Impact of specific long chain acyl CoA synthetases on plant development

PhD Thesis

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submitted by

Dirk Jessen

born in

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Affidavit

Herewith I affirm that I wrote this PhD Thesis independently and with no other sources and aids than quoted.

Dirk Jessen

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Göttingen, Germany

List of Publications

Journal article

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Abstract

Fatty acids are essential components of cellular life. However, in their natural form they cannot enter metabolism but in fact they need enzymatic activation by coenzyme A to become metabolically accessible. One enzyme class catalyzing such a reaction is called long chain acyl-CoA synthetases (LACS). Although the chemical aspects of this reaction are well understood the biological impact remained largely unknown, since pronounced phenotypes of *LACS* mutants have not been reported so far.

However, in this project it has been shown that deletion of these activities can lead to severe phenotypes affecting processes throughout the whole plant life cycle. The observations were made by the establishment of a LACS mutant collection with dozen of double and triple knock-out lines. Some lines showed reduced or even absent fertility and reduced surface wax levels caused by a reduced synthesis of very long chain lipids. Other mutants showed light dependent phenotypes resulting in severely altered plant morphology. These modifications were most likely due to perturbations of the lipid metabolism. In this context, a reduced flux between prokaryotic and eukaryotic pathway is assumed based on radiolabeled flux analysis. In addition, embryo development and storage lipid synthesis were reduced in some lines indicating important roles of specific LACS enzymes also in these processes.

All these phenotypes are caused by specific changes in various parts of lipid metabolism, suggesting that fatty acids require repeated activation, after being transported out of the chloroplast. Evidences for such processes have been found in surface wax synthesis, storage lipid synthesis, fatty acid degradation and in part also for eukaryotic lipid synthesis. Deactivation of acyl-CoA and subsequent reactivation of the free fatty acid seem to be contra productive; however, it might allow a better more efficient regulation of the connected metabolic pathways or transport processes through membranes. Furthermore, the results are suggesting the existence of different acyl CoA pools for specific pathways. Such different pools might also be the reason for the existence of nine LACS isogenes in *Arabidopsis*.

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Abbreviation

(RT)-PCR	Real time polymerase chain reaction
(w/v)	(weight / volume)
°C	Degree Celsius
ACBP	Acyl-CoA binding protein
ACP	Acyl carrier protein
AMP	Adenosine monophosphate
ANS	8-anilino-1-naphthalene-sulphonic acid
Approx	Approximately
ATP	Adenosine triphosphate
BHT	2,6-Di-tert-butyl-4-methylphenol
BSA	Bovine serum albumin
BSTFA	N,O-bis(trimethylsilyl) trifluoroacetamide
cDNA	Copy deoxyribonucleic acid
CDP	Cytidine diphosphate
CER	*Arabidopsis* mutant „*Eceriferum*“
Ci	Curie
CO_2	Carbon dioxide
CoA	Coenzyme A
Col-0	*Arabidopsis* ecotype columbia
CPT	CDP-choline:diacylglycerol cholinephosphotransferase
CTAB	Cetyltrimethylammoniumbromid
DAG	Diacylglycerol
DGD	Digalactosyldiacylglycerol
DIR	*Arabidopsis* mutant „Defective in induced resistance“

DNA	Deoxyribonucleic acid
e.g.	For example
EDAC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediaminetetra acetic acid
ER	Endoplasmic reticulum
et al.	et alii, et aliae; and others
FAD	Fatty acid desaturase
FAE	Fatty acid elongases
FAME	Fatty acid methyl ester
FAS	Fatty acid synthase
FAT	Fatty acyl-acyl carrier protein thioesterase
FFA	Free fatty acid
FID	Flame ionization detector
Fig	Figure
g	Gramm
GC	Gas chromatography
GPAT	Glycerol-3-phosphate acyltransferase
GUS	β - glucuronidase
h	Hour
HIP	Hexane: Isopropanol: 2,6-Di-tert-butyl-4-methylphenol (BHT)
HPLC	High-performance liquid chromatography
l	Liter
LACS	Long chain acyl CoA synthetases
LPAAT	Lysophosphatidic acid acyltransferase
m	Meter
M	Mol

MAG	Monoacylglycerol
MAH	Mid-chain alkane hydroxylase
Mbp	Mega basepairs
MES	2-(*N*-morpholino)ethanesulfonic acid
MGD	Monogalactosyldiacylglycerol
min	Minutes
mRNA	Messenger ribonucleic acid
MS	Immature microspores
OD	Optical density
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidlyethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PM	Plasma membrane
PMC	Premature cell layer
PPC	Primary parietal cell
PPi	Pyrophosphate
PS	Phosphatidylserine
PSC	Primary sporogenous cell
RNA	Ribonucleic acid
Rpm	Rounds per minute
s	Second
SDS	Sodium Dodecyl Sulfate
SEM	Scanning electron microscope
SPC	Parietal cells

SQD	Sulfoquinovosyl diacylglycerol
Tab	Table
TAG	Triacylglycerol
TEM	Transmission electron microscope
TLC	Thin layer chromatography
UDP	Uridine diphosphate
UV	Ultra violette
V	Volt
WBC	White-brown complex
WSD	Wax ester synthase/diacylglycerol acyltransferase
Wt	Wild type

1. Introduction

1.1 Arabidopsis as a model organism

Arabidopsis thaliana is a small herbaceous plant, which naturally occurs in the moderate climate zones of the northern hemisphere and grows in areas between forest and grassland. Although it is an unimposing plant it has become one of the best established model organisms for plant science during the last decades (Koornneef *et al.,* 1997). One of the main reasons for this development was the small size of the plant. It reaches a maximal height of 30 cm therefore thousands of plants can be grown in one greenhouse. Furthermore it has a short generation time; after 6 - 8 weeks a germinating seed has become an adult plant with ripe siliques producing more than 1000 seeds per plant (approx. 50 seeds / silique). Breeding of Arabidopsis is very convenient due to its self-pollination. Hence fertilization occurs independently of exogenous factors like wind or insects. Furthermore, it belongs to the *Brassicacae* family (Tab.1), which is of high economic relevance due to members like *Brassica napus* or *Sinapis alba*.

Beside these biological aspects *Arabidopsis* has also many advantages on the molecular level. Due to its relatively small genome with 157 mega base pairs (Mbp) genetic studies are easier compared to studies with *Brassica napus* (2,4 giga base pairs) or *Triticum* (16 Giga base pairs). It was the first plant with a completely sequenced genome, done in 2000 by the Arabidopsis Genome Initiative (Ausubel 2000). So far 27.000 genes and 35.000 proteins have been identified (Koornneef *et al.,* 1997). Furthermore genetic manipulations are well established; the common technique is transformation with *Agrobacterium tumefaciens.*

Tab.1: Scientific classification of *Arabidopsis thaliana*

Scientific classification of *Arabidopsis thaliana*	
Kingdom	*Plantae*
Subphylum	*Angiosperms*
Class	*Eudicots*
Subclass	*Rosids*
Order	*Brassicales*
Family	*Brassicacae*
Genus	*Arabidopsis*

1.2 Lipids

Lipids belong together with carbohydrates, proteins and nucleic acids to the basic building blocks of life. It is a very heterogeneous chemical group with many diverse hydrophobic molecules like fatty acids, fats, waxes, terpenoides and sterols. A common feature is their insolubility in water and their solubility in organic solvents. In contrast membrane lipids have an amphiphilic character, due to a hydrophobic and hydrophilic part; thus they are important for compartmentation in general. However, a plant cell consists only to 5 % - 10 % out of lipids. Most of them are located in membranes, where they function as main building blocks. Additionally, lipids are also important for carbon and energy storage, anchoring of enzymes, signaling and sealing processes (Buchanan *et al.,* 2000). Furthermore, they have become important for biotechnology projects like bio fuel production and synthesis of lubricants but also for quality improvement of nutrition.

