylsulfinylheptyl
7MTH	7-Methylthioheptyl-GLS
8MSOO	8-Methylsulfinyloctyl
8MTO	8-Methylthiooctyl-GLS

Introduction

Arabidopsis thaliana contains many secondary metabolites, and glucosinolates (GLSs) are considered to be among the most characteristic (D'Auria and Gershenzon, 2005). GLSs are synthesized from glucose and amino acids and can be classified into three groups: aliphatic, indole and aromatic GLSs. The ecotype Columbia (Col-0) contains a set of about 30 different GLSs (Brown et al. 2003). The main function of GLSs, plant defense against herbivores and pathogens, is mediated by their breakdown products, mainly simple nitriles, epithionitriles and isothiocyanates (ITCs) (Bones and Rossiter, 1996; Kliebenstein et al., 2001). In particular the toxic product ITC serves as important defense compound against various plant pests (Brown and Morra, 1997; Fahey et al., 2001). In addition to their function in plant pathogen resistance, GLSs and their breakdown products are also highly relevant for medical research. In cancer cell lines, ITCs were shown to inhibit phase I enzymes, increase the activity of phase II enzymes and to induce apoptotic alterations and cell cycle arrest (Das et al., 2000; Hayes et al., 2008; Hecht, 1999). GLS breakdown is initiated by myrosinase (thioglucoside glucohydrolase, TGG), which hydrolyses the thioglucoside bond to cleave off the glucose group (Halkier and Gershenzon, 2006; Rask et al., 2000). The remaining instable aglucone spontaneously converts to ITC, or in the presence of specifier proteins is metabolized either to a simple nitrile or an epithionitrile (Wittstock and Burow, 2010). Since GLSs and myrosinases are stored separately in the leaves, this breakdown is thought to occur mainly as a reaction to tissue damage after pathogen attack (Koroleva et al., 2000; Ueda et al., 2006). However, several studies also indicate that there might be an internal turnover of GLSs in intact plant tissues (Burow and Halkier, 2017; Grubb and Abel, 2006; Neilson et al., 2013; Wittstock and Burow, 2010; Yan and Chen, 2007). First, the tissue GLS content as well as composition constantly changes throughout the life span of *A. thaliana*. Seeds have a particularly high GLS content with a characteristic composition. They contain mainly GLSs without secondary

modifications while the modified versions are more abundant in vegetative tissues (Petersen et al. 2002, Brown et al. 2003, Barth and Jander 2006). Conversion from the seed to the leaf GLS set requires degradation of the seed GLSs during the early seedling developmental process, which has been confirmed using a radiolabeled GLS (Petersen et al. 2002). GLS contents then increase in leaves until the bolting stage and decrease again during senescence, which most likely also involves internal degradation (Brown et al., 2003; Petersen et al., 2002). Secondly, the total GLS content of two-week-old Arabidopsis leaves shows diurnal fluctuations, and the decrease detected during the night might be due to internal turnover (Huseby et al. 2013). Thirdly, a decrease in GLS content observed during sulfur depletion indicates that GLSs might serve as sulfur storage compounds (Falk et al., 2007; Hirai et al., 2004; Kutz et al., 2002; Zhao et al., 1994).

In the roots, atypical myrosinases and GLSs are stored in two independent compartments of the same cell, the ER bodies and vacuoles, respectively (Nakano et al., 2014). Thus, GLS turnover could take place without tissue disruption following translocation of myrosinases into vacuoles, co-secretion of myrosinases and GLSs out of the cell or a single cell collapse. In leaves, GLSs are stored primarily in specialized S-cells localized in the midvein close to the phloem. The main leaf myrosinase isoforms TGG1 and TGG2 are expressed in scattered myrosin cells as well as in phloem-associated cells, and TGG1 in addition is highly abundant in stomatal guard cells (reviewed by Wittstock and Burow 2010). Subcellular localization is not entirely clear yet. Binding of MVP1 (Modified vacuolar phenotype 1; At1g54030) to TGG2 was suggested to translocate TGG2 relative to GLSs and thus enable turnover in intact leaves (Wittstock and Burow, 2010).

For efficient nutrient remobilization, the GLS breakdown products should be non-toxic and easy to integrate into primary plant metabolism. Thus, the degradation pathway via simple nitriles seems to be the most likely option for internal GLS turnover. Nitriles can be further catabolized via nitrilases into carboxylic acids. Via this pathway glucose, sulfate, elemental sulfur, and nitrogen in form of ammonium are released, and all of these nutrients can be reused in primary metabolism or as energy supply (James and Rossiter, 1991; Neilson et al., 2013; Schonhof et al., 2007).

The aim of the present study was to analyze internal turnover of GLSs in Arabidopsis leaves under clearly defined conditions. Total myrosinase activity in combination with the protein abundance of the major myrosinase isoforms expressed in leaves (TGG1 and TGG2) were used as a marker for the induction of GLS breakdown. We also measured nitrilase activity in

order to estimate the flux through this branch of the GLS degradation pathway. A developmental timeline and a diurnal setup were included since changes in the Gls profile have been reported under these conditions. In addition, we used extended darkness as a tool to induce carbohydrate starvation. In the absence of photosynthesis, plants remobilize nutrients such that they can use amino acids and fatty acids as alternative substrates for ATP production (Araújo et al., 2011; Hildebrandt et al., 2015). Our results indicate that GLS turnover is also induced during extended darkness to provide nitrogen and sulfur in addition to glucose and carboxylic acids as substrates for ATP production most likely via the nitrile pathway.

Material & Methods

Plant material and growth conditions

All plants used for this study were *Arabidopsis thaliana* ecotype Columbia (Col-0). The plants were grown under long day (LD; 16 h light/8 h dark) and short day (SD; 8 h light/16 h dark) conditions at 22 °C with a light intensity of 85 µmol $s^{-}1$ m^{-2} light and 65 % humidity.

