Plant thermotolerance: The role of heat stress-induced triacylglycerols in

Arabidopsis thaliana

Thermotoleranz in Pflanzen: Die Rolle von Hitzestress induzierten

Triacylglycerolen in *Arabidopsis thaliana*

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Summary

Plants are exposed to high temperature, especially during hot summer days. Temperatures are typically lowest in the morning and reach a maximum in the afternoon. Plants can tolerate and survive short-term heat stress even on hot summer days. *A. thaliana* seedlings have been reported to tolerate higher temperatures for different time periods, a phenomenon that has been termed basal thermotolerance. In addition, plants have the inherent capacity to acclimate to otherwise lethal temperatures. *Arabidopsis thaliana* seedlings acclimate at moderately elevated temperatures between 32–38° C. During heat acclimation, a genetically programmed heat shock response (HSR) is triggered that is characterized by a rapid activation of heat shock transcription factors (HSFs), which trigger a massive accumulation of heat shock proteins that are chiefly involved in protein folding and protection.

Although the HSF-triggered heat-shock response is well characterized, little is known about the metabolic adjustments during heat stress. The aim of this work was to get more insight into heat-responsive metabolism and its importance for thermotolerance.

In order to identify the response of metabolites to elevated temperatures, global metabolite profiles of heat-acclimated and control seedlings were compared. Untargeted metabolite analyses revealed that levels of polyunsaturated triacylglycerols (TG) rapidly increase during heat acclimation. TG accumulation was found to be temperature-dependent in a temperature range from 32–50° C (optimum at 42° C). Heat-induced TG accumulation was localized in extra-chloroplastic compartments by chloroplast isolation as well as by fluorescence microscopy of *A. thaliana* cell cultures.

Analysis of mutants deficient in all four HSFA1 master regulator genes or the HSFA2 gene revealed that TG accumulation occurred independently to HSF. Moreover, the TG response was not limited to heat stress since drought and salt stress (but not short-term osmotic, cold and high light stress) also triggered an accumulation of TGs.

In order to reveal the origin of TG synthesis, lipid analysis was carried out. Heat-induced accumulation of TGs does not derive from massive *de novo* fatty acid (FA) synthesis. On the other hand, lipidomic analyses of *A. thaliana* seedlings indicated that polyunsaturated FA from thylakoid galactolipids are incorporated into cytosolic TGs during heat stress. This was verified by lipidomic analyses of *A. thaliana fad7/8* transgenic seedlings, which displayed altered FA compositions of plastidic lipids. In addition, wild type *A. thaliana* seedlings displayed a rapid conversion of plastidic monogalactosyldiacylglycerols (MGDGs) into oligogalactolipids,

acylated MGDGs and diacylglycerols (DGs). For TG synthesis, DG requires a FA from the acyl CoA pool or phosphatidylcholine (PC). Seedlings deficient in phospholipid:diacylglycerol acyltransferase1 (PDAT1) were unable to accumulate TGs following heat stress; thus PC appears to be the major FA donor for TGs during heat treatment. These results suggest that TG and oligogalactolipid accumulation during heat stress is driven by post-translationally regulated plastid lipid metabolism.

TG accumulation following heat stress was found to increase basal thermotolerance. *Pdat1* mutant seedlings were more sensitive to severe heat stress without prior acclimatization, as revealed by a more dramatic decline of the maximum efficiency of PSII and lower survival rate compared to wild type seedlings. In contrast, *tgd1* mutants over-accumulating TGs and oligogalactolipids displayed a higher basal thermotolerance compared to wild type seedlings. These results therefore suggest that accumulation of TGs increases thermotolerance in addition to the genetically encoded heat shock response.

Zusammenfassung

Pflanzen sind besonders während der Sommerzeit hohen Temperaturschwankungen ausgesetzt. Temperaturen sind am Morgen meist niedrig und erreichen ihr Maximum während des Nachmittags. Pflanzen können Hitzestress im Sommer jedoch für eine kurze Zeit tolerieren. *Arabidopsis thaliana* Keimlinge können höhere Temperaturen für verschiedene Zeitspannen tolerieren, was als Basale Thermotoleranz beschrieben wird. Zusätzlich können Pflanzen durch Akklimatisierung eine Toleranz zu andernfalls letalen Temperaturen erwerben. *A. thaliana* Keimlinge beginnen sich bereits bei moderat erhöhten Temperaturen zwischen 32–38° C zu akklimatisieren. Während der Hitzeakklimatisierung wird eine genetisch programmierte Hitzeschockantwort (HSR) ausgelöst, welche durch eine rasche Aktivierung von Hitzeschock-Transkriptionsfaktoren (HSF) eingeleitet wird. Dies führt wiederum zu einem enormen Anstieg von einer Reihe von Hitzeschockproteinen (HSP), welche an der Faltung und dem Schutz der Proteine beteiligt sind.

Obwohl die HSF-induzierte Hitzeschockantwort bereits gut charakterisiert ist, ist über die metabolomische Anpassung während des Hitzestress nur wenig bekannt. Das Ziel dieser Arbeit war es mehr Kenntnisse von hitze-respondierenden Metaboliten zu erhalten sowie deren Bedeutung für die Thermotoleranz. Zur Identifizierung von thermosensitiven Metaboliten, wurden die Metabolitprofile von Hitze akklimatisierten und Kontrollkeimlingen miteinander verglichen. Mittels ungerichteter Metabolit Analyse wurde ein rascher Anstieg von vielfach ungesättigten Triacylglycerolen (TG) während der Hitzeakklimatisierung nachgewiesen. Der TG Anstieg ist temperaturabhängig in einem Bereich von 32–50° C (Optimum bei 42° C).

Der hitzeinduzierte TG Anstieg konnte mittels Chloroplastenisolierung sowie der separaten Analyse von Wurzel und Spross in den extrachloroplastidären Kompartimenten lokalisiert werden. Dies konnte durch Fluoreszenz Mikroskopie in Zellkulturen von *A. thaliana* bestätigt werden.

Die Analyse von Mutanten, die einen Defekt in allen vier HSFA1 Masterregulatoren oder in dem HSFA2 Gen besitzen, zeigte, dass der Anstieg der TGs keine Abhängigkeit von den HSFs aufweist. Zudem ist der TG Anstieg nicht nur auf die Hitzestressantwort begrenzt, sondern auch durch Trockenheit und Salzstress induzierbar, jedoch nicht durch kurzzeitigen osmotischen-, Kälte- und Hochlichtstress.

Zur Aufklärung des Ursprungs der TG Synthese wurde eine Lipidanalyse durchgeführt. Die hitzeinduzierte TG Akkumulation durch eine massive *De Novo* Fettsäuresynthese konnte ausgeschlossen werden. Die Untersuchung des Lipidoms von *A. thaliana* Keimlingen nach Hitze bot jedoch Hinweise auf einen Einbau von vielfach ungesättigten Fettsäuren aus thylakoiden Galaktolipiden in zytosolische TGs. Dies konnte durch die Untersuchung des Lipidoms von *fad7/8* transgenen *A. thaliana* Keimlingen mit veränderter Fettsäure Komposition der plastidären Lipide bestätigt werden.

Der Wildtyp von *A. thaliana* wies zudem eine rasche Umwandlung von plastidärem Monogalactosyldiacylglycerolen (MGDGs) zu Oligogalaktolipiden, acylierten MGDGs und Diacylglycerolen (DGs) auf. Für die TG Biosynthese wird eine Fettsäure aus dem Acyl-CoA Pool oder von Phosphatidylcholin (PC) auf ein DG übertragen. Keimlinge, die einen Defekt in der Phosolipid:Diacylglycerol Acyltransferase (PDAT1) aufweisen, waren nicht in der Lage TGs nach Hitzestress zu akkumulieren, auf PC als der wesentliche Fettsäure-Donor für TGs nach Hitzestress hinweist. Die Ergebnisse deuten auf einen TG und Oligogalaktolipid Anstieg durch einen posttranskriptionell regulierten Lipidumbau während des Hitzestress hin.

Es konnte gezeigt werden, dass der TG Anstieg nach Hitzestress zu einer erhöhten Thermotoleranz führt. Keimlinge der *pdat1* Mutanten waren ohne Akklimatisierung empfindlicher gegenüber massiven Hitzestress, da sowohl ein dramatischer Abfall der maximalen Effizienz des Photosystems II und eine niedrigere Überlebensrate im Vergleich zu Keimlingen des Wildtyps nachgewiesen wurden. Im Gegensatz dazu zeigten *tgd1* Mutanten, welche eine Überakkumulation von TGs und Oligogalaktolipiden aufweisen, eine höhere Thermotoleranz auf als Keimlinge des Wildtyps. Diese Ergebnisse weisen darauf hin, dass die TG Akkumulation die Thermotoleranz zusätzlich zu der genetisch kodierten Hitzeschockantwort erhöht.

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1 Introduction

1.1 Heat Stress in Plants

Elevation of temperature above the optimal growth range is one of the major abiotic stress factors faced by all organisms. Plants are continuously exposed to daily and seasonal temperature fluctuations and require therefore robust mechanisms to sense and respond to aberrations from their optimal growth conditions. Heat can have a negative effect on the stability of proteins, enzymes, nucleic acids, bio membranes and cytoskeletal structures (Asthir, 2015). Due to the relevance of global warming and its possible detrimental effects on the productivity of crop plants, many studies have focused on plant response to heat stress. Substantial reductions in yield have already been documented in various important food crops (Lobell et al., 2008; Lobell and Field, 2007). In rice, a yield loss of 4-14 % was recorded due to a 1° C increase in South-East Asia. In cereals, a 1° C increase in the average seasonal temperature leads to a reduction in productivity of 4–10 % (Wang et al., 2012). In addition, temperature trend analysis reported a decrease of 5.5 % in wheat production (Lobell et al., 2011) along with a loss of eight million tons per year from 1981 to 2002 caused by an usual warming in this time period. This loss was estimated to have costed approximately $1.0 billion (Lobell and Field, 2007). The future will see more challenging environmental conditions. Following a scenario of a 2° C temperature increase, worldwide yield reductions can be expected for wheat, rice and maize in both tropical and temperate regions (Challinor et al., 2014). The average temperature is estimated to increase by 0.2° C over the next decade. Especially heatwaves, i.e. an increase of several degrees over the seasonal temperature for a sustained number of days, are predicted to occur more frequently (Field et al., 2014; Meehl and Tebaldi, 2004). Temperature and yield relationship analysis predict a nearly 46 % yield loss of soy bean in USA before year 2100 (Schlenker and Roberts, 2009) as well as an estimated decline by 45 % of the global maize yield by 2080 compared to 1980, caused by heat shock during anthesis (Deryng, 2014).

Plants have evolved a series of mechanisms to survive heat stress, including temperature sensing, changes of metabolism and cellular structures. Understanding these processes will be essential in creating strategies to improve plants to overcome heat stress more effectively.

1.1.1 Definition of thermotolerance

Basal thermotolerance is the capacity of plants to survive temperatures above the optimal growth temperature without prior acclimation (Larkindale et al., 2005). The optimum growth temperature varies strongly in the plant kingdom. For example, the optimum temperature, measured by the pace of new leaf formation throughout the vegetative development, ranges from 26° C for wheat to 37° C for cotton (Hatfield, 2011). The optimal temperature of *Arabidopsis thaliana*, measured by the rate of leaf initiation, leaf expansion and the duration of expansion, increases linearly in the range of 6 to 26° C (Granier et al., 2002). However, *A. thaliana* seedlings have been reported to tolerate higher temperatures for different time periods. They can survive 30° C for up to 5 days (Chen et al., 2006), 38° C for 16 h (Clarke et al., 2009) and 45° C for up to 45 min (Yeh et al., 2012).

In addition to basal thermotolerance, plants have the ability to cope with lethal high temperatures following acclimation at sublethal high temperatures (known as acquired thermotolerance) (Larkindale et al., 2005). Short term acquired thermotolerance (SAT) has been observed following acclimation of *A. thaliana* seedlings at moderately elevated temperatures of 31-39° C for 1 h (Liu and Charng, 2012). Following acclimation at 38° C for 1.5 h, *A. thaliana* seedlings can survive exposure to 45° C for 2 h, which is a lethal temperature for non-acclimatized plants (Hong and Vierling, 2000; Larkindale and Vierling, 2008). It has been suggested that SAT evolved as a coping mechanism for plants during the gradual rise of temperature during the day. Typically, there is sufficient time to acclimate in the temperature range from 31–38° C before potentially lethal temperatures above 40° C are reached in the afternoon, lasting a few hours before decreasing during the night. Seedlings, which were gradually heated from 38° C to 45° C over a time period of 6 h, can survive longer periods of 45° C exposure compared to seedlings acclimated at 38° C for 90 minutes (Larkindale and Vierling, 2008). The thermotolerance acquired through short-term treatment is fully reversible, lasts for up to 72 h and decays gradually (Charng et al., 2006).

1.1.2 Heat Shock Response

Plants possess an highly conserved evolutionary salvage mechanism called heat shock response (HSR) to help cells cope with stress situations and to protect them against cell death (Lindquist, 1986). The HSR leads to a global transition of gene expression in the plant. The rapid activation of heat stress transcription factors (HSFs) plays a central role in the HSR. HSFs

enhance the expression of many genes that lead to an accumulation of heat shock proteins (HSPs).

1.1.2.1 Heat Shock Transcription Factors

HSFs, the core regulators of the HSR, have a modular structure and are evolutionary conserved among eukaryotes. Despite demonstrating a variability in size and sequence, they show high similarities in the mode of promoter recognition and their basic structure (Baniwal et al., 2004; Bjork and Sistonen, 2010; Fujimoto and Nakai, 2010). *A. thaliana* has 21 HSFs belonging to three classes (A, B and C), which include 14 groups (A1 to A9, B1 to B4 and C1) (Nover et al., 2001; Scharf et al., 2012). The A1 group includes four members, HSFA1a, b, d and e, which have been termed 'master regulators' of the HSR since *hsfa1abd* triple and *hsfa1abde* quadruple mutants are unable to induce the HSR or to acquire thermotolerance (Liu et al., 2011; Yoshida et al., 2011). Among the four master regulators, HSFA1a and d are the strongest effectors, whereas HSFA1b has demonstrated moderate effects and HSFA1e showed the least response in conferring thermotolerance (Liu et al., 2011). The HSF class A has short peptide motifs and is enriched in aromatic and large hydrophobic amino acid residues embedded in an acidic surrounding, which was shown to be essential for their activation (Kotak et al., 2004). Following heat stress, the master regulators HSFA1a, b, d, and e activate a series of transcription factors, including HSFA2, HSFB, dehydration-responsive element-binding protein 2A (DREB2A) and multiprotein bridging factor 1 (MBF1c) (Liu and Charng, 2013; Liu et al., 2011; Yoshida et al., 2011). HSFA1-inducible transcription factors include both enhancers and repressors of the HSR. DREB2A and MBF1c are known enhancers, whereas HSFBs are negative regulators of the HSR (Charng et al., 2007; Czarnecka-Verner et al., 2004; Ikeda et al., 2011; Larkindale and Vierling, 2008; Nishizawa-Yokoi et al., 2011; Sakuma et al., 2006; Schramm et al., 2008; Suzuki et al., 2011; Yoshida et al., 2008).

HSFA2 is the most heat-inducible transcription factor of the HSF family (Busch et al., 2005). In *hsfa1a* knockdown tomato plants HSFA2 expression was suppressed (Mishra et al., 2002). In contrast, in *A. thaliana,* HSFA2 was not affected in the *hsfa1a/1b* double knockout line (Busch et al., 2005). The crucial role of HSFA2 in the late phase of HSR has been determined (Charng et al., 2007; Schramm et al., 2006; Wunderlich, 2007). These results suggest that HSFA2 is a secondary regulator under the control of at least one master regulator. The early and late expression of heat shock genes can be mediated through this transcriptional cascade (Banti et

al., 2010; Nishizawa, 2006). Among the HSFA1-induced transcription factors, HSFA2 is required for the extension of short-term heat acclimation (Charng et al., 2007) and triggers an up-regulation of a subset of heat-responsive genes such as HSPs (including HSP101, HSP26.5, HSP22-ER, HSP18.1, HSP70b), ascorbate-peroxidase 2 (APX2) and galactinol-synthase 1 (GOLS1) as well as some drought- and cold-regulated genes (Schramm et al., 2006).

1.1.2.2 Heat Shock Proteins

In addition to heat stress, almost all kinds of stresses can lead to gene expression and synthesis of heat-shock proteins; however, to a lesser extent (De Maio, 1999; Feige, 1996). HSPs play an essential role in the maintenance of cellular homeostasis by preventing protein denaturation and aggregation, assisting in the correct folding of nascent as well as stress accumulated, misfolded proteins and degrading nonnative proteins (Morimoto, 1993; Saluja and Dudeja, 2008; Schlesinger, 1990; Sikora and Grzesiuk, 2007). In addition, HSPs can also interact with various components of the programmed-cell death machinery (Garrido et al., 2001; Lanneau et al., 2008; Parcellier et al., 2003). HSPs can be grouped into five major families corresponding to their molecular weight, amino acid sequence homologies and functions: HSP100, HSP90, HSP70, HSP60 and the small HSP family, ranging from 15 to 28 kDa (Gupta, 2010).

The best studied members of the HSP100 family is the cytosolic/nuclear ClpB1, a product of the HOT1 gene, which is known as *At*HSP101 in *A. thaliana*. The highest expression of HSP101 is induced 1 to 2 h following moderate heat treatment and showed subsequently a rapid decrease (Muench et al., 2016). HSP101, displaying dramatic accumulation during high temperature stress, plays an essential role in basal and acquired thermotolerance in *A. thaliana* (Hong and Vierling, 2000; Larkindale et al., 2005; Lee et al., 2007; Queitsch et al., 2000). Deficiency of other HSPs leads to lower thermotolerance (Dafny-Yelin et al., 2008; Su and Li, 2008; Yamada et al., 2007).

1.1.2.3 Heat sensing

In contrast to the regulation of HSFs and HSPs, heat perception has yet to be fully understood. It is most likely that heat perception and signal transduction is regulated by a complex interwoven network of sensors and proteins (Saidi et al., 2011). Several experiments regarding signal transduction of the HSR have suggested an interaction between both HSFs and HSPs, as described by the "chaperon-titration" model (Saidi et al., 2011). The chaperone titration

model assumes that HSF1 is kept inactive in unstressed cells through its interaction with chaperones. The presence of misfolded proteins activates HSF1 by "titrating" (or attracting) the chaperones away from HSF1 (Hahn et al., 2011; Le Breton and Mayer, 2016; Nishizawa-Yokoi et al., 2010; Saidi et al., 2011; Sugio et al., 2009).

In addition, exposure of hydrophobic residues of proteins leads to an accumulation of chaperones, known as the unfolded protein response UPR, and has been suggested to trigger the HSR (Che et al., 2010; Mittler et al., 2012).

Membranes also play a central role in heat-sensing. They can enclose sensory devices which can detect specific signals and are able to transduce them into appropriate gene expression (Los and Murata, 2004; Saidi et al., 2011; Vígh et al., 1998).

Furthermore, H2A.Z, an alternative Histone of H2A, has been found to act in nucleosomes as a direct temperature sensor and to mediate temperature response-associated gene expression (Clapier and Cairns, 2009; Erkina et al., 2008; Erkina et al., 2010; Kumar and Wigge, 2010; Saidi et al., 2011).

1.2   Metabolism during heat stress

In addition to heat perception and the regulation of HSFs and HSPs, an important part of heat stress response is the production of metabolites which help plants to survive high temperatures. It has been predicted that plants synthesize between 200.000 and 1.000.000 different metabolites. In *A. thaliana* the number of metabolites has been estimated to be at least 5.000 (D'Auria and Gershenzon, 2005; Davies et al., 2010; De Luca and St Pierre, 2000; Saito and Matsuda, 2010). Technical innovations have made metabolomics a more available technique and, with its broad applicability and great yield of information, it is becoming more and more important in plant physiology and biochemistry.

However, only one large metabolite profiling analysis of heat-induced metabolome changes in *A. thaliana* has been reported. 143 metabolites could be identified to be affected from heat (40° C for up to 4 h), including several pyruvate- and oxaloacetate-derived amino acids, oxaloacetate precursors (malate and fumarate), amine-containing metabolites (β-alanine, GABA and putrescine) and several carbohydrates.

From those metabolites, 80 showed a significant increase ($p < 0.05$), from which only four showed a strong accumulation with a higher than 4-fold change. Those metabolites could be identified as galactinol, raffinose, pipecolic acid and digalactosylglycerol (Kaplan et al., 2004).

Accumulation of raffinose and galactinol following moderate heat stress has already been described in detail (Panikulangara et al., 2004). The knockout mutants of GOLS1, the key enzyme in the biosynthesis of the raffinose family oligosaccharides, including raffinose, stachyose and verbascose, showed, in contrast to the wild type, no accumulation of raffinose following heat treatment. However, no heat tolerance phenotype could be detected in the knock-out mutant lines compared to the wild type (Panikulangara et al., 2004). In addition to raffinose and galactinol, an accumulation of maltose, glycerol, sucrose and trehalose could also be detected following 6 hours of heat treatment (Rizhsky et al., 2004).

I previously completed an untargeted metabolics study, in which an increase in raffinose and galactinol could also be observed (Mueller et al., 2015). The response was further investigated in this thesis. Phytohormones, cytokinins, ethylene, jasmonic acid and methyl jasmonate have also been reported to play a role in basal thermotolerance (Clarke et al., 2009; Larkindale et al., 2005; Xing, 2009; Xu, 2008). Abscisic acid and salicylic acid have been shown to be important in both basal and acquired thermotolerance (Clarke et al., 2004; Kaplan et al., 2004; Larkindale et al., 2005).

1.2.1 Lipidomic changes during heat stress

High temperature stress leads to higher membrane fluidity (Quinn, 1988). Temperature related changes have a major impact on the activity of membrane-localized proteins as well as on membrane permeability to water, solutes and proteins (Lande et al., 1995; Whiting et al., 2000). Plants counteract by regulating the saturation level of membrane glycerolipids, as unsaturated lipids lead to a decrease in the phase transition temperature (Nishida and Murata, 1996). Following long-term heat stress, higher amounts of saturated FAs could be determined in the grass *Agrostis stolonifera,* while tolerant varieties already exhibited a greater rate of saturated lipids at basal conditions (Larkindale and Huang, 2004).

The reorganization of membrane lipids to a higher saturation level in *A. thaliana* could be determined following 108 h at 29° C. After this timespan, 16:3-acyl chains in thylakoid lipids are less abundant and 16:0-acyl chains are more abundant. Replacement of 18:3-acyl chains by 18:2-acyl chains does not take place before 204 hours (Falcone et al., 2004). In agreement with this data, no change in lipid composition was observed following 6 h at 32° C. It has also been reported, that high light, leading to a higher saturation level in membrane levels, has a bigger impact on membrane remodeling than temperature (Burgos et al., 2011). A shift

towards higher saturation levels in membrane lipids has been reported following long-term heat exposition; short-term heat treatment does not appear to be sufficient for the remodeling of lipids (Balogi et al., 2005).

A higher thermotolerance following short-term heat treatment in comparison to the wild type has been reported in the *A. thaliana fad3.2/fad7.2/fad8* triple mutant. This triple mutant contains no detectable trienoic FAs. Higher levels of saturated FAs improves membrane stability, especially in thylakoid membranes, which results in a higher thermotolerance of the photosystem II (Routaboul et al., 2012).

Changes in the membrane composition were determined following short-term heat treatment in *Synechocystis* thylakoids. It was shown that higher temperatures (42° C, 3 h), led to an increase in monoglucosyldiacylglycerol (MGlcDG), a precursor of the chloroplast lipid MGDG. MGlcDGs have been suggested to act as a novel "heat shock lipid", which may stabilize the membranes at an early stage via highly saturated FAs and through its ability to interact with HSP17 (Balogi et al., 2005; Horvath et al., 2012). Temperature-dependent activation of MGlcDG-synthases could be observed, whereas protein levels remained unchanged, suggesting a post-translationally-regulated activity (Horvath et al., 2012; Shimojima et al., 2009). However, this has not been shown in higher plants until now.

Moreover, in my previous studies, untargeted metabolomic analysis revealed a link between heat stress and triacylglycerols (TGs) (Mueller et al., 2015). TGs are lipids derived from glycerol and three fatty acids (FAs) and are used as energy storage. Rapid accumulation of polyunsaturated TGs, in particular TG54:9, 54:8, 54:7 and 54:6, was observed following heat stress. The functional significance and origin of TG accumulation following heat treatment shall be addressed in this thesis.

1.2.2   Triacylglycerol (TG) biosynthesis

Triacylglycerols (TGs) are highly concentrated stores of metabolic energy. They are the most abundant components of Arabidopsis seed oils (Li-Beisson et al., 2013). In addition, TGs are also present in leaves, roots and stems but in smaller concentrations (Yang and Ohlrogge, 2009). Several studies have shown that environmental stress, such as ozone exposure, drought stress and nitrogen deprivation, can lead to an increase of TGs in leaves (Gaude et al., 2007; Sakaki et al., 1990).

1.2.2.1   Acyl-CoA-dependent *de novo* TG synthesis pathway: The Kennedy pathway

The Kennedy pathway is located at the ER and is believed to be the most important pathway for *de novo* TG synthesis in most organisms (Chapman and Ohlrogge, 2012). The pathway involves a sequential acylation and dephosphorylation of glycerol 3-phosphate (G3P). G3P acyltransferase (GPAT), which is ER-associated, catalyzes the first acylation at the *sn1* position of G3P (Li-Beisson et al., 2013). It was recently shown that *AtGPAT9* is a single-copy and essential gene in *A. thaliana,* demonstrated by both male and female gametophyte lethality of the *gpat9* mutant. GPAT9 knockdowns demonstrated a reduction in the oil content. Due to the fact that GPAT9 has been shown to be essential for gametophyte function, GPAT activity, involved in seed TG synthesis, is probably also essential for ER membrane lipid synthesis in other tissues (Shockey et al., 2016).

The next acylation step at the *sn*-2 position – the conversion of lysophosphatidic acid to phosphatidic acid (PA) - is catalyzed by lysophosphatidic acid acyltransferase (LPAAT) (Korbes et al., 2015). Until now, five *LPAAT* genes have been described in *A. thaliana*: one plastidial isoform-encoding gene (*AtLPAAT1*) and four cytoplasmatic genes (*AtLPAAT2-5*). The role of the enzymes in the TG synthesis remains to be confirmed.

The de-phosphorylation of PA to form diacylglycerol (DG) is catalyzed by a phosphatidic acid phosphatase (PAP), also known as phosphatidic acid hydrolase (PAH). This step in the Kennedy pathway is the least studied, as *A. thaliana* contains at least 11 genes with a predicted homology to PAP (Li-Beisson et al., 2013). In mammals and yeasts the enzyme has been shown to control the flux between membrane lipid synthesis and TGs. In both systems, loss-of-function mutations led to an increased membrane lipid proliferation and decreased TG content (Carman and Han, 2009; Reue and Dwyer, 2009). Two homologs of the yeast phosphatidic acid hydrolase (*AtPAH1* and *AtPAH2*) have been reported in *A. thaliana.* Disruption of *PAH1* and *PAH2* affects the eukaryotic pathway of galactolipid biosynthesis and membrane remodeling, suggesting a role in the eukaryotic pathway of DG formation (Nakamura et al., 2009). However, *Atpah1* and *Atpah2* seeds displayed only a slight decrease in TG levels (Eastmond et al., 2010), suggesting that additional uncharacterized genes are involved in TG synthesis (Chapman and Ohlrogge, 2012).

After DG is formed, diacylglycerol-acyltransferase (DGAT) catalyzes the acylation of the *sn*-3 position to yield TGs. Acyl-CoA used for acylation originates either directly from plastid FA export or from the acyl-CoA pool derived from acyl exchange with PC or other glycerolipids.

Three different classes of DGAT have been identified in *A. thaliana*: DGAT1, DGAT2 and a soluble DGAT (Chapman and Ohlrogge, 2012; Hernandez et al., 2012; Routaboul et al., 1999; Saha et al., 2006; Shockey et al., 2006). Biochemical and genetic experiments have provided evidence that DGAT1 is relevant for TG biosynthesis in developing and senescent leaves of *A. thaliana* (Kaup et al., 2002; Routaboul et al., 1999; Slocombe et al., 2009; Zou et al., 1999). Mutations in the *DGAT1* gene caused a decrease in TG content in seed oil, confirming that DGAT1 is essential for TG biosynthesis in seeds (Katavic et al., 1995; Routaboul et al., 1999; Zou et al., 1999). The role of DGAT2 in *A. thaliana* remains unclear.
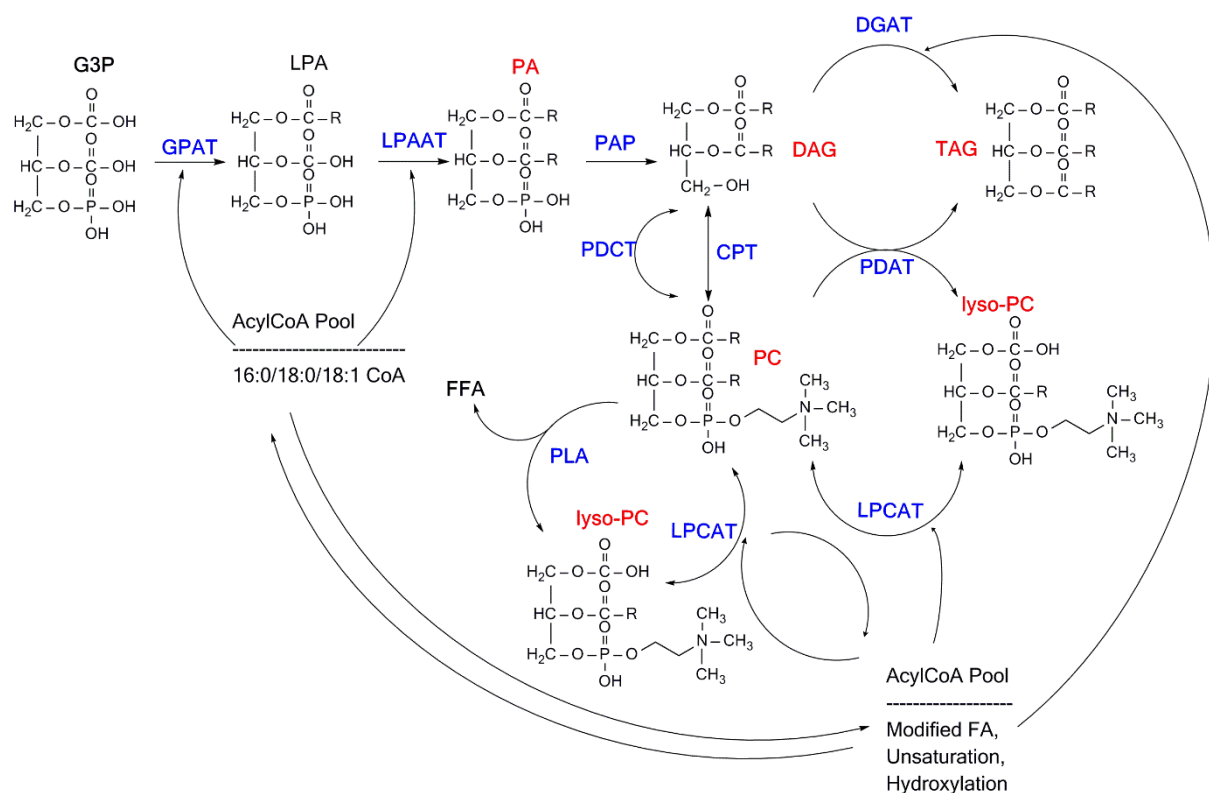
Figure 1. Modell of acyl-CoA dependent and acyl-CoA independent pathway for TG biosynthesis in plants adapted from (Chapman and Ohlrogge, 2012).
The Kennedy pathway involves a sequential acylation and dephosphorylation of glycerol 3-phosphate (G3P) through acyl-CoA-dependent acyltransferases (GPAT, LPAAT, and DGAT) resulting in PA that is then hydrolyzed by PAP to form DG. In addition, TG can be synthesized from DG and PC by PDAT in an acyl CoA independent way, yielding LPC, which can be re-acylated to form PC. Newly synthesized FA from the plastids enters an acyl-CoA pool that is incorporated into PC by acyl-editing reactions (LPCAT). While esterified to PC acyl groups are desaturated or modified before being released back to the acyl-CoA pool. PCs are used to generate DG via PDCT (or CPT).