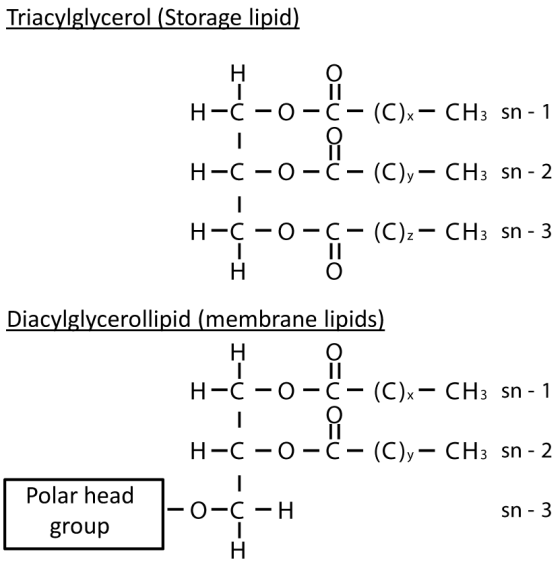

Fig.1. Storage lipids are composed of a glycerol backbone and three fatty acids linked via an ester bondage at each sn position. Membrane lipids are also composed of a glycerol backbone but having only two fatty acids at sn – 1 and sn -2 and a polar head group at position sn – 3. The hydrophilic headgroup can be choline, inositol. ethanolamine or serine in case of phospholipids; in glycolipids one (MGD) or two galactose moieties (DGD) can be found.

In general, membrane lipids consist of a glycerol backbone with two fatty acids linked via an ester bondage to the position sn-1 and sn-2 (Fig.1). A variable head group is added to the position sn-3, this can be a sugar or a phosphate group. The biophysical properties of membrane lipids are often based on their fatty acids, they can vary in chain length, the degree of saturation or their architecture (e.g. branching). All these factors have a significant

influence on membrane fluidity, thickness and curvature. Therefore, modulation of fatty acid composition is used by many organisms for adaptation to changing environmental conditions (Buchanan *et al.,* 2000).

Although lipids and their metabolism have an enormous impact on the life cycle of any organism, many aspects are still unknown. The so far present knowledge regarding plant lipid synthesis, transport and utilization will be presented in the following.

1.3 Fatty acid synthesis

The fatty acid synthesis can lead to a variety of products with fatty acids varying in size between 4 to 32 carbon atoms and being rationed into four subgroups:

Short chain fatty acids:	4 to 9 carbon atoms
Mid chain fatty acids:	10 to 14 carbon atoms
Long chain fatty acids:	16 to 22 carbon atoms
Very long chain fatty acids:	longer than 22 carbon atoms

De novo synthesis of these compounds is localized in plants exclusively within the plastids and is catalyzed by an enzyme complex called fatty acid synthase (FAS) (Buchanan *et al.,* 2000). It is thought that this complex leads to a higher efficiency in comparison to spatially separated enzymes. The committed step in the synthesis is the formation of malonyl-CoA from acetyl-CoA and CO_2, being catalyzed by the acetyl-CoA carboxylase (Sasaki *et al.,* 1995) (Fig 2a, 2b). This enzyme is tightly controlled and regulating the whole fatty acid synthesis (Ohlrogge *et al.,* 1997). The resulting malonyl-CoA is turned into a malonyl-ACP which enters a four step series of reactions (Fig.2b).

In the first step acetate from acetyl-CoA is bound to a cysteine of a condensing enzyme (3-ketoacyl-ACP synthase) and is transferred to malonyl-ACP to form β –Ketoacyl-ACP. During the reaction CO_2 is released via decarboxylation. In the second step the keto group at position C-3 is reduced leading to β–D–Hydroxyacyl-ACP, which is subsequently transformed into trans-Δ^2-Enoyl–ACP by the release of water. In the last step trans-Δ^2-Enoyl–ACP is reduced a second time leading to the saturation of the remaining double bond. Hence

the acyl chain is elongated by a two carbon unit (Fig.2b). In general, elongation continues until the growing acyl chain consists of 16 to 18 carbon atoms (Buchanan *et al.,* 2000).

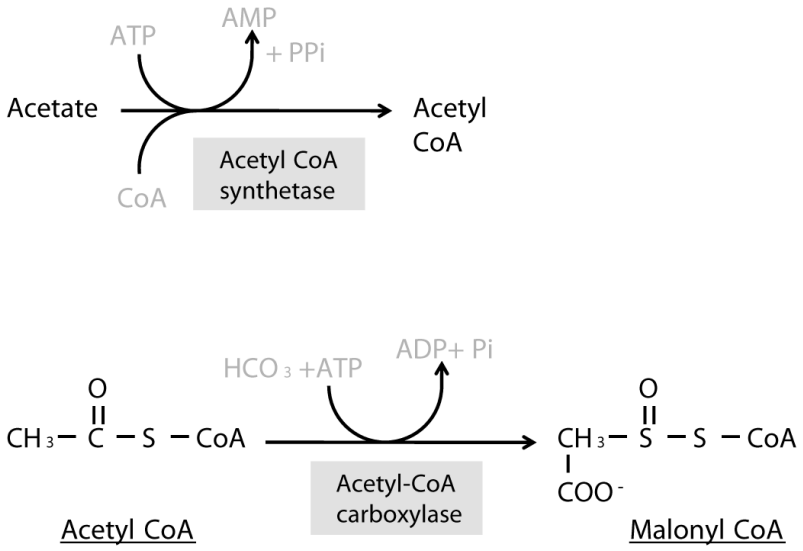

Fig.2a. Acetyl-CoA can be formed from acetate and CoA in a reaction catalyzed by the enzyme acetyl-CoA synthetase under the consumption of one ATP. A high activity of this enzyme was detected in chloroplasts, thus acetate is rapidly incorporated into fatty acid synthesis in the plastids.
The resulting acetyl CoA is carboxylated to malonyl-CoA by an acetyl-CoA carboxylase under consumption of one ATP. This reaction is the committed step of fatty acid synthesis. Modified version from "Plant Biochemistry"; Heldt and Piechulla; 4th Edition; Academic Press; 2011

Mainly 16:1-ACP and 18:0-ACP are released from the chloroplast, but fatty acid elongation and modification can be continued in the ER as well as in the chloroplast (Hamilton 2007). During the export process of acyl-ACPs a thioesterase is catalyzing the cleavage of fatty acid and ACP (Pollard *et al.,* 1999). Afterwards the deactivated fatty acid is transported by an unknown mechanism through the plastidial membrane and is reactivated on the cytosolic site by the addition of a CoA group, being catalyzed via a long chain acyl CoA synthetase (LACS) (Hamilton 2007; Koo *et al.,* 2004; Schnurr *et al.,* 2002). At this point the eukaryotic lipid synthesis sets in, presented in chap. 1.5.2.