The circadian setup was performed with two-week-old LD plants (pooled to four to five replicates) and the four harvest points were: (i) at the beginning of the light period, (ii) after 8 h of light, (iii) at the end of the light period and (iv) after 4 h of darkness. Plants at different developmental stages were harvested at the beginning of the light period. For the measurements four to five plants from each developmental stage were taken and pools collected of two- and three-week-old LD and three- and four-week-old SD plants, respectively.

Extended darkness experiments were performed in duplicate with 42 d old SD plants (four to five replicates). The plants were harvested after 3 d (45 d old plants) and 7 d (49 d old plants) of darkness. Metabolite data were obtained from plants grown for 42 d under SD conditions followed by one week of darkness. All plant material was frozen in liquid nitrogen immediately after harvest, ground to a fine powder, and stored at -80°C until use.

LC-MS

Profiling of GLSs by LC-MS was performed in negative/positive ion detection mode (Tohge and Fernie, 2010). Chromatographic data were processed using Xcalibur 2.2 software (Thermo Fisher Scientific). The processed data matrix was normalized using an internal standard (Isovitexin; CAS 29702-25-8) in extraction buffer (5 µg ml^{-1}). Metabolites were identified and annotated based on previously published data (Tohge et al., 2016), as well as

the properties of purified compounds obtained from Arabidopsis extracts (Nakabayashi et al., 2009).

Myrosinase activity

The method from Palmieri et al., 1982 was used to determine the myrosinase activity. For extraction 600 µl of ice-cold potassium phosphate buffer (25 mM, pH 7.0) was added to 40 mg of frozen and ground rosette leaves, vortexed and kept on ice for 10 min. After centrifugation (10 min; 10000 *g*), 450 µl of the supernatant was loaded onto Microcon® Centrifugal Filter Devices. After two filtering steps and filling up the supernatant in the filter devices exactly till the marking of the tubes in between with potassium phosphate buffer, 75 µl of the extract was used for the activity measurement. A total volume of 300 µl was composed of extraction buffer, 0.13 mM ascorbic acid, 0.5 mM sinigrin and 75 µl of the extract. The absorbance of sinigrin was measured photometrically in a UV-permeable 96 well plate at 227 nm for 15 min. Each 9 sec a data point was collected and all samples were measured in duplicate. The control did not contain the GLS sinigrin. The activity was calculated using an extinction coefficient of ε_{227} = 7273 M^{-1} cm^{-1} for sinigrin (Barth and Jander 2006).

Nitrilase activity

For the measurement of nitrilase activity, the production of ammonia was measured by the Berthelot reaction (Hiller and Van Slyke 1933). Approximately 60 mg of frozen and ground plant material was extracted with 500 µl of 100 mM sodium phosphate buffer (ice cold) and kept on ice for 10 min. After centrifugation, the supernatant (100 µl) was taken directly or heated for 10 min at 100 °C (control). The nitrilase reaction was started by addition of the substrate 6-heptenenitrile (2.5 mM) and samples were incubated at 30 °C for 45 min. For detection of the produced NH_3, 330 mM sodium phenolate trihydrate, 20 mM sodiumhypochloride and 0.01 % disodium pentacyanonitrosyl ferrate(III) dihydrate (sodiumprussid) were added and heating at 99 °C for 2 min was performed. Samples were diluted with 600 µl H_2O and analysed in a 96 well plate at 640 nm. Each sample was measured in triplet. The activity was calculated using a standard curve with NH_4Cl. The protein content of the samples was determined using the Pierce Coomassie (Bradford) Protein Assay (Thermo Fisher Scientific).

Western blots

A denaturing SDS-PAGE (Biorad; Mini-PROTEAN® TGX Stain-Free™ Precast Gel) was performed (45 mA; 40 min) with 6 µg of leaf extract. As a marker, the Amersham ECL High-

Range Rainbow marker (GE Healthcare) was used. The ensuing blotting to a nitrocellulose membrane was performed for 1.5 h at 440 mA and the proteins were detected by anti-TGG1 and anti-TGG2 antibodies (Ueda et al., 2006). As a control and for quantification, a polyclonal anti-actin (rabbit) antibody (Agrisera; AS13 2640) was used. Detection was performed by the usage of a secondary antibody goat-anti-rabbit conjugated with horseradish-peroxidase (Agrisera) and the Amersham ECL Western Blotting Detection Reagent (GE Healthcare). All experiments were performed in triplicate.

Results

Myrosinase activity during plant development

In order to identify a possible turnover of GLSs in rosette leaves, we tested the myrosinase activity in two to five-week-old plants grown under long-day (LD) conditions and additionally in plants grown under short-day (SD) conditions for three to nine weeks (**Fig. *1***, **Fig. S4**). Plants grown under LD conditions showed an increased myrosinase activity at the age of three-week-old compared to two-week-old plants and this activity subsequently decreased during further development (**Fig. *1***). We tested abundances of the myrosinase isoforms TGG1 and TGG2 to estimate if the higher activity was due to a higher protein content in leaves. The abundance of TGG1 remained stable in two- to five-week-old plants, while TGG2 was increased in three- to five-week-old plants (**Fig. *2***).

Col-0 plants produced more leaf biomass under SD conditions compared to LD conditions and started to bolt much later, such that nine-week-old SD plants had not yet entered the bolting stage. The leaf myrosinase activity was constant in three- to nine-week-old plants except for a peak at the age of four weeks (**Fig. *1***). Taking together the results from the myrosinase activity measurement and the phenotype, five- to nine-week-old plants grown under SD conditions provide a stable background to investigate the effect of stress treatments on myrosinase activity in *A. thaliana*.