1.2.2.2   Acyl-Co independent TG synthesis

In addition to the acyl-CoA dependent pathway, DG can also be synthesized in an acyl-CoA-independent pathway. Phospholipid-diacylglycerol acyltransferase (PDAT), identified in yeast and developing oil seeds, transfers an acyl-group from the *sn*-2 position of PC to the *sn*-3

hydroxyl of DG to produce TG and lysophosphatidylcholine (LPC) (Dahlqvist et al., 2000). LPC can then be re-acylated by acyl-CoA:lysophophatidylcholine acyltransferase (LPCAT) (Xu et al., 2012; Zhang et al., 2009).

The importance of PDAT and DGAT in TG synthesis seems to differ during the development of *A. thaliana*. *Pdat1* mutant plants were reported to display 57 % lower levels of TGs in developing leaves and 39 % in senescing leaves, whereas *dgat1* mutant plants displayed only a 31 % decrease in developing leaves but a 63 % decrease in senescing leaves. The data suggests that PDAT1 is of greater importance in developing leaves while DGAT1 has a greater role in senescing leaves (Fan et al., 2013b).

Interestingly, double mutants of *PDAT1* and *DGAT1* are lethal. RNAi suppression of each gene in a mutant background of the other gene leads to a reduced seed oil content and number of oil bodies. These results and the overlapping functions of *dgat1* and *pdat1* mutant plants in pollen and embryo development strongly suggest that DGAT1 and PDAT1 can compensate for each other (Zhang et al., 2009).

1.2.2.3   *De novo* fatty acid (FA) synthesis for TG synthesis

The FA building blocks for TG synthesis as well as for membrane synthesis are produced in the chloroplastic compartments of the plants. In the first step, malonyl-CoA is formed from acetyl-CoA and bicarbonate by acetyl-CoA carboxylase (ACC) (Konishi et al., 1996). The malonyl group from malonyl-CoA is then transferred to an acyl carrier protein (ACP), catalyzed by a malonyltransferase (MCMT), before entering the FA pathway (Li-Beisson et al., 2013). C18 and C16 acyl chains are assembled through an FA synthase (Brown et al., 2006), using acetyl-CoA as a starting unit and malonyl-ACP as a carbon donator at each step of elongation (Li-Beisson et al., 2013). Following desaturation of C18:0 to C18:1, FA are released from ACP in the plastid and are subsequently activated to CoA and exported to the ER. The remaining reactions of the TG synthesis occur in the eukaryotic pathway outside of the plastids (Browse and Somerville, 1991; Voelker and Kinney, 2001). FA can then be directly incorporated into DGs through the sequential acylation of glycerol-3-phosphate as mentioned above. Alternatively, it has been shown in *A. thaliana* seeds that 18:1-CoA can be elongated into 20:1-CoA and further into 22:1-CoA on the ER membrane by a FA elongase (FAE1) and can then be incorporated into TGs (Kunst and Samuels, 2003; Kunst et al., 1992).

In addition, newly synthesized FAs can enter PCs, where extra-plastidial desaturation takes place. 18:1 may then be desaturated to linoleic acid at the *sn*-2 position of PC by the oleate desaturase FAD2 and further to linolenic acid by the linoleate desaturase FAD3 (Arondel et al., 1992; Browse et al., 1993; Okuley et al., 1994; Sperling et al., 1993; Stymne and Appelqvist, 1978). Acyl groups from PC, enriched in polyunsaturated FAs (PUFAs), can then be channeled into DGs, the precursor of TGs, on different metabolic routes.

1.2.2.4   Acyl Flux through phosphatidylcholine (PC)

There are two broad metabolic routes of acyl-flux through PC which provide the fatty acyl chains for TG synthesis in the acyl-CoA independent pathway. The first route is acyl editing, which involves the exchange of fatty acyl chains of PCs, whereas the other route describes *de novo* synthesis of DG into PC and the subsequent net flux of PC-derived DG into TG.

Acyl editing

There are three different reactions that can occur in acyl editing. One reaction is known as the "Lands" cycle. It involves the hydrolysis of a FA from the *sn*-2 position of a PC by a phospholipase A$_2$, generating LPC and free FA, which is then activated to acyl-CoA. The re-acylation of LPC to PC is catalyzed by LPCAT. The Lands cycle is one mechanism for enriching the acyl-CoA pool with PC-modified FAs, including the hydrolysis of 18:2 and 18:3 from PC by PLA as well as the incorporation of 18:1 into PC through LPCAT for desaturation and the activation of free FA with CoA by a long chain acyl-CoA synthetase (Bates, 2016; Lands, 1958; Lands, 1960). A more efficient mechanism is the direct transfer of FA from PC onto CoA by the reverse LPCAT reaction, in contrast to the energy intensive re-acylation of acyl-CoA in the Lands cycle (Bar-Tana et al., 1973; Bates, 2016; Jasieniecka-Gazarkiewicz et al., 2016; Lager et al., 2013; Shockey et al., 2003; Stymne and Stobart, 1984).

The third mechanism for acyl editing is the direct transfer of an acyl group from the *sn*-2 position of PC to the *sn*-3 hydroxyl of DG by PDAT1, producing TG and LPC. The re-acylation of LPC by LPCAT completes the PDAT1 based acyl-editing cycle, channeling acyl groups from the acyl-CoA pool into PC, for modification, and further into TG (Dahlqvist et al., 2000; Xu et al., 2012; Zhang et al., 2009).

Acyl flux of DG into PC

The acyl flux of DG into PC can also lead to changes in the acyl compositions of TGs. The synthesis of PC from DG through CDP-choline:diacylglycerol cholinephosphotransferase (CPT)

is important for this flux (Goode and Dewey, 1999; Kennedy, 1961; Kennedy and Weiss, 1956; Li-Beisson et al., 2013; Liu et al., 2015). In addition, the CPT reaction seems to be reversible and can therefore lead to an equilibrium of the DG moiety between PC and DG pools (Slack et al., 1983; Slack et al., 1985). This reversible reaction may play a role in the exchange between PC and DG, but remains to be investigated. Two *A. thaliana* genes (*AtAAPT1* and *AtAAPT2*) are known to encode enzymes with CPT activity. While the double mutant is lethal, mutations in the single genes lead to a disturbance of lipid homeostasis (Liu et al., 2015).

The conversion of PC into DG can also be performed by a phospholipase C (PLC), generating DG by cleaving the phosphocholine headgroup from PC. Six non-specific PLC enzymes are known in *A. thaliana*, of which two indicate playing a role in phosphate or nutrient stress; however, no involvement in oil biosynthesis has thus far been detected (Chen, 2011; Gaude et al., 2008; Li-Beisson et al., 2013; Nakamura et al., 2005; Peters et al., 2010). PC turnover can also take place through the combined action of a phospholipase D (PLD) and PAP, in which choline and then phosphate is cleaved from PC to obtain DG. PLD suppression has been reported to lead to a reduction of PC-modified FAs in TGs (Lee et al., 2011).

The highest exchange of the DG moiety between PC and DG seems to be catalyzed by the phosphatidylcholine diacylglycerol cholinephosphotransferase (PDCT). The PDCT enzyme, encoded by reduced oleate desaturation1 (*ROD1*), transfers a phosphocholine headgroup from PC to DG, creating a new molecular species of PC and DG. The mutation reduces the 18:3 and 18:2 accumulation in seed TG by 40 % (Lu et al., 2009). Flux analysis proved that the majority of DG precursors for TG synthesis are not derived from *de novo* synthesis but from PC (Bates and Browse, 2011). Moreover, recent studies could show that the major fluxes between unsaturated PC to TG in *A. thaliana* seeds are controlled through LPCAT-mediated acyl-editing with PDCT-based PC-*sn*-1,2-DG interconversion (Bates et al., 2012).

1.2.2.5 TG synthesis in chloroplasts

In addition to the enzymes DGAT1 and PDAT1, which synthesize the last step of TG synthesis localized at the ER, additional enzymes which can synthesize TG could be detected in the chloroplasts. Chloroplastic phytyl ester synthase 1 and 2 (PES1, PES2), belonging to the esterase/lipase/thioesterase family of acyltransferases from *Arabidopsis thaliana,* are normally involved in FA phytyl ester synthesis in chloroplasts. However, heterologous expression of *PES1* and *PES2* in yeast revealed that PES1 and PES2 have phytyl ester synthesis

as well as diacylglycerol acyltransferase activities. The enzymes display wide substrate specificities and can use acyl-CoAs, acyl carrier proteins and galactolipids as acyl donors. The highest substrate specificity was identified towards acyl-CoAs. It seems that PES1 and PES2 are involved in the removal of free phytol and free fatty acids (FFAs) in the form of phytyl esters in the chloroplast during abiotic stress and are thereby involved in the maintenance of the photosynthetic membrane (Lippold et al., 2012). However, it is unclear what role PES1 and PES2 play in TG biosynthesis, in contrast to DGAT1 and PDAT1.

1.2.2.6   TG synthesis in the *A. thaliana* transgenic *tgd1.1* line

Some transgenic lines of *A. thaliana* show changes in TG synthesis and understanding of these processes could advance current knowledge of fluxes of FA into TGs. For example, not only fluxes of FAs from the chloroplasts into the cytosol and FA fluxes in the cytosol seem to be important for TG formation, also a disturbance in lipid transfer from the ER to the chloroplast can lead to changes in TG levels (Xu et al., 2005; Xu et al., 2003). The Arabidopsis *trigalactosyldiacylglycerol1* mutant (*tgd1.1*) displays a disruption in the transport of lipid precursors derived from the ER into the chloroplasts. The mutant has a point mutation in a gene which encodes a permease-like component of an ABC transport complex (Xu et al., 2003). The *tgd1* mutation leads to a decline of thylakoid lipid levels synthesized by the eukaryotic pathway and a compensatory increase in galactolipids produced by the prokaryotic pathway. Interestingly, the *tgd1.1* mutants show an accumulation of TG in oil droplet structures in the cytosol of leaf cells (Xu et al., 2005).

As the model in Figure 2 shows, high levels of PC are normally transported into the chloroplast for thylakoid glycolipid synthesis. This eukaryotic pathway of glycolipid synthesis is disturbed in the tgd1.1 mutant, which leads to an increase in FA synthesis in the chloroplasts and FA peroxisomal β-oxidation through PC and TG (Fan et al., 2014).
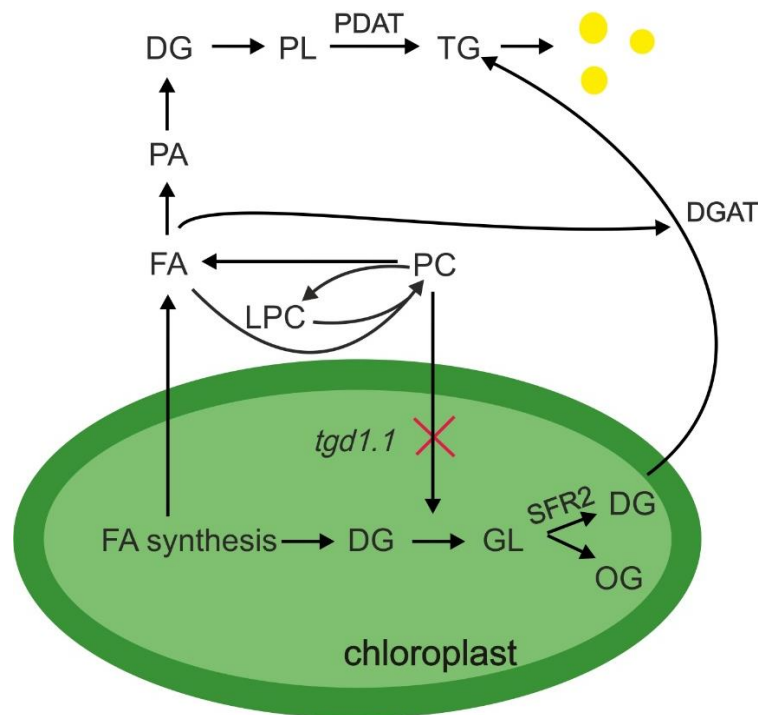
Figure 2. Model for the proposed pathways of FA oxidation in leaves of wild type and *tgd1.1* mutant, adapted from (Fan et al., 2014).

Model proposes that PDAT is crucial for diverting FA from membrane lipid synthesis to TG storage. FA are exported from the plastid and are incorporated into PCs through an acyl editing cycle. After acyl groups are released from PC acyl editing cycle, FA are used to produce PA. PA is synthesized further to DG, used for *de novo* synthesis of extra-plastidic phospholipids, including PC. A major amount of PC derivates are returned to the plastids for the eukaryotic pathway of thylakoid glycolipid synthesis.

In the tgd1.1 mutant, eukaryotic glycolipid pathway is defect, through which FA synthesis but also FA peroxisomal β-oxidation through PC and TG is enhanced. Acyl chains in *tgd1.1* mutants are also channeled into peroxisomal β-oxidation through SFR2-mediated DG.

1.2.2.7   TG synthesis in the *A. thaliana* transgenic *sfr2* line

SFR2 is an enzyme which is able to lead to changes in TG levels after being activated. As shown in Figure 2, SFR2 can synthesize DGs and oligogalactolipids out of galactolipids in the chloroplast. SFR2 was identified in a screen for freezing tolerance in *A. thaliana* and was reported to act as galactolipid:galactolipid galactosyltransferase (GGGT) involved in lipid remodeling (Moellering et al., 2010; Thorlby et al., 2004). As shown in Figure 3, SFR2 transfers a galactose head group from a MGDG to a second MGDG, leading to the formation of DGDG and DG, which might then be further synthesized to TG. Moreover, the galactose head group of MGDG can be transferred to an oligogalactolipid, such as di- or trigalactosyldiacylglycerol (DGDG, TGDG), possessing two or three galactosyl groups and can thereby increase the number of galactosyl moieties in a processive manner. Freezing stress can lead to organelle

shrinkage due to apoplastic ice formation and cellular dehydration. It has been hypothesized that SFR2 activation during freezing leads to the compensation of organelle volume and a stabilization of the chloroplast membrane by replacing non-bilayer-forming lipids (MGDG) with bilayer-forming lipids (DGDG, oligogalactosyl-DG) (Moellering et al., 2010).

In addition, it has been reported that only freezing, but not cold acclimation, causes plastid membrane leakage resulting in a cytosolic acidification and, potentially, Mg^{2+} release. This result suggests that the cytosolic acidification and the Mg^{2+} release lead to the activation of SFR2. Interestingly, *A. thaliana* plants deficient in SFR2 are still able to accumulate TGs, leading to the conclusion that another enzyme seems to be activated that channels MGDGs to TGs following freezing stress. However, an increase in 16:3 FAs in TGs could not be detected in *sfr2* plants in contrast to the wild type, making this change SFR2-dependent (Barnes et al., 2016).

SFR2 activation is not restricted to freezing stress. In tomato plants, in contrast to *A. thaliana*, SFR2 can confer salt-/drought stress resistance but cannot rescue the plants from freezing stress. It seems that *A. thaliana* plants are more sensitive to salt-/drought-stress than tomato plants; the stress is lethal for *A. thaliana* before the SFR2-based lipid remodeling is activated. Tomato plants can activate SFR2 during salt-/drought-stress before irreversible damage occurs, whereas freezing seems to be lethal before SFR2 can be activated. Results suggest that SRF2 constitutes a first line of defense against cellular dehydration following different abiotic stresses by a common molecular membrane lipid remodeling (Wang et al., 2016).
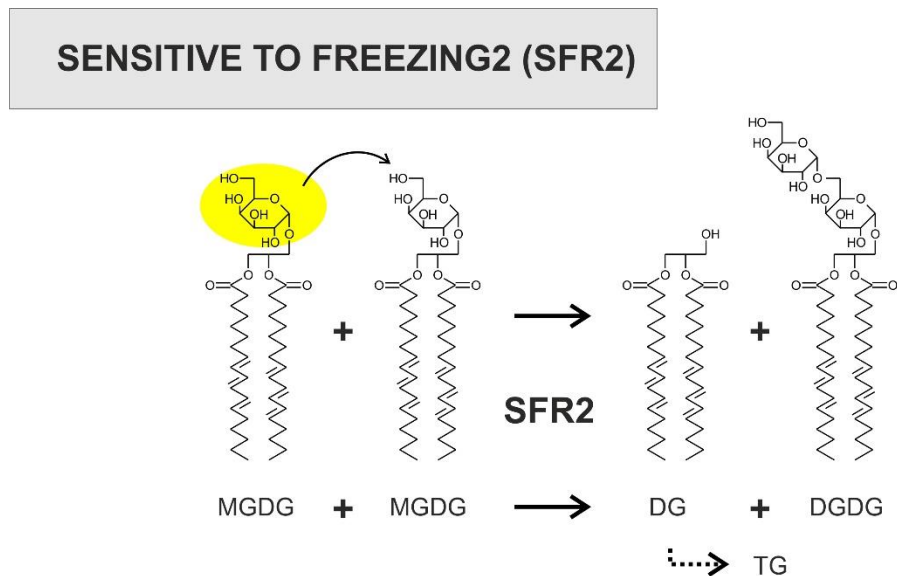
Figure 3. Modell for reaction of Sensitive to Freezing 2 (SFR2).
SFR2 transfers a galactose head group from a MGDGs to a second MGDG, forming DGDG and DG. The galactose head group of MGDG can be also transferred to an oligogalactolipid, i.e. di- or triglalctosyldiacylglycerol (DGDG, TGDG), possessing two or three galactosyl groups, and thereby increasing the number of galactosyl moieties in a processive manner.

1.3   Aims

In my preliminary work, untargeted metabolite analysis of *A. thaliana* seedlings revealed an 8- to 10-fold accumulation of polyunsaturated TGs following a heat acclimation phase (37° C, 2 h) in *A. thaliana*. The following work shall identify regulatory mechanisms and biosynthetic pathways involved in the heat-induced TG synthesis.

First, I characterized TG accumulation compared to the heat response of the sugar markers. The correlation between time and temperature and the response following different abiotic heat treatments were investigated. Furthermore, it was tested if heat-induced TG synthesis, like raffinose synthesis, is regulated through the genetically programmed HSR and if the canonical master regulators, namely the HSFA1 transcription factors, are involved in this process.

The localization of the TG accumulation is important for the analysis of TG biosynthesis following heat treatment. A number of approaches were used to find out if TG accumulation following heat shock is located in the cytosol or in the plastids. Chloroplasts were isolated and the TG content was compared to the whole seedling. Moreover, TG content of heat-treated

roots and shoots was analyzed separately, with roots containing proplastids but no functional chloroplasts. In addition, visualization was carried out using a Nile Red stain of the lipid droplets and fluorescence microscopy.

A lipidomic approach to study TG synthesis in *A. thaliana* was used to clarify the mechanisms with which TGs are produced under heat stress conditions. Total FA levels were measured to test if TG synthesis derives from *de novo* synthesis. Potential lipid remodeling was investigated by analyzing the total membrane lipid classes as well as the membrane lipid species. Another aim was to identify mutants deficient in heat-induced TG synthesis. Using these mutants, the biological significance of TG synthesis with respect to basal and acquired thermotolerance was clarified.

Lipidomic analysis of transgenic Arabidopsis lines deficient in different key genes involved in TG synthesis was performed in order to identify putative genes important for heat-induced TG synthesis.

Following the identification of mutants specifically deficient in heat-induced TG synthesis, these mutants were compared to wild type plants using different functional assays to evaluate the fitness of the plants under heat stress to test the physiological function of the TG response for basal and acquired thermotolerance.

2   Results

2.1   Analysis of TG species after heat treatment

A significant increase in TG54:9, 54:8, 54:7 and 54:6 could be identified by performing an untargeted analysis with 14-day-old *A. thaliana* seedlings following 37° C treatment for 2 h in my master thesis. The aim of this work was to further investigate TG response and its functional significance.

To characterize the TG response, it was verified whether other TG species, in addition to the identified TG markers (TG54:9, 54:8, 54:7, 54:6), accumulate due to heat treatment (37° C, 2 h). Therefore endogenous TG species were identified and quantified with the help of an in-house developed database (unpublished). Our database consists of 160 TG species with 36 to 66 carbons and 0 to 3 double bonds. The mass to charge ratio (m/z) of the ammonium adduct of the parent ion and the m/z of the ammonium adduct of a fragment ion of each TG species has been identified. It was confirmed that the peaks of the parent and fragment ions were eluting at the same retention time (RT).

In total 48 TG species could be identified on the basis of RT-aligned molecule and fragment spectra (Table 7). TGs contain three FA esters linked to a single glycerol. The identified TG species were plotted according to their number of carbons (rows) and double bonds (columns) (Figure 4):

C/DB	0	1	2	3	4	5	6	7	8	9
46	nd	nd	nd	0.4	nd	nd	nd	nd	nd	nd
48	nd	1.1	1.0	1.1	nd	nd	nd	nd	nd	nd
50	nd	1.0	1.2	1.1	2.1	2.5	3.1	nd	nd	nd
52	nd	1.0	1.0	1.2	2.8	4.0	3.5	4.1	8.8	15.8
54	nd	1.1	1.0	1.0	1.3	2.4	5.5	6.1	6.4	7.6
56	nd	nd	1.1	1.0	1.1	1.1	1.1	1.7	3.1	nd
58	nd	nd	1.2	1.0	1.1	1.2	1.2	1.1	nd	nd
60	nd	nd	1.0	1.1	1.2	1.3	1.4	1.2	nd	nd
62	nd	nd	nd	nd	1.5	nd	nd	nd	nd	nd

Figure 4. Fold increase of identified TGs after heat acclimation (Mueller et al., 2015).
TGs were plotted according to the number of carbons (rows) and double bonds (columns) of the FA esterified to glycerol. Fold changes after heat acclimation (37° C, 2 h) are shown. C= number of carbon atoms in the fatty acyls. DB=number of double bonds in the fatty acyls. nd = not detected. Data represents means ± SD, n= 9.

Investigating all 48 TG species, 17 demonstrated an increase of at least 1.5-fold to a maximum of 16-fold. TG 52:9 and 52:8, with very low basal levels, displayed the highest increases (15.8- and 8.8-fold changes). Amongst the most abundant TG species, which had levels higher than 2.5 mol %, TG 54:9, 54:8, 54:7, 54:6, showed the highest accumulation (5.5- to 7.6-fold changes). No induction could be detected in TGs with higher saturation of fatty acyl chains (lower than 4 double bonds). TG species with carbon numbers from 50 to 56 showed an induction, whereas TG species with both higher and lower carbon numbers did not accumulate.

2.2 Characterization of TG and raffinose accumulation

For further characterization of heat-induced TG response, the time and temperature dependency was investigated. Moreover, experiments, addressing the TG response after different abiotic stress treatments, were carried out. In addition, the dependency of TG accumulation on HSFs was analyzed.

Raffinose and galactinol, termed sugar markers, were also identified as heat-responsive markers during my master thesis. Much is already known about their heat-dependent increases, time and temperature dependency as well as a possible regulation by heat shock transcription factors. Therefore, sugar markers were analyzed alongside with TG markers.

2.2.1 Time and temperature dependency

To investigate time and temperature dependency, sugar and TG markers were quantified in 14-day-old *A. thaliana* seedlings following 37° C treatment at different exposure times as well as following different temperature profiles for 2 h (Figure 5).
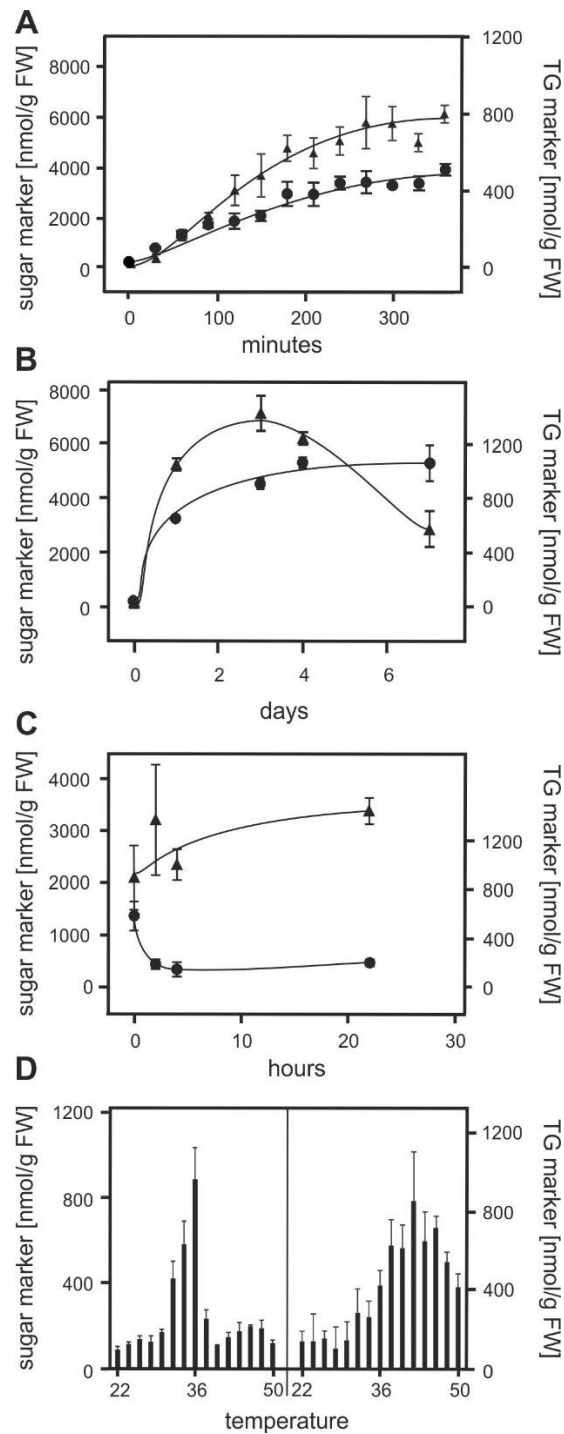
Figure 5. Time- and temperature-dependent responses of sugar (▲) and TG (●) markers in Arabidopsis seedlings (Mueller et al., 2015).

14-day-old seedlings exposed to 37° C for the times indicated were analyzed (A and B). Heat-acclimated seedlings (37° C, 2 h) were transferred back to the growth condition (22° C) for the times indicated (C). Sugar and TG markers were determined in seedlings treated at different temperatures (22° C to 50° C) for 2 h (D). Data represent means ± SD, n = 4.

In order to analyze the time dependency of sugar and TG markers, a kinetic was carried out for 6 h, with samples being harvested in 30 min increments (Figure 5A). Already 30 min after the onset of the heat treatment, a 6-fold increase in sugar and TG markers could be observed. Sugar and TG markers increased steadily for six hours to 6 µmol/g and 0.5 µmol/g FW, respectively. In addition, markers were quantified during long-term exposure at 37° C for 7 days (Figure 5B). TG markers remained high at 0.5 µmol/g FW for the whole experiment, whereas the sugar markers reached a maximum after 3 days (7 µmol/g FW) and decreased afterwards. For investigation of sugar and TG markers after heat acclimation (37° C, 2 h), levels were measured following the return of the seedlings to 22° C (Figure 5C). TG marker levels dropped back to basal levels after 2 h, whereas sugar markers stayed on the same high level for at least 24 h. For analyzing temperature dependency of the markers, seedlings, kept in liquid media, were treated at temperatures ranging from 22° C to 50° C for 2 h, in 2° C increments (Figure 5D). Both sugar and TG markers still accumulated above 30 ℃. Sugar markers displayed the highest accumulation at 36° C (0.8 µmol/g FW), TG markers at 42° C (0.85 µmol/g FW). TG markers still accumulated at 50° C, levels of sugar markers, on the contrary did not differ from the control when seedlings were treated above 38° C.

Summing up, a rapid increase was determined in TG and sugar markers following heat stress. TG levels, however, stayed high under long-term exposure, and decreased back to basal levels after heat treatment in contrast to sugar markers. TG markers increased at high temperatures, whereas sugar markers did not.

2.2.2   TG response after different abiotic stress treatments

Raffinose accumulation was not only observed after moderate heat treatment, but also after various abiotic stresses, like heat, cold, drought, salt, and oxidative stress (Nishizawa et al., 2008; Panikulangara et al., 2004; Taji et al., 2002). To determine whether TG accumulation is restricted to heat stress or is a general stress response, TG marker levels were quantified following various stress treatments (Figure 6).
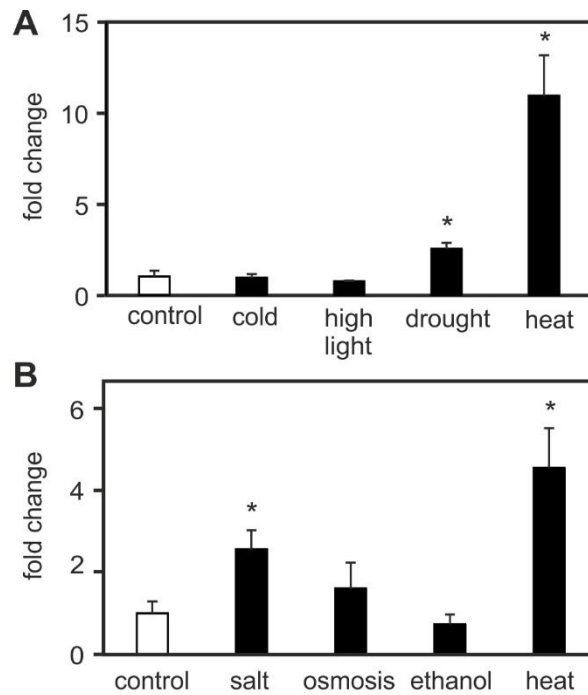
Figure 6. Accumulation of TG markers after different abiotic stresses (Mueller et al., 2015).
14-day-old seedlings grown on agar plates were exposed to cold (4° C, 2 h), high light (900 µE/m²s, 2 h), drought (seedlings placed on filter paper), or heat (37° C, 2 h) stress (A). Seedlings grown in liquid Murashige & Skoog medium were treated with salt (125 mM NaCl, 2 h), mannitol (250 mM, 2 h), ethanol (1 %, 2 h), or heat (37° C, 2 h) stress (B). Data represent means ± SD, n = 4.