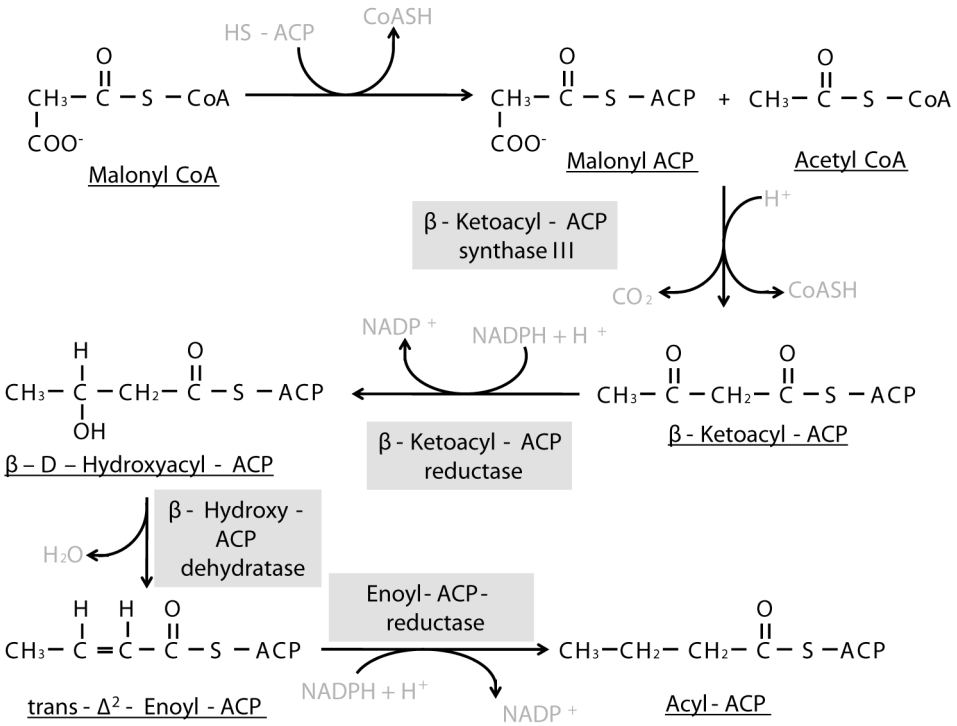

Fig.2b. Fatty acid synthesis is a four step reaction. In this first reaction CoA is exchanged and substituted by an acyl carrier protein (ACP). The second reaction is a condensation of malonyl ACP and acetyl CoA, being irreversible due to the release of CO_2. The resulting β–ketoacyl–ACP is reduced by the β–ketoacyl–ACP reductase, the following release of water is catalyzed by β–Hydroxy–ACP – dehydratase. In the last step the remaining carbon – carbon double bond is reduced by the enoyl–ACP–reductase leading to acyl-ACP. Modified version from "Plant Biochemistry"; Heldt and Piechulla; 4th Edition; Academic Press; 2011

1.4 Acyl – CoA Synthetases

Acyl CoA synthetases play a critical role in the metabolism. They are activating free fatty acids via esterification with CoA (Fig. 3) (Groot *et al.,* 1976). This activation step is crucial for the utilization of fatty acids in any downstream application. The catalyzed activation reaction follows a two step mechanism (Groot *et al.,* 1976); in the first step the free fatty acid is turned into an acyl-AMP intermediate called adenylate. In the second step the adenylate is coupled to the thiol group of CoA, resulting in the formation of acyl-CoA. This reaction mechanism is well conserved and found not only for acyl-CoA synthetases but for many other metabolic reactions like algal polyketide synthetases (Bibb *et al.,* 1994; Conti *et al.,* 1996), arthropod luciferase (Conti *et al.,* 1996) and bacterial peptide antibiotic synthetases (Conti *et al.,* 1997).

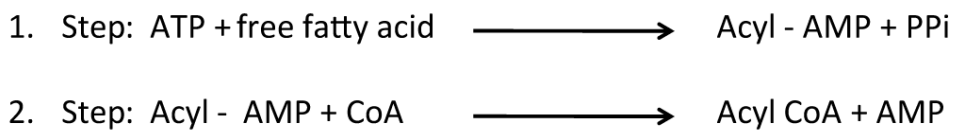

Fig.3. Activation of free fatty acids catalyzed by acyl-CoA synthetases. In the first reaction the free fatty acid substitutes diphosphate at an ATP moiety leading to the formation of acyl–AMP. In the second reaction the acyl chain is bound to CoA. The free energy of this two step reaction is near zero, however due to the exothermic hydrolysis of the released PPi (catalyzed by inorganic pyrophosphatase) the reaction becomes irreversible.

1.4.1 Long chain acyl CoA synthetases (LACS)

LACS are an important subclass of the acyl-CoA synthetases family. So far 9 LACS genes have been identified in *Arabidopsis*. The identification was based on *in silico*, *in vitro* and *in vivo* studies (Shockey *et al.* 2003). Hereby it had been shown that all LACS enzymes are able to produce acyl-CoA (Knoll *et al.,* 1995; Shockey *et al.,* 2002; Shockey *et al.,* 2003). Based on heterologous expression studies it was concluded that all nine enzymes are membrane associated. (Overath *et al.,* 1969)

The substrate specificity of the 9 enzymes was analyzed by (Shockey *et al.,* 2002) showing just minor differences. All proteins were active with saturated and unsaturated C16 and C18 fatty acids. In addition, LACS6 and LACS7 showed a relatively high specificity for C20:1, which is a seed specific fatty acid (Fulda *et al.,* 2002; Fulda *et al.,* 2004; Shockey *et al.,* 2002). The second special feature was detected for LACS1 showing high activity with C30 fatty acids (Lu *et al.,* 2009; Weng *et al.,* 2010). These results are mainly reflecting the overall fatty acid combination of *Arabidopsis* with 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3 as the main components. Consequently, possible differences in the biological impact of the 9 LACS proteins are probably not caused by their substrate specificity.

The tissue specific expression pattern of the 9 LACS genes were analyzed by histo-chemical GUS staining, DNA array analysis and RT-PCR showing only minor differences (Shockey *et al.,* 2002). Interestingly all LACS genes showed high expression levels in flowers; indicating that this organ has a very active lipid metabolism. Furthermore it was the only tissue type with *LACS5* expression. In developing seeds transcripts of *LACS1, LACS2, LACS4, LACS9* and also a weak level of *LACS8* have been detected; moreover almost all LACS genes (with the exception of *LACS3* and *LACS5*) were expressed in germinating seedling (Lu *et al.,* 2009; Schnurr *et al.*). These results are emphasizing the importance of LACS genes for seed

ripening and germination. Similarly, almost all LACS genes were expressed in leaf whereas *LACS1* and *LACS6* were the only genes having high expression levels in stems (Lu *et al.,* 2009; Shockey *et al.,* 2002). Interestingly *LACS4* and *LACS9* were also intensively expressed in roots (Zhao *et al.,* 2010); consequently it is almost not possible to assign a specific LACS activity exclusively to one tissue type.