Myrosinase activity during the diurnal cycle

Huseby et al. (2013) demonstrated a circadian synthesis of GLSs in leaves of two-week-old Arabidopsis plants grown under LD conditions. We tested, whether GLS catabolism also followed a circadian rhythm under these growth conditions and detected a significant decrease in myrosinase activity during the light period and an increase in the dark (**Fig. *3***A). Western blot analyses showed no significant variation in the protein abundance for the myrosinase

isoforms TGG1 and TGG2 during the diurnal cycle (**Fig. *3***B), indicating post-translational activation of myrosinases in the light or deactivation in the dark.

Glucosinolate turnover under extended darkness conditions

Carbohydrate starvation was induced by extended darkness (ED) treatment of Arabidopsis plants for three days (3 d) or seven days (7 d). Plants grown for six weeks under short-day conditions were selected for this experiment since myrosinase activity is stable (**Fig. *1***) and plants have produced sufficient leaf material for analysis (**Fig. S5**). Myrosinase activity was equally enhanced after 3 d and 7 d of ED (**Fig. *4***A). The protein abundance of the isoform TGG1 was increased seven-fold already after 3 d of ED and was equally high after 7 d. TGG2 showed a more time-dependent increase in protein abundance of four-fold after 3 d and six-fold after 7 d (**Fig. *4***B). It has to be considered that the protein abundance of actin, which was used for normalization, on our blots showed a tendency to decrease during ED conditions, indicating an increased degradation of this protein as energy source. This would lead to an over-estimation of the fold-increase in TGG1 and TGG2 abundance (**Fig. S*6***). However, normalization to the actin control also showed higher protein abundances for both myrosinase isoforms under ED conditions indicating that the strong relative increase of myrosinase compared to actin abundance was not only due to a lower degradation rate but most likely caused by an induction of synthesis.

The enhanced leaf myrosinase activity and the higher abundance of TGG1 and TGG2 strongly indicated a turnover of GLSs. To provide further evidence, the contents of GLSs in leaves of *A. thaliana* were measured by LC-MS. Results showed up to 89 % reduced contents mainly of aliphatic GLSs in rosette leaves after one week of darkness (**Fig. *5***). The strongest decrease (89 %) was observed for 4-methylthiobutyl-GLS (4MTB), followed by 5-methylthiopentyl-GLS (5MTP, 66 % decrease) and 7-methylthioheptyl-GLS (7MTH, 54 % decrease). This shows that especially methylthio-GLS contents were decreased in leaves. In addition, the content of 7-methylsulfinylheptyl-GLS (7MSOH) was reduced by 41 % and 8-methylsulfinyloctyl-GLS (8MSOO) was reduced by 31 %. Indol-3-ylmethyl-GLS (I3M) was the only indole GLS which showed a decreased content, being depleted by 46 %.

The decrease of specific GLSs in addition to a strong increase in protein abundance of TGG1 and TGG2 and an enhanced myrosinase activity in leaves leads to the conclusion that turnover of GLSs took place under ED conditions. We also measured nitrilase activity in order to estimate, whether breakdown into carboxylic acids via simple nitriles might be relevant for this process. Nitrilase activity was indeed significantly induced in leaves kept for one week under ED conditions compared to the control (**Fig. *4***C).

Discussion

Myrosinase activity but not protein abundance peaks in young Arabidopsis plants

Our results indicate that total leaf myrosinase activity reaches a maximum when plants are approximately at the ten-leaf rosette stage (stage 1.10, Boyes et al., 2001), (**Fig. *1***, **Fig. S4**). Since growth and development of Arabidopsis plants is delayed under SD compared to LD conditions, this developmental stage is reached after four and three weeks, respectively. A previous study addressing developmental changes in myrosinase activity under LD conditions produced similar results (Barth and Jander, 2006). Remarkably, the protein abundance of the myrosinase isoform TGG2 was significantly enhanced from three to five weeks compared to two-week-old plants possibly indicating a growing importance of this isoform in mature plants. Petersen and colleagues (2002) also showed a high increase in myrosinase content from young to mature bolting Col-0 plants using a 3D7 antibody against *Brassica napus* myrosinase. This antibody turned out to be specific for TGG2 (Ueda et al., 2006) and therefore the results of Petersen et al. (2002) were comparable with the findings we report here. Furthermore, Barth and Jander (2006) demonstrated an increased myrosinase activity in mature plants in the *tgg1* mutant. Thus, not only the protein abundance, but also the activity of TGG2 was enhanced in mature Col-0 plants, indicating possible regulation at the transcript level. Using specific antibodies against TGG1 and TGG2, respectively, we could additionally determine the protein abundance of TGG1, which was not altered during development. Total myrosinase activity decreased in four- and five-week-old plants, while the protein abundances of TGG1 and TGG2 remained stable. Therefore, myrosinase activity of TGG1 and TGG2 would additionally appear to be regulated post-translationally.

Diurnal regulation of leaf myrosinase activity

The synthesis of GLSs requires amino acids, glucose, activated sulfur and ATP. Sulfate assimilation as well as GLS synthesis show a diurnal regulation pattern in two week old Col-0 plants grown under long-day conditions with maximal rates during the day when precursors and reducing power are available from photosynthesis (Huseby et al., 2013). Our dataset indicates that GLS turnover might be regulated reciprocally leading to increased degradation rates during the night. At least under certain growth or developmental conditions GLS breakdown could thus contribute to the reallocation of energy or nutrients during the dark period, as has been shown for amino acid metabolism (Izumi et al., 2013; Peng et al., 2015). Indeed, the content of a selected set of GLSs measured in two week old Col-0 seedlings

increased during the day and decreased again during the night supporting this hypothesis (Huseby et al. 2013). Given that neither TGG1 nor TGG2 showed a diurnally altered protein abundance, we postulate that these changes are mediate by an as yet unknown post-transcriptional regulation mechanism.