In detail, 14-day-old seedlings, grown on agar plates, were treated with cold (4° C), elevated light intensity (900 µE/m²s) and drought (seedlings were placed on filter paper) for 2 h each (Figure 6A). In another set of experiments, seedlings were transferred to liquid Murashige & Skoog media and exposed to salt (125 mM sodium chloride), osmotic (250 mM mannitol), and 1 % ethanol stress for 2 h (Figure 6B). In both series of experiments, heat led to the highest increase in TG markers under the given test conditions. In addition, a significant increase can be determined after drought and salt stress (2.6- and 2.5-fold). TGs do not increase significantly following cold, high light, mannitol and ethanol treatment.

Interestingly, heat-responsive TG accumulation was dependent on the growth conditions, as *A. thaliana* seedlings, which were kept on agar plate demonstrated an 11-fold increase, seedlings in liquid media only a 4.5-fold change at 37° C for 2 h.

2.2.3   Dependency on heat shock transcription factors

In order to investigate whether HSFs induce sugar and TG markers, *Arabidopsis* mutant lines deficient in HSFA1 or HSFA2 were treated at 37° C for 2 h and compared to the wild type.

Additionally, *golS1* Arabidopsis mutants were analyzed, to investigate whether TG markers are dependent on raffinose biosynthesis. *GolS1* transgenic plants display a deficiency in heat-induced raffinose synthesis (Panikulangara et al., 2004)
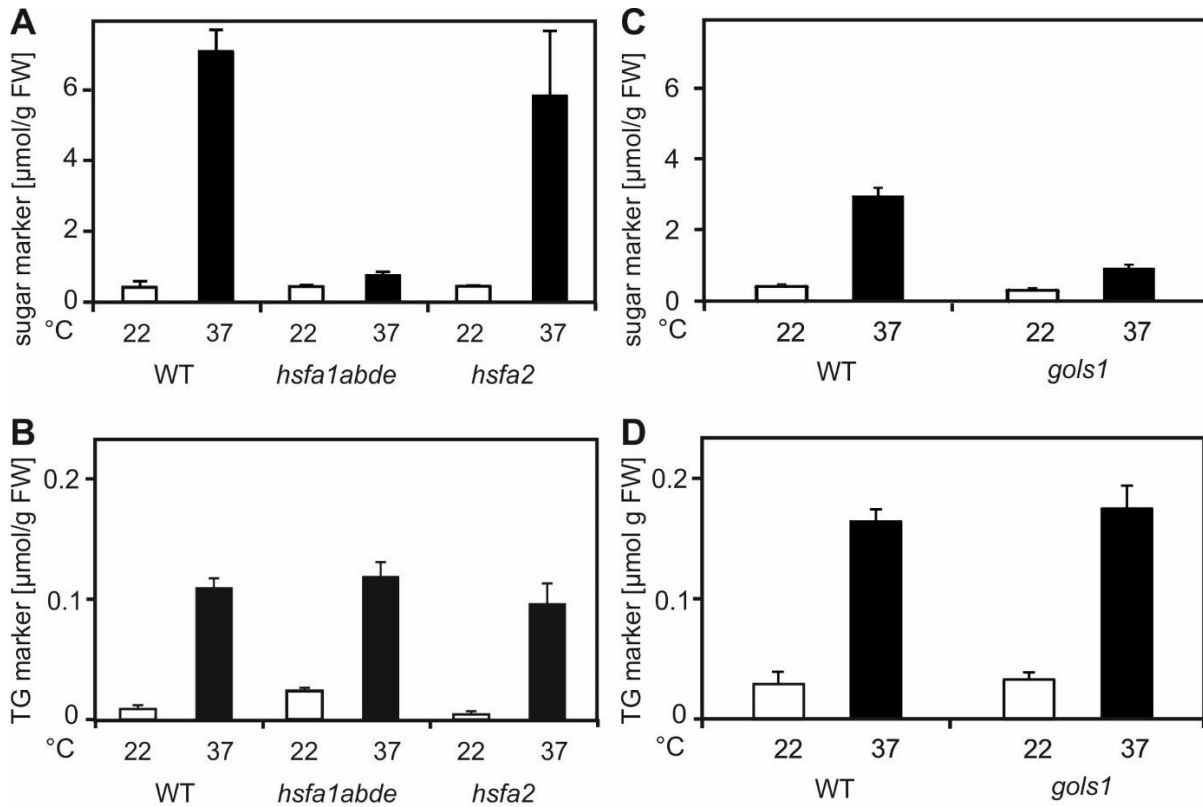

Figure 7. Accumulation of TG markers in *hsfa1abde*, *hsfa2*, and *golS1* mutant Arabidopsis seedlings after heat acclimation (Mueller et al., 2015).
Levels of sugar and TG markers were determined in 14-day-old *hsfa1abde*, *hsfa2*, and *golS1* seedlings kept at 22° C (white bar) and 37° C for 2 h (black bar). Data represent means ± SD, n = 4.

Analyzing 14-day-old *hsfa1abde* quadruple *Arabidopsis* mutants, raffinose and galactinol did not accumulate following heat acclimation (37° C, 2 h). Wild type seedlings showed an increase to 700 nmol/g FW (Figure 7A). Interestingly, *hsfa2* mutants, did rise to wild type like levels following heat acclimation. TG markers accumulated to wild type levels in *hsfa1abde* quadruple and *hsfa2* mutants (Figure 7B). *GolS1* mutants revealed no accumulation of the sugar markers following 37° C (Figure 7C), whereas the TG marker accumulation was similar to the wild type (Figure 7D). Comprising, TG markers display an accumulation independent on HSFs and raffinose biosynthesis.

2.3 Localization of TG accumulation

2.3.1 Chloroplast isolation

TG accumulation can take place either in the cytosol or in the chloroplast. To identify the location of TG accumulation following heat acclimation, chloroplasts were isolated from 14-day-old *A. thaliana* seedlings. TG markers were quantified in the chloroplasts compared to whole seedlings. Beside TG markers, phosphatidylethanolamines (PEs) and MGDGs were quantified. PEs occur exclusively in the extra-chloroplastic membranes, MGDG in the plastid membranes. With analysis of these compartment specific lipids, a proper chloroplast isolation could be assured.
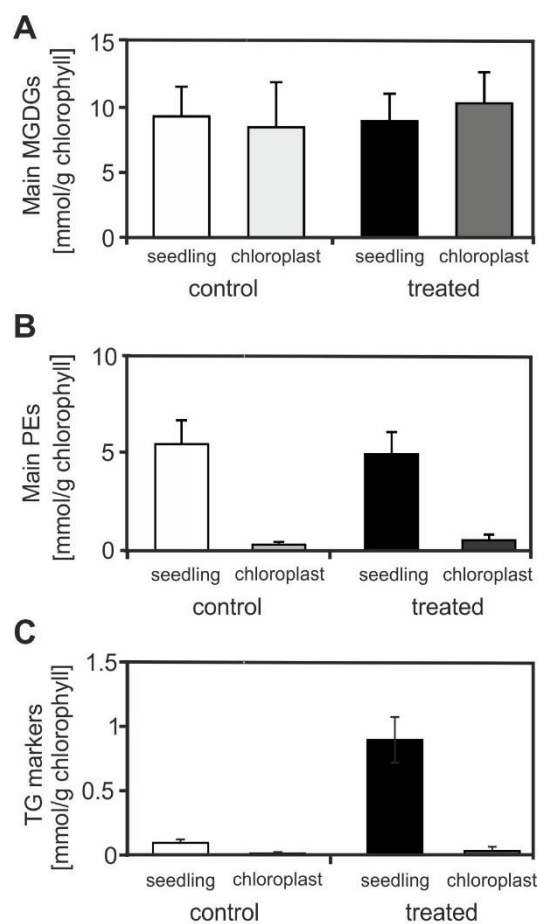
Figure 8. TG markers accumulated predominantly in extra-chloroplastic compartments after heat acclimation (Mueller et al., 2015).
Chloroplasts of control (white columns) and heat-acclimated (37° C, 2 h; black columns) seedlings (14-day-old) were isolated and most abundant MGDGs (A), PEs (B), and TG markers (C) were determined relative to chlorophyll levels. The determined MGDGs and PEs were MGDG 36:6, MGDG 34:4, PE 36:5, PE 36:4, PE 34:3, and PE 34:2. Data represent means ± SD, n = 3.

MGDG 18:3-18:3 and 18:3-16:3, are the most abundant chloroplast-specific lipids. As expected, the two most abundant MGDG species (summed up), had similar levels in the isolated chloroplasts and the whole seedling (Figure 8A). Isolation of chloroplasts from seedlings was efficient. To confirm the purity of the chloroplast isolate, the most abundant PEs (PE36:5, PE36:4, PE34:3, PE34:2) were quantified (Figure 8B). Nearly no PEs could be detected in the chloroplast fraction, the contamination was beyond 5 %.

Analyzing TG markers, a 9-fold increase to 9 mmol/g chlorophyll could be detected in the seedlings after 37° C for 2 h. In contrast, a 3.5-fold rise to 3.2 mmol/g chlorophyll was determined in the chloroplast after heat treatment. Taking into account that only a contamination below 5 % of the extra-chloroplastic fraction could be detected in the chloroplastic fraction, it can be concluded that the highest amount of TG marker accumulation is localized in the cytosol and not in the chlorplastic compartments.

To confirm this results, TG markers in shoots and roots were quantified, separately.

2.3.2   Analysis of roots and shoots

To provide further evidence that TGs accumulate in extra-chloroplastic compartments, TG markers were quantified in shoots and roots, separately, as roots contain only low levels of plastids (Figure 9).
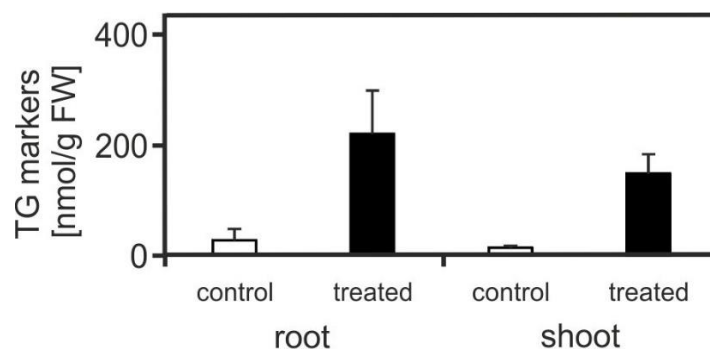
Figure 9. Levels of TG markers in roots and shoots (Mueller et al., 2015). Roots and shoots of control (22° C, white columns) and heat-acclimated (37° C, 2 h, black columns) seedlings (14-day-old) were harvested; shoots were separated from roots and TG markers were analyzed. Data represent means ± SD, n = 4.

After treating 14-days-old *A. thaliana* seedlings with 37° C for 2 h, roots and shoots were harvested separately and TG markers were analyzed (Figure 9). In line with the results of the chloroplast isolation, a similar increase (8- to 10-fold) was determined in roots and shoots.

The absolute levels with 220 and 150 nmol/g FW in roots and shoots after heat treatment, were comparable.

2.3.3   Visualization of heat responsive TG accumulation

To confirm the chloroplastic localization of TGs, fluorescence microscopy with heat treated *A. thaliana* cell culture was carried out. Cell cultures, while shaking, were treated with 37 ° C and 45° C as well as kept under control conditions (22° C) for 2 h. After treatment, cell culture was stained with Nile Red and samples were analyzed using a Zeiss Elyra S.1 SIM Super Resolution Microscope (Figure 10). Nile Red was used to localize and quantitate lipids, in particular neutral lipid droplets, in which TGs are stored. In addition, chlorophyll fluorescence was monitored.
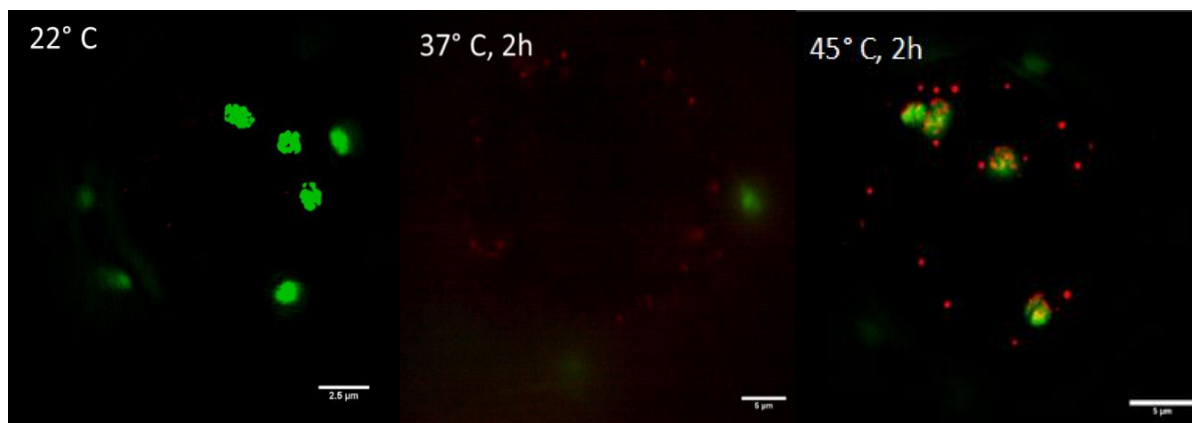

Figure 10. Localization of lipid droplets in *A. thaliana* cell culture.
Representative fluorescence microscope images of 22° C, 37° C and 45° C treated *A. thaliana* cell culture. Chloroplast auto fluorescence is green and lipid droplets stained with Nile Red are red. Bar = 2.5 µm, 5 µm and 5 µm.

At 22° C only auto fluorescence of chloroplasts could be detected (green). At 37° C and more clearly at 45° C treatment, *A. thaliana* cells displayed many small lipid droplets (red). By overlapping the fluorescence of lipid droplets and the auto fluorescence of the chloroplasts, lipid droplets could be localized in the cytosol and not in the chloroplastic compartments. Therefore, cytosolic localization of TG accumulation, determined in chloroplast isolation and root/shoot analysis of *A. thaliana* seedlings can be confirmed by fluorescence microscopy of *A. thaliana* cell culture.

2.4   Origin of TG accumulation after heat stress

An important question, which has yet to be fully understood is, where TG accumulation derives from, especially where FAs for TG synthesis origin from following heat treatment. TG accumulation might be due to an increased TG biosynthesis or TG break-down. Changes of TG biosynthesis following heat treatment, can on the one hand be due to higher *de novo* FA synthesis and integration into TGs via the Kennedy pathway. On the other hand, lipid remodeling, meaning a channeling from structural membrane lipids to TGs, might occur. To investigate these hypothesis FA acid levels and changes in complex lipids in wild type seedlings and transgenic lines deficient in TG accumulation were analyzed following heat treatment.

2.4.1   Analysis of transgenic lines of TG biosynthesis

To understand biosynthesis of TG accumulation during heat stress, transgenic Arabidopsis plants, which are deficient in TG accumulation, were analyzed. DGAT1 and/or PDAT1, both localized in the cytosol, are potentially involved in the acylation of DGs, the last step of TG synthesis. To reveal which biosynthetic pathway is responsible for TG accumulation, TG levels of the Arabidopsis *dgat1.1* (AS11), *pdat1a* and *pdat1b* mutants following heat treatment (45° C, 90 min) were quantified. *Dgat1.1* (Zou et al., 1999) has an ethylmethanesulfonate–induced lesion in the DGAT1 locus, *pdat1a* (SALK_065334) and *pdat1b* (SALK_032261C) have T-DNA insertions in the PDAT1 gene.

In addition, a transgenic line with higher basal levels of TGs compared to the wild type was analyzed. Therefore, Arabidopsis *tgd1.1* seedlings, displaying a point mutation in a gene, encoding a permease-like component of an ABC transporter complex and which is therefore defective in the transport of ER derived lipid precursors into plastids, were investigated (Xu et al., 2003). This mutation leads to a decrease in thylakoid lipid levels produced by the eukaryotic pathway, and a compensatory increase in galactolipids of the prokaryotic pathway as well as an additional accumulation of TGs in the cytosol of leaves (Xu et al., 2005).
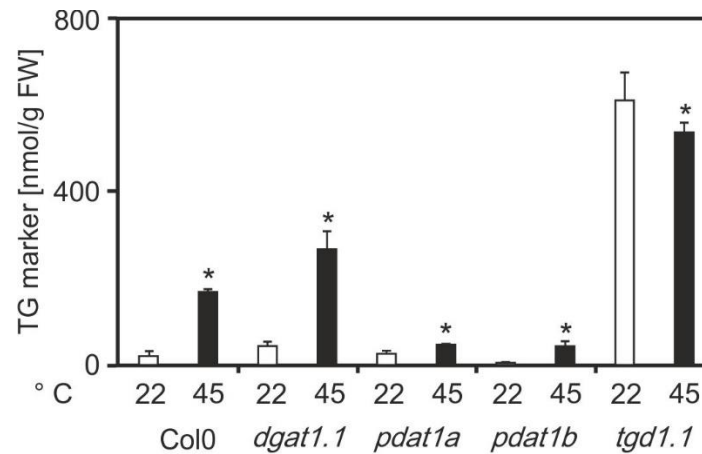
Figure 11. Response of TG markers in *dgat1.1, pdat1a, pdat1b* and *tgd1.1* transgenic *A. thaliana* seedlings after heat treatment.
Levels of TG markers were determined in 14-day-old *dgat1.1, pdat1a, pdat1b* and *tgd1.1* seedlings kept at 22° C (white bar) and 45° C for 90 min (black bar). Data represent means ± SD, n = 4.

14-day-old *dgat1.1, pdat1a, pdat1b* and *tgd1.1* transgenic Arabidopsis seedlings were treated at 45° C for 90 min (Figure 11). The high temperature was chosen to obtain maximal levels of TGs following the treatment. Basal levels of TGs were comparable in wild type, *dgat1* and *pdat1* lines. Seedlings, deficient in a permease-like protein trigalactosyldiacylgycerol (TGD1) displayed 30-fold higher TG levels compared to the wild type.

After the heat treatment TG markers of *dgat1* seedlings accumulated to wild type levels. In contrast, nearly no increase (1.8-fold) could be detected in *pdat1* lines, reaching 43–46 nmol/g FW, while wild type seedlings displayed 170 ng/g FW after the heat stress. This results suggest that PDAT1, but not DGAT1 is involved in the synthesis of TGs during heat stress.

Tgd1.1 mutant seedlings showed no further accumulation of TGs after heat stress, the levels were 3-fold higher compared to the wild type following heat treatment.

2.4.2   Hydrolysis of lipids after heat treatment

To determine whether TGs derive from *de novo* FA synthesis, a hydrolysis of glycerolipids was carried out to compare if FA levels in heat treated seedlings were higher than in non-treated (Figure 12).
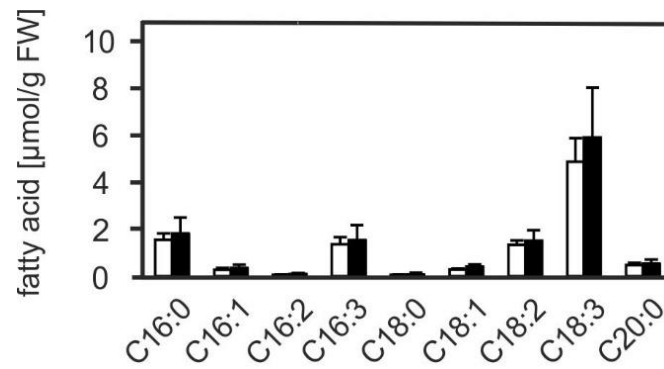
Figure 12. Levels of total FAs after heat acclimation (Mueller et al., 2015).
Total FAs from control (22° C; white columns) and heat-acclimated (37° C, 2 h; black columns) seedlings (14-day-old). Data represent means ± SD, n = 4.

FAs were quantified in 14-day-old *A. thaliana* seedlings following 37° C treatment for 2 h and FA levels were compared to control seedlings (22° C) (Figure 12). Levels of total FA, meaning esterified FA and FFA, demonstrated no significant differences due to heat acclimation. Therefore, TG accumulation does not seem to derive from massive *de novo* FA synthesis.

2.4.3 Analysis of lipidome after heat stress

Furthermore, the question was addressed, whether FAs for TG synthesis after heat treatment might originate from the degradation of lipids.

2.4.3.1 Analysis of lipid classes

For analyzing whether lipids are degraded during heat treatment, total levels of membrane lipid classes were quantified. After 37° C treatment for 2 h, total levels of MGDG, DGDG, PE and PC as well as TG and its direct precursor DG were determined (Figure 13).
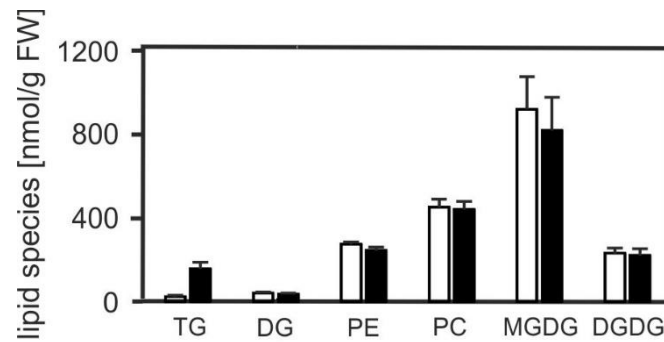
Figure 13. Levels of neutral lipids and membrane lipids after heat acclimation (Mueller et al., 2015).
TG markers and complex lipids (B) from control (22° C; white columns) and heat-acclimated (37° C, 2 h; black columns) seedlings (14-day-old). Data represent means ± SD, n = 9.

As expected TG levels increased (6.5-fold) after 37° C treatment. A trend towards degradation, which was not significant, was observed in MGDGs. The other lipid classes showed no changes after heat treatment. Therefore, no dramatic disturbance of membrane lipid homeostasis did occur in the major lipid classes. However, FA of TGs add up to only 1-8 % compared to the FA pool of structural lipids and therefore slight changes of lipid pools might have escaped detection.

2.4.3.2 Analysis of species of lipid classes

As no changes in lipid classes could be observed, all lipid species of different lipid classes have been analyzed. Compositions of lipid classes of 14-day-old Col0 *A. thaliana* seedlings were determined at 22° C and following heat treatment (45° C, 90 min) in comparison to the wild type. In addition, *pdat1a* line was analyzed to investigate lipidomic changes in seedlings with no TG accumulation following heat stress. The species of TGs, DGs, cytosolic lipids PCs, PEs and PAs as well as chloroplastic lipids MGDG and DGDG were analyzed (Figure 14) according to m/z and RT in Table 7.
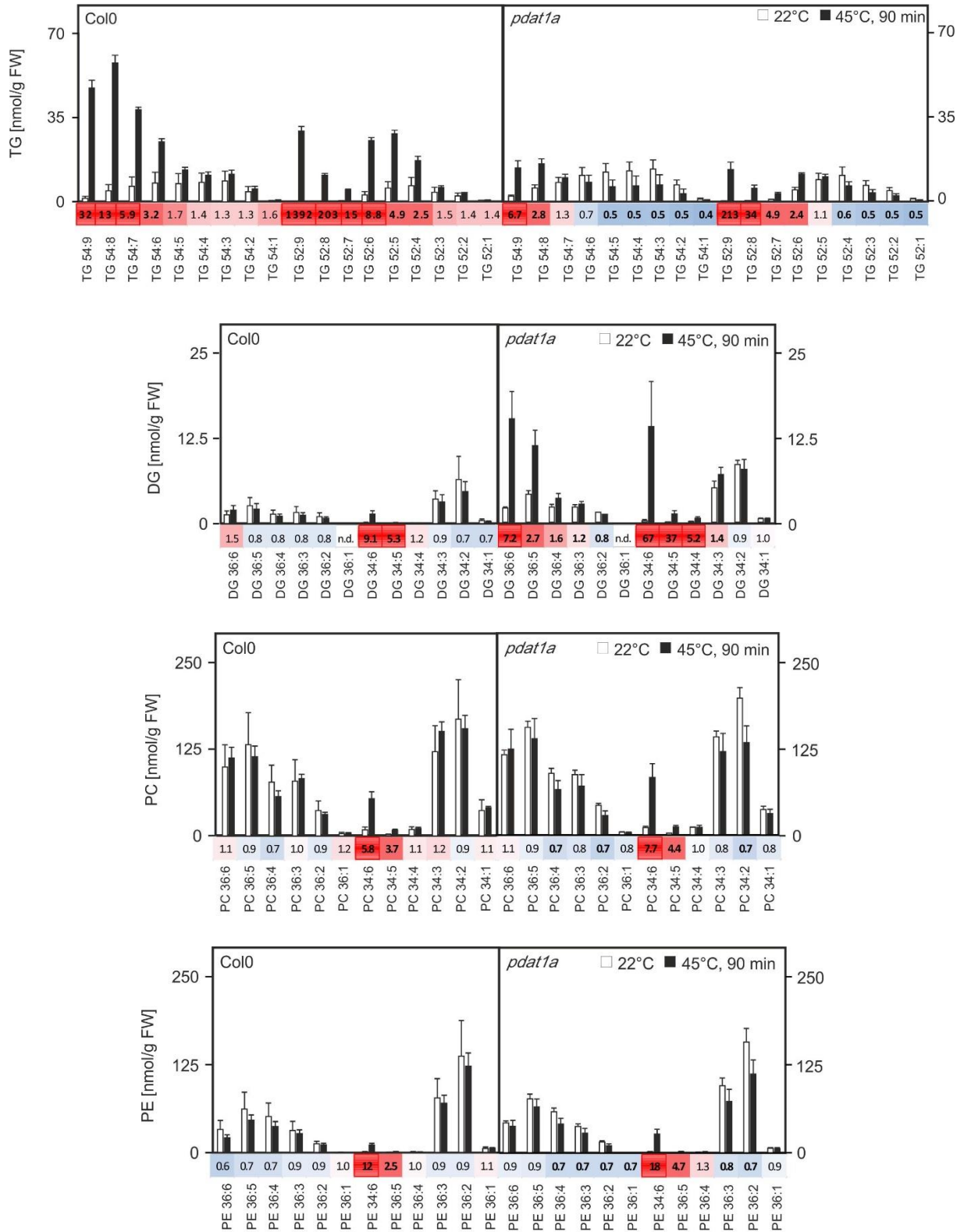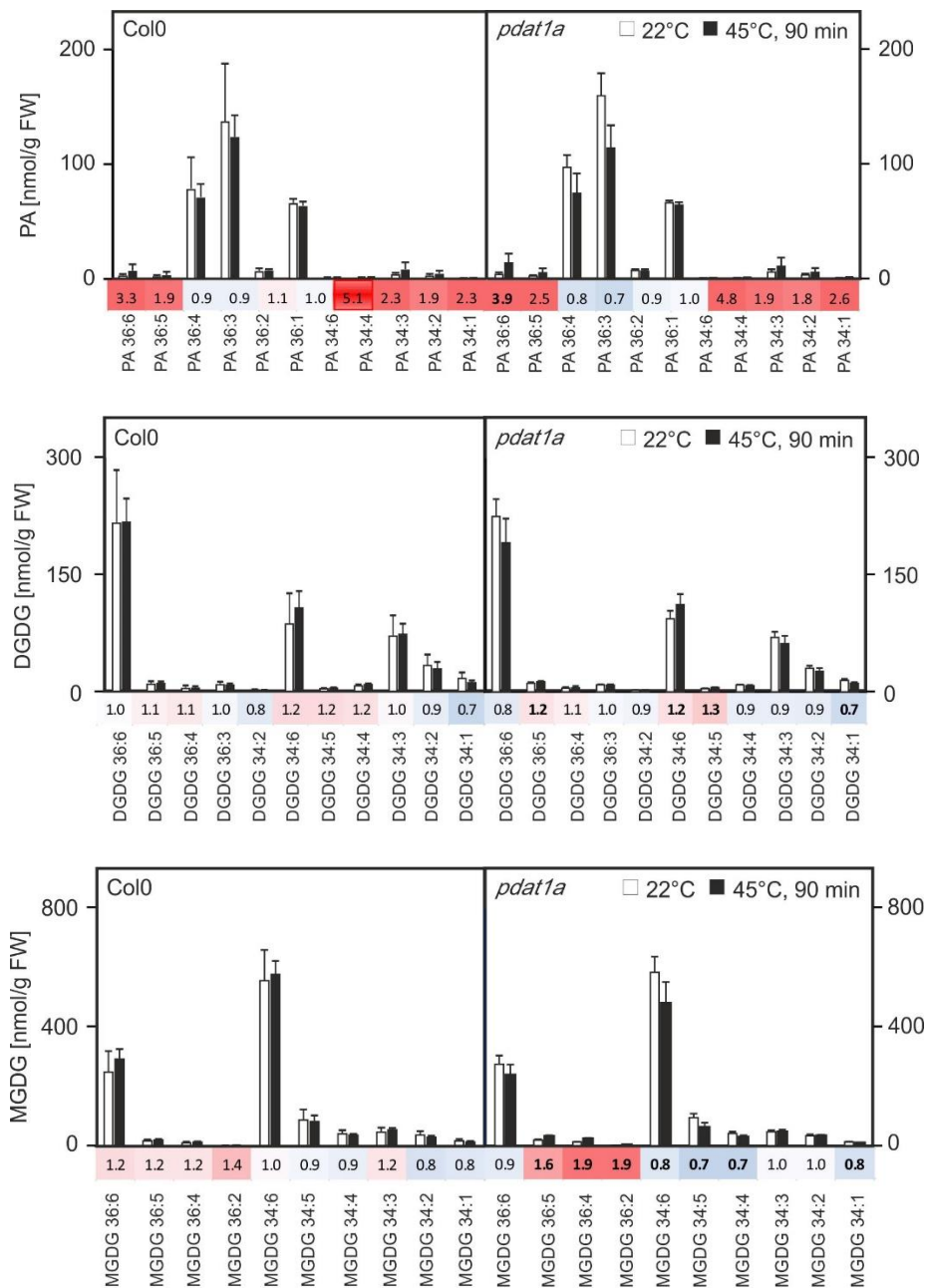
Figure 14. Heat-induced changes of lipidome.
Levels of lipids (TGs, DGs, PAs, PCs, PEs, MGDGs and DGDGs) were determined in 14-day-old wild type and *pdat1a* seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. The heat maps display the fold-changes of lipid species (characterized by their head group, total number of acyl carbons: total number of double bonds) after the heat-shock compared to control seedlings at 22° C. Statistical significant changes of lipid levels (p<0.05) are indicated by bold numbers.

Unsaturated TG species demonstrated the highest increases of TG species in wild type plants, confirming the results shown in Figure 4. As expected, TG accumulation was higher than detected in Figure 4 as heat treatment was now performed at a higher temperature (45° C, 90 min compared to 37° C, 2 h).

The most abundant TG species following heat treatment in wild type seedlings were TG 54:9 and TG 54:8, showing fold changes of 32 and 12 to levels of 47 and 57 nmol/g FW, respectively. TG 52:9, nearly not detectable under basal conditions, increased even 1400-fold following heat treatment. Interestingly, TG 52:9 is composed of two 18:3- and one 16:3-FA. The FA 16:3 does occur almost exclusively in the plastidic lipids, with the highest levels in MGDG. Compared to wild type TG levels, levels of *pdat1a* seedlings did increase to only very low amounts.