Hence the intracellular localization has become of great interest since it is one of the last factors for a potential discrimination of the biological impact. However, so far there is only an incomplete picture for intracellular LACS localization. It has been shown that LACS1 as well as LACS8 are localized at the ER (Lu *et al.,* 2009; Zhao *et al.,* 2010), Fulda and colleagues identified the peroxisomal localization of LACS6 and LACS7 (Fulda *et al.,* 2004) and is has been shown for LACS9 that it is localized at the outer plastidial envelope (Schnurr *et al.,* 2002). For the remaining 5 enzymes the subcellular localization is still under investigation.

1.4.2 Phenotypes of LACS

The loss of acyl-CoA synthesizing activity is thought to have an enormous influence on plant metabolism, since acyl-CoA is a central intermediate for many pathways. However, single knock out of LACS genes are leading to no or just subtle phenotypes. So far only *lacs1* and *lacs2* are known to show changes in appearance and morphology (Lu *et al.,* 2009; Schnurr *et al.,* 2004; Weng *et al.,* 2010). The knockout of LACS1 is affecting surface wax synthesis and also fertility (Lu *et al.,* 2009; Weng *et al.,* 2010), together with its specificity for C30 fatty acids and its localization at the ER it seems to be an important factor for wax synthesis in general. LACS2 is also essential for the development of a proper cuticle, however the *lacs2* line has, in contrast to *lacs1,* a reduced cutin layer and a higher resistance against a specific pathogen (Bessire *et al.,* 2007; Schnurr *et al.,* 2004). When these single knock outs were combined the resulting *lacs1 lacs2* had a more pronounced surface wax reduction, however the cutin layer was similar to *lacs2* (Lu *et al.,* 2009; Weng *et al.,* 2010). Moreover the mutant developed also an intensive fertility phenotype; but the published data about this aspect is inconsistent. Data from the study by (Lu *et al.,* 2009) is suggesting that fertility of *lacs1 lacs2* is restored in high humidity, whereas results of (Weng *et al.,* 2010) indicating that humidity

has no impact on fertility of this plant. Rather, it seems that seed development is independently restored during very late developmental stages.

All remaining 7 single knock out lines are indistinguishable from wild type and show no obvious phenotypes. Interestingly, the single knock out of LACS9 was demonstrated to exhibit a biochemical phenotype with a 95 % reduction of LACS activity at the chloroplast (Schnurr *et al.,* 2002). This observation correlates with the localization of LACS9 to the outer envelope and raises the possibility that LACS9 is important for the reactivation of fatty acids after leaving the chloroplast (Fig 4). However, since *lacs9* showed no lipid, growth or morphological phenotype it is thought that the remaining 5 % of LACS activity are sufficient for a normal lipid metabolism (Schnurr *et al.,* 2004).

So far no LACS mutants have been described showing a compromised fatty acid transport form chloroplast to ER although it was believed that LACS activity might be involved in this process (Benning 2009) (Fig.4).

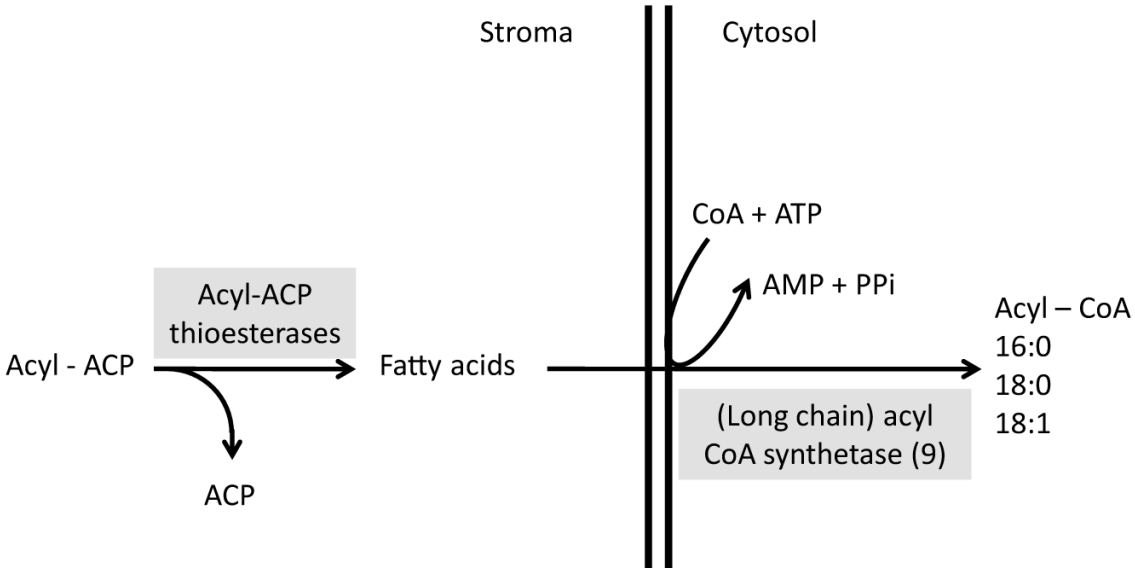

Fig.4. Acyl–ACP is cleaved by an acyl–ACP thioesterase, the resulting free fatty acids are directly transported from the stroma through the inner and outer envelope of plastids to the cytosol. This transport process is still poorly understood; so far it is not known whether it is catalyzed by a transporter or just due to diffusion. Only three specific fatty acids are transported to the cytosol, these are primarily 16:0 and 18:1 and low amounts of 18:0. It is thought that the reactivation to acyl-CoAs at the outer plastidial membrane is catalyzed by LACS9. Modified version from "Plant Biochemistry"; Heldt and Piechulla; 4th Edition; Academic Press; 2011

The absence of intensive phenotypes could indicate that LACS enzymes have overlapping functions. This assumption is supported by two examples in the literature describing double knock outs with more severe phenotypes.

(Fulda *et al.,* 2004) reported that the double knock out of LACS6 and LACS7 is compromised in seedling establishment under normal conditions; only the presences of sucrose could rescue the phenotype and led to normal post-germinative growth. It was shown that LACS6 and LACS7 are required for the utilization of fatty acids in the peroxisomes which is of special importance during post-germinative growth. This correlates with the localization of LACS6 and LACS7 and with their substrate specificity showing high preference for the seed specific 20:1 fatty acid.

Another described double knock is *lacs1 lacs2* showing reduced amounts of surface wax and a reduced fertility (Lu *et al.,* 2009; Weng *et al.,* 2010). The pronounced reduction in surface wax can be due to an overlapping function in the wax synthesis of both enzymes, but it is also possible that the reduced cutin layer is inhibiting higher wax loads due to structure and stability reasons. However, the results of this line are supporting the important role of LACS1 for wax synthesis.

Furthermore, a recent publication demonstrated that LACS1 in combination with LACS8 seem to be involved in seed oil biosynthesis (Zhao *et al.,* 2010). Seeds of the *lacs1 lacs8* double mutant were shown to contain about 10 % less triacylglycerol compared to wild type.

1.5 Synthesis of lipids

As mentioned in chap. 1.3 plants bear two distinct pathways for synthesizing membrane lipids, shown by radiotracer experiments with spinach chloroplasts (Roughan *et al.,* 1982). One synthesis route is the prokaryotic pathway in the plastids; the other route is located in the ER and called the eukaryotic pathway (Somerville *et al.,* 1991).