Extended darkness induces internal turnover of glucosinolates in Arabidopsis leaves

Extended darkness leads to carbohydrate starvation, and as a consequence plants degrade proteins and lipids in order to oxidize amino acids and fatty acids as alternative substrates for mitochondrial ATP production (Araujo et al. 2011). Our results strongly suggest that GLS breakdown in the leaves is an additional component of the carbohydrate starvation response in Arabidopsis. Total leaf myrosinase activity was moderately increased after 3 and 7 days of darkness. This was accompanied by a strong increase in TGG1 and TGG2 protein content. By contrast, the leaf content of actin was reduced by approximately 70 %, which was expected due to increased protein degradation rates in the dark (**Fig. S6**B). The finding that TGG1 and TGG2 are not only excepted from degradation but even synthesized during the extended darkness period shows that leaf myrosinases have a crucial function for survival during carbohydrate starvation. The most obvious potential benefit from GLS turnover, the supply of oxidizable substrates for ATP production, could best be achieved via the nitrilase-dependent degradation pathway. GLS breakdown by this pathway releases glucose and carboxylic acids without producing toxic by-products such as ITCs. Col-0 plants express five different isoforms of nitrile-specifier protein and four nitrilases (Wittstock and Burow, 2010). Our data reveal a significant increase in total leaf nitrilase activity during extended darkness and, taken together with the elevated myrosinase activity, strongly support a potential role of this pathway in internal GLS turnover.

Several scenarios are possible that would enable internal GLS degradation within intact leaves. First, there might be distinct pools of myrosinase, e.g. a large one in the M-cells for fast GLS breakdown after tissue disruption (the “mustard bomb”, Matile, 1980) and a second smaller, potentially inducible pool in the S-cells which catalyzes a more gradual GLS turnover in intact tissue. Secondly, translocation of myrosinase by specific mediator proteins such as MVP1 has already been suggested to affect the subcellular localization of TGG2 (Wittstock and Burow 2010). Thirdly, internal turnover might be a specific function of one of the myrosinase isoforms. However, the finding that both major leaf myrosinase isoforms TGG1 and TGG2 are strongly up-regulated during carbohydrate starvation renders this scenario rather unlikely.

A study addressing the role of light in the regulation of GLS biosynthesis in Arabidopsis had previously detected a decrease in total GLS content by 25 % after 44 hours of darkness (Huseby et al. 2014). Since the plants were still in an early developmental stage, which is characterized by net synthesis and accumulation of GLSs, this effect might have been caused by a decrease in GLS synthesis rates in the dark treated plants compared to the light grown controls, increased GLS turnover, or a combination of both factors. In our approach, plants grown under short-day conditions for 6 weeks were used. These are in a vegetative growth stage with a stable myrosinase abundance and activity and therefore most likely no major changes in the GLS profile occur at this time point. Thus, the strong decrease in relative leaf GLS levels after 7 days of extended darkness can likely be attributed predominantly to internal degradation. Interestingly, we detected large differences between the relative turnover rates of individual GLSs (**Fig. *5***). Short-chain methylthio-GLSs representing the unmodified GLS core structure (4MTB, 5MTP, 7MTH) were reduced most markedly, whilst a less pronounced but still significant decrease was detected for the long-chain methylsulfinyl-GLSs (7MSOH and 8MSOO) and indolic I3M. Strikingly, this set of GLSs corresponds exactly to the characteristic seed GLSs profile, where 4MTB is most abundant (Petersen et al. 2002, Brown et al. 2003, Barth and Jander 2006). Unbiased degradation of a mixture of GLSs would be anticipated to lead to equal relative decreases in all glucosinolates. Thus, our results indicate that the enzyme set catalyzing internal GLS turnover might be optimized for converting the seed GLS profile to the totally different composition present in the leaves during early seedling growth. This process would require a certain substrate specificity of myrosinases. TGG1 and TGG2 expressed in *E. coli* or *Pichia pastoris* have been shown to metabolize different GLSs with different rates (Chung et al., 2005). However, preferences seem to be similar in both isoforms and seed specific GLSs have not been tested (Chung et al., 2005). TGG1 and TGG2 also do not show specificity for particular GLSs in crushed tissue (Barth and Jander 2006), so additional regulatory proteins might be involved in internal GLS turnover *in vivo*. It has to be borne in mind that, while GLS breakdown in leaf homogenate was severely decreased in *tgg1*/*tgg2* double knockout mutants, the developmental decrease in GLS content during germination and senescence was unaffected (Barth and Jander 2006). Thus, there must be an alternative route to catalyze the much slower internal GLS degradation process, which could also be relevant during extended darkness. The fate of the indole GLS I3M during internal degradation might be another interesting aspect to address in future studies given that in the presence of nitrile specifier proteins myrosinase can catabolize I3M to the nitrile indole-3-acetonitrile, which is further degraded by nitrilases into indole-3-acetic

acid (IAA) the plant hormone auxin (Agerbirk et al., 2009; Ljung et al., 2002; Woodward and Bartel, 2005).

Conclusion

In this study, we demonstrated that leaf myrosinase activity changes during plant development as well as diurnally most likely due to a combination of transcriptional and post-translational regulation mechanisms. Extended darkness strongly induces both major leaf myrosinase isoforms TGG1 and TGG1 as well as total nitrilase activity and leads to internal GLS degradation. The knowledge concerning GLS turnover and its regulation is of special interest regarding the chemo-preventive properties of GLS breakdown products (for review see Cheung and Kong, 2010; Hayes et al., 2008). We identified extended darkness as a means to induce turnover of specific GLSs. Further elucidation of the mechanism conveying myrosinase substrate specificity will be a useful aid in producing plants that contain a particularly beneficial GLS profile for human health.

Authors contribution

SB and SF performed experiments concerning myrosinase activity, TT and ARF performed glucosinolate measurements. SB planned and performed all other experiments. SB and TMH designed the experiments, evaluated the data and wrote the manuscript. HPB contributed to the scientific context.