Analysis of DGs, the direct precursor of TGs, in wild type seedlings, did not display significant changes with exception of DG 34:6 (18:3/16:3) and DG 34:5 (mostly 18:2/16:3), showing a 9- and 5-fold increase, respectively. In *pdat1* transgenic seedlings, nearly all DG species showed a significant increase compared to basal levels. *Pdat1a* cannot convert DGs into TGs via the acyl-CoA independent pathway, and therefore seems to accumulate DGs. DG 36:6 (18:3/18:3) demonstrated the highest amount of DGs with 15 nmol/g FW and a 7-fold increase. The highest accumulation was detected in DG 34:6 (18:3/16:3) and DG 34:5 (mostly 18:2/16:3) with 67-fold and 37-fold higher levels compared to 22° C. Again, as already observed in the wild type seedlings, TGs with the acyl-combinations 18:3 and 16:3 seem to be the predominant species following the heat treatment. Results suggests, that FAs might originate from MGDG and/or DGDG, as well. The highest occurring lipid species in MGDG and DGDG are combinations with 18:3/16:3 and 18:3/18:3. The combination 18:3/16:3 is almost exclusively present in the lipid classes of MGDG and DGDG.

Besides DG, PC is another important precursor of TG in the acyl-CoA independent pathway in which PDAT1 catalyzes the formation of DG, using PC as immediate acyl donor and DG as acyl acceptor. In wild type plants, no significant changes where determined in the PC species, with exception of the species PC34:6 (18:3/16:3) and PC34:5 (mostly 18:2/16:3), showing a 6-fold and 4-fold increase, respectively. In *pdat1a* seedlings, fold changes of these two species were slightly higher compared to the wild type with a nearly 8-fold increase in PC 34:6 and a 4-fold increase in PC 34:5.

In PAs, a precursor of DG in the Kennedy pathway, a 3- and 4-fold increase in PA 36:6 can be detected in both wild type and *pdat1a* seedlings, which was significant in *pdat1a.* However, PA 34:6 and PA 34:5, could not be detected in both wild type and the transgenic line.

In addition, PE was analyzed, an extra-chloroplastic membrane lipid, which is not directly involved in TG synthesis, but can be synthesized out of PAs. Similar results of PE analysis can

be observed compared to PCs and DGs. The only changes in wild type seedlings could be detected in PE 34:6 and PE 34:5, with a 12- and 2.5-fold increase, respectively. *Pdat1a* seedlings demonstrated higher fold changes following heat treatment compared to the wild type with an 18-fold increase in PE 34:6 and a 5-fold increase in PE 34:5 following heat stress. Plastidic lipids, on the other hand, did not show increases in lipid species. In *pdat1a* little, but significant decreases of MGDG 34:6 (0.8-fold), MGDG 34:5 (0.7-fold) and MGDG 34:4 (0.7-fold) were determined. The MGDG pool (1116 ± 105 nmol/g FW) is very large, compared to the heat-induced TG pool (336 ± 15 nmol/g FW) and relative changes of TG levels are within the experimental error of MGDG quantification. However, increases of lipid species with 18:3/16:3 compositions in the cytosolic lipids match the hypothesis that FA for TG synthesis might derive from MGDGs.

Interestingly, in both wild type and *pdat1a* seedlings, a slight increase in DGDG 34:6 and DGDG 34:5 (1.2 to 1.3-fold) could be detected, which was significant in *pdat1a*.

2.4.4   Analysis of transgenic lines with altered FA compositions of lipid classes

To investigate, if FAs for TG synthesis following heat treatment origin from cytosolic or chlorplastic lipids, FA compositions of neutral lipids were analyzed in both *fad3* and *fad7/8* transgenic lines. *Fad3* transgenic *A. thaliana* seedlings display a deficiency in the ER-localized ω-3 desaturase, leading to reduced levels of 18:3 fatty acyls and accordingly higher levels of 18:2 fatty acyls in cytosolic lipids in comparison to wild type plants (Browse et al., 1993). *Fad7/8* double transgenic *A. thaliana* seedlings are deficient in two chloroplastic ω-3 desaturases, resulting in a reduction of 18:3 and 16:3 fatty acyl levels and correspondingly higher levels of 16:2 and 18:2 in plastidic lipids (McConn et al., 1994). Through knock-out of ω-3 desaturase in both plastidic and chloroplastic compartments, however, no total lack of 18:3 fatty acyls can be achieved, due to the exchange of lipids and FAs between chloroplast and ER.

For analysis of FA compositions of lipids in both *fad3* and *fad7/8* transgenic lines, total lipid extraction was carried out, followed by separation through solid phase extraction chromatography. Neutral lipids, phospholipids and galactolipids were isolated from each other, hydrolyzed and FAs were analyzed.

For determination of the altered FA compositions of the transgenic lines, FA compositions and lipidome data of galactolipids in *fad7/8* and phospholipids in *fad3* were analyzed under basal conditions.

Fad3.2 displayed the anticipated FA composition changes in cytosolic lipids. FA compositions of phospholipids showed that *fad3* had higher 18:2 and lower 18:3 level compared to the wild type (Figure S 1). Changes in FA composition of *fad3* compared to the wild type were determined by lipidome data of PCs and PEs. *Fad3* displayed lower levels of PE and PC 36:6, compared to the wild type, but had higher levels of PC 36:4 and PC 34:2, composed of 18:2/18:2 and 18:2/16:0 (Figure S 5, Figure S 6).

Furthermore, FA compositions of plastidic galactolipids in *fad7/8* displayed higher levels of 18:2 and 16:2, and lower levels of 18:3 and not detectable levels of 16:3, compared to wild type and *fad3* mutants (Figure S 2). Lipidome data of MGDGs and DGDGs determined the changed FA compositions of *fad7/8* seedlings, with lower levels of lipids with FA compositions 36:6 and 34:6 and higher levels of lipids with 36:4 and 34:4 FA compositions (Figure S 3, Figure S *4*).
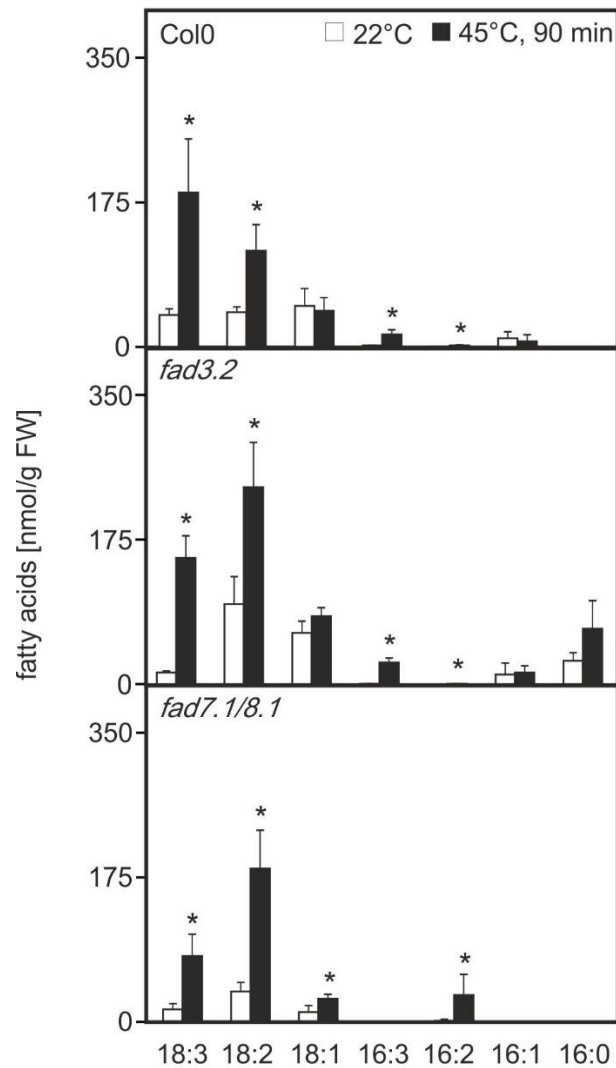
Figure 15. FA levels in neutral lipids in wild type, *fad7.1/8.1* and *fad3.2* seedlings.
14-day-old seedlings were kept at 22° C (white bars) and or treated with a heat shock (45° C for 90 min, black bars). Thereafter, neutral lipids were separated, hydrolyzed and FAs were determined. Data represent means ± SD, n = 4. Statistical significant changes of lipid levels ($p<0.05$) are indicated by asterisks.

At control conditions (22° C) *fad3* transgenic plants showed higher 18:2 levels (2-fold) and lower levels of 18:3 compared to the wild type. *Fad7/8* seedlings revealed only slightly higher 18:2 and lower 18:3 levels as Col0. These analyses hint towards a plastidic and cytosolic origin of FAs for TG synthesis at basal conditions.

Following 45° C treatment for 90 minutes, wild type and *fad3* seedlings displayed a 2-to 3-fold accumulation. In contrast, 18:2 levels in *fad7/8* seedlings increased 5-fold compared to control conditions. In addition, *fad3* showed similar levels of 18:3, while levels were strongly

decreased in *fad7/8.* Even more strikingly, 16:3 accumulated 9- to 17-fold in *fad3* and wild type plants, respectively, whereas no increase could be determined in *fad7/8* seedlings. Analysis of complex lipids without hydrolysis in *fad3* and *fad7/8,* displayed the same results. TG levels of *fad3* seedlings demonstrated only slightly lower levels of polyunsaturated and higher levels of saturated TG species after heat stress, due to already lower basal 18:3-FA levels in the *fad3* seedlings (Figure S 7). In *fad7/8* seedlings the highest fold changes can be detected in TG compositions with 6 and 7 double bonds, leading to the assumption that TG species with higher 18:2 and 16:2 level occur in the *fad7/8* seedlings following heat treatment (Figure S 7). Additionally, lipidome data revealed, that accumulation of PC 34:6, DG 34:6 and PE34:6 (18:3/16:3) cannot be detected in *fad7/8* seedlings in comparison to wild type and *fad3* mutants (Figure S 5, Figure S 6, Figure S 8).

In summary, results confirmed that polyunsaturated FA for TG synthesis during heat stress origin from plastidic compartments.

2.4.5   Origin of diacylglycerol (DG) building blocks for heat-induced TG synthesis

It has yet to be fully understood where DG for heat-induced TG synthesis derive from. Therefore, different transgenic lines deficient in different DG synthesis pathways were investigated.

2.4.5.1   Origin of DG building blocks for heat-induced TG synthesis is not synthesized via PAH and PDCT

DGs for TG synthesis may be synthesized *de novo* via the Kennedy pathway through de-phosphorylation of PA catalyzed by a phosphatidic acid phosphatase (PAP), also known as phosphatidic acid hydrolase (PAH). For addressing the question where the DG moiety for TG synthesis following heat stress derives from, *pah1, pah2* and *pah1pah2* seedlings were analyzed under basal conditions as well as after 45° C, 90 min treatments (Figure 16).
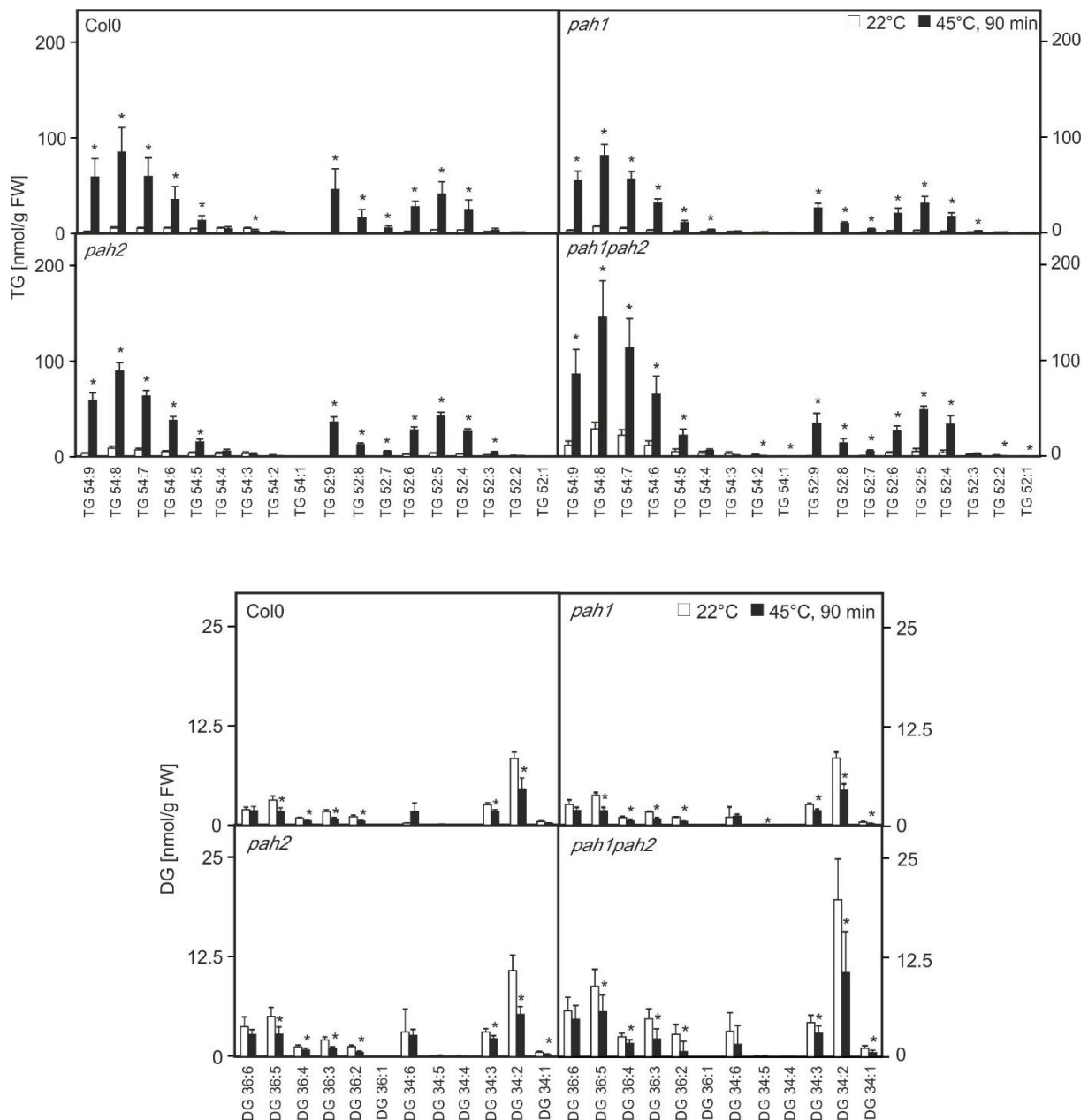
Figure 16. Heat-induced changes of TGs and DGs in wild type, *pah1, pah2* and *pah1pah2* seedlings.
Levels of lipids were determined in 14-day-old wild type, *pah1, pah2* and *pah1pah2* seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid levels (p<0.05) are indicated by asterisks.

No changes were determined in TG levels in *pah1, pah2* and *pah1pah2* seedlings following heat stress in comparison to Col0 seedlings (Figure 16). Moreover, no accumulation could be detected in seedlings of all transgenic lines of DG 34:6 after heat stress compared to the wild type seedlings.

Meanwhile, decreases for all other DG species following heat treatment can be determined in wild type as well as in *pah1, pah2* and *pah1pah2* seedlings. The species of the other lipid classes (PC, PE, PA, MGDG, and DGDG) displayed also no changes in the transgenic lines on

basal levels and following heat treatment compared to the wild type (Figure S 9-13). Therefore, *pah* lines are not involved in DG synthesis as substrate for TG synthesis after heat stress.

In addition, basal levels showed no changes of DG levels between *pah* lines and the wild type. It seems very likely, that PAH1 and PAH2 are not the only phosphatidic acid phosphatases involved in DG synthesis. Other phosphatidic acid phosphatases might substitute for deficiency in PAH1 and PAH2.

As shown above, PDAT1 is essential for TG synthesis after heat stress. Therefore FAs at the *sn*-3 positons of TGs seem to be derived from PCs. DGs, the second substrate for PDAT1 can as well be liberated from PCs (Bates, 2016). This reaction, catalyzed by the PDCT enzyme and encoded by the *ROD1* gene, exchanges a phosphocholine headgroup from PC to DG, creating new molecular species of PC and DG. For *A. thaliana* seed TG synthesis, PDCT seems to play a major part of guiding acyl flux through PC. A mutation of the *ROD1* gene reduces 18:3 and 18:2 accumulation in seed TGs by 40 % (Lu et al., 2009).

FA analysis of neutral lipids revealed that *rod1* seedlings displayed two times higher 18:1 levels under basal conditions compared to 18:2 and 18:3 FA, whereas levels of these FAs are nearly equal in Col0 (Figure S 14). Higher levels of 18:1 compared to levels of 18:2 and 18:3 were already described in the literature (Lu et al., 2009).
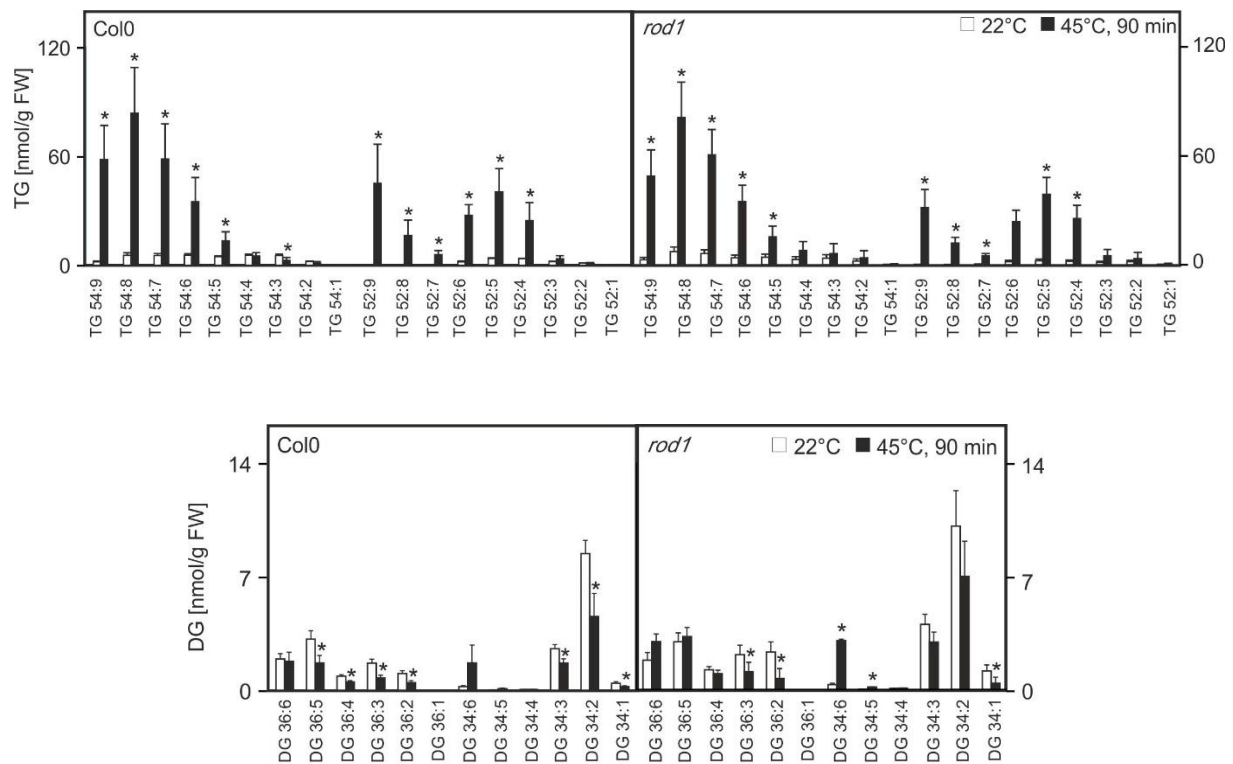
Figure 17. Heat-induced changes of TGs and DGs in wild type and *rod1* seedlings.
Levels of lipids were determined in 14-day-old wild type and *rod1* seedlings kept at 22°C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid levels (p<0.05) are indicated by asterisks.

Analysis of 14-day-old *rod1* seedlings, revealed that TG and DG levels were similar to the wild type following heat treatment (45° C, 90 min) (Figure 17). FA analysis of neutral lipids (TGs, DGs) displayed the same accumulations compared to the wild type. In detail, a higher accumulation of polyunsaturated FA (18:3 and 16:3) compared to the saturated FA was determined (Figure S *14*). In addition, other cytosolic lipids (PC, PE and PA) and plastidic lipids (MDGD, DGDG) showed no changes after heat treatment compared to the wild type (Figure S 15-19).

In addition to PDCT, CPT activity could as well lead to an equilibrium of the DG moiety between PC and DG pools. Furthermore, DG can be produced from PC via phospholipases (Bates, 2016). The DG moiety might also be directly generated through degradation of galactolipids in the plastidic compartments.

2.4.5.2 Analysis of DG synthesis in plastidic compartments

As shown in Figure 14, acyl compositions of DGs in *pdat1* seedlings following heat treatment, resemble acyl compositions of MGDG lipid species. Therefore, MGDGs might be the direct

source for DGs utilized for TG synthesis. After freezing stress, it was shown that monolayer forming MGDGs are synthesized to bilayer forming DGDGs, TGDGs and higher oligogalactosyl diacylglycerols, probably for stabilization of chloroplast membranes. This reaction is synthesized via post-translational regulation of SFR2, which transfers galactosyl residues from MGDGs to galactolipids. In addition, DGs are formed, which could serve as direct precursors for TG synthesis in the cytosol (Moellering et al., 2010).

To analyze if SFR2 is active after heat stress, levels of TGDGs were determined in 14-day-old *pdat1* seedlings after heat treatment (45° C, 90 min).

Furthermore, enzymatic products of an acyltransferase, the acylated galactolipid associated phospholipase 1 (AGAP1) were analyzed. *AGAP1* expression was shown to be induced after infection with *P. syringae* strains and powdery mildew *Golovinomyces orontii*. Among abiotic treatments an induction was determined after salt, osmotic and heat stress as well as after phosphorous deficiency (Hruz et al., 2008; Nilsson et al., 2015). AGAP1 transfers a FA from MGDG to galactose residues in galactolipids thereby producing acyl-MGDG and lyso-MGDG (Nilsson et al., 2015). As degradation of lyso-MGDG could potentially contribute to FA delivery for TG synthesis, acyl-MGDGs and lyso-MGDGs were profiled in heat treated wild type and *pdat1* seedlings (Figure 18).
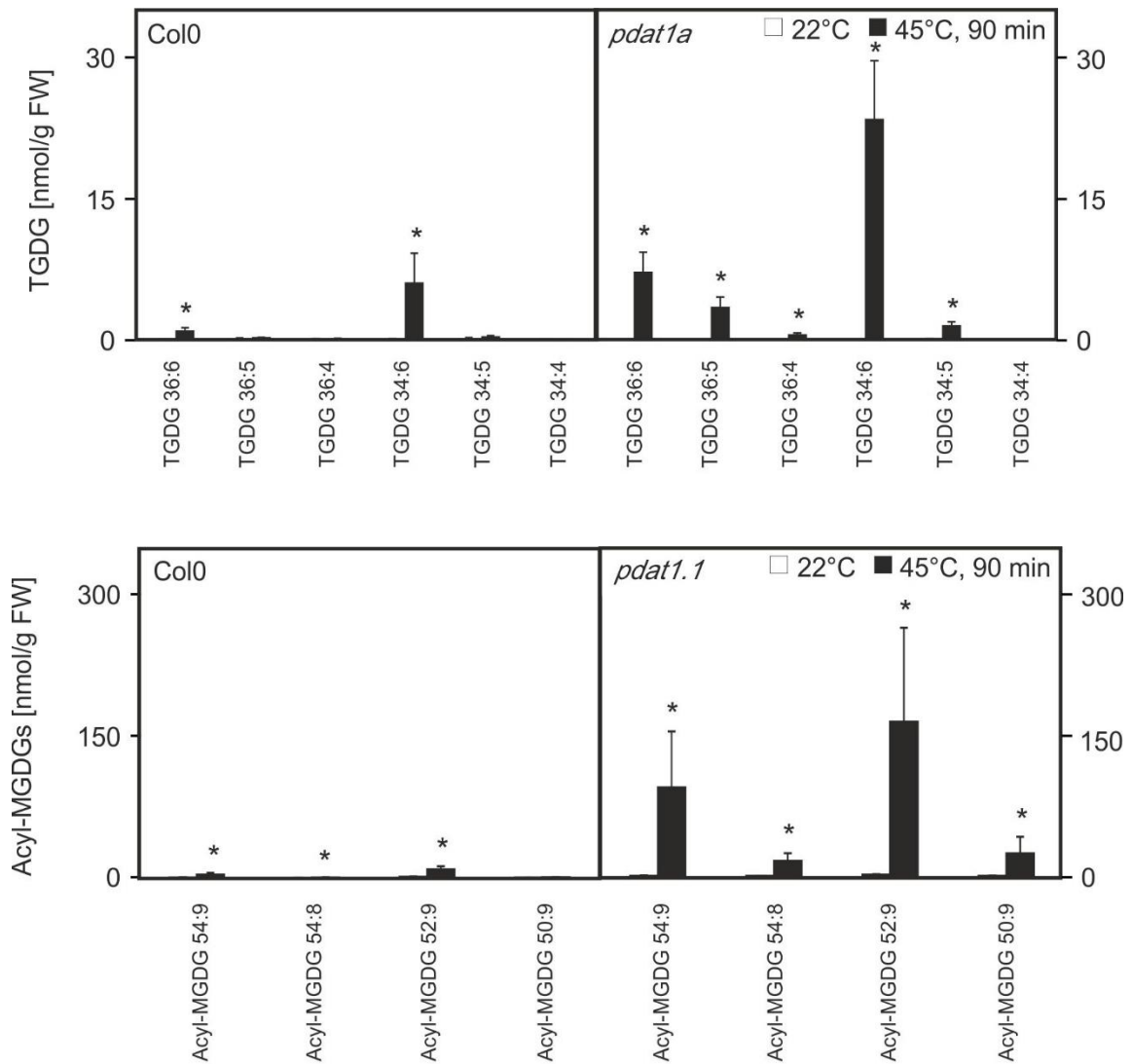
Figure 18. Heat-induced changes of TGDGs and acyl-MGDGs in wild type and *pdat1a* seedlings.
Levels of lipids were determined in 14-day-old seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid levels (p<0.05) are indicated by asterisks.

SFR2 seems to be activated by heat stress, as accumulation of TGDGs can be determined following heat treatment (45°C, 90 min). Interestingly, increases of TGDGs, as already observed in DG levels (Figure 14), are more significant in *pdat1* lines, which are unable to convert DG into TG.

Furthermore, accumulation of acyl-MGDG, which can be produced by AGAP1, could be detected in heat treated wild type plants. Especially acyl-MGDG 54:9 and acyl-MGDG 52:9 showed high increases. Again the accumulation of acyl-MGDGs was even more notably in *pdat1a* seedlings compared to the wild type (Figure 18). Lyso-MGDGs could not be detected in neither untreated nor treated seedlings.

The data suggests, that MGDG turnover leads to an accumulation of TGDG and acyl-MGDG during heat stress. This accumulation is not compromised in *pdat1* seedlings unable to accumulate TGs. The even higher increase of TGDG and acyl-MGDG in *pdat1* seedlings leads to the suggestion that MGDG remodeling caused by heat stress is even more induced in plants, which are deficient in TG synthesis. Another possibility is that plants impaired in storing FAs in TGs, are comprised in MGDG turnover and degradation of remodeling products.

2.4.6   Thermotolerance assay of transgenic lines of TG biosynthesis

TG accumulation due to abiotic or biotic stress factors is commonly speculated to be initiated through mechanisms removing products of lipid metabolism, which might be detrimental for plant cells, such as FAs and DGs. TGs might as well act as storage molecule, helping the plants to recover after heat treatment during the recovery phase, e.g. as FA provider for reversing heat-induced lipid metabolism. For analyzing the relevance of TG accumulation regarding the thermotolerance, transgenic *A. thaliana* plants with altered TG levels were analyzed. If TG accumulation has a biological relevance *pdat1* transgenic plants, lacking TG accumulation after heat treatment, might display a lower thermotolerance. Therefore thermotolerance tests after acquired and basal thermotolerance were performed with wild type, *pdat1*, *dgat1* and *tgd1* seedlings. First, differences in the survival rates after short term acquired thermotolerance were investigated. Therefore, 14-day-old *dgat1.1, pdat1a, pdat1b* and *tgd1.1* mutant Arabidopsis seedlings, were acclimated at 37° C for 2 h and treated, after a recovery phase at 22° C for 2 h, at 45° C for 90 min (Figure 19).
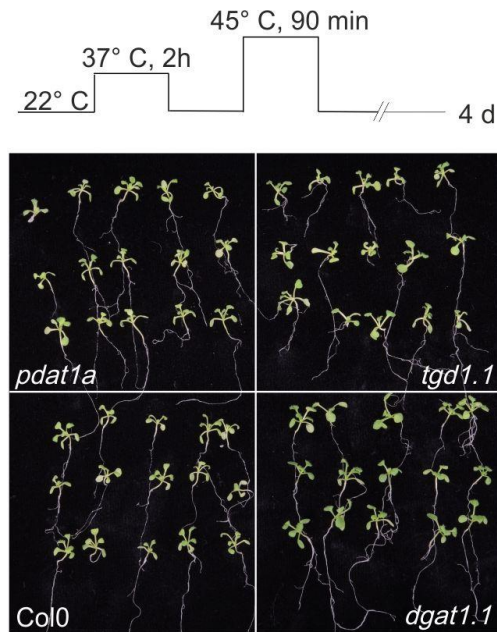
Figure 19. Acquired thermotolerance of *dgat1.1, pdat1a, pdat1b* and *tgd1.1* mutant Arabidopsis seedlings after heat treatment compared to Col0. Schemes of heat stress regimes for the thermotolerance assays are shown. 14-day-old *A. thaliana* seedlings (100 seed/plate) were treated with 37° C for 2 h and after a recovery phase of 2 h at 22° C they were treated with 45° C for 90 minutes. Photographs show representative seedlings of Col0, *pdat1a, dgat1.1* and *tgd1.1* 4 days after recovery at 22° C.

No changes could be detected following short term acquired thermotolerance between Col0 and *Arabidopsis* mutant seedlings. After 4 days the seedlings of all genotypes survived and showed no chlorotic necrosis (Figure 19). All genotypes were able to acquire thermotolerance.