In course of the prokaryotic pathway MGD, DGD, SQD as well as PG are synthesized; the other phospholipids (PC, PE, PI and PS) and neutral lipids (TAG) are formed by the eukaryotic pathway. Lipids like PA, MAG and DAG are important intermediates which can be found in both metabolic pathways (Fig.5) (Browse *et al.,* 1986; Somerville *et al.,* 1991).

The prokaryotic and the eukaryotic pathways are tightly connected, since lipids from the ER can be transported to the chloroplast being used for the synthesis of glycolipids and fatty acids from plastids are needed as starting point for synthesis at the ER (Browse *et al.,* 1991). However products of both pathways are distinguishable based on their fatty acid composition and their stereo specific distribution. A clear sign for a plastidial origin is a C16 fatty acid on the sn-2 position due to the specificity of the second acyl transferases in the plastids. In contrast, lipids from the eukaryotic pathway contain a C18 fatty acid at the sn-2 position (Fig.5). Furthermore the presence of 16:3 is indicative for a plastidial origin. Consequently it is possible to identify the metabolic origin of a lipid and therefore metabolic flux analyses of both pathways are possible despite their intensive interaction (Arondel *et al.,* 1992; Browse *et al.,* 1986; Browse *et al.,* 1986; Ohlrogge *et al.,* 1995; Okuley *et al.,* 1994).

1.5.1 Prokaryotic pathway

The synthesis of prokaryotic lipids occurs at the envelope membranes. In the first step an activated fatty acid is transferred onto the sn-1 position of a glycerol molecule via a glycerol-3-phosphate-acyltransferase (GPAT) (Kunst *et al.,* 1988). The resulting lyso-PA is acylated a second time via the lyso-phosphate acyl transferase (LPAAT) (Fulda *et al.,* 1997; Murata *et al.,* 1997; Yu *et al.,* 2004). A phosphatase is converting the obtained PA into DAG, which can be used as a substrate for the synthesis of the main plastid lipids like MGD, DGD and SQD (Joyard *et al.,* 1994) (Fig. 5). However, PA can also be converted into PG using CDP-DAG synthase, PG phosphate synthase and PG phosphate phosphatase (Andrews *et al.,* 1985).

For the synthesis of galactolipids, which are the main plastidial membrane components, different galactosyl transferase activities are known. Three enzymes have been identified catalyzing the UDP-Gal dependent synthesis of MGD (MGD1, MGD2, MGD3) (Jarvis *et al.,* 2000; Miege *et al.,* 1999; Moellering *et al.,* 2011; Ohta *et al.,* 1997). It has been shown, that for the supply of thylakoid membranes with MGD the enzyme MGD1 is essential, which is localized at the inner envelope (Kobayashi *et al.,* 2007). In contrast to that MGD2 and MGD3 are localized at the outer envelope and involved in membrane remodeling under stress conditions e.g. phosphate deprivation (Kobayashi *et al.,* 2009). For the UDP-Gal dependent synthesis of DGD two enzymes have been identified (DGD1, DGD2), which are both located at the outer envelope (Moellering *et al.,* 2011).

DGD1 is important for glycolipd supply of thylakoid membrane, by converting MGD1 derived MGD into DGD. DGD2 is known to be important for membrane remodeling under stress conditions (Kelly *et al.,* 2002; Kelly *et al.,* 2003; Moellering *et al.,* 2011).

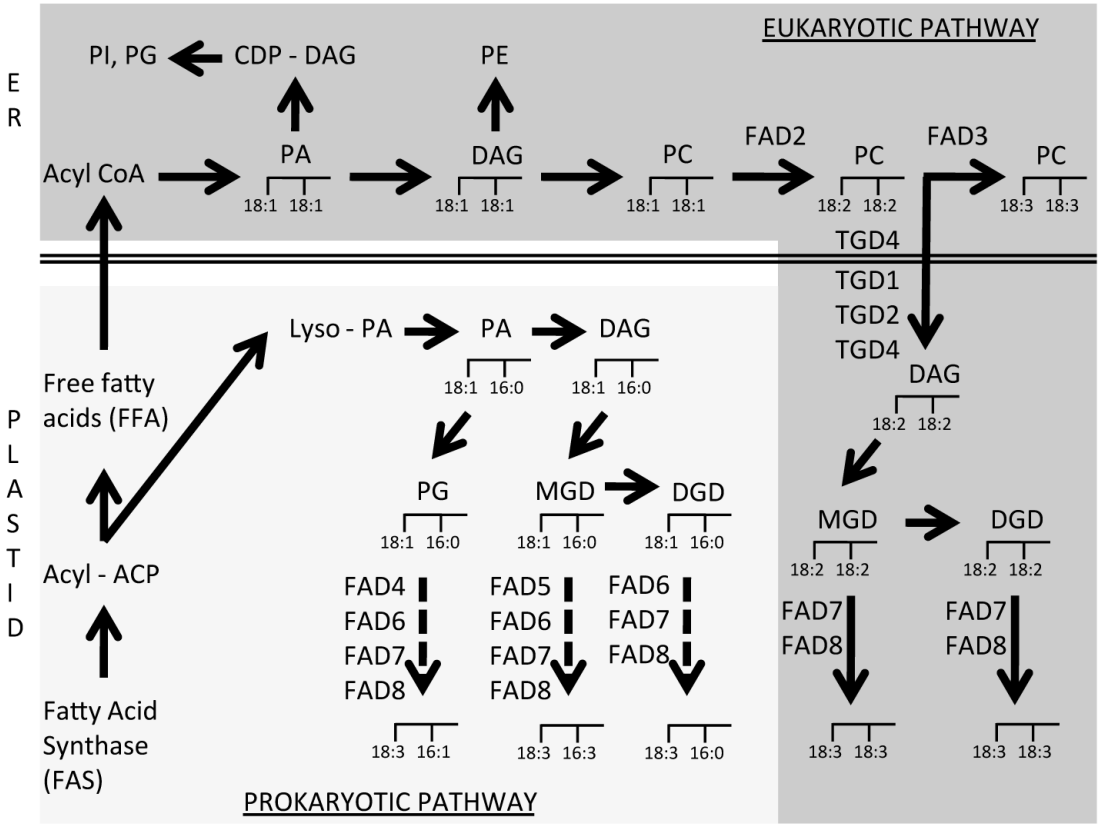

Fig.5. Overview about lipid synthesis in the pro- and eukaryotic pathway. The prokaryotic pathway is depicted in light gray, whereas the eukaryotic pathway is shown in dark gray. After their synthesis fatty acids can be released into the cytosol and being transported as acyl-CoA to the ER; or fatty acids remain in the plastids as acyl–ACP and enter the prokaryotic lipid synthesis with the formation of MGD, DGD and PG. Acyl-CoA being transported to the ER lead to the formation of PA, DAG, PI, PE and PC. It is known that PC containing fatty acid 18:2 at position sn – 1 and sn – 2 is the precursor of eukaryotic lipid molecule being shuttled to the plastids. According to recent data this transport is catalyzed by TGD1, TGD2, TGD3 and TGD4. In the plastids MGD and DGD are synthesized using the imported eukaryotic precursors. Desaturation of fatty acids is catalyzed by a various number of enzymes, at the ER only two (FAD2, FAD3) desaturases are identified. However, inside the plastids 6 desaturases are known (FAB2, FAD4, FAD5, FAD6, FAD7, FAD8). This is a simplified figure, taken from Wallis, J.G. and Browse, J. Mutants of Arabidopsis reveal many roles for membrane lipids. *Prog. Lipid Res*., **41**, 254-278 (2002). PA = Phosphatidic acids, PI = Phosphatidylinositol, PG = Phosphatidylglycerol, DAG = diacylglycerol, PE = Phosphatidylethanolamine, PC = Phosphatidylcholine, MGD = Monogalactosyldiacylglycerol, DGD = Digalactosyldiacylglycerol, FAD = Fatty acid desaturases