Supplemental Material

Fig. S1 Phenotype of developmental stages under long day and short day conditions

Fig. S2 Plants grown under extended darkness (ED) conditions for 3 d and 7 d

Fig. S3 Western Blots of TGG1 and TGG2 (A) and protein abundance of actin (B) in leaves of plants transferred to extended darkness after six weeks under short day conditions

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Figures

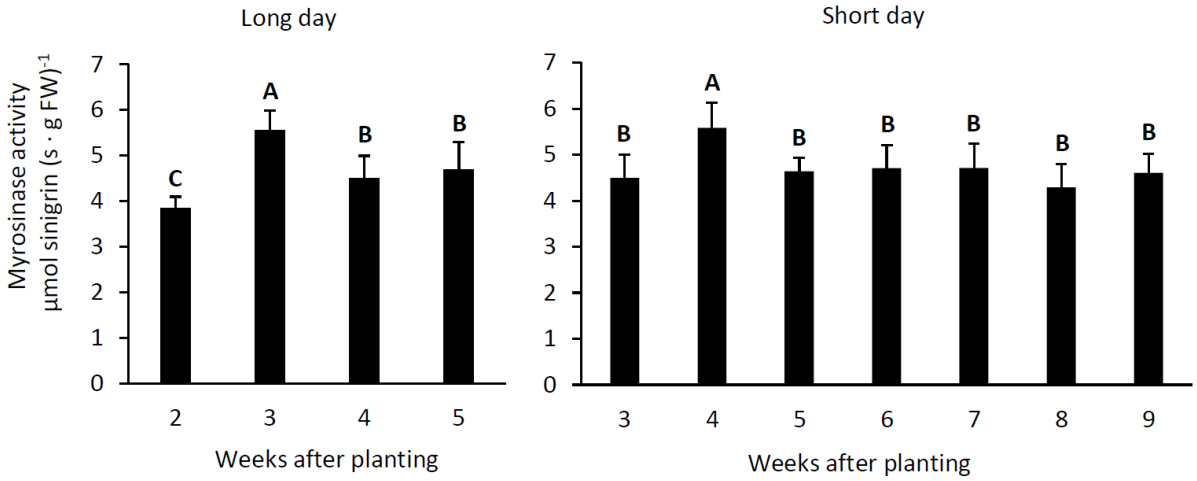

Fig. 1 Myrosinase activity in rosette leaves during development of *A. thaliana*. Complete rosettes were harvested at the beginning of the light period after 2-5 weeks of growth under long-day conditions (16/8h light/dark), and 3-9 weeks of growth under short-day conditions (8/16 h light/dark). Significantly different means are indicated by different letters (Student Newman Keuls; $P < 0.05$)

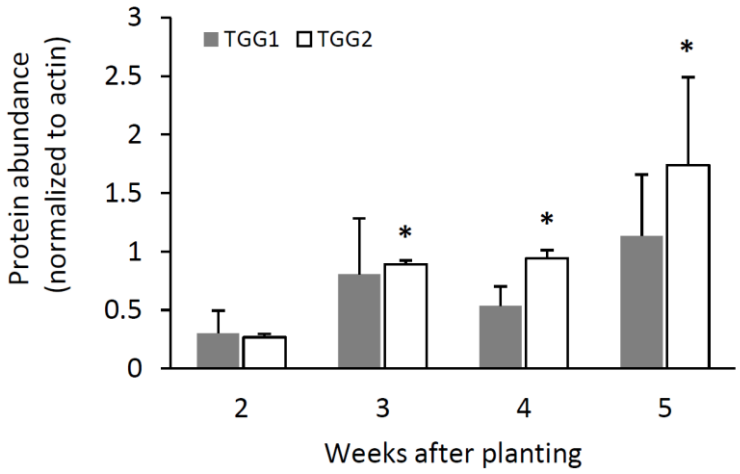

Fig. 2 Protein abundance of TGG1 and TGG2 during development of Arabdiopsis plants grown under long-day conditions. Rosette leaves were harvested at the beginning of the light period and protein abundance was quantified after western-blotting and immunodetection using ImageJ. Asterics indicate significant differences (Student's T-test; $P < 0.01$) compared to the protein abundance in two-week-old plants