Next, possible changes in the basal thermotolerance were investigated. Plants normally die after a heat stress of 45° C for 90 min (as applied in the experiments above). A thermotolerance assay was established in which shoots of seedlings, which were kept on agar plate, were treated at 45° C, while roots were cooled down to approximately 40° C. Thereby, a recovery after the normally lethal heat stress was enabled.
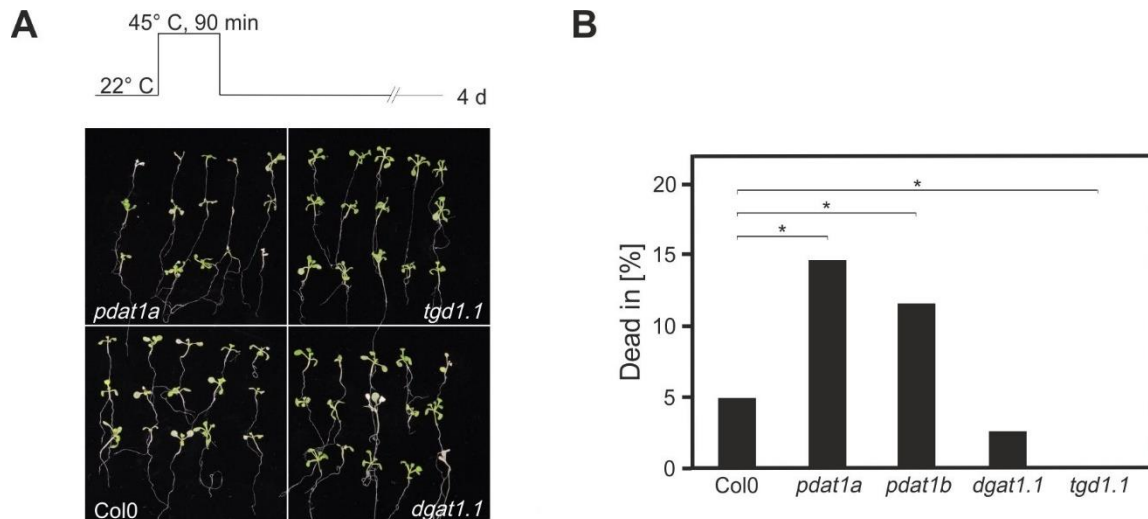
Figure 20. Basal thermotolerance of *dgat1.1, pdat1a, pdat1b* and *tgd1.1* mutant Arabidopsis seedlings after heat treatment.
Schemes of heat stress regimes for the thermotolerance assays are shown in panel A. 14-days-old Arabidopsis seedlings were treated with 45° C for 90 min from above, while roots were cooled to 40° C. Photographs show representative seedlings of Col0, *pdat1a, dgat1.1* and *tgd1.1* four days after recovery at 22° C (A) The rate of dead plants were collected from four replicates, statistical analysis were performed with chi square test with n ≥ 85, p<0.05.

Significant changes in survival could be determined due to altered TG levels following heat treatment (Figure 20). *Pdat1a* and *pdat1b* mutant *Arabidopsis* seedlings, which are unable to accumulate TGs during heat treatment (Figure 11), showed a significant higher lethality rate in comparison to the wild type. In line with these observations, *tgd1.1* seedlings, which showed higher levels of TGs before and after heat treatment, demonstrated a significantly higher thermotolerance compared to the wild type at the conditions applied. *Dgat1.1* seedlings, displaying the same levels as the wild type (Figure 11), displayed no differences to the wild type regarding its thermotolerance. Comprising these results, TG accumulation following heat treatment has a functional relevance and is important for acquiring basal thermotolerance.

2.4.7 Chlorophyll fluorescence of transgenic lines of TG biosynthesis

In addition to the survival assay, changes in thermotolerance of 14-day-old *dgat1.1, pdat1a, pdat1b* and *tgd1.1* seedlings were analyzed by measuring the maximal quantum yield (Fv/Fm) of PSII photochemistry to estimate the decline of photosynthetic quantum efficiency. Photosynthesis is the most heat sensitive mechanism in plant cells and photosystem II was shown to be the primary site of heat damage in the photosynthetic apparatus (Routaboul et al., 2012). The maximum quantum yield is a valid factor for heat damage as it represents the efficiency of electron transport in PSII. For measurements *A. thaliana* seedlings were

acclimated with 37° C for 2 h, and treated, after a recovery phase at 22° C for 2 h, at 45° C for 90 min (Figure 21). One leaf per seedling was measured following a recovery phase of 1 h.
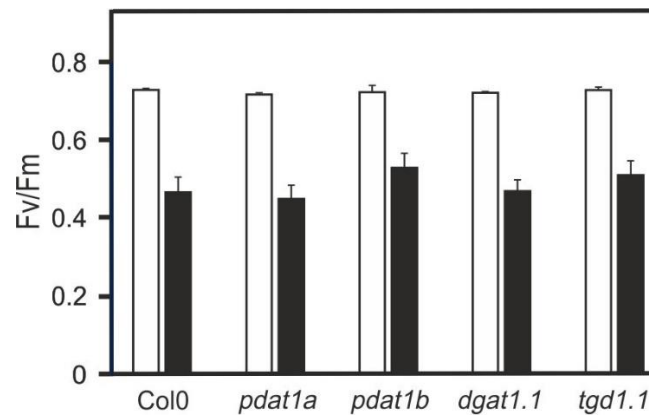

Figure 21. Chlorophyll fluorescence of *dgat1.1, pdat1a, pdat1b* and *tgd1.1* mutant Arabidopsis seedlings after heat treatment.
Pulse-Amplitude-Modulation (PAM) fluorometry in 14-day-old *dgat1.1, pdat1a, pdat1b* and *tgd1.1* seedlings kept at 22° C (white bar) as well as acclimated with 37° C for 2 h, and treated, after 22° C for 2 h, at 45° C for 90 minutes (black bar). Samples were measured after 1 h at 22° C. Data represent means ± SE, n = 50, p<0.05 .

Measurements showed that photosynthetic efficiency of all transgenic lines and Col0 are very stable under basal conditions (Figure 21). Following heat acclimation (37° C, 2 h; 22° C, 2 h; 45° C, 90 min) and a recovery phase at 22° C for 1 h a reduction of the quantum yield of photosystem II in comparison to control plants was clearly detectable in all lines. Photosynthesis was compromised in acclimated seedlings following heat treatment (45°C, 90 min). Wild type plants and all mutant lines were affected to a similar degree.

In addition, seedlings were treated with 45° C for 90 min from above, while roots were cooled to 40° C. Following a recovery phase at 22° C for 1 h, one leaf per seedling was measured (Figure 22).
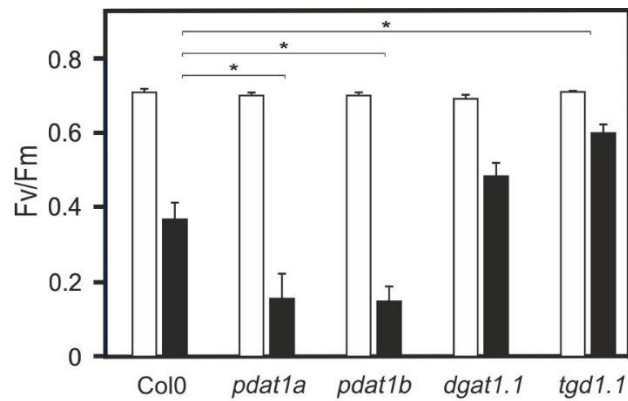
Figure 22. Chlorophyll fluorescence of *dgat1.1, pdat1a, pdat1b* and *tgd1.1* mutant Arabidopsis seedlings after heat treatment. Pulse-Amplitude-Modulation (PAM) fluorometry in 14-day-old *dgat1.1, pdat1a, pdat1b* and *tgd1.1* seedlings kept at 22°C (white bar) and heated at 45° C for 90 min from above, while roots were cooled to 40° C (black bar). Samples were measured after 1 h at 22° C. Data represent means ± SE, n = 10, $p < 0.05$.

Heat treatment at 45° C for 90 min (followed by a recovery phase of 1 h at 22° C) lead to a reduction of the quantum yield of photosystem II in comparison to control plants of all lines. In contrast to acclimated plants (Figure 21), wild type and mutant lines were not affected to a similar degree. *Pdat1* lines showed a significant stronger drop in photosynthetic efficiency compared to the wild type (Figure 22). On the contrary, a significant higher yield could be observed in *tgd1.1* in comparison to the wild type after heat stress and followed by a recovery phase of 1 h. Therefore, the extent of photosystem II damage correlated with the survival assay 4 days following heat treatment. The results confirm that accumulation of TGs result in a protection of photosynthetic apparatus and higher thermotolerance.

3 Discussion

3.1 Accumulation of TGs, raffinose and galactinol during heat acclimation

Analysis of the consequences of heat stress has become more important due to the increasing relevance of climate change. Notably, even short heat periods can have enormous damaging effects on crop yield. Temperature increases of 1-2° C are not detrimental to plants; however, harmful temperature extremes have been predicted to occur more frequently (Alexander et al., 2006).

During exposure with elevated temperatures a HSR is activated in plants that comprises a battery of processes. The HSR is mostly characterized by a rapid activation of HSFs triggering a massive accumulation of HSPs. In addition to the activation of HSFs and HSPs, the metabolism of plants needs to adjust to higher temperatures. However, metabolites, which are potentially important for surviving elevated temperatures, have been hardly investigated. This work deals with the metabolic pathways that are regulated during heat acclimation. In my work, the application of untargeted metabolite analysis following the treatment of *A. thaliana* seedlings at 37° C for 2 h detected a rapid accumulation of the sugars raffinose and galactinol as well as an increase in the most abundant TG species (Mueller et al., 2015). Already after 30 min a massive accumulation of both sugars (raffinose and galactinol) and TG markers (TG54:9, TG54:8, TG54:7, TG54:6) could be detected (Figure 5A). The increase in raffinose and galactinol following moderate heat treatment has previously been described (Kaplan et al., 2004; Panikulangara et al., 2004).

In addition to these metabolic studies, only one large metabolite profiling analysis has been performed (Kaplan et al., 2004). Following a 2 h exposure at 40° C, 5-week-old *A. thaliana* plants displayed a significant increase of 80 metabolite features ($p<0.05$). A strong accumulation (> 4-fold change) was detected in four identified metabolites and in four unidentified compounds. In addition to raffinose and galactinol an increase in pipecolic acid and digalactosylglycerol following a 40° C treatment in 5-week-old *A. thaliana* plants could be detected. This increase could not be detected in two-week-old *A. thaliana* seedlings treated with 37° C for 2 h (Mueller et al., 2015). Heat treatment at 40° C for 1 h can already be lethal for *A. thaliana* and therefore the metabolic changes measured following a 40° C treatment could mirror defects of cellular homeostasis (Larkindale and Knight, 2002).

The TG increase following heat acclimation in *A. thaliana* seedlings observed in our study has not been previously characterized (Mueller et al., 2015). However, in parallel to us, the finding

has been independently confirmed through a lipidome study following short-term heat stress (Higashi et al., 2015).

In addition to the four TG markers identified during our untargeted analysis, 48 TGs could be detected during control conditions (22° C) and following short-term heat treatment (37° C, 2h), specified through the number of carbons and double bonds (Figure 4). TG markers were shown to be the most abundant TG species (TG54:9, TG54:8, TG54:7 and TG54:6). TG species with higher saturation levels and with higher carbon numbers showed no accumulation following heat treatment, whereas the highest accumulation could be determined in polyunsaturated TGs. TG species containing 54 and 9 to 6 carbons and TGs with 52 carbons and 9 to 5 carbons increased 3.5- to 16-fold following heat treatment (37° C, 2 h). This was also confirmed by the lipidome study of Higashi *et al.,* in which TG54:9, TG54:8 and TG54:7 displayed the highest increase after 38° C for 1 day. Polyunsaturated TG species containing 52 carbons were not monitored (Higashi et al., 2015).

Levels of TG markers were shown to be very sensitive to temperature increases - accumulation could already be determined at 30° C and the maximum was reached at 42° C for 2 h. (Figure 5D). Moreover, in contrast to sugar markers, TG markers dropped back to nearly basal levels 2 h after *A. thaliana* seedlings were returned to the optimal growth temperature (22° C) (Figure 5C). TG levels were observed to be very sensitive to the heating method, growth conditions and harvest duration. Only light differences during experiments or growth conditions could be reflected in variations of mean TG levels in different experiments .

3.2   TG accumulation not limited to heat treatment

TG response might be limited to heat treatment or it could be a general response to stress. An accumulation of raffinose has not only been described during heat acclimation, but up-regulation of the raffinose pathway has also been reported during drought, salt and cold stress. These abiotic stresses induce the expression of *GOLS1* and *GOLS3*, resulting in an increase in raffinose and galactinol (Taji et al., 2002). When TG levels were quantified following different abiotic stress treatments, a significant accumulation could be determined after short term drought and salt stress. Cold treatment, high light and osmotic stress did not result in a TG accumulation (Figure 6). Therefore, TG accumulation, similar to the raffinose response, does not seem to be limited to heat stress. In addition to the abiotic stress treatments tested, TG accumulation was already detected after long term freezing stress (Moellering et al., 2010),

desiccation (Gasulla et al., 2013), senescence (Watanabe et al., 2013) and *Pseudomonas* infection (Zoeller et al., 2012) in *A. thaliana.*

3.3   TG accumulation is no part of the genetically programmed HSR

Accumulation of TGs and raffinose after heat treatment leads to the question as to whether the metabolite responses are connected or regulated through the same pathways. A possible connection of both responses might be e.g. a channeling of carbons from the sugar pathway to the TG pathway to synthesize heat-induced TGs.

Galactinol synthase 1 was described to be the key enzyme in the biosynthesis of raffinose family oligosaccharides. Galactinol is synthesized via GOLS1 from uridine diphosphate galactose (UDP-Gal) and myo-inositol, serving as a galactosyl donor to build raffinose, stachyose and verbascose (Liu et al., 1998). GOLS1 itself is induced through HSFA1abde transcription factors (Liu et al., 2011). HSFA1abde are termed the master regulators of the heat shock response as plants deficient in these HSF are unable to induce the HSR and to acquire thermotolerance (Liu et al., 2011; Yoshida et al., 2011). HSFA1abde induce a series of heat-inducible transcription factors, including HSFA2, HSFB, DREB2A and MBF1c (Liu and Charng, 2013; Liu et al., 2011; Yoshida et al., 2011). HSFA2 is induced through HSFA1 and is required for the extension of short-term heat acclimation (Charng et al., 2007). In addition, HSFA2 induces the up-regulation of HSPs, antioxidative enzymes, such as APX2, and metabolic enzymes, such as GOLS1. Raffinose and TG markers were analyzed in *hsfa1abde, hsfa2* and *gols1* transgenic seedlings to investigate a possible connection of the heat-induced accumulation. *Hsfa1abde* quadruple mutants did not accumulate raffinose during heat treatment (Figure 7A). These are in line with previous results and suggests that HSFA1a and HSFA1b are essential for *GOLS1* transcription after heat as *hsfa1ab* double mutants displayed a reduced *GOLS1* expression (Busch et al., 2005). *Hsfa2* transgenic *A. thaliana* seedlings displayed a wild type-like accumulation of raffinose after heat treatment under our conditions (Figure 7A). Previous experiments showed that plants deficient in *hsfa2* displayed reduced expression levels of *GOLS1* (Busch et al., 2005; Nishizawa, 2006). In addition, a transgenic line overexpressing *HSFA2* demonstrated an increased expression of *GOLS1* as well as a higher accumulation of raffinose and galactinol compared to the wild type (Nishizawa et al., 2008). However, our results indicated that *hsfa1abde* seem to be more important for the induction of *GOLS1* expression and raffinose accumulation after heat treatment when compared to

hsfa2. TG levels were analyzed in *hsfa1abde* and *hsfa2* seedlings to investigate if the TG response after heat treatment is also regulated through the genetically-regulated HSR. TG levels increased to wild type levels after heat treatment in both the *hsfa1* and the *hsfa2* transgenic lines (Figure 7B). In contrast to raffinose, TG response after heat is not coupled to the genetically-programmed HSR.

In addition, raffinose and TG marker levels were investigated in *gols1* transgenic lines. As expected, raffinose did not accumulate in *golS1* mutant seedlings (Figure 7C). TG levels increased after heat treatment in *golS1* mutant plants to wild type-like levels (Figure 7D). Data suggests that TG and raffinose responses after heat treatment are not connected and that the carbons used for TG synthesis are not redirected from the sugar pathway to the TG pathway.

3.4 TG localization during heat acclimation

Following synthesis, TGs coalesce to form oil bodies, consisting of a TG core, which is surrounded by a phospholipid monolayer that is coated with different proteins. The most abundant proteins, oleosins, caleosins and steroleosins, determine the size of oil droplets, the mobilization of TGs and the signal transduction (Jolivet et al., 2004; Lin et al., 2002; Poxleitner et al., 2006; Shimada et al., 2008; Siloto et al., 2006). Vital Nile Red stain was used to localize oil droplets in heat-treated *A. thaliana* cell cultures. Nile Red is a fluorescent, lipophilic dye that detects intracellular neutral oil droplets. Extra-chloroplastic lipid droplets after heat stress were determined using fluorescence microscopy (Figure 10). No lipid droplets could be detected in the chloroplasts of *A. thaliana* cell cultures. In contrast to these results, a chloroplast swelling and plastoglobule formation was observed after moderate heat treatment (Zhang et al., 2010).

Metabolic analysis of isolated chloroplasts in comparison to whole seedlings was carried out to confirm a cytosolic and not a plastidic localization of heat-induced TGs. A cytosolic TG accumulation with only small increases of chloroplastic TG levels was determined (Figure 8). In addition, separate analysis of shoots and roots from heat-treated *A. thaliana* seedlings revealed that roots, containing proplastids but no functional chloroplasts, also accumulate TGs (Figure 9). Results of fluorescence microscopy and metabolic analysis suggest that the majority of heat-induced TGs are located in the cytosol.

3.5   PDAT1 involved in heat-induced TG accumulation

Lipidomic analyses were carried out and transgenic lines lacking the capacity for heat-induced TG synthesis were searched to identify the functional significance of heat-induced TGs. Plants with a deficiency in enzymes, which might be involved in heat-induced TG synthesis, were investigated.

The Kennedy pathway is reported to be the most important pathway of *de novo* TG synthesis in plant tissues. It involves a sequential acylation and dephosphorylation of glycerol 3-phosphate (G3P) through acyl-CoA-dependent acyltransferases (GPAT, LPAAT and DGAT) resulting in PA, which is then hydrolyzed by PAP to form DG. DGAT1, an acyltransferase, catalyzes the transfer of an acyl chain from acyl-CoA to DG to form TG. These steps are localized in the ER (Chapman and Ohlrogge, 2012). An alternative acyl-CoA independent pathway involves PDAT1, an acyltransferase transferring an acyl group from PC to DG, which produces TG and LPC, that are then be re-acylated to form PC. Enzymes of the acyl-CoA-independent pathway are also localized in the ER (Chapman and Ohlrogge, 2012; Dahlqvist et al., 2000). PES1 and PES2 are localized in the chloroplast and have been reported to possess diacylglycerol acyltransferase activity and can therefore synthesize TGs in the chloroplastic compartments (Lippold et al., 2012). TGs are stored in thylakoid-associated plastoglobuli in the chloroplasts (Lippold et al., 2012; Martin and Wilson, 1984). Plastoglobuli, which contain mainly TGs, carotenoids and prenyl quinones, were shown to enlarge upon abiotic stress, such as drought, nitrogen starvation and most interestingly heat stress (Eymery and Rey, 1999; Gaude et al., 2007; Steinmueller and Tevini, 1985; Zhang et al., 2010).

Due to the fact that most heat-induced TGs are localized in the cytosol, the involvement of the ER-localized enzymes DGAT1 and PDAT1 in TG accumulation following heat stress was tested. Transgenic seedlings deficient of the ER-localized enzymes DGAT1 and PDAT1 were analyzed. Interestingly, is was found that only PDAT1 is essential for TG assembly (Figure 11). It has been reported that PDAT1 activity is crucial for TG synthesis in seedlings while DGAT1 plays an important role in older leaves (Fan et al., 2013). However, analysis of 6-week-old *A. thaliana* plants deficient in either PDAT1 or DGAT1 revealed PDAT1 as the essential enzyme in TG accumulation after heat treatment also in older leaves (data not shown). Since PDAT1 is crucial for heat-induced TG accumulation, DGs and PCs are the direct precursors of TGs during heat stress (Figure 23).

Recently, microarray analysis has revealed an upregulation of the expression of both DGAT1 and PES1 genes after heat treatment. Gene expression of PDAT1 did not show an increase after heat treatment (Higashi et al., 2015). Therefore, PDAT1 seems to be upregulated on a posttranslational level after heat treatment.

3.6   Origin of FAs used for TG synthesis

3.6.1   Heat-induced TGs derive from lipid remodeling and not from *de novo* FA synthesis

It could be determined that PDAT1, which can form TGs out of PC and DG, is essential for the heat-induced accumulation of TGs. The building blocks for DGs and PCs originate either from the *de novo* synthesis of FA or by channeling FA from structural lipids through the direct precursors to TGs (lipid remodeling). Heat-induced TGs originating from the *de novo* synthesis of FA might suggest a direct benefit of high TG levels during heat stress or during the recovery phase by providing building blocks for the repair of membrane damages. Alternatively, if TGs derive from the remodeling of structural lipids, heat-induced TGs might serve as a storage for detrimental degradation products following heat stress.

Levels of free and esterified FA were quantified to analyze whether TGs originate from the *de novo* synthesis of FAs. Total FA levels did no accumulate after heat treatment at 37° C for 2 h, indicating that TGs do not derive from a massive *de novo* FA synthesis during heat treatment (Figure 12).

To analyze whether TG accumulation reflects channeling of FA from membrane lipids to TGs, the most abundant membrane lipid classes, MGDGs, DGDGs, PCs, PEs and DGs, were measured after heat acclimation (Figure 13). No dramatic disturbance of membrane lipid homeostasis was observed. Considering that the FA pool in TGs is only 1-8 % of the total FA pool, small changes in the lipid pools might not have been detected.

Heat-induced TGs comprise predominantly polyunsaturated FA acyl residues, suggesting that polyunsaturated FAs are released from structural lipids and channeled into TGs during membrane remodeling. Therefore, all lipid species of different lipid classes were analyzed.

The importance of DG and PC as precursors for TG assembly catalyzed by PDAT1 after heat treatment has already been discussed. After heat stress, wild type seedlings displayed little, but significant, accumulation of PC and DG with the acyl compositions 34:6 and 34:5, containing mostly 18:3 and 16:3 fatty acyls (Figure 14). Acyl flux in and out of these precursors is mostly in steady state. In addition, an increase in 18:3 and 16:3 FAs could also be detected

in other cytosolic lipid species, such as PEs. The occurrence of 16:3 FAs in cytosolic lipids suggests a chloroplastic origin of FA used for TG accumulation. MGDGs and DGDGs contain nearly exclusively 16:3 FAs. However, they show no significant changes in their lipid species after heat treatment.

Previously, radiolabel experiments of lipids revealed that TGs are synthetized using a specific DG and acyl-CoA sub-pool in *A. thaliana* leaves (Tjellstrom et al., 2015). This might explain the increase in DGs in *pdat1* seedlings. DGs, normally demonstrated a fast turnover, are not able to be channeled into *pdat1* in TGs after heat stress and might remain in the sub-pool. Radiolabel experiments were carried out during normal conditions in which *dgat1* appears to be the most important enzyme for TG synthesis in three-week-old *A. thaliana* leaves (Tjellstrom et al., 2015). PC is also an important substrate for TG synthesis in heat treated plants. Due to the fact that no heat-induced PC increase could be revealed in *pdat1* in contrast to DGs, PC for TG synthesis might not derive from a distinct sub-pool or the sub-pool of PCs is too small to reveal its changes in the transgenic line after heat treatment compared to the overall amount of PCs.

Interestingly, *pdat1* seedlings showed a high increase in DG species containing 18:3/18:3 and 18:3/16:3 FAs, reflecting the lipid composition of MGDGs (Figure 14). Therefore, the sub-pool of DGs seems to origin from chloroplastic lipids.

3.6.2   FAs for TG synthesis derive from chloroplastic lipids

The cytosolic enzyme PDAT1 was confirmed to be essential for TG accumulation. PDAT1 directly transfers an acyl group from the *sn*-2 position of PC to the *sn*-3 hydroxyl of DG, producing TG and LPC. However, TGs of Col0 seedlings showed an accumulation of lipid species mostly composed of 18:3 and 16:3 FAs, reflecting the lipid composition of MGDGs and not the composition of PCs (Figure 14). Therefore, FAs for TG accumulation do not seem to derive directly from cytosolic PCs and DGs but seem to be channeled from chloroplastic lipids through PCs and DGs into TGs. This hypothesis could be confirmed by the increase in PC 16:3/18:3 and DG 16:3/18:3 after heat stress - compositions that occur in only small amounts under basal conditions. Therefore, a chloroplastic origin of the FA, channeled through PCs and DGs into TGs, seems to be the most probable.

To confirm the plastidic origin of FAs for TG synthesis, FA composition of neutral lipids were analyzed in *fad3* and *fad7/8* mutant seedlings before and after heat treatment (Figure 15).

Fad3 mutant plants, deficient in the ER-localized ω-3 desaturase, displayed reduced levels of linolenate (18:3) and correspondingly elevated levels of linoleate (18:2) in cytosolic lipids (Browse et al., 1993). On the other hand, *Fad7/8* mutant seedlings contain reduced 18:3 and 16:3 levels in cytosolic lipids as well as higher levels of 18:2 and 16:2 in chloroplastic lipids (McConn et al., 1994).

Under basal conditions *fad3* mutant seedlings showed a higher 18:2 FA content compared to the wild type and *fad7/8* seedlings, suggesting that most TGs are synthesized from cytosolic lipids. Most interesting, heat treatment led to a higher increase in 18:2 levels in neutral lipids of *fad7/8* mutant seedlings compared to *fad3* mutant and wild type seedlings. Moreover, 18:3 levels in neutral lipids were strongly reduced and 16:3 could not be detected in *fad7/8* mutant seedlings, while a strong accumulation was determined in *fad3* and Col0 plants. These results suggest that a major amount of polyunsaturated FA originates from plastids for the TG synthesis. However, FA desaturated in PCs seem to be incorporated into TGs after heat treatment since *fad3* seedlings displayed slightly altered changes in the FA composition compared to the wild type.

The reasons why polyunsaturated FA might be channeled from the chloroplasts to TG synthesis are diverse. Firstly, high temperature stress leads to higher membrane fluidity (Quinn, 1988). As changes of the membrane fluidity have a major impact on the activity of membrane-localized proteins as well as the membrane permeability to water, solutes and proteins, the plant counteracts these reactions by regulating the saturation levels of membrane glycerolipids (Lande et al., 1995; Nishida and Murata, 1996; Whiting et al., 2000). A decrease in trienoic FA levels in membrane glycerolipids could be determined during a long-term acclimation to high temperatures (Pearcy, 1978; Raison et al., 1982). However, the replacement of unsaturated lipids by more saturated lipids in thylakoid lipids begins to occur after 100 h at 29° C (Falcone et al., 2004). Changes in lipid composition could not be detected after 6 h of heat treatment at 32° C (Burgos et al., 2011). These results could be confirmed in our experiments as the major *A. thaliana* thylakoid lipids, MGDG and DGDG, displayed no changes in the lipid compositions at 45° C after 90 min (Figure 13). Changes in saturation levels after heat stress were also demonstrated in seed oil triacylglycerol composition. Being matured under high temperatures, *A. thaliana* seeds showed an inhibition of PC desaturation, leading to a lower desaturation level in TGs (Menard et al., 2017). Due to the fact that 94 % of FA being found in seed oil of *A. thaliana*, the composition and plasticity of

the seed oil could be a subject for natural selection (Li-Beisson et al., 2013; Menard et al., 2017). Plasticity changes of the low amounts of TGs in seedlings do not seem to be important for the plant. Another reason for the channeling of polyunsaturated FAs out of the plastids might be their negative effect on the efficiency of photosynthesis during heat stress. It has been reported that seedlings of the triple mutant *fad3/7/8*, containing less than 0.1 % 16:3 and 18:3 FA, showed an enhanced efficiency in photosynthesis (Routaboul et al., 2012). A change in the ratio of unsaturated to saturated FAs in thylakoid membranes to gain higher membrane stability could not be detected in our experiments. However, local changes of the saturation level in photosystem II, which might be too small to detect, could occur. In addition, it was proposed that highly unsaturated FAs are targets for highly reactive oxygen species and free radicals, which occur as side products of oxygenic photosynthesis (Routaboul et al., 2012). During heat stress, reactive oxygen species lead to detrimental effects and degradation of highly unsaturated plastidic lipids could protect the cell against reactive oxygen species (Legeret et al., 2016; Sgobba et al., 2015).

3.7 Origin of DG building blocks for TG synthesis after heat treatment

3.7.1 DG building block for TG synthesis after heat treatment is not derived from PC via PDCT and PAH1/PAH2

The plastidic origin of the building blocks for heat-induced TGs has already been confirmed. How these building blocks are channeled into TGs has yet to be clarified. Therefore, the origin of DG, the direct precursor of TG, was further investigated. DG might derive from *de novo* synthesis via the Kennedy pathway, in which they utilize plastidic lipid FAs, which are then channeled into the acyl-CoA pool.

Moreover, DGs for TG synthesis could also be derived from PCs via PDCT. PDCT, encoded by the *ROD1* gene, transfers the phosphocholine headgroup from PC to DG, forming a new molecular species. PDCT was reported to control the major flux through PC in *A. thaliana* TG synthesis under basal conditions (Bates, 2016; Lu et al., 2009). The PDCT enzyme catalyzes the major reaction for transferring 18:1-FA into PC for desaturation and transfers 18:2- and 18:3-FAs into the TG synthesis pathway. In *rod1* 18:2 and 18:3-FA accumulation of seed TGs is reduced by 40 % (Lu et al., 2009).

Another possibility might be that the DG moiety is directly formed through the degradation of galactolipids and that it is not of cytosolic origin.

To address the question as to whether DGs might derive from *de novo* synthesis, transgenic seedlings deficient in PAH1 and PAH1, which synthesize the de-phosphorylation of PA to DG, were analyzed. *Pah1/pah2* displayed no changes compared to the wild type, suggesting that DGs are not synthesized *de novo*. It has been reported that PAH1 and PAH2 are responsible for the eukaryotic pathway of galactolipid synthesis (Nakamura et al., 2009). In the absence of PAH1 and PAH2 other PA phosphohydrolase isozymes seem to produce DGs to fulfill the overall demand for glycerolipids during vegetative and reproductive phases (Nakamura et al., 2009; Nakamura et al., 2007; Pearce and Slabas, 1998; Pierrugues et al., 2001). To that end, it cannot be ruled out that other phosphatidic acid hydrolases are synthesizing DGs *de novo* via the Kennedy pathway.

However, it has been reported that DGs for TG synthesis originate predominantly from PC turnover (Bates et al., 2009). Therefore, plants deficient in the PDCT enzyme, which synthesizes DGs out of PCs, were investigated (Figure 17). However, after heat treatment no differences in TG levels and in the acyl compositions of TG, DG and PC lipid species could be determined in *rod1* seedlings in comparison to the wild type (Figure 17).

In addition to PDCT, DGs and PCs could also be equilibrated via the reversed reaction of CPT (Slack et al., 1983). As previously mentioned, the DG moiety might also be derived directly through the degradation of galactolipids.

3.7.2   DG building block for TG synthesis is provided by heat induced remodeling of MGDGs

Profiling of MGDG metabolites was carried out to test the hypothesis that the DG building blocks for TG synthesis after heat treatment are provided by degradation of galactolipids (Figure 18).

Lipidomic analysis revealed a strong and statistically significant accumulation of acyl-MGDGs and TGDGs. These two degradation products indicate a partial degradation of MGDGs, catalyzed by at least two enzymes: AGAP1 and SFR2.

AGAP1 is phylogenetically conserved and occurs ubiquitously in the plant kingdom. AGAP1, localized in the cytosol and associated to the outer chloroplast envelope, acylates MGDG with a third FA to the galactose head group, thereby forming acyl-MGDG and lyso-MGDGs (Figure 23) (Heemskerk et al., 1986; Nilsson et al., 2015). Microarray data revealed that the *AGAP1* gene is highly expressed in green tissues and is induced after several abiotic treatments, such

as salt stress, osmotic stress, heat stress and phosphorous deficiency (Hruz et al., 2008; Nilsson et al., 2015).