In each of these reactions a galactose moiety from uridine diphosphate galactose (UDP-Gal) is transferred to the sn-3 position of DAG or MGDG (Kelly *et al.,* 2004). Sulfoquinovosyl diacylglycerol **(**SQDG) is also synthesized at the envelope membrane similar to MGD with a transfer of sulfoquinovosyl from a UDP-conjugate to the head group of DAG (Weissenmayer *et al.,* 2000). The products of those syntheses can be modified by desaturases FAD4, FAD5, FAD6, FAD7 or FAD8 leading to the typical plastidial fatty acid composition with the specific 16:3 and 16:1 $^{\Delta 3}$ fatty acids (Fig.5) (Browse *et al.,* 1991; Heinz 1993; Ohlrogge *et al.,* 1995).

FAD4 and FAD5 show a very specific substrate profile, since PG is the only substrate for FAD4 resulting in the specific 16:1 $^{\Delta 3}$ fatty acid. FAD5 has a similar specificity for MGD with the desaturation of 16:0 to 16:1 at the sn-2 position (Somerville *et al.,* 1996).

The remaining pastidial acyl-lipid desaturases FAD6, FAD7 and FAD8 have a broader substrate profile and are not so restricted to specific head groups. PG, MGD, DGD and SQD are equivalent substrates for these enzymes. When analyzed more precisely it turned out that FAD6 is catalyzing the desaturation of fatty acids 16:1 and 18:1 into 16:2 and 18:2. The next desaturation to 16:3 and 18:3 is catalyzed by FAD7 and FAD8 (Browse *et al.,* 1986; Falcone *et al.,* 1994; Gibson *et al.,* 1994; McConn *et al.,* 1994) (Fig.5).

1.5.2 Eukaryotic pathway

The eukaryotic pathway is located at the ER and is the major route of phospholipid biosynthesis in plants. Fatty acids used in this pathway are exported from the chloroplast, reactivated by long chain acyl CoA synthetases at the outer envelope and transported to the ER (Browse *et al.,* 1991) (Fig.4). The transport process of these fatty acids to the ER is so far unknown, but it is discussed whether acyl-CoA binding proteins (ACBPs), other enzymes or diffusion are involved (Xiao *et al.,* 2009).

After reaching the ER it is thought that fatty acids are transferred to a glycerol backbone catalyzed by GPATs similar to the prokaryotic pathway. Afterwards a second acylation step is catalyzed by a LPAAT leading to PA formation (Fig.5) (Mongrand *et al.,* 1997; Mongrand *et al.,* 2000). However recent data raised the possibility that fatty acids could be transferred directly to a lyso-PC and subsequently the resulting PC is providing the substrate pool for PA formation (Bates *et al.,* 2007).

Further synthesis of phospholipids can occur via two different pathways (Athenstaedt *et al.,* 1999). PA is turned into DAG via a phosphatase reaction and modified with activated head groups (CDP-choline or CDP-ethanolamine) being added to the sn-3 position resulting in PC or PE (Arondel *et al.,* 1992; Okuley *et al.,* 1994). Those reactions are catalyzed by CDP-choline: DAG-phosphocholine transferase (CPT) or CDP-ethanolamine: DAG-phosphoethanolamine transferase. The second possibility is found for the synthesis of PI and PS. The headgroup of PA is exchanged by cytidine triphosphate resulting in CDP-DAG, in the next step inositol or serine are added leading to PS or PI formation (Browse *et al.,* 1991; Kinney *et al.,* 1990; Ohlrogge *et al.,* 1995).

As shown in Fig.5 desaturation of lipids is also occurring in the eukaryotic pathway. However, in contrast to the plastids, the ER contains only two enzymes called FAD2 and FAD3 catalyzing this reaction. These enzymes are mainly acting on fatty acids attached to PC and catalyzing the desaturation of 18:1 to 18:2 (FAD2) and from 18:2 to 18:3 (FAD3) (Arondel *et al.,* 1992; Okuley *et al.,* 1994). As mentioned before lipids from the eukaryotic pathway are used in many plant species also for glycolipid biosynthesis in the plastids (Browse *et al.,* 1991). It is still under discussion how this transport is achieved. Although much data was obtained during the last years many aspects of this transport are still under investigation (Benning 2009)(chap.1.6). Glycolipids synthesized with eukaryotic precursors are easily to detect by their fatty acid ratio of 16:3 / 18:3 and their stereo specificity at the sn-2 position. Lipids synthesized in this pathway contain C18 fatty acids at the sn-2 position, whereas lipids from the prokaryotic pathway contain C16 fatty acids in their sn-2 position (Ohlrogge *et al.,* 1995).

Interestingly, most of the ER derived components being transported to the plastids are used for DGD synthesis whereas MGD is mostly only an intermediate (Benning 2009; Ohlrogge *et al.,* 1995).

1.6 Lipid Transport

Transport of lipid components is a crucial process for plant cells and their development but also for other processes like defense reactions or signal transduction. In the following different lipid transport systems will be presented focusing on the routes between plastids and ER as well as plasma membrane and ER.

1.6.1 Export of fatty acids from chloroplasts to ER

For the export of fatty acids out of the chloroplast ACP must be removed from acyl–ACP by a thioesterase. The resulting free fatty acid can be transported through the envelope membranes. The detailed mechanism of this transport is unknown; however *in vitro* studies have shown that proteins are not necessarily required indicating that this process can also be based on diffusion (Hamilton 2007; Kamp *et al.,* 2006).

After transport to the cytosolic site fatty acids are activated to acyl-CoA by long chain acyl CoA synthetases. A central candidate for catalyzing this reaction is LACS9 since it is localized at the plastids and its inactivation leads to a 95 % reduction of the plastidial LACS activity. However, the *lacs9* mutant line showed no visual phenotype and also its biochemical profile of membrane lipids showed no significant differences (Schnurr *et al.,* 2002). After transport to the ER fatty acids are assembled to PA being the substrate for many different pathways (Ohlrogge *et al.,* 1995). In contrast to this established model it is now discussed according to recent data whether acyl groups are first incorporated into PC by acyl editing (Bates *et al.,* 2007).

1.6.2 Transport of Lipids from ER to chloroplast

During the last years the knowledge about the transport of lipids from ER to the plastid increased dramatically, but many aspects remain still unclear and need further investigation. So far one protein called TGD4 is known to be important for the transport of lipids from the ER to the chloroplast (Xu *et al.,* 2010). It is thought that TGD4 is localized at the ER or inside the outer envelope at contact sites between ER and plastids. The exact role of this protein is not fully understood, but it is discussed whether TGD 4 is acting directly in the transport process or indirectly by protein recruitment (e.g. phospholipase D) (Benning 2009).

When PC is transported from the ER to the outer envelope, it is converted into PA by the action of a predicted phospholipase D. Also other models are discussed for this process suggesting that lyso-PC is a potential cargo between ER and plastids too (Browse *et al.,* 1993; Miquel *et al.,* 1992; Roughan *et al.,* 1982; Xu *et al.,* 2010).