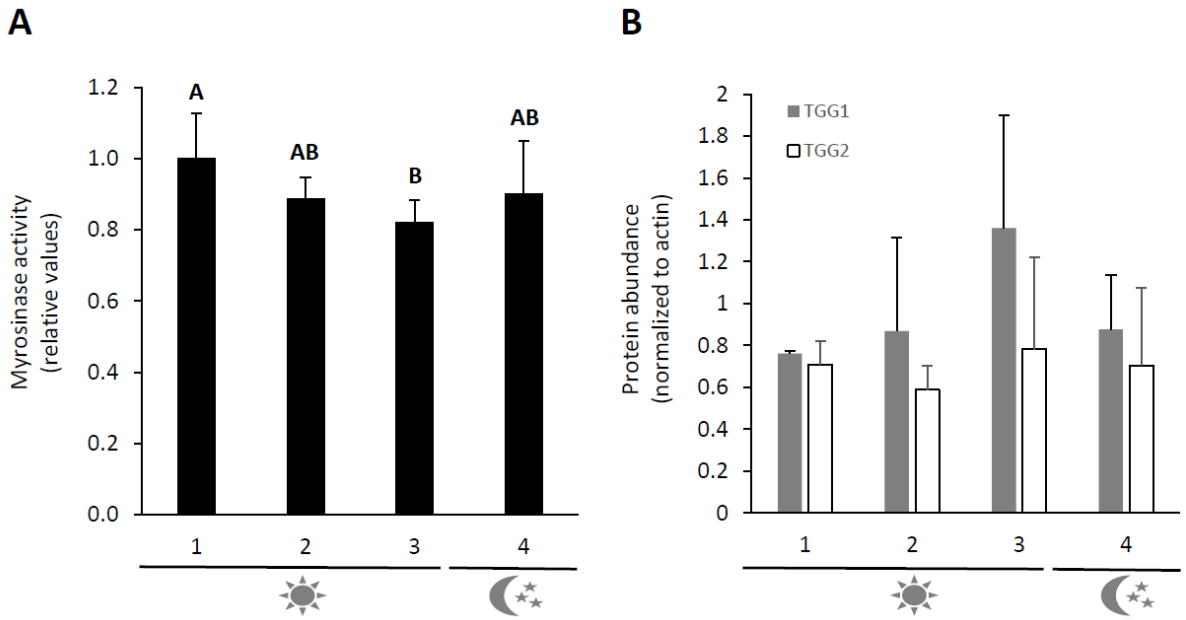

Fig. 3 Circadian myrosinase activity and protein abundance in two-week-old Arabidopsis plants grown under long-day conditions. The plants were harvested at four different time points: **1** Beginning of the light period; **2** Middle of the day; **3** End of the light period; **4** Middle of the night. The myrosinase activity (**A**) was measured photometrically and normalized to the maximal activity. For protein abundance of TGG1 and TGG2 (**B**), immunoblotting and normalization with actin was performed. Significantly different means are indicated by different letters (Student Newman Keuls; $P < 0.05$)

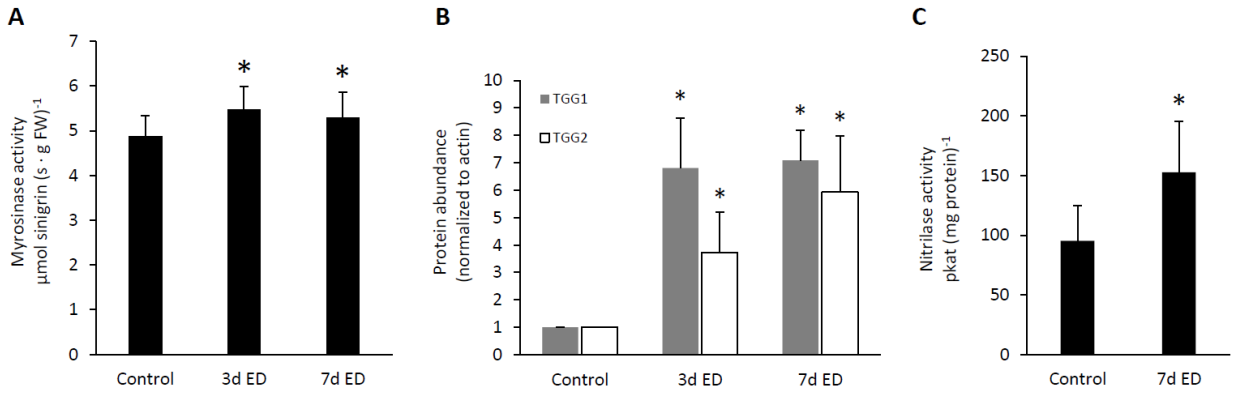

Fig. 4 Myrosinase activity, myrosinase protein abundance and nitrilase activity in rosette leaves after extended darkness (ED). The plants were transferred to complete darkness for 3 d (**3d ED**) or 7 d (**7d ED**) after six weeks of growth under short day conditions. Myrosinase (**A**) as well as nitrilase (**C**) activity was measured photometrically. For protein abundance of TGG1 and TGG2 (**B**), immunoblotting and normalization with actin was performed. Asterics indicate significant differences (Student's T-test; $P < 0.05$) compared to the control