Furthermore, accumulation of acyl-MGDG could be detected after stress conditions, such as freezing stress and inoculation with *P. syringae,* leading to tissue disruption. Plants deficient in AGAP1 appeared to be unaffected by the severe decrease in acyl-MGDGs. Moreover, no defects were perceptible in *agap1* under stress conditions, as a deficiency of AGAP1 does not affect feeding behavior of the generalist herbivore and does not confer reduced HR or a higher susceptibility to *P. syringae* infection*.* However, the highly-conserved stress-induced activation of AGAP1 throughout so many different plant species suggests its significant, yet unknown, biological role (Nilsson et al., 2015). AGAP1 might be activated through heat-associated cell damage. In contrast to acyl-MGDGs, lyso-MGDGs do not accumulate after heat stress (Figure 18). This suggests that lyso-MGDGs are either rapidly re-acylated to MGDG or hydrolyzed and could thereby deliver FAs, which could potentially enter TGs via PCs (Figure 23).

Some plant species showed a decrease in high levels of MGDGs after freeze-thawing, which could not be accounted for in the amount of increase in acyl-MGDGs. In addition to AGAP1, several degradation pathways, which compete for MGDG as a substrate, seem to be triggered after tissue disruption (Nilsson et al., 2015). One of these enzyme, competing with AGAP1 for MGDG as substrate, might be SFR2.

SFR2, a glycosyl hydrolase family1 protein is activated following tissue damage. Deficiency of SFR2 leads to severe damages upon freezing (Fourrier et al., 2008; Thorlby et al., 2004). SFR2 is expressed constitutively and does not appear to be upregulated by various stresses; therefore, SFR2 seems to be post-translationally activated by severe stresses. Similar to AGAP1, SFR2 is ubiquitously present in all plant tissues and is associated with the outer chloroplastic membrane (Barnes et al., 2016). SRF2 transfers a galactosyl residue from MGDG to galactolipids, thereby producing DGDGs, TGDGs and higher oligogalactolipids as well as DGs, the direct precursor for TG synthesis (Figure 23) (Moellering et al., 2010). After heat treatment, a significant increase in TGDG levels could be determined, suggesting an activation of SFR2 following severe heat stress. Oligogalactolipids, produced by SFR2, remain in the plastid membrane, whereas DGs might be discarded from the membrane and can then be used for TG synthesis (Moellering and Benning, 2011).
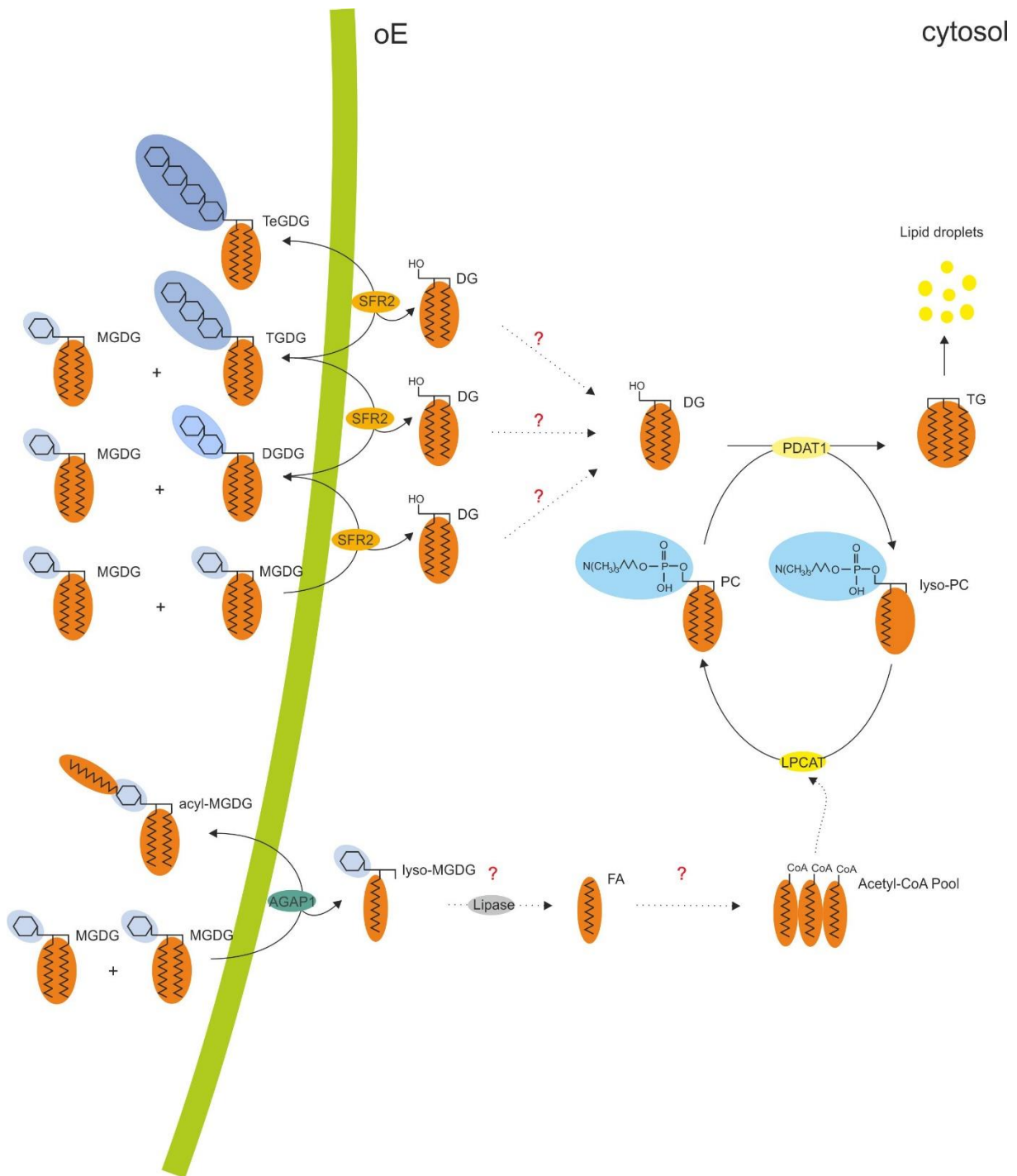
Figure 23. Model for predicted TG accumulation after heat treatment.
SFR2, associated to the outer chloroplast envelope (oE), transfers a galactose head group from a MGDG to a second MGDG, forming DGDG and DG. The galactose head group of MGDG can be also transferred to an oligogalactolipid, i.e. di- or triglalctosyldiacylglycerol (DGDG, TGDG), possessing two or three galactosyl groups, and thereby increasing the number of galactosyl moieties in a processive manner. Oligogalactolipids remain in the plastid membrane, DGs might be discarded from the membrane and can then be used for TG synthesis in the cytosol. AGAP1, associated to the outer chloroplast envelope, acylates MGDG with a third FA to the galactose head group, forming acyl-MGDG and lyso-MGDG. Through a lipase FA may be cleaved from lyso-MGDG, the FA incorporated into the acyl CoA pool, which can then be incorporated into PCs. Following heat TGs are synthesized from DGs and PCs by PDAT in an acyl CoA independent way, yielding LPC, which can be reacylated to form PC by LPCAT.

3.8   Functional significance of heat-induced TGs

3.8.1   Heat-induced TGs confer basal thermotolerance

Heat-induced TGs seems to origin through the degradation of MGDG by AGAP1 and/or SFR2 in the chloroplast. Whether these reactions have a functional significance for the thermotolerance of the plants remains to be investigated.

Heat-induced TGs could be important for the protection against degradation products of plastidic lipids. For example, accumulated DGs, which favor the formation of inverted micellar structures, may introduce small areas with unstable negative curvatures into bilayers, leading to the fusion of apposed membrane bilayers (Gasulla et al., 2013; Goni and Alonso, 1999). Therefore, channeling DGs into TGs might prevent harmful membrane structures. Another possibility is that heat-induced TGs may provide building blocks during recovery to form membrane lipid synthesis or for energy production through FA ß-oxidation.

The thermotolerance of *pdat1* seedlings, unable to induce TG accumulation after heat stress, was tested to analyze the functional significance of heat-induced TGs. After acclimation at 37° C, *pdat1* seedlings could survive a heat shock at 45° C for 90 min like wild type seedlings, suggesting that the genetically encoded HSR is sufficient to acquire thermotolerance. The increase in TG levels is not of functional significance under these conditions.

However, testing the basal thermotolerance in *pdat1* seedlings at 45° C for 90 min without acclimation, differences could be detected compared to the wild type. *Pdat1* seedlings displayed a significantly compromised survival (Figure 20). Lipidome analysis revealed higher levels of DG in *pdat1* seedlings compared to the wild type (Figure 11). If DGs cannot be converted into TGs in *pdat1* seedlings, membrane structures might be damaged, leading to a lower survival rate after heat stress. Treatment of detached leaves with exogenous DGs did not lead to phenotypical changes (Fan et al., 2013a). However, exogenous DGs might not even reach chloroplastic membranes, where they could lead to negative curvatures, but might stick to cell membranes or cuticle.

The survival rate of *tgd1* mutant seedlings compared to the wild type, over accumulating TGs, increased following heat treatment (Figure 20). SFR2 was reported to be constitutively activated in *tgd1* mutant seedlings, leading to an accumulation of oligogalaytosyldiacylglycerols and DGs. In *sfr2.3 tgd1.1* double knockout mutants, the TG content was decreased by around 27 % compared to *tgd1.1,* suggesting that SFR2 contributes

to TG synthesis by providing DGs (Fan et al., 2014). Interestingly, high amounts of DGs did not lead to detrimental phenotypical changes in *tgd1* mutant seedlings.

In addition to determining the survival rate 4 days after heat treatment (45°C, 90 min), the immediate effects on photosynthetic performance were analyzed. *Pdat1* displayed a higher level of damage of PSII, whereas damage in *tgd1* seedlings was shown to be lower compared to the wild type already 1 hour after heat treatment (Figure 21).

Data suggests that lipid remodeling leading to TG accumulation has indeed functional significance regarding the thermotolerance of non-acclimated seedlings that were exposed to severe heat stress. It still needs to be unraveled how lipid metabolism leads to an increase in heat resistance.

3.8.2   Correlation of heat-induced TGs and activation of enzymes involved in MGDG degradation

Heat-induced TG accumulation was determined to originate from the degradation of plastidic lipids. Furthermore, heat-induced TGs were beneficial for non-acclimated seedlings exposed to heat stress that led to severe damages.

Lipidomic analysis revealed an accumulation of MGDG degradation products, acyl-MGDGs and TGDGs, with the degradation being catalyzed by AGAP1 and SFR2. Therefore, activation of AGAP1 and SFR2 might play an important role in the accumulation of heat-induced TGs during heat stress and for conferring thermotolerance after basal heat stress.

Both enzymes, AGAP1 and SFR2, catalyze a net removal of glycerolipids from plastid membranes, thereby reducing the total surface area and accommodating for the shrinkage of the organelle due to loss of water. Since MGDGs do not decrease significantly during heat stress, MGDG degradation does not seem to be important for thermotolerance (Figure 13).

During freezing, and activation of both enzymes, SFR2 and AGAP1, ice crystals form outside of the cell (Moellering et al., 2010; Nilsson et al., 2015). Water is drawn out of the cell until the water potential across the plasma membrane is in equilibrium (Steponkus, 1984; Thomashow, 1999). Therefore, freezing leads to cellular dehydration, which results in damage to membranes through the formation of non-lamellar lipid phases, such as the inverted hexagonal II phase (HII) (Uemura et al., 1995). Mechanisms that maintain lamellar membrane bilayers under a dehydrated state are believed to be essential to survive freezing stress (Steponkus, 1984; Thomashow, 1999; Uemura et al., 1995).

Freezing stress in *A. thaliana* (Moellering et al., 2010) as well as salt and drought stress in tomato (Wang et al., 2016) all lead to cellular dehydration and membrane leakage, resulting in a lower cytosolic pH and an increase in the cytosolic Mg^{2+} concentration.

3.8.2.1   Correlation of heat-induced TGs and activation of SFR2

High concentrations of Mg^{2+} were in turn reported to activate SFR2 (Barnes et al., 2016). After activation, SFR2 converts MGDGs to oligogalactolipids, which display a lower tendency for the transition to HII phase structures. Moreover, the polar surface area of oligogalactolipids gets larger, preventing fusion of apposed bilayers and subsequent membrane damage (Browse, 2010; Moellering and Benning, 2011).

TG accumulation after freezing stress was shown to be partially dependent upon SFR2. Previous experiments revealed a 50 % reduction of TG accumulation in *sfr2* plants compared to wild type plants after freezing stress (Moellering et al., 2010). Another set of experiments showed that after freezing stress *sfr2* does not accumulate TGs containing 16:3 in comparison to treated wild type plants (Barnes et al., 2016). Therefore, DG produced by the activation of SFR2 is further acylated to form TG (Barnes et al., 2016; Moellering and Benning, 2011). These results suggest that SFR2 is involved in DG synthesis as a precursor for heat-induced TGs but that it is not responsible for providing the majority of building blocks for TG synthesis during heat stress. It can be speculated that SFR2 might be important for thermoprotection since *tgd1* mutant seedlings, showing high constitutive oligogalactolipid and TG levels, display a higher basal thermotolerance compared to the wild type. SFR2 might confer thermoprotection due to the high oligogalactolipid levels, which stabilize the membrane during heat stress. Heat-induced TGs seems to be partially synthesized out of DGs, which are formed by SFR2 activity, to prevent membrane damage following DG formation. On the contrary, an increase in oligogalactolipids can be determined in *pdat1* mutant seedlings in comparison to the wild type, which show a reduced basal thermotolerance. High amounts of DGs in *pdat1* might justify the reduction of thermotolerance since the integration of DGs in the membrane can lead to apposed membrane bilayers. High amounts of DGs in *tgd1,* which have an elevated thermotolerance in comparison to the wild type, oppose this theory. However, *tgd1,* displaying constitutively high DG levels, might have already developed other mechanisms to cope with the high DG amounts.

3.8.2.2 Correlation of heat-induced TGs and activation of AGAP

Since SFR2 is not responsible for providing the majority of building blocks for TG synthesis during heat stress, other enzymes involved in the degradation of glycerolipids and channeling of its fatty acyls into TGs need to be investigated. In addition to SFR2, AGAP1 is also activated by severe stresses, such as freezing stress and *P. syringae* inoculation, leading to tissue disruption (Nilsson et al., 2015). AGAP1 activation leads to increases in acyl-MGDGs, which also accumulate during heat stress. Physiological properties of head group acylated lipids have been poorly investigated. An increase in lipophilic surface properties might increase the tendency for membrane fusion. However, since AGAP1 is conserved throughout so many different plant species, its protein products are speculated to play a significant biological role (Nilsson et al., 2015). Furthermore, accumulation of acyl-MGDGs could also be detected in non-lethal abiotic conditions (Vu et al., 2014).

Alternatively, it has been hypothesized that AGAP1 might act as a phospholipase, releasing FA for signaling purposes. AGAP1 activation might occur incidentally during stress. De-esterification of polyunsaturated FA might occur following severe stresses, cell disruption and membrane damage, leading to lipid stress (Farmer and Mueller, 2013; Nilsson et al., 2015). AGAP1 might act to sequester these FA before they are released from galactolipids and to help in the protection from FFA damages, which can disrupt cell homeostasis (Nilsson et al., 2015). Each round of head group acylation produces a lyso-MGDG. It can be speculated that lyso-MGDGs are cleaved by a lipase to provide FAs, which could then be integrated into the acyl-CoA pool, followed by their incorporation into PCs for DG and TG synthesis. Physiochemical properties of lyso-MGDGs are poorly understood. As lyso-MGDGs are not detected after heat stress, their negative influence on membrane properties is unlikely.

However, beside a possible thermoprotective role of AGAP1 and SFR2, heat-induced TG accumulation might simply be required for avoiding negative effects of MGDG degradation products. Lipid remodeling by SFR2 and AGAP1 could potentially be an extremely fast mechanism to change the membrane composition to a more heat-resistant and saturated state. However, this appears not to take place to a significant extent as MGDG levels do not change after short-term heat exposure.

Therefore it seems more likely that TGs accumulating during heat stress are beneficial during the recovery from heat stress, through degradation of TGs and provision of building blocks for membrane lipid synthesis or energy production through FA ß-oxidation.

3.9   Outlook

To this end, it has yet to be fully understood whether SFR2 and AGAP1 play a thermoprotective role or if heat-induced TGs are intermediates in the ß-oxidation of FAs, which are important for metabolism during heat stress and/or recovery. The question whether AGAP1- or SFR2-induced membrane remodeling or AGAP1 and SFR2 products have a positive effect on thermotolerance of *A. thaliana* could be addressed by investigating plants deficient in those enzymes. Survival assays and the effects on photosynthetic performance after heat stress for mutant seedling should be carried out to test whether they confer thermotolerance after basal heat treatment.

Lipase activity for TG degradation should be investigated to determine whether TGs are important storage molecules in metabolism during and after heat stress. If TGs are indeed important for the maintenance of metabolism, a decrease in heat-induced degradation should lead to a lower thermotolerance. Therefore, the lipidome of mutant plants deficient in SDP1, a lipase that controls fat storage breakdown, should be analyzed during heat stress and during the recovery phase (Eastmond, 2006). Higher levels of TGs during and after heat stress in *sdp1* seedlings would suggest a fast degradation of TG molecules after heat stress, proposing an involvement of TGs in heat recovery. Furthermore, survival assays after heat stress should be carried out to investigate whether TG degradation during the recovery phase is important for conveying thermotolerance.

4 Material and Methods

4.1 Material

4.1.1 Chemicals

Table 1. Chemicals

Name	Manufacturer
2-Propanol "LC-MS grade"	Biosolve, Dieuze (FR)
Acetonitrile "gradient grade"	Biosolve, Dieuze (FR)
Acetonitrile "LC-MS grade"	Biosolve, Dieuze, FR
Ammonium acetate	Biosolve, Dieuze (FR)
Butylated Hyrdoxytoluene	Sigma Aldrich, St. Louis (USA)
Chloroform normapur	VWR, Radnor (USA)
Diethylether	Merck, Darmstadt
Ethylenediaminetetraacetic acid (EDTA)	AppliChem, Darmstadt
Formic acid 99 %	LC-MS Biosolve, Dieuze (FR)
Hydroxyethyl piperazineethanesulfonic acid (HEPES)	Sigma Aldrich, St. Louis (USA)
Hydrochloric acid conc.	AppliChem, Darmstadt
Potassium hydroxide	AppliChem, Darmstadt
Magnesium chloride	AppliChem, Darmstadt
Magnesium sulfate	AppliChem, Darmstadt
Mannitol	Merck, Darmstadt
Methanol „gradient grade"	Merck, Darmstadt
Murashige and Skoog medium incl. MES buffer	Duchefa Biochemie, Haarlem, (NL)
myo-Inositol	Merck, Darmstadt
Phyto agar	Duchefa Biochemie, Haarlem (NL)
Potassium hydroxide	Roth, Karlsruhe
Sepra Silica (50 µm, 65 A)	Phenomenex, Aschaffenburg
Sodium bicarbonate	AppliChem, Darmstadt
Sodium chloride	Sigma Aldrich, St. Louis (USA)
Sorbitol	AppliChem, Darmstadt
Tween 21 [10 %-ig]	AppliChem, Darmstadt
Vitamin B5 pantothenic acid	Roth, Karlsruhe

4.1.2   Consumables

Table 2. Consumables

Name	Manufacturer
Cell culture dishes	Greiner, Frickenhausen
Pipette tips	Sarstedt, Nürnberg
Reaction tube (1.5 ml)	Sarstedt, Nürnberg
Reaction tube (2 ml)	Sarstedt, Nürnberg
Screw cap reaction tubes (2 ml)	Hartenstein, Würzburg
UPLC-Vials	Chromacol, Herts (UK)
Zirconium balls	Retsch, Haan

4.1.3   Devices

Table 3. Devices

Device	Manufacturer
- 20° C Freezer "Comfort"	Liebherr, Ochsenhausen
- 80° C Freezer "Ultra Low Temperature"	New Brunswick Scientific, Edison
Analytical balance "XPE 205 Delta Range"	Mettler Toledo, Columbus (USA)
Autoclave "5075ELV"	Tuttnauer Syter, New York (USA)
Ball mill "MM400"	Retsch, Haan
Centrifuge "541712"	Eppendorf, Hamburg
Climate cabinet "Binder"	Tuttlingen
Climate cabinet "Percival AR-36 L"	CLF-Laborgeräte, Hamburg
Climate cabinet "Percival AR-66 L"	CLF-Laborgeräte, Hamburg
Cross flow sterile bench	Ceag Envirco, Dortmund
Imaging-PAM *M-Series* Chlorophyll Fluorometer	Heinz Walz GmbH, Effeltrich
pH-meter "inolab 720"	Hartenstein, Sarsted
Quadrupole Time-of-flight mass spectrometer "SYNAPT HDMS G2"	Waters, Milford (USA)
Quattro Premier™ Triple Quadrupol Massenspektrometer	Waters, Milford (USA)

Single-channel pipettes (100-1000 µl, 10-100 µl, 0.5-10 µl) "Research variable"   Eppendorf, Hamburg

Ultrapure water system "RiOs Water Purification System"   Millipore, Billerica (USA)

Ultrasonic bath   VWR, Darmstadt

UPLC "Acquity Ultra Performance LC"   Waters, Milford (USA)

Vacuum rotary evaporator "RVC 2-18"   Christ, Osterode

Vortex shaker "Vortex Genie 2"   Scientific Industries, Bohemia (USA)

4.1.4   Solutions

Table 4. Solutions

Name	Composition
Bleach solution:	15 ml Sodium bicarbonate
	5 ml Millipore water
	10 µl 10 % Tween 21
Chloroplast isolation buffer:	20 mM HEPES KOH, pH 8.0
	5 mM EDTA
	5 mM $MgCl_2 \times H_2O$
	10 mM $NaHCO_3$
	0.3 M Sorbitol
Chloroplast isolation wash buffer:	50 mM HEPES KOH, pH 8.0
	3 mM $MgSO_4 \times H_2O$
	0.3 M Sorbitol
Chloroplast isolation gradient mix:	25 mM HEPES KOH, pH 8.0
	10 mM Sorbitol

4.1.5   Media

Table 5. Media

Name	Composition
<u>Murashige & Skoog Media:</u>	4.8 g * l^{-1} Basal salt mixture incl. MES Buffer
	1.0 ml * l^{-1} Vitamin B_5
	0.1 g * l^{-1} *myo*-Inositol
	30 g * l^{-1} Sugar
	12 g * l^{-1} Phytoagar

4.1.6   Internal Standards

Table 6. Internal Standards

Name	Composition
D2-Trehalose	Sigma Aldrich, St. Louis (USA)
DG 30:0	Avanti Polar Lipids, Alabster (USA)
DGDG 36:0	Matreya LLC, State College (USA)
FA 17:0	Roth, Karlsruhe
MGDG 36:0	Matreya LLC, State College (USA)
PA 34:0	Sigma Aldrich, St. Louis (USA)
PC 34:0	Sigma Aldrich, St. Louis (USA)
PE 34:0	Sigma Aldrich, St. Louis (USA)
TG 30:0	Larodan, Solna (Sweden)

4.1.7   Plant material

Arabidopsis thaliana ecotypes "Columbia 0" and "Wassilevskija" were used as wild type plants. The mutant line *gols1* was kindly provided by Schoeffel (Panikulangara et al., 2004) the *hsfa1abde* quadruple mutant was kindly provided by Ohama and Yamaguchi-Shinozaki (Yoshida et al., 2011). The mutant lines *tgd1.1, dgat1.1* and *pdat1a* (pdat1-2, SALK_065334) were kindly provided by Xu (Fan et al., 2013b; Xu et al., 2003), *pah1, pah2, pah1pah2* by Ohta (Nakamura et al., 2009) and *rod1* by Browse (Lu et al., 2009). Additionally, *pdat1b* (SALK_032261C), *fad3.2* (LK70), *fad7.1/8.1* (SH1) were used (Browse et al., 1993; McConn et al., 1994).

4.2   Methods

4.2.1   Growth conditions

Seeds of *A. thaliana* were sterilized with 1 ml ethanol for 2 min and 1 ml bleaching solution for 20 min (Table 4). Afterwards, seeds were washed in 1 ml H_2O, five times in succession. The seeds (100 per plate) were grown on agar plates containing Murashige & Skoog (MS) medium (Table 5). Seedlings were grown in a growth chamber under an 8 h/16 h short day cycle at 22° C and 160 µE for 2 weeks.

4.2.2   Heat treatment and abiotic stress treatments

For heat treatment, plants grown on agar plates (100 seeds per plate) were transferred 2 h after the onset of light to a growth chamber set at the temperature and time indicated. For investigation of basal thermotolerance and Pulse-Amplitude-Modulation (PAM) fluorometry, shoots of the seedlings were heated at 45° C for 90 min, while roots were cooled to 40° C. For phenotypical analysis, plants were transferred back to growth conditions (8 h / 16 h short-day cycle at 22° C) and plants were classified into the categories alive and dead after 4 days. For metabolite extraction, all seedlings grown on a plate were harvested following heat treatment, immediately shock frozen with liquid nitrogen, and stored at −80° C until extraction. In particular, to determine the temperature dependency of the sugar and TG markers, seedlings were transferred to liquid Murashige & Skoog media (Table 5) 1 day before the treatment. Eight seedlings were pooled and heated at different temperatures (22–50°C) in a heating block for 2 h.

For cold treatment, plates were transferred to a refrigerator set to 4° C for 2 h. For high light treatment, seedlings were irradiated with high quantum flux density (650 µE/m²s) for 2 h. Dehydration treatments were carried out by gently removing the plants were gently from the plates and allowed to dry on whatman paper for 2 h. For osmotic and salt stress, seedlings were transferred to liquid Murashige & Skoog media 1 day before the experiment. Seedlings (eight seedlings were pooled) were treated with 125 mM sodium chloride, 250 mM mannitol or water as a control for 2 h.

4.2.3   Pulse-Amplitude-Modulation (PAM) fluorometry

In addition to phenotypical analysis, survival of seedlings after heat stress was measured by photosynthetic activity of plants. One leaf (cotyledon) from ten seedlings per genotype were measured after control (22° C) and heat treatment. PAM fluorometry was carried out after a one hour recovery phase at 22° C. An Imaging PAM M-Series MAXI version was used, generating amplitude modulated light, for capturing fluorescence, which is produced by chlorophyll during photosynthesis. For determining the quantum yield in photosystem II during conversion of light quantum into energy, Fv/Fm was calculated after 10 minutes of dark adaption:

$$Fv/Fm = \frac{Fm-Fo}{Fm} \; ,$$

where Fm corresponds to the maximal fluorescence yield, being reached after dark adaptation, Fo equals the minimal fluorescence yield, after all pigment complexes are open due to dark adaptation, Fv (variable fluorescence) represents the difference between maximal and minimal fluorescence yield.

4.2.4   Metabolite analysis

4.2.4.1   Modified Bligh and Dyer extraction

For metabolite extraction, seedlings (100 mg, pooled from one plate) were shock-frozen in liquid nitrogen. Both hydrophilic and hydrophobic metabolites were extracted with 600 µl of chloroform/methanol/water (3:2:1, v/v) modified according to Bligh and Dyer (Bligh and Dyer, 1959). Seedlings were homogenized by adding a zirconia ball and using a ball mill at 21 Hz for

10 min. The organic phase, used for lipid analysis, was evaporated in a vacuum concentrator at 40°C and the residue was resuspended in 100 µl isopropanol.

4.2.4.2   Lipidome analysis

The organic phase was analyzed using an ACQUITY UPLC system coupled to a Synapt G2 HDMS qTOF-MS (all Waters, Eschborn, Germany). For chromatographic separation a BEH C18 column (2.1 × 100 mm, 1.7 µm; Waters) at 60 ° C was used. A linear binary solvent gradient was applied using 30–100 % eluent B over 10 min at a flow rate of 0.3 ml/min. Eluent A consisted of 60:40 water/acetonitrile with 10 mM ammounium acetate and eluent B consisted of 90:10 isopropanol/acetonitrile with 10 mM ammonium acetate.

After chromatographic separation, lipophilic metabolites were ionized with an electrospray ionization (ESI) source operated in positive and negative modes and detected with a time-of-flight mass spectrometer. The ESI capillary voltage was set to 0.8 kV and nitrogen (at 350° C, flow rate of 800 l/h) was used as desolvation gas. The quadrupole was operated in a wide-band RF mode, and data was acquired over the mass range of 50–1200 Da. Two discrete and independent interleaved acquisition functions were automatically created. The first function collected the low energy data where molecule ions were acquired while the second function collected the fragments of the molecule ion (high energy data) by using a collision energy ramp from 15 to 35 eV (MSE). MassLynx, MarkerLynx, and QuanLynx (version 4.1; all Waters) were used to acquire and process chromatograms.

For the targeted analysis of the defined lipid species, TG 30:0 (0.24 µg/sample), PE 34:0, PC 34:0, MGDG 36:0, DGDG 36:0, and DG 20:0 (2.4 µg/sample) were used as IS for each lipid class. For semi-quantitative analysis, peak areas of the analytes and ISs were determined in the extracted total ion chromatogram and lipid concentrations were calculated by using a response factor of 1 for each analyte/IS pair. The retention time (RT) and mass-to-charge ratios (m/z) of the lipids are included in Table 7.