After reaching the plastid, a protein complex called TGD 1 - 3 is catalyzing the transport of lipid cargo from the outer envelope to the inner envelope. This complex consists out of three building blocks being similar to the permease, the substrate binding unit and the ATPase of an ABC transporter (Xu *et al.,* 2005 Xu *et al.,* 2003). Inside the plastid the transported lipid is formed into a DAG moiety, being the substrate for eukaryotic glycolipid synthesis leading to the formation of MGD and DGD (Awai *et al.,* 2006; Awai *et al.,* 2006; Lu *et al.,* 2007; Xu *et al.,* 2003; Xu *et al.,* 2005).

1.6.3 Transport of surface wax to the cuticle

Beside the transport of lipids between plastids and ER there are many more essential lipid transport processes in a plant cell. One of these processes is the shuttling of surface wax components from ER to the cuticle for the establishment of a protection barrier against uncontrolled loss of water and pathogen attack. This process can be divided into three steps:

1. Transport from the ER to the plasma membrane
2. Transport through the plasma membrane
3. Transport through the cell wall

The mechanism of the first transport process is still not completely understood. It is controversially discussed whether wax components are transported via golgi-mediated vesicle transport or via direct transport at ER - plasma membrane (PM) contact sites (Kunst *et al.,* 2003; Schulz *et al.,* 2004). Until today evidence for both hypotheses have been reported; e.g. vesicles filled with lipophilic components in epidermal cells (Jenks *et al.,* 1996), or contact sites between PM and ER having an enrichment of lipid biosynthetic proteins (Hoffmann-Benning *et al.,* 1994).

ABC transporters are essential for the second transport process through the plasma membrane. In *Arabidopsis* CER5 and WBC11 have been identified as flexible heterodimer forming an ABC transporter which is responsible for the export of wax components. Their inactivation led to reduced wax load on the surface and also to an accumulation of surface wax inside epidermal cells (Beaudoin *et al.,* 2009; Bird *et al.,* 2007; Luo *et al.,* 2007; McFarlane *et al.,* 2010; Panikashvili *et al.,* 2007; Pighin *et al.,* 2004; Ukitsu *et al.,* 2007).

For the third and also last transportation step of surface waxes, lipid transport proteins (LTP) were shown to be involved (Beisson *et al.,* 2003; Thoma *et al.,* 1993). This enzyme class has a relatively high expression rate in the epidermis and is known to be secreted into the apoplast. It was demonstrated that LTPs contain a hydrophobic pocket and have the ability to bind long chain fatty acids; however this has only been shown in *in vitro* studies (Samuels *et al.,* 2008). Although 72 potential LTP genes are identified in *Arabidopsis* so far only one mutant (*dir1*) has been characterized. The mutant plants showed a reduced resistance against pathogen attack accompanied by changes in cuticle composition (Maldonado *et al.,* 2002; Suh *et al.,* 2005; Zachowski *et al.,* 1998).

1.7 Wax Synthesis

As mentioned in chap 1.6.3 the establishment of a proper cuticle is very important for the plant to avoid uncontrolled water loss but also as a protection against pathogens. The transport of these cuticle wax components was already presented in the proceeding chapter hence only their synthesis is shown in more detail in the following.
Surface wax synthesis starts with the formation of C16 and C18 fatty acids in the leucoplasts of the epidermis (Samuels *et al.,* 2008). These components are synthesized and transported to the ER according to the processes mentioned before (Voelker 1996). It is remarkable that a thioesterase has been identified being especially important for wax synthesis (FATB), since inactivation led to a severe reduction of wax load on stem and leaf (Bonaventure *et al.,* 2003).

After transport to the ER fatty acids are elongated to chain lengths with a maximum of C32 catalyzed by fatty acid elongases (FAE). These elongated fatty acids are the substrates for the acyl reduction and the decarbonylation pathway (Fig.6) (Kunst *et al.,* 2003; Xu *et al.,* 2002; Zheng *et al.,* 2005).

The acyl reduction branch is leading to the synthesis of primary alcohols and wax esters. The conversion of VLCFA to primary alcohol is catalyzed by a fatty acyl CoA reductase (FAR) (Samuels *et al.,* 2008). In *Arabidopsis* eight FAR genes have been identified, but so far only one of them (CER4) is thought to be responsible for the primary alcohol formation, since *cer4* has only trace amounts of primary alcohol in the surface wax (Jenks *et al.,* 1995; Rowland *et*

al., 2006). The products of CER4 can be directly transported to the cell surface or used as substrate in the wax ester synthesis. The second step of the acyl reduction branch is catalyzed by a wax synthase (WSD1) being exclusively expressed in the epidermis. Mainly C16 acyl-CoAs and primary alcohols with a chain length between 26 and 32 are used as educts by this enzyme for wax ester formation, leading to a huge variety of different species (Fig.6) (Lai *et al.,* 2007; Lardizabal *et al.,* 2000; Li *et al.,* 2008).

The second branch in the wax synthesis is called decarbonylation pathway and leads to the formation of aldehydes, alkanes, secondary alcohols and ketones (Fig.6). The central reaction of the pathway is the loss of one C atom leading to alkane formation via an aldehyde intermediate (Bognar *et al.,* 1984). However, the potential enzymes (aldehyde-forming acyl CoA reductase and aldehyde decarbonylase) catalyzing these reactions still need to be identified.

Alkanes are formed into secondary alcohol and ketones by a mid-chain hydroxylation catalyzed by mid-chain alkane hydroxylase (MAH1) (Greer *et al.,* 2007). The described biosynthesis is resulting in the characteristic wax composition of *Arabidopsis* with alkane C29, secondary alcohol C29 and ketone C29 as the predominant components (Chibnall *et al.,* 1934; Jenks *et al.,* 1995; Kolattukudy and Walton 1972; Kolattukudy, Buckner *et al.,* 1972; Kolattukudy *et al.,* 1973).