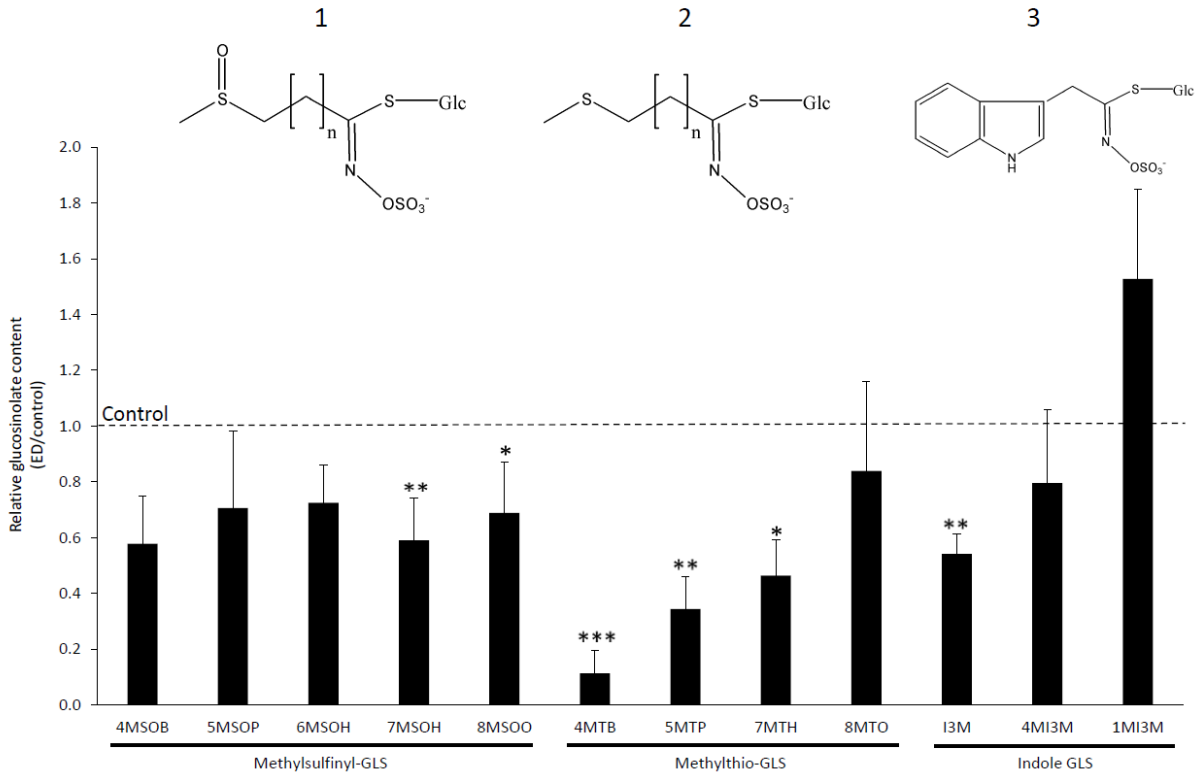

Fig. 5 Glucosinolate content in rosette leaves after extended darkness (ED). Plants were grown for six weeks under short day condition and transferred to darkness for 7 d. Glucosinolate content of rosette leaves was measured via LC-MS. Values are means of three to five replicates and Student's T-test shows *P < 0.05; ** P < 0.01; ***P < 0.001. **1**: general structure of methylsulfinyl-GLS; **2**: general structure of methylthio-GLS; **3**: structure of I3M. **4MSOB** (4-Methylsulfinylbutyl-GLS); **5MSOP** (5-Methylsulfinylpentyl-GLS); **6MSOH** (6-Methylsulfinylhexyl-GLS); **7MSOH** (7-Methylsulfinylheptyl); **8MSOO** (8-Methylsulfinyloctyl); **4MTB** (4-Methylthiobutyl-GLS); **5MTP** (5-Methylthiopentyl-GLS); **7MTH** (7-Methylthioheptyl-GLS); **8MTO** (8-Methylthiooctyl-GLS); **I3M** (Indol-3-ylmethyl-GLS); **4MI3M** (4-Methoxy-indol-3-ylmethyl-GLS); **1MI3M** (N-Methoxy-indol-3-ylmethyl-GLS)

Supplement Figures

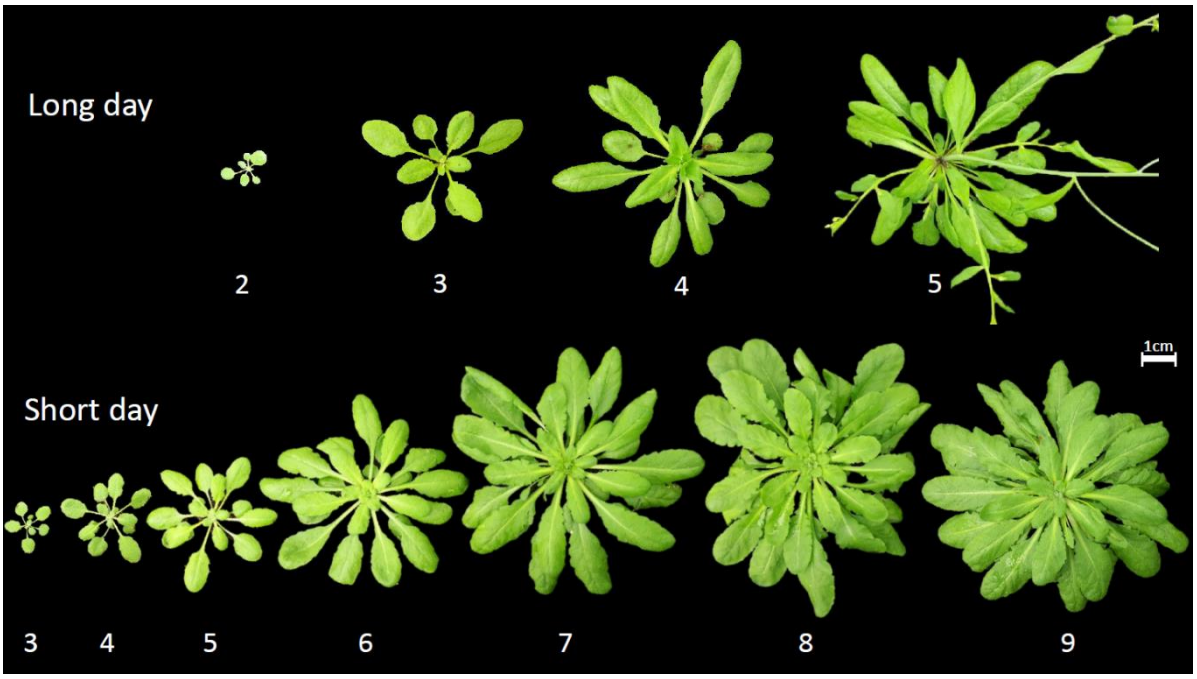

Fig. S4 Phenotype of developmental stages under long day and short day conditions. The plants were grown at 22 °C, 85 µmol s^{-1} m^{-2} light and 65 % humidity for 16 h light/8 h dark (long day) and 8 h light/16 h dark (short day). Numbers indicate the age of the plants in weeks

Fig. S5 Plants grown under extended darkness (ED) conditions for 3 d and 7 d. Phenotype of six-week-old plants grown under short-day conditions (22 °C, 85 µmol s^{-1} m^{-2} light, 65 % humidity) and transferred to darkness (22 °C, 65 % humidity) for 3 d and 7 d