Table 7. m/z and RT of TG, DG, PA, PC, MGDG, DGDG

	m/z	RT [min]
Triacylglycerol (TG) species ionized as [M+ NH$_4^+$]$^+$		
TG 30:0 (IS)	572.488	7.65
TG 46:2	792.706	9.51
TG 46:3	790.691	9.34
TG 48:1	822.754	9.85
TG 48:2	820.739	9.70
TG 48:3	818.724	9.54
TG 50:1	850.789	10.01
TG 50:2	848.772	9.86
TG 50:3	846.757	9.71
TG 50:4	844.740	9.54
TG 50:5	842.727	9.38
TG 50:6	840.710	9.22
TG 50:7	838.694	9.03
TG 50:8	836.676	8.84
TG 50:9	834.664	8.65
TG 52:1	878.813	10.15
TG 52:2	876.804	10.01
TG 52:3	874.790	9.86
TG 52:4	872.774	9.71
TG 52:5	870.756	9.56
TG 52:6	868.741	9.41
TG 52:7	866.727	9.22
TG 52:8	864.711	9.05
TG 52:9	862.693	8.86
TG 54:1	906.846	10.28
TG 54:2	904.834	10.14
TG 54:3	902.820	10.01
TG 54:4	900.805	9.86
TG 54:5	898.788	9.71
TG 54:6	896.773	9.56
TG 54:7	894.757	9.40
TG 54:8	892.741	9.24
TG 54:9	890.726	9.07
TG 56:1	934.881	10.41
TG 56:2	932.866	10.28
TG 56:3	930.850	10.13
TG 56:4	928.836	10.01
TG 56:5	926.821	9.86
TG 56:6	924.804	9.73
TG 56:7	922.789	9.59

TG 56:8	920.773	9.43
TG 58:1	962.910	10.52
TG 58:2	960.895	10.40
TG 58:3	958.883	10.26
TG 58:4	956.867	10.14
TG 58:5	954.854	10.02
TG 58:6	952.837	9.89
TG 58:7	950.820	9.75
TG 58:8	948.805	9.59
TG 60:2	988.929	10.50
TG 60:3	986.910	10.38
TG 60:4	984.900	10.26
TG 60:5	982.883	10.15
TG 60:6	980.866	10.03
TG 60:7	978.849	9.92
TG 62:4	1012.931	10.39
Diacylglycerol (DG) species ionized as [M+ NH$_4^+$]$^+$		
DG 34:0 (IS)	614.572	8.63
DG 34:1	612.557	8.32
DG 34:2	610.541	8.04
DG 34:3	608.525	7.77
DG 34:4	606.510	7.59
DG 34:5	604.494	7.19
DG 34:6	602.478	6.74
DG 36:0	642.604	8.90
DG 36:2	638.572	8.36
DG 36:3	636.557	8.07
DG 36:4	634.541	7.77
DG 36:5	632.525	7.48
DG 36:6	630.510	7.17
Phosphatidic acid (PA) species ionized as [M+ NH$_4^+$]$^+$		
PA 34:0 (IS)	694.538	8.73
PA 34:1	692.523	7.64
PA 34:2	690.507	7.25
PA 34:3	688.491	6.76
PA 34:4	688.476	6.45
PA 34:6	682.444	6.26
PA 36:1	720.554	8.03
PA 36:2	718.538	7.69
PA 36:3	716.523	7.36
PA 36:4	714.507	7.05
PA 36:5	712.491	6.63
PA 36:6	710.476	6.26

Phosphatidylethanolamine (PE) species ionized as [M+H$^+$]$^+$		
PE 34:0 (IS)	720.554	8.03
PE 34:1	718.539	7.69
PE 34:2	716.523	7.36
PE 34:3	714.507	7.05
PE 34:4	712.492	6.63
PE 34:5	710.476	6.27
PE 34:6	708.460	5.88
PE 36:1	746.570	8.06
PE 36:2	744.554	7.77
PE 36:3	742.539	7.40
PE 36:4	740.523	7.07
PE 36:5	738.507	6.72
PE 36:6	736.492	6.37
Phosphatidylcholines (PC) species ionized as [M+H$^+$]$^+$		
PC 34:0 (IS)	762.601	7.96
PC 34:1	760.586	7.61
PC 34:2	758.570	7.27
PC 34:3	756.554	6.95
PC 34:4	754.539	6.53
PC 34:5	752.523	6.15
PC 34:6	750.507	5.76
PC 36:0	790.633	8.31
PC 36:1	788.617	7.99
PC 36:2	786.601	7.64
PC 36:3	784.586	7.31
PC 36:4	782.570	6.97
PC 36:5	780.554	6.62
PC 36:6	778.539	6.26
Monogalactosyldiacylglycerol (MGDG) species ionized as [M+NH$_4$$^+$]$^+$		
MGDG 36:0 (IS)	804.657	8.53
MGDG 34:0	776.625	8.21
MGDG 34:1	774.610	7.88
MGDG 34:2	772.594	7.57
MGDG 34:3	770.578	7.27
MGDG 34:4	768.563	6.88
MGDG 34:5	766.547	6.52
MGDG 34:6	764.531	6.15
MGDG 36:2	800.625	7.97
MGDG 36:4	796.594	7.29
MGDG 36:5	794.578	6.96
MGDG 36:6	792.563	6.62

Digalactosyldiacylglycerol (DGDG) species ionized as [M+NH$_4^+$]$^+$		
DGDG 36:0 (IS)	966.710	8.29
DGDG 34:0	938.670	7.92
DGDG 34:1	936.662	7.59
DGDG 34:2	934.647	7.26
DGDG 34:3	932.631	6.95
DGDG 34:4	930.615	6.53
DGDG 34:5	928.560	6.15
DGDG 34:6	926.594	5.77
DGDG 36:2	962.678	7.67
DGDG 36:3	960.663	7.40
DGDG 36:4	958.647	6.98
DGDG 36:5	956.631	6.62
DGDG 36:6	954.615	6.26

4.2.4.3   Carbohydrate analysis

The aqueous phase was used for the analysis of hydrophilic metabolites. Chromatographic separation was performed on a BEH amide column (1.7 μm, 2.1 × 100 mm; Waters) according to the Waters application note WA60126 with modifications. Briefly, elution was performed using a linear solvent-strength gradient (0.2 ml/min at 35° C) from 75 % to 45 % acetonitrile containing 1 % ammonium hydroxide in 10 min.

For the determination of raffinose and galactinol in the aqueous phases, multiple reaction monitoring (MRM) was applied using a Waters Micromass Quattro Premier triple quadrupole MS coupled to UPLC (Waters). The operational parameter of chromatographic separation was identical to the ones used for the untargeted metabolite analysis. The sugars were ionized in an ESI source, operated in the negative mode. The collision-induced dissociation of each compound for MRM was performed using argon as collision gas with a flow rate of 0.3 ml/min and a pressure of 3.0×10^{-3} mbar. The following MRM transitions were monitored: m/z 503→179 at a retention time (RT) of 5.33 min for raffinose, m/z 341→179 at RT of 5.79 min for galactinol, and m/z 343→180 at RT of 4.51 min for D2-trehalose (IS).

4.2.4.4   FA analysis

For total FA analysis, 100 mg seedlings of one agar plate were shock frozen in liquid nitrogen and extracted with 500 μl of isopropanol containing 10 % potassium hydroxide and 5 μg heptadecanoic acid. For homogenization a zirconia ball was added and seedlings were ground using in a ball mill (21 Hz, 10 min). The supernatant was incubated at 60° C for 1 h in a water

bath. The samples were centrifuged and the pH of the supernatant was adjusted to 6. Afterwards the samples were analyzed by UPLC–qTOF-MS in the negative ESI mode. Therefore a BEH C18 column (1.7 µm, 2.1 × 100 mm; Waters) was used at 40° C. A linear binary solvent gradient was applied using 50–100 % eluent B over 10 min at a flow rate of 0.3 ml/min. Eluent A consisted of water acidified with 0.1 % formic acid and eluent B was acetonitrile. The RTs of the FAs, mass-to-charge ratios (m/z), and response factors are included in Table 8.

Table 8. m/z and RT of FAs

Fatty acids (FAs) ionized as [M-H$^+$]$^-$		
	m/z	RT [min]
C17:0 (IS)	269.2481	9.93
C16:0	255.2324	9.02
C16:1	253.2168	7.58
C16:2	251.2011	6.57
C16:3	249.1855	5.52
C18:0	283.2637	10.34
C18:1	281.2481	9.20
C18:2	279.2324	8.07
C18:3	277.2168	7.04

4.2.4.5   FA analysis after solid phase extraction

For analyzing FAs compositions of different lipid classes, a separation of lipid classes through solid phase extraction (SPE) was carried out. Afterwards, the separated lipid classes were hydrolyzed to measure the FA compositions (see 4.2.4.4). After total lipid extraction with modified Bligh and Dyer extraction (4.2.4.1) the lower evaporated lipophilic phase was resolved in 80 µl of chloroform. For separation, a vertical glass column was filled with 500 mg silica gel and equilibrated with two column volumes (5 ml) of methanol and chloroform each. Afterwards, lipids were separated with solvents as follows (Table 9):

Table 9. Elution scheme solid-phase-extraction

Column Volumes	Solvent	Lipids
2	chloroform	neutral lipids (TGs, DGs)
2	diethyl ether	DGs
3	acetone	DGDG
3	acetone/methanol (9.9/0.1)	DGDG
3	acetone/methanol (9.75/0.25)	-
3	acetone/methanol (9.5/0.5)	MGDG
3	acetone/methanol (9.25/0.75)	MGDG
3	methanol	phospholipids

Fractions were mixed, and divided in half and evaporated. One half of each fraction was resuspended in 50 µl of isopropanol and centrifuged at 14.000 rpm. Afterwards lipids of the fractions were measured according to 4.2.4.1 to determine if lipids were clearly separated. The other half of each fraction was hydrolyzed to analyze the FA compositions of the lipid classes. Fractions which contained the same lipid class were combined. The samples were hydrolyzed and measured as described in 4.2.4.2.

4.2.5 Chloroplast isolation

For chloroplast isolation, 3-week-old *A. thaliana* seedlings, grown on Murashige & Skoog agar containing 1 % sucrose, were used. The chloroplasts were isolated according to (Aronsson and Jarvis, 2002). All steps were carried out at 4° C or on ice. Plants were transferred into 15 ml of isolation buffer, ground, and filtered three times in succession. The lysate was centrifuged at 950 g for 4 min and the pellet was gently resuspended in 2 ml of isolation buffer. The sample was then layered on top of a discontinuous Percoll gradient (75 % Percoll in gradient mix, 28 % Percoll in gradient mix with 14 % water) and centrifuged at 1300 g for 6 min. Afterwards the green chloroplast-rich fraction that appeared at the interface of the Percoll layers was collected, diluted with 10 ml wash buffer and centrifuged at 950 g for 4 min to remove the Percoll. Chloroplasts were resuspended in 200 µl wash buffer. Lipid extraction and measurement of lipids with a UPLC-qTOF was carried out as explained in 4.2.4.2.

4.2.6 Fluorescence microscopy

To confirm the localization of TG accumulation after heat stress *Arabidopsis* cell culture was analyzed with fluorescence microscopy. Cell culture was incubated on a shaker at 45° C for 2

h and at 22° C as control. After heat treatment cells were diluted 1:100 with Nile Red stain and incubated for 15 minutes. Afterwards fresh medium was applied. Nile Red is used to localize and quantitate lipids, in particular neutral lipid droplets in the cells and has an excitation/emission maxima of ~552/636 nm in methanol. Samples were then analyzed using a Zeiss Elyra S.1 SIM Super Resolution Microscope with a PCO Edge 5.5 sCMOS-Camera system. Chlorophyll fluoresces at 620 nm when excited with a 642 nm laser and a filter with cut-out wavelength above 655 nm was used. Nile Red fluoresces at 560 and 600 nm when excited with a 488 nm laser and a filter with cut-out wavelength from 570 - 620 nm was used.

5   List of Abbreviations

°C	Centigrade
ACC	Acetyl-CoA carboxylase
ACN	Acetonitirile
ACP	Acyl carrier protein
AGAP1	Acylated Galactolipid Associated Phospholipase1
APX	Ascorbate Peroxidase
ARP	Actin related protein
At	Arabidopsis thaliana
CPT	CDP-choline:diacylglycerol cholinephosphotransferase
DG	Diacylglycerol
DGAT	Diacylglycerol-Acyltransferase
DGDG	Digalactosyldiacylglycerols
ER	Endoplasmic reticulum
FA	Fatty acid
FAD	Fatty acid desaturase
FAE	Fatty acid elongase
FAS	Fatty acid synthesis
FW	Fresh Weight
G3P	Glycerol 3-phosphate
GGGT	Galactolipid:galactolipid galactosyltransferase
GPAT	G3P-Acyltransferase
GABA	γ-aminobutyric acid
GolS	Galactinol Synthase
HSF	Heat shock transcriptionfactor
HSP	Heat shock protein
HSR	Heat shock response
IS	Internal standard
kDa	kilo Dalton
LPAAT	Lysophosphatidic acid acyltransferase
LPC	lysophosphatidylcholine
LPCAT	acyl-CoA:lysophophatidylcholine acyltransferase

LPC	Lysophosphatidylcholine
μ	Mikro
MCMT	Malonyltransferase
MGDG	Monogalactosyldiacylglycerols
MGlcDG	Monoglucosyldiacylglycerol
min	Minutes
nd	not detected
PA	Phosphatidic acid
PAH	Phosphatidic acid hydrolase
PAP	Phosphatidic acid phosphatase
PC	Phosphatidylcholine
PDAT	Phospolipid:Diacylglycerol Acyltransferase 1
PDCT	Phosphatidylcholine diacylglycerol cholinephosphotransferase
PE	Phosphatidylethanolamine
PES	Phytyl ester synthase
PLC	Phospholipase C
PLD	Phospholipase D
PUFA	Polyunsaturated fatty acid
ROD1	Reduced Oleate Desaturation1
SD	Standard deviation
SFR2	Sensitive to freezing2
TG	Triacylglycerol
TGD1	Trigalactosyldiacylglycerol1
TGDG	Trigalactosyldiacylglycerol
UDP-Gal	Uridine diphosphate galactose
UPR	Unfolded protein response

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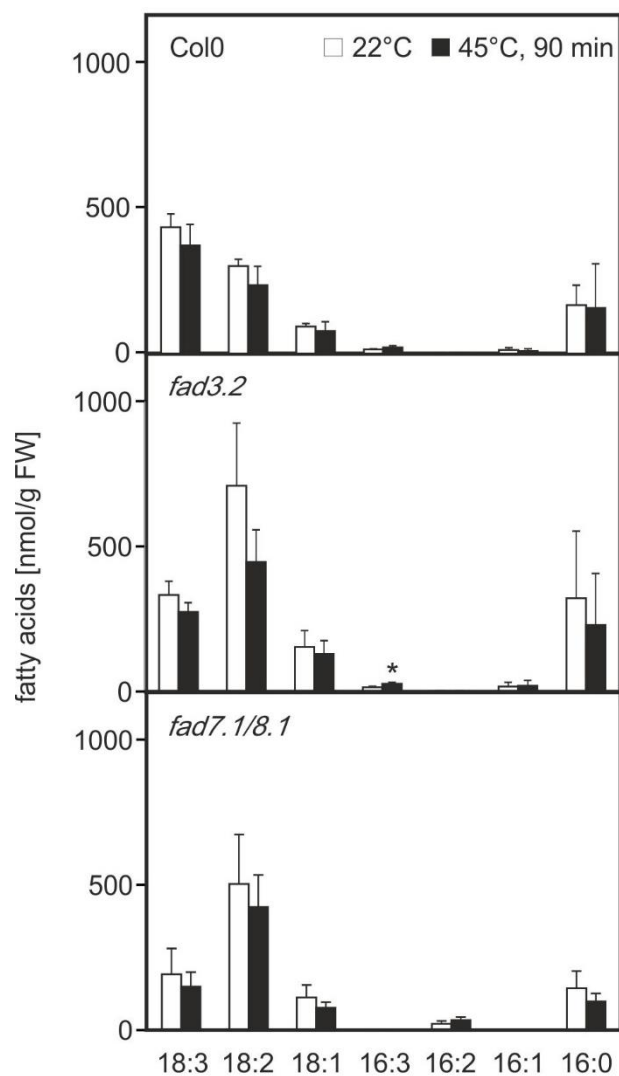
Figure S 1. FA levels in phospholipids in wild type, *fad7.1/8.1* and *fad3.2* seedlings.

14-day-old seedlings were kept at 22° C (white bars) and or treated with a heat shock (45° C for 90 min, black bars). Thereafter, phospholipids were separated, hydrolyzed and FAs were determined. Data represent means ± SD, n = 4. Statistical significant changes of FA levels (p<0.05) are indicated by asterisks.
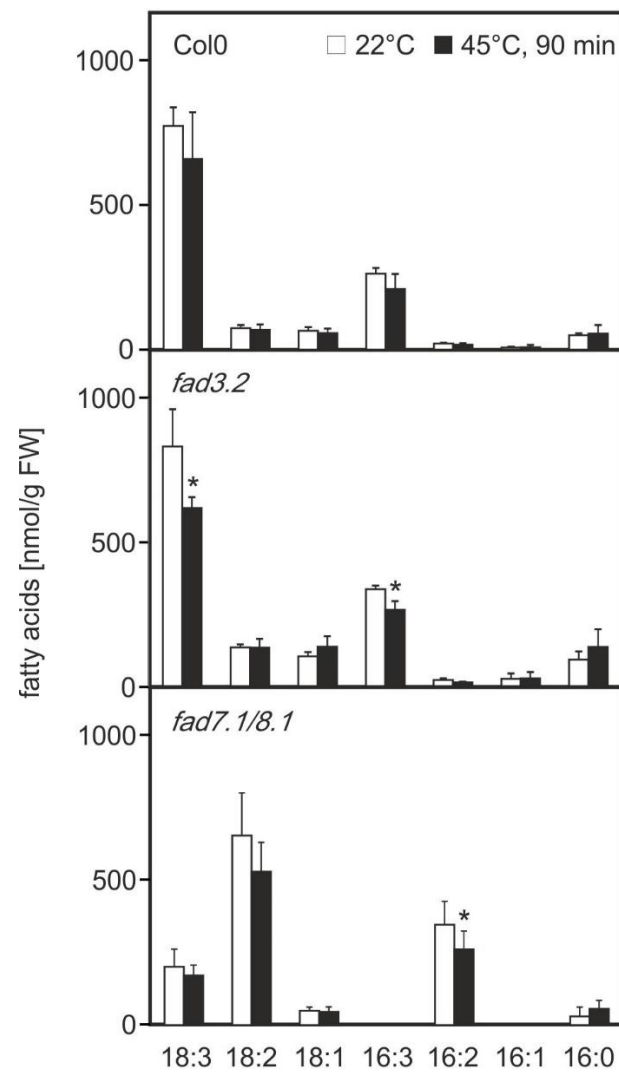
Figure S 2. FA levels in galactolipids in wild type, *fad7.1/8.1* and *fad3.2* seedlings.

14-day-old seedlings were kept at 22° C (white bars) and or treated with a heat shock (45° C for 90 min, black bars). Thereafter, galactolipids were separated, hydrolyzed and FAs were determined. Data represent means ± SD, n = 4. Statistical significant changes of FA levels (p<0.05) are indicated by asterisks.
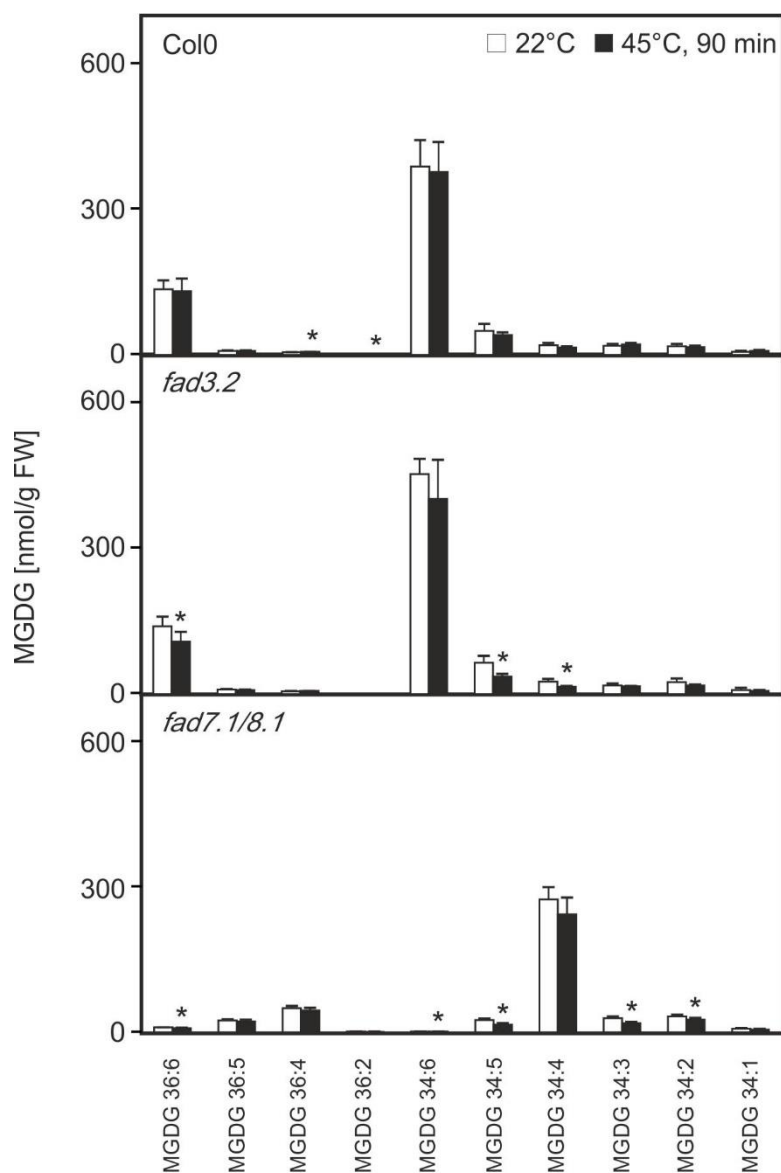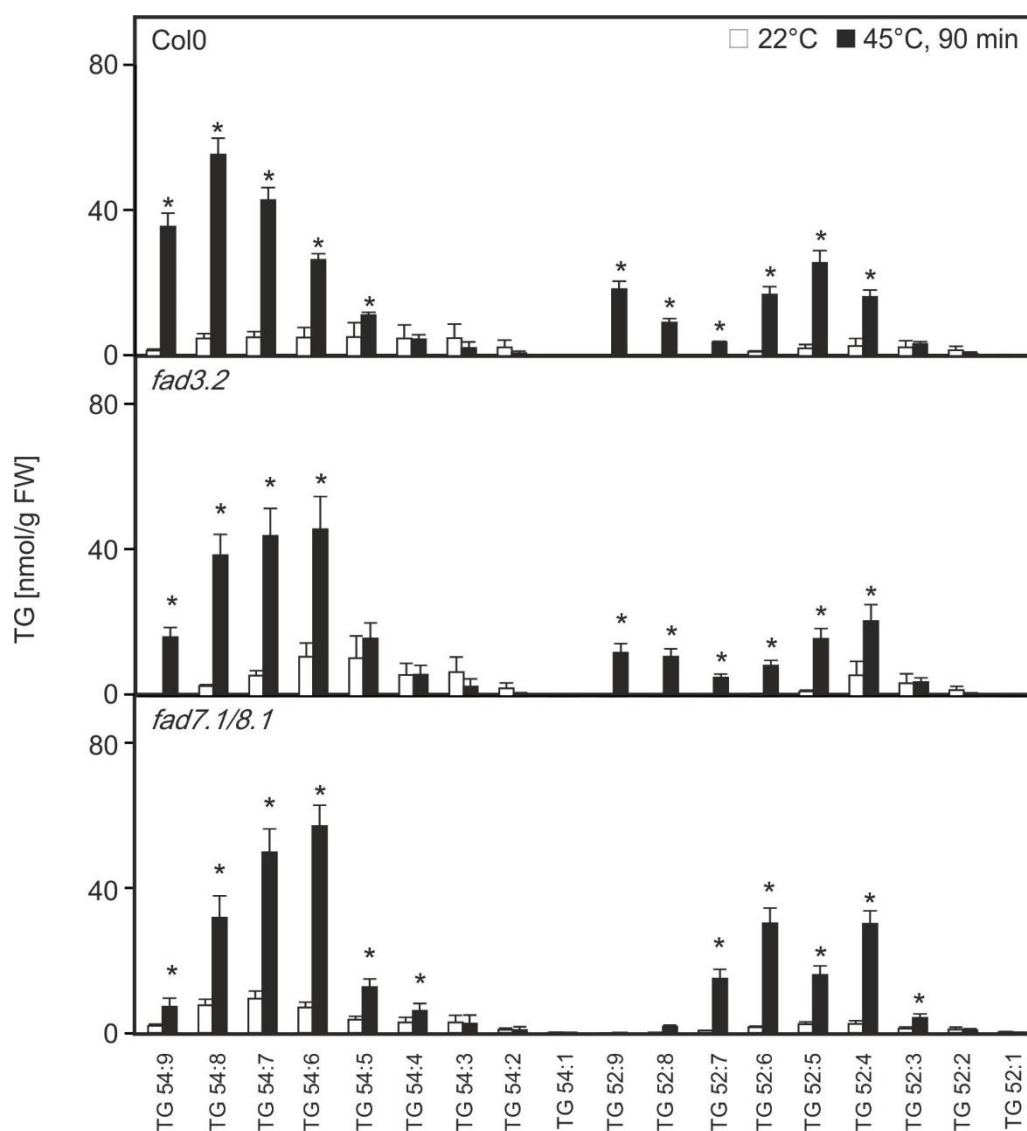
Figure S 3. Heat-induced changes of MGDGs in wild type, *fad3.2* and *fad7.1/8.1* seedlings.
Levels of lipids were determined in 14-day-old wild type, *fad3.2* and *fad7.1/8.1* seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid levels ($p < 0.05$) are indicated by asterisks.
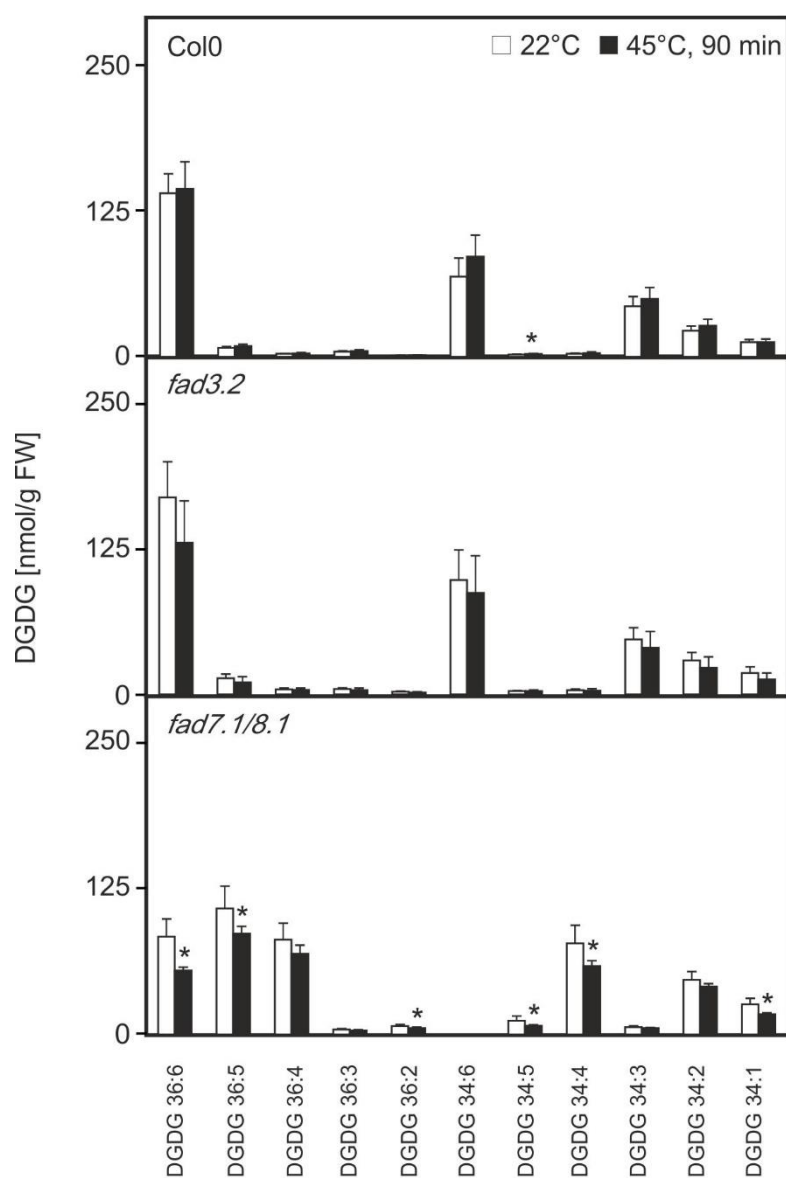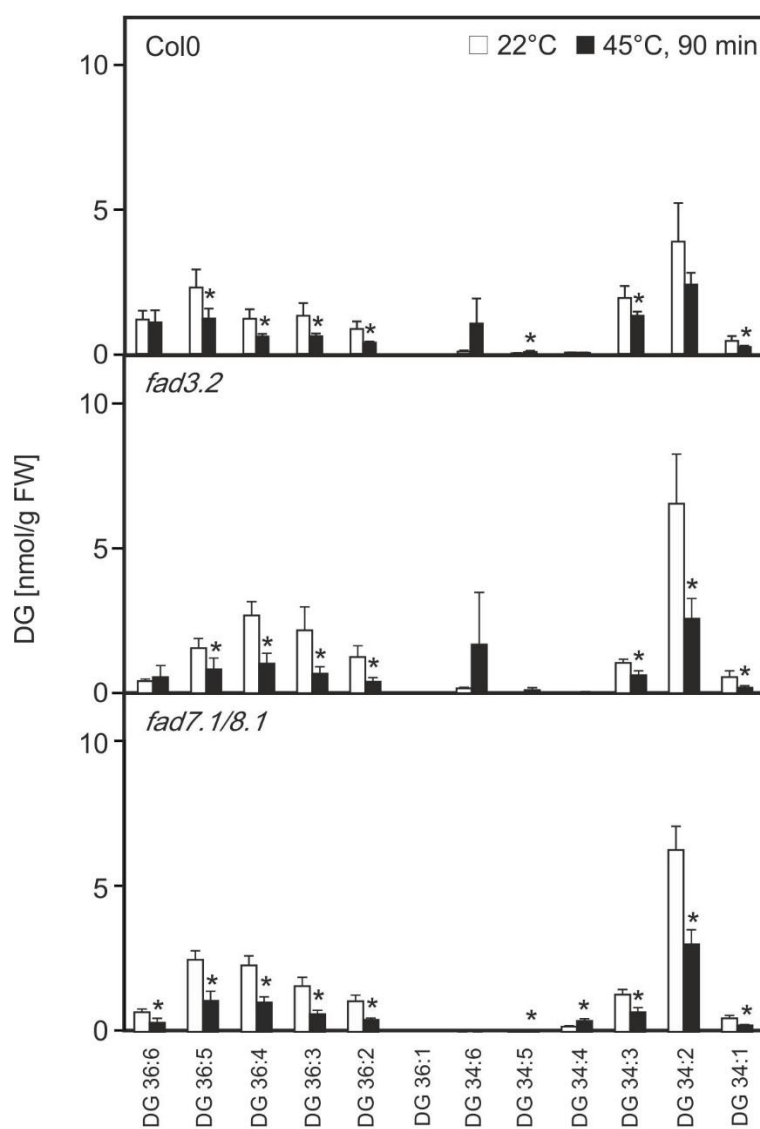
Figure S 4. Heat-induced changes of DGDGs in wild type, *fad3.2* and *fad7.1/8.1* seedlings.
Levels of lipids were determined in 14-day-old wild type, *fad3.2* and *fad7.1/8.1* seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid levels (p<0.05) are indicated by asterisks.
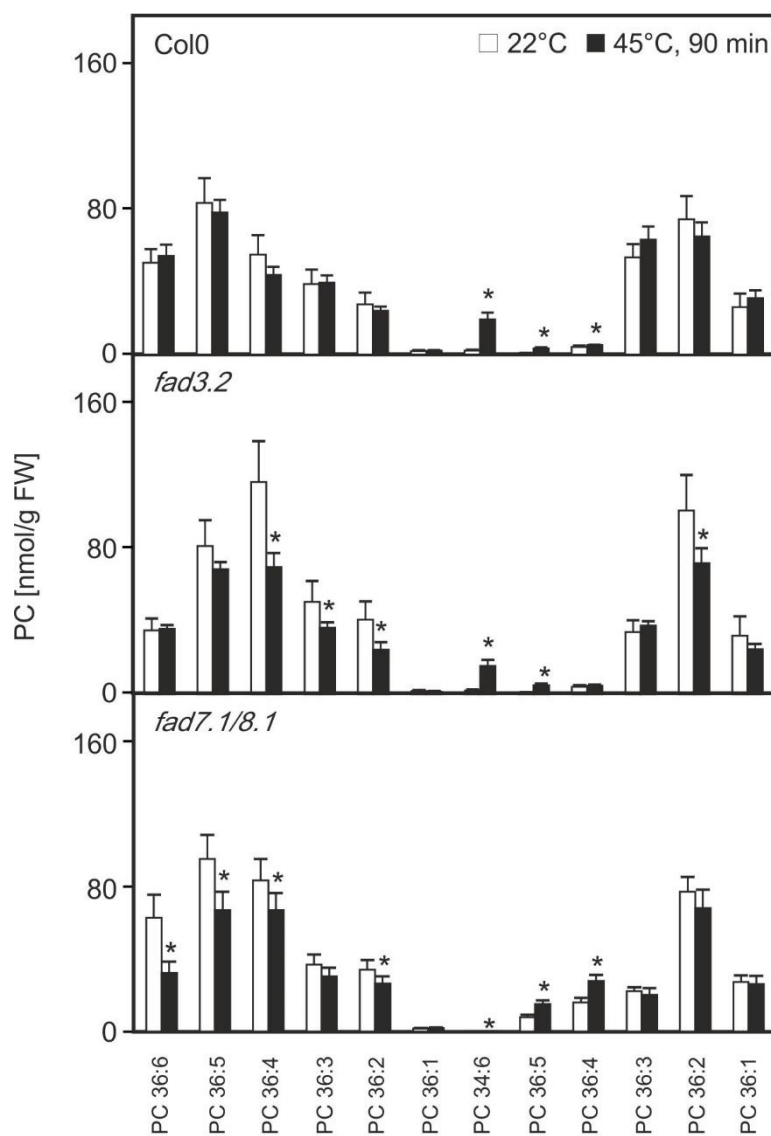
Figure S 5. Heat-induced changes of PCs in wild type, *fad3.2* and *fad7.1/8.1* seedlings.

Levels of lipids were determined in 14-day-old wild type, *fad3.2* and *fad7.1/8.1* seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid levels (p<0.05) are indicated by asterisks.
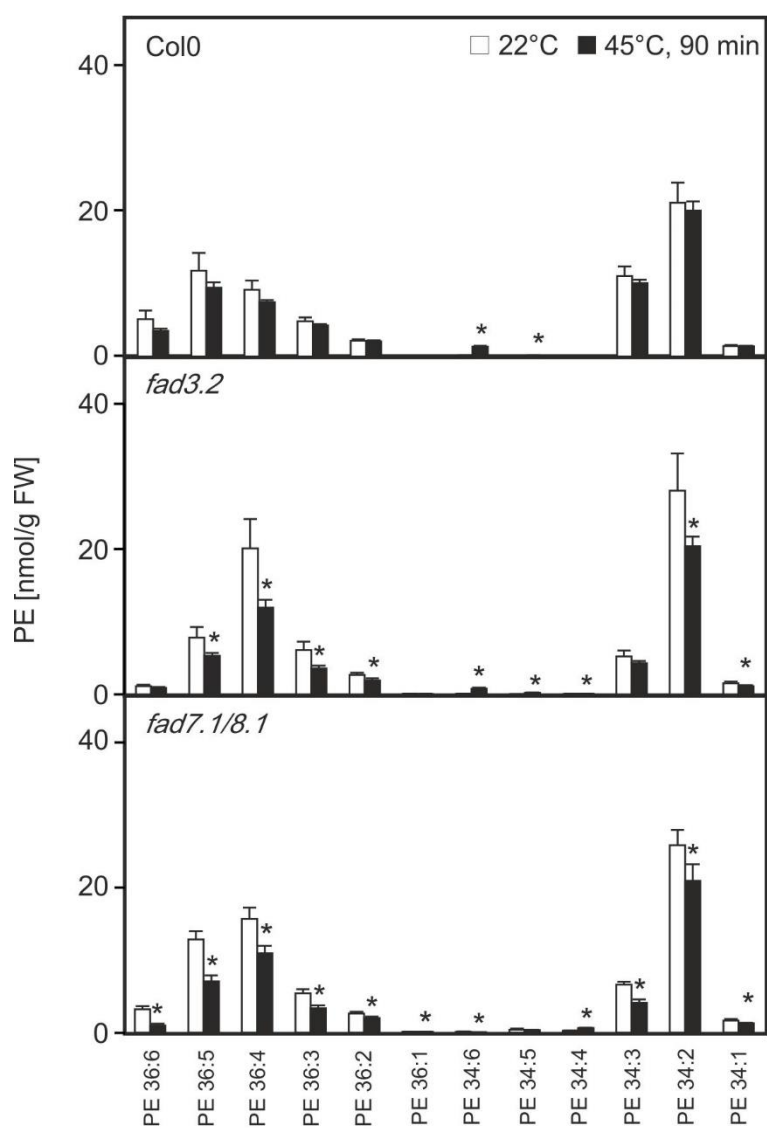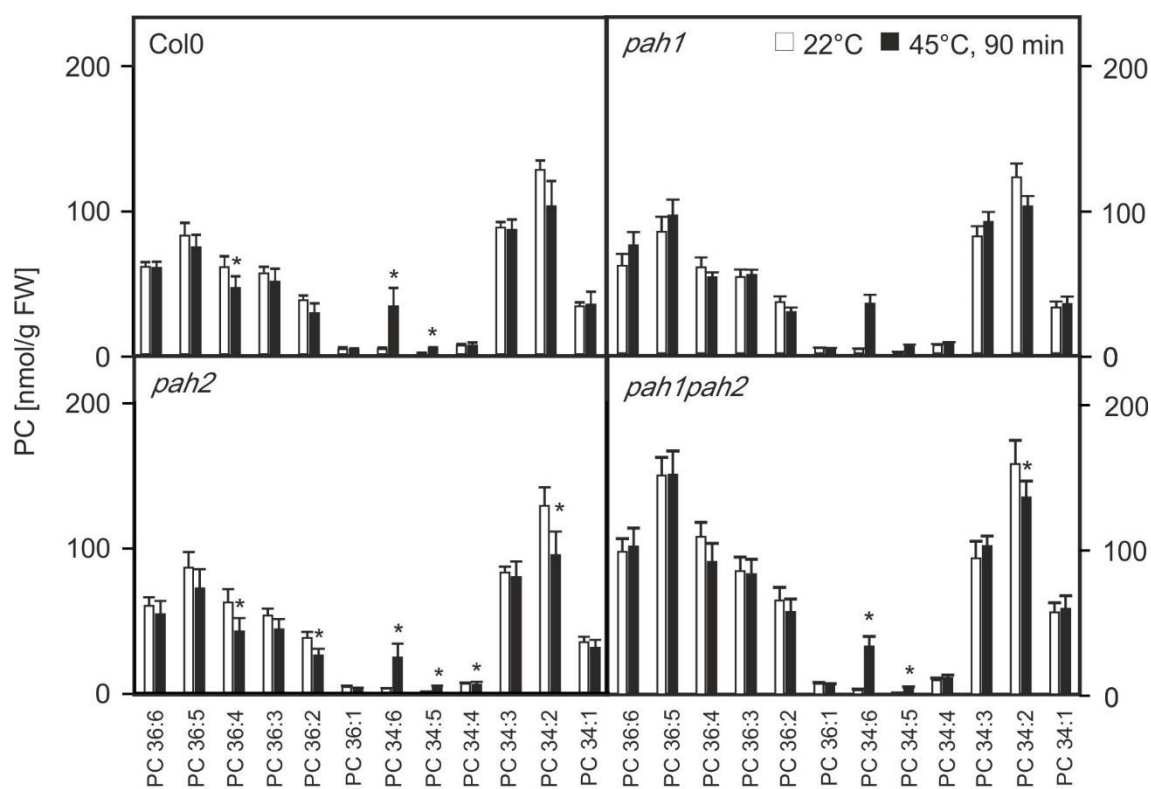
Figure S 6. Heat-induced changes of PEs in wild type, *fad3.2* and *fad7.1/8.1* seedlings.

Levels of lipids were determined in 14-day-old wild type, *fad3.2* and *fad7.1/8.1* seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid levels (p<0.05) are indicated by asterisks.
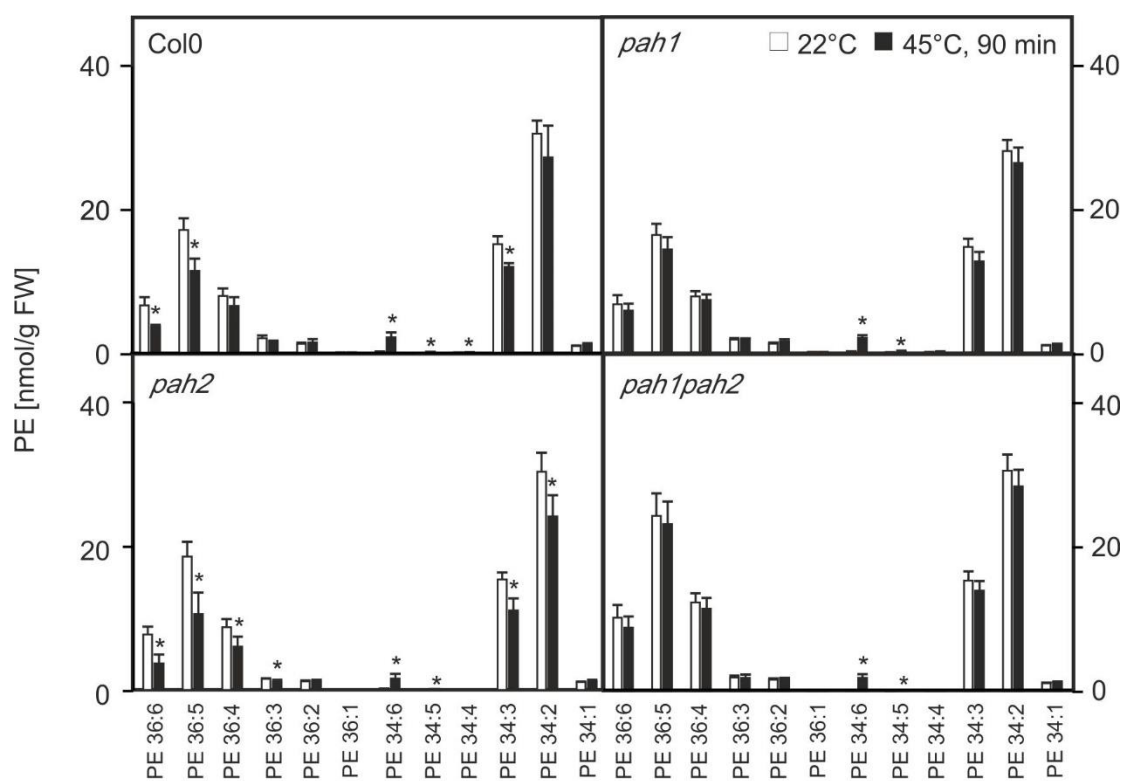
Figure S 7. Heat-induced changes of TGs in wild type, *fad3.2* and *fad7.1/8.1* seedlings.
Levels of lipids were determined in 14-day-old wild type, *fad3.2* and *fad7.1/8.1* seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid levels (p<0.05) are indicated by asterisks.

Figure S 8. Heat-induced changes of DGs in wild type, *fad3.2* and *fad7.1/8.1*
seedlings.
Levels of lipids were determined in 14-day-old wild type, *fad3.2* and *fad7.1/8.1*
seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black
bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid
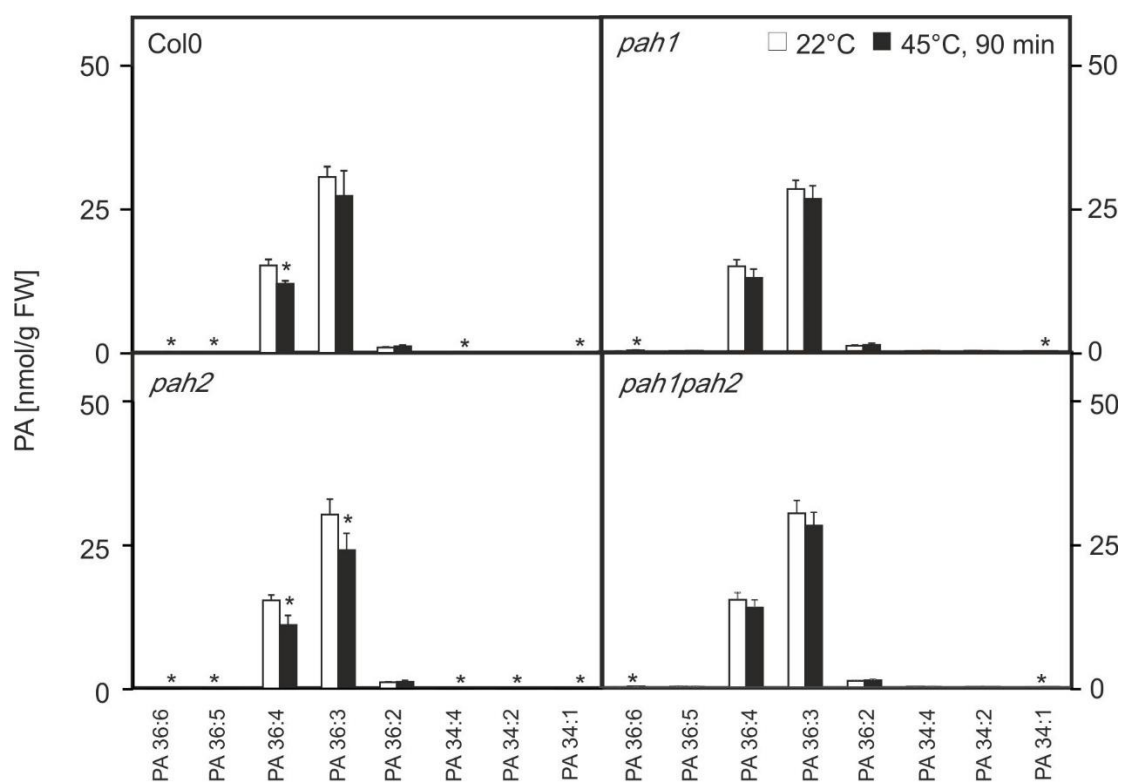levels (p<0.05) are indicated by asterisks.

Figure S 9. Heat-induced changes of PCs in wild type, *pah1, pah2* and *pah1pah2* seedlings.
Levels of lipids were determined in 14-day-old wild type, *pah1, pah2* and *pah1pah2* seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid levels ($p < 0.05$) are indicated by asterisks.
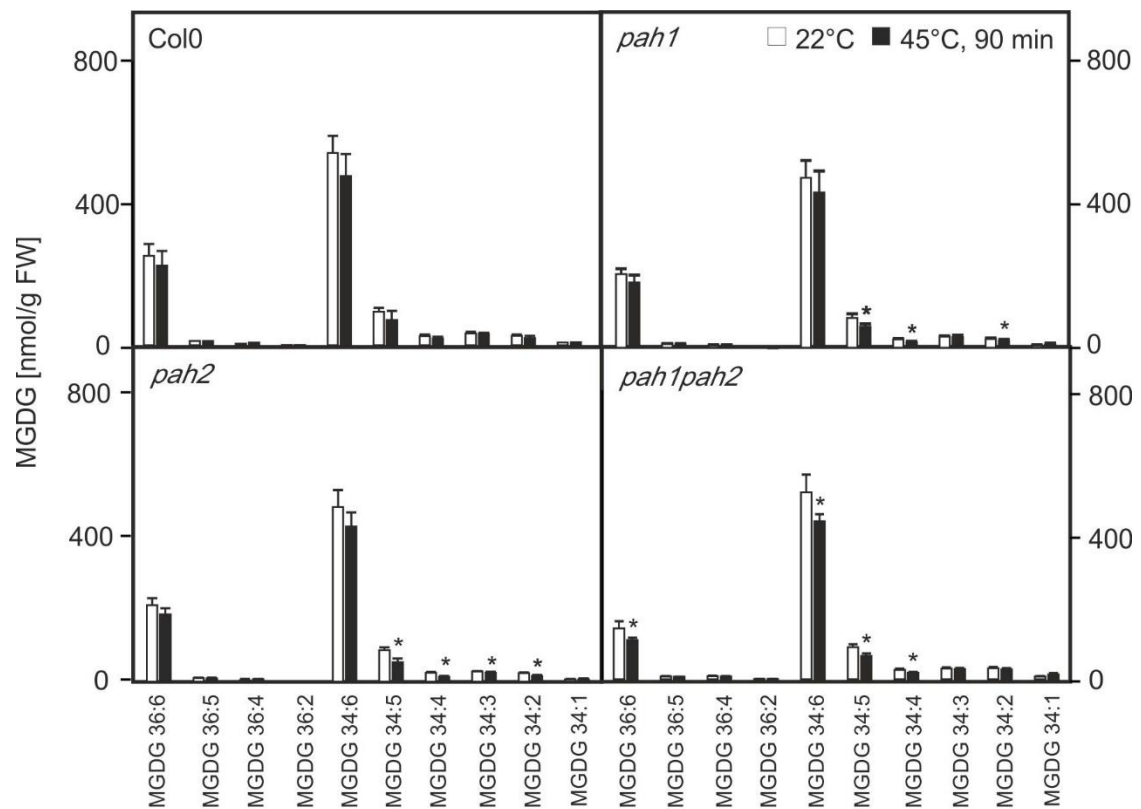
Figure S 10. Heat-induced changes of PEs in wild type, *pah1, pah2* and *pah1pah2* seedlings.
Levels of lipids were determined in 14-day-old wild type, *pah1, pah2* and *pah1pah2* seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid levels (p<0.05) are indicated by asterisks.
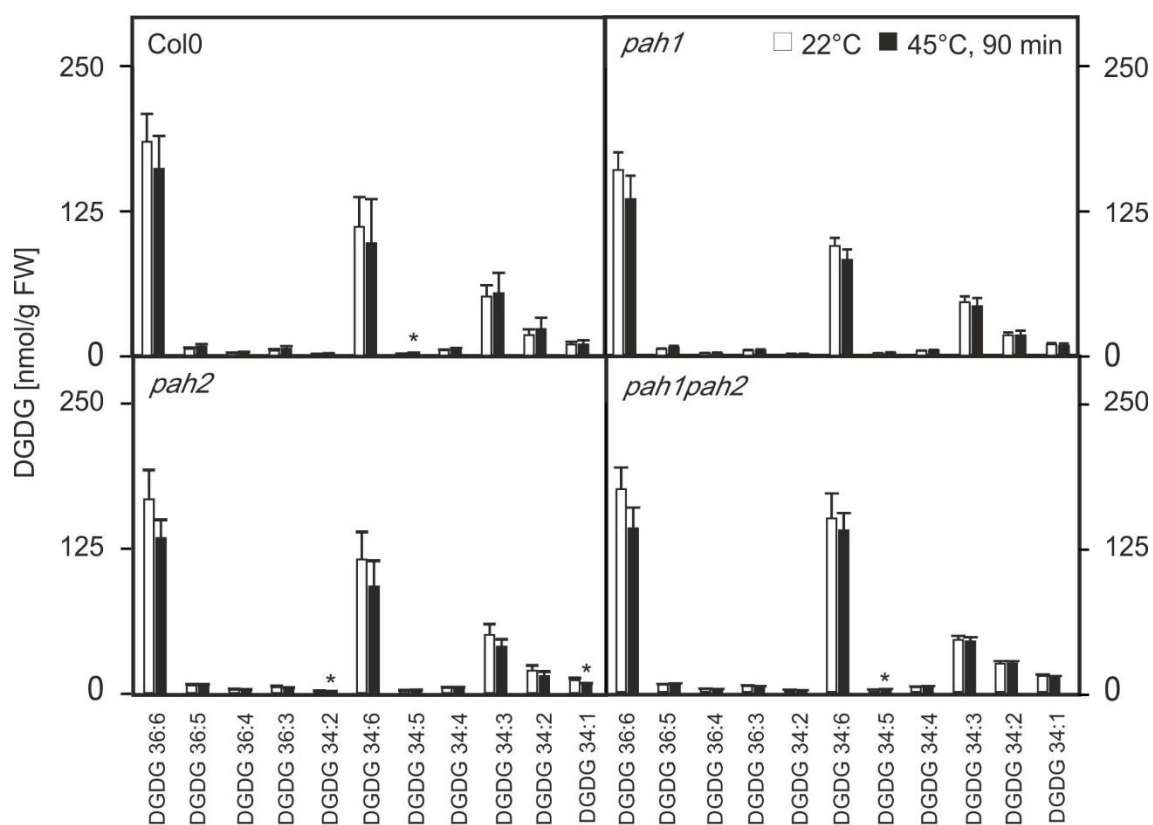
Figure S 11. Heat-induced changes of PAs in wild type, *pah1, pah2* and *pah1pah2* seedlings.
Levels of lipids were determined in 14-day-old wild type, *pah1, pah2* and *pah1pah2* seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid levels (p<0.05) are indicated by asterisks.

Figure S 12. Heat-induced changes of MGDGs in wild type, *pah1, pah2* and *pah1pah2* seedlings.
Levels of lipids were determined in 14-day-old wild type, *pah1, pah2* and *pah1pah2* seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid levels (p<0.05) are indicated by asterisks.
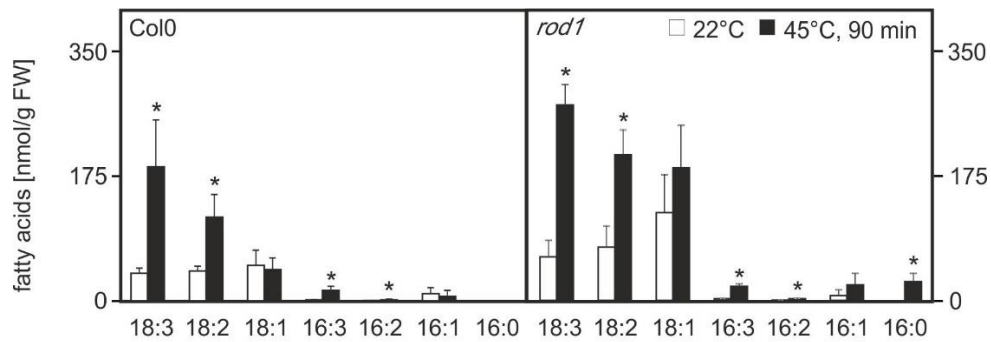
Figure S 13. Heat-induced changes of DGDGs in wild type, *pah1, pah2* and *pah1pah2* seedlings.
Levels of lipids were determined in 14-day-old wild type, *pah1, pah2* and *pah1pah2* seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid levels (p<0.05) are indicated by asterisks.

Figure S 14. FA levels in neutral lipids in wild type and *rod1* seedlings.
14-day-old seedlings were kept at 22° C (white bars) and or treated with a heat shock (45° C for 90 min, black bars). Thereafter, neutral lipids were separated, hydrolyzed and FAs were determined. Data represent means ± SD, n = 4. Statistical significant changes of FA levels (p<0.05) are indicated by asterisks.
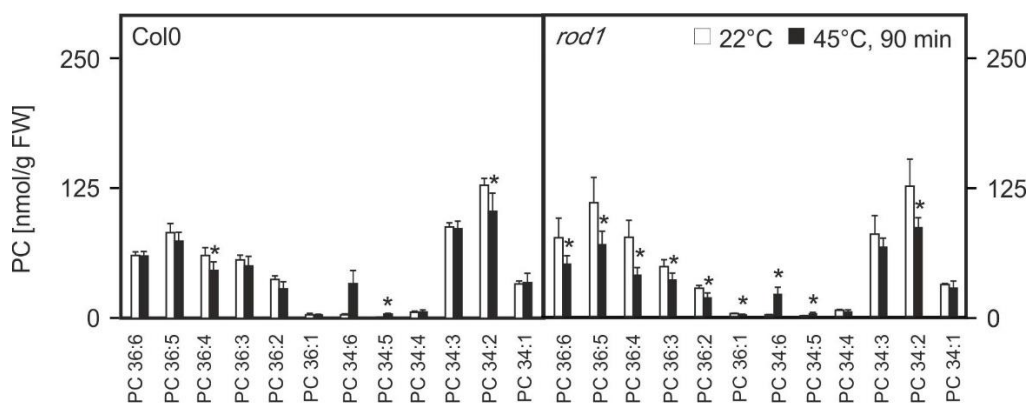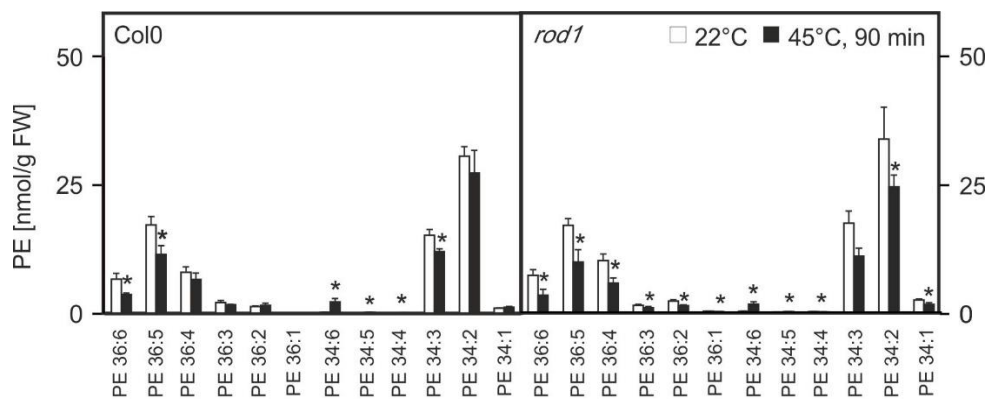

Figure S 15. Heat-induced changes of PCs in wild type and *rod1* seedlings.
Levels of lipids were determined in 14-day-old wild type and *rod1* seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid levels (p<0.05) are indicated by asterisks.
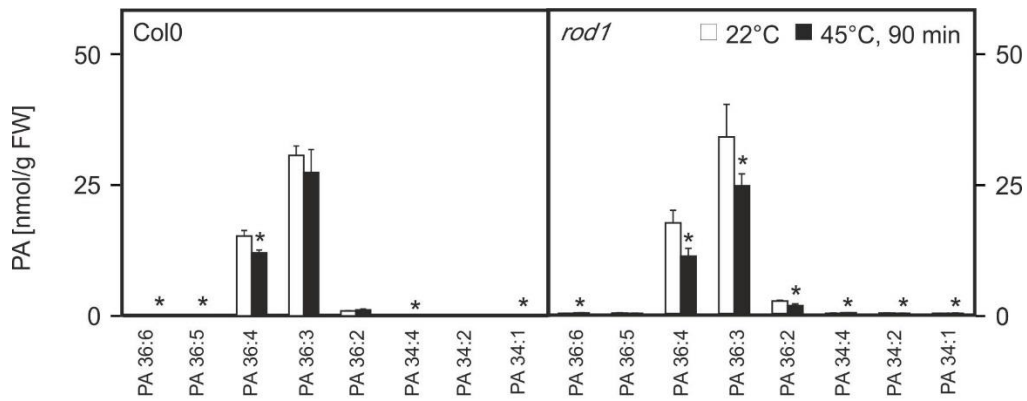

Figure S 16. Heat-induced changes of PEs in wild type and *rod1* seedlings.
Levels of lipids were determined in 14-day-old wild type and *rod1* seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid levels (p<0.05) are indicated by asterisks.

Figure S 17. Heat-induced changes of PAs in wild type and *rod1* seedlings.
Levels of lipids were determined in 14-day-old wild type and *rod1* seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid levels (p<0.05) are indicated by asterisks.
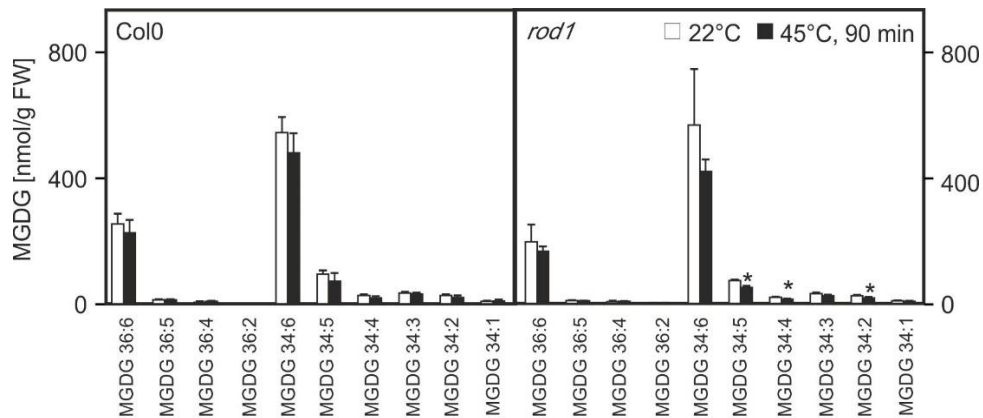

Figure S 18. Heat-induced changes of MGDGs in wild type and *rod1* seedlings.
Levels of lipids were determined in 14-day-old wild type and *rod1* seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid levels (p<0.05) are indicated by asterisks.
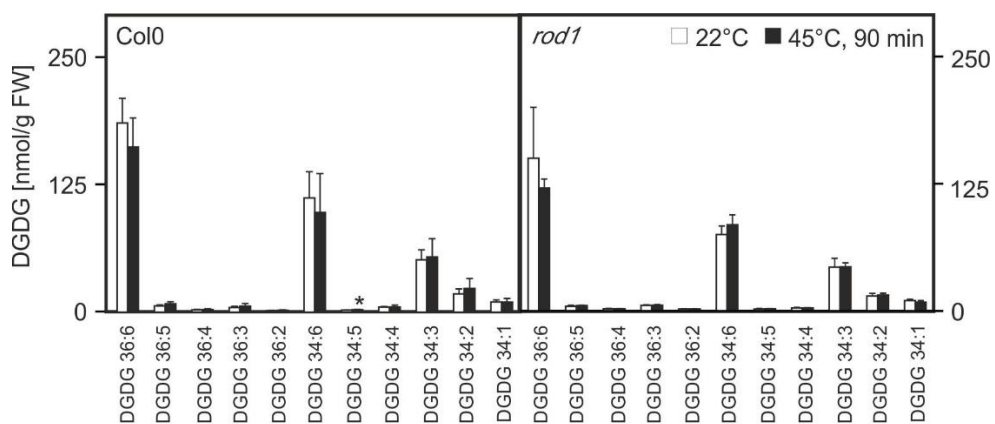

Figure S 19. Heat-induced changes of DGDGs in wild type and *rod1* seedlings.
Levels of lipids were determined in 14-day-old wild type and *rod1* seedlings kept at 22° C (white bars) or after a heat shock (45° C for 90 min, black bars). Data represent means ± SD, n = 4. Statistical significant changes of lipid levels (p<0.05) are indicated by asterisks.

Affidavit

I hereby confirm that my thesis entitled "Plant thermotolerance: The role of heat stress-induced triacylglycerols in *Arabidopsis thaliana* is the result of my own work". I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in this thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, date   Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Thermotoleranz in Pflanzen: Die Rolle von Hitzestress induzierten Triacylglycerolen in *Arabidopsis thaliana*" eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum   Unterschrift