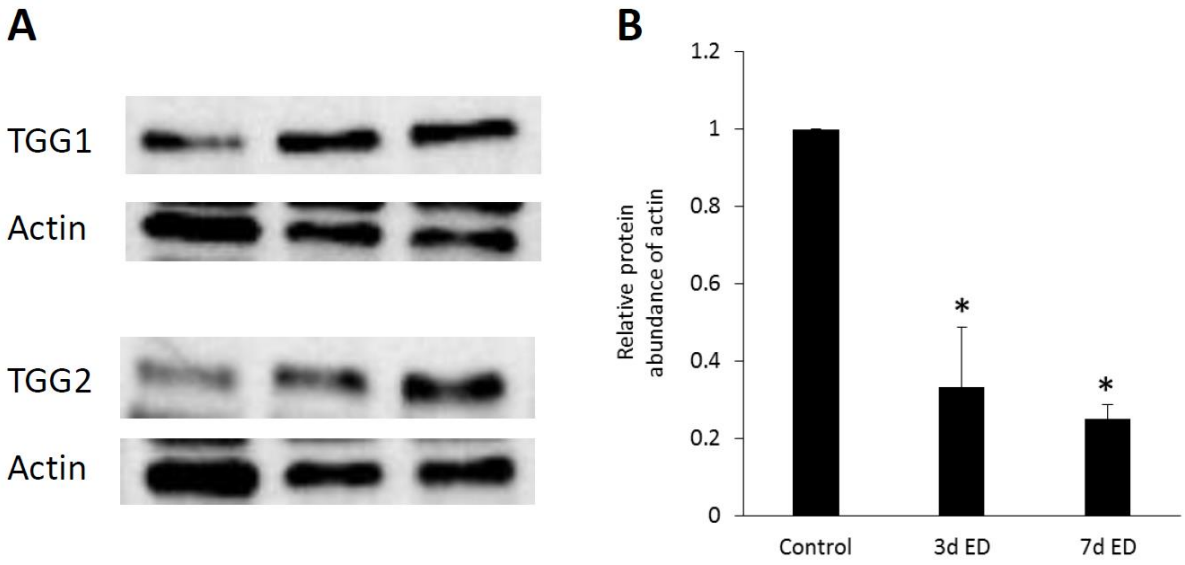

Fig. S6 Western Blots of TGG1 and TGG2 (A) and protein abundance of actin (B) in leaves of plants transferred to extended darkness after six weeks under short day conditions. Leaves were harvested after 3 d (**3 d ED**) and 7 d (**7 d ED**) of extended darkness, directly frozen and ground to powder. 6 µg of denatured leaf extract was load on a SDS-PAGE and further transferred to a nitrocellulose membrane. Specific antibodies against the myrosinase isoforms TGG1 and TGG2 and actin were used. Asterics indicate significant differences (Student's T-test; $P < 0.001$) compared to the control

Curriculum Vitae

Personal data

Name	Saskia Brandt, née Höfler
Date of Birth	14.07.1989
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Citizenship	Citizen of Germany

School education

09.1996 – 07.2000	Primary school in Wäschenbeuren
08.2000 - 06.2009	Secondary school Mörikegymnasium Göppingen Qualification: Abitur

Academic education

Since 01.2015	PhD student in the frame of the "Hannover School for Biomolecular Drug Research" (HSBDR) at the Leibniz Universität Hannover, Institute of Plant Genetics, Department of Plant Proteomics, Prof. Dr. Hans-Peter Braun • Thesis: Cysteine catabolism and glucosinolate turnover in *Arabidopsis thaliana*
10.2012 – 09.2014	M. Sc. student at the Leibniz Universität Hannover, Plant Biology (Grade: "with honor") • Thesis: Secondary compounds of seagrasses and their use as biofilm-inhibiting agents
10.2009 – 09.2012	B. Sc. student at the Universität Osnabrück, Cell Biology (Grade: A) • Thesis: Funktionelle Analyse von rekombinanter Alternativer Oxidase (AOX1D) aus *Arabidopsis thaliana* sowie ihrer Cystein-Mutanten

Publications

1. Brandt S., Fachinger S., Tohge T., Fernie A.R., Hans-Peter Braun H.P. and Tatjana M. Hildebrandt T.M. (in preparation) Extended darkness induces internal turnover of glucosinolates in Arabidopsis leaves.

2. Lorenz C., Brandt S., Borisjuk L., Rolletschek H., Heinzel N., Tohge T., Fernie A.R., Braun H.-P., Hildebrandt T.M. (in preparation): Amino acid metabolism and the role of ETHE1 in Arabidopsis thaliana seeds.

3. Höfler, S., Lorenz, C., Busch, T., Brinkkötter, M., Tohge, T., Fernie, A., Braun, H.P. and Hildebrandt, T.M. (2016): Dealing with the sulfur part of cysteine: four enzymatic steps degrade l-cysteine to pyruvate and thiosulfate in Arabidopsis mitochondria., Physiologia Plantarum 157, 352-366

4. Selinski J, Hartmann A, Höfler S, Deckers-Hebestreit G, Scheibe R. (2016): Refined method to study the post-translational regulation of alternative oxidases from Arabidopsis thaliana in vitro., Physiologia Plantarum 157, 264-279

5. Nguyen, XV., Höfler, S., Glasenapp, Y., Thangaradjou, T., Lucas, C., Papenbrock, J. (2015): New insights into DNA barcoding of seagrasses., Systematics and Biodiversity 13, 496-508

Conference contributions

1. Plant Sulfur Conference in Goslar, Germany (September 2015)
   Poster: “Cysteine catabolism in mitochondria of Arabidopsis thaliana”

2. HSBRD Symposium in Warberg, Germany (June 2016)
   Talk: “Glucosinolate turnover in *Arabidopsis thaliana*”

3. 23. Arbeitstagung Mikromethoden in der Proteinchemie in Dortmund, Germany (June 2016)

4. 109th International Workshop on HRR and O2k-Fluorometry in Schröcken, Vorarlberg, Austria (April 2016)

5. HSBRD Symposium in Warberg, Germany (June 2017)
   Talk: “Myrosinase activity in *Arabidopsis thaliana*”

6. Plant and Human Sulfur Biology Conference in Balatonfüred, Hungary (September 2017)
   Poster: “Highlights of the myrosinase activity of TGG1 and TGG2 under abiotic stress conditions”

7. Glucosinolate Summit in Berlin, Germany (September 2017)
   Poster: “Highlights of the myrosinase activity of TGG1 and TGG2 under abiotic stress conditions”

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