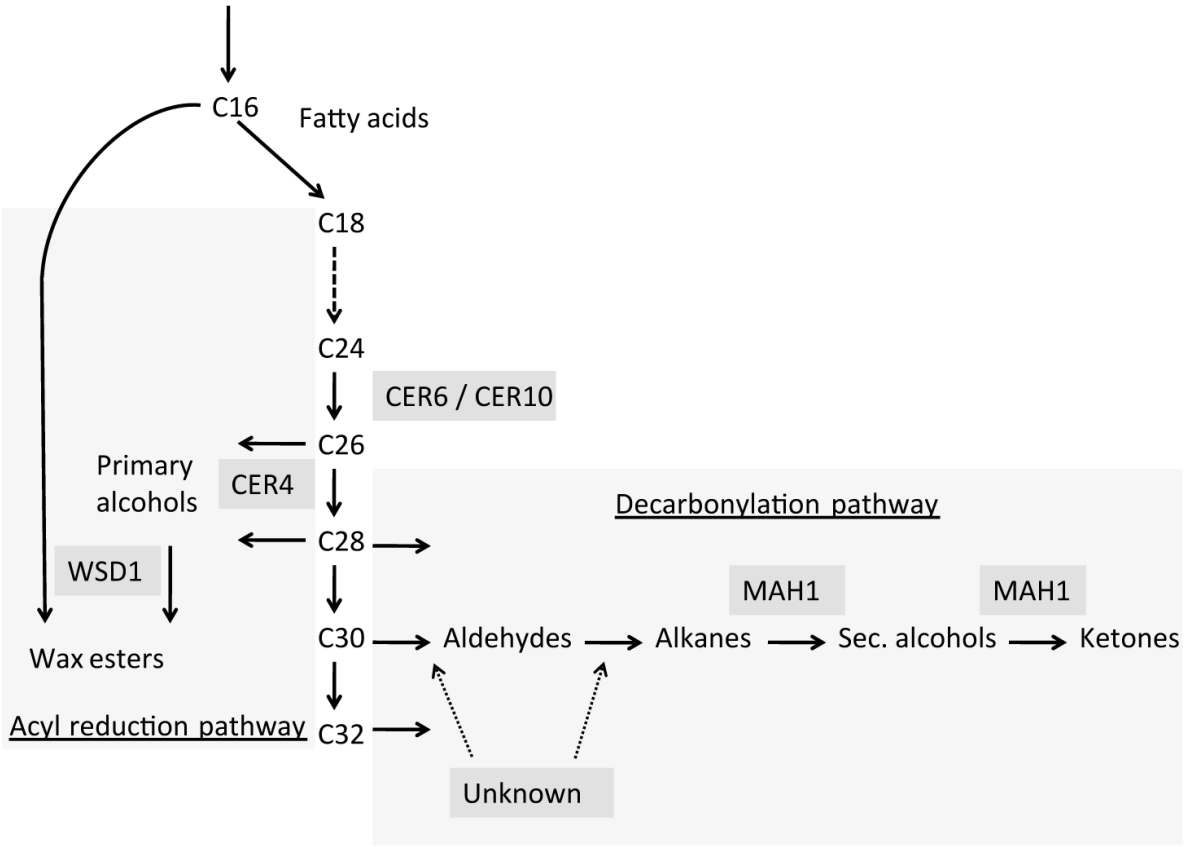

Fig.6. Overview about surface wax biosynthesis. Fatty acids are elongated at the ER to a maximum chain length of C32 catalyzed by CER6 and CER10. When fatty acids have reached a minimum chain length of C28 they might become a substrate for the decarbonylation pathway leading to the formation of alkanes, sec. alcohols and ketones. The enzymes required for the synthesis of aldehydes and alkanes are still unknown but it has been shown that the following reaction steps for the synthesis of sec. alcohols and ketones are catalyzed by MAH1. The second pathway shown in this figure is the acyl reduction pathway. In this branch C16 fatty acids are fused to C26 or C28 primary alcohols forming wax esters catalyzed by the wax ester synthase WSD1. The primary alcohols are synthesized by CER4 using VLCFA as substrate. The figure is simplified and taken from Lacey Samuels *et al.* Annu. Rev. Plant Biol. (2008).

1.8 Plant fertility

Plant fertilization is a highly regulated and very complex process. One crucial aspect is the species specific pollen grain recognition. The interaction between pollen grains and stigma (the first part of the flower having contact with pollen grains during fertilization) is very important in this context and will be presented in more detail in the following.

1.8.1. Interaction between pollen grain and stigma

A central characteristic of *Arabidopsis* is siphonogamy, since unflagellated sperm cells are transported through the pollen tube to the female gametophyte. Hence delivery and recognition of pollen grains by the gynoecium is crucial for a proper fertilization. Angiosperms have developed two different strategies for this process:

- Plants with a dry stigma; Recognition and discrimination of pollen grains begins with the first physical contact
- Plants with a wet stigma; All pollen grains can germinate on the stigma, recognition and discrimination occur at a later time point.

Since *Arabidopsis* belongs to the first group, the focus will be on plants with dry stigmas.

The first step of pollen-stigma interaction in plants with a dry stigma is pollen capture and adhesion. For this purpose proteinous layers of the pollen grain (exinic outer layer) and the stigma (pellicle) are essential (Edlund *et al.,* 2004; Hiscock *et al.,* 2008). They establish an early but very stable connection so that pollen grains are fixed and further development is enabled.

As the second step hydration of pollen grains occurs, requiring a lipophilic layer on the surface of pollen grains called pollen coat (Edlund *et al.,* 2004). The chemical composition of this layer is very similar to surface wax however the main difference is the presence of alkenes as a new class of compounds. Synthesis of these pollen coat components is thought to take place inside the tapetum, a nursing tissue for gametophyte development (Ariizumi *et al.,* 2011). Upon apoptosis of tapetal cells those lipophilic components are released and transported to the surface of pollen grains (Elleman *et al.,* 1986; Preuss *et al.,* 1993; Wolters-Arts *et al.,* 1998).

The hydration mentioned above is essential since pollen grains have a water content of only 15 % to 35 % (Hiscock *et al.,* 2008), therefore germination can only take place after the supply with exogenous water from the stigma. This critical release of water is triggered by the pollen coat floating onto the stigma to form a foot like structure (Edlund *et al.,* 2004). So far the molecular basis of this process is not completely understood, but it has been shown that also simple lipophilic compounds are able to rescue the hydration of mutants lacking a proper pollen coat (Fiebig *et al.,* 2000; Jenks *et al.,* 1995; Preuss *et al.,* 1993; Wolters-Arts *et al.,*

1998). It is believed, that the lipids are establishing a water gradient guiding pollen tubes to the stigma. However, more data is needed to explain this process in detail (Hiscock *et al.,* 2008).

After water release and hydration pollen grains are able to germinate and penetrate the stigma. The key player in this process is the enzyme cutinase, making the cuticle of the stigma permeable (Hiscock *et al.,* 2008). When pollen tubes have penetrated the stigmatic tissue, pollen tube guidance become important as the last step before sperm cells can be delivered to the ovule. A great variety of compounds like plant hormones are essential for a proper guidance, however also nutrition gradients are involved (Hiscock *et al.,* 2008).

In the end of the intensive interaction between stigma and pollen grain a double fertilization takes place leading to the development of an embryo and nursing tissue.

1.8.2. Pollen grain development

The microsporogenesis leading to pollen grain development in *Arabidopsis* can be divided into different steps (Fig.6). It starts with premature cells in the anther; forming a three cell layer tissue. The outer and inner most layers have, in contrast to the second layer a distinct developmental pattern. Layer one (outer most layer) is becoming anther epidermis and layer 3 (inner most layer) develops into vascular and connective tissue (Liu *et al.,* 2008).

The second layer undergoes a complex development. During developmental stage 2 and 3 this layer is differentiating into primary parental (PPC) and primary sporogenous cells (PSC) (Armstrong *et al.,* 2001). The PSCs will develop during stage 4 and 5 to microspores whereas the PPCs are dividing into two layers of parietal cells (SPC). The inner layer is becoming the tapetum, the outer layer forms the endothecium and the middle layer (Fig.6) (Armstrong *et al.,* 2001; Yang *et al.,* 2000; Yang *et al.,* 1999).
The immature microspores, being formed out of primary parental cells, are forming a tetrad, kept together by callose. After the degradation of callose immature pollen grains are released into the locule of the pollen sack. As a last step in development lipophilic components are released by the tapetum to become the pollen coat on the pollen surface essential for fertilization.(Dong *et al.,* 2005; Enns *et al.,* 2005; Suzuki *et al.,* 2008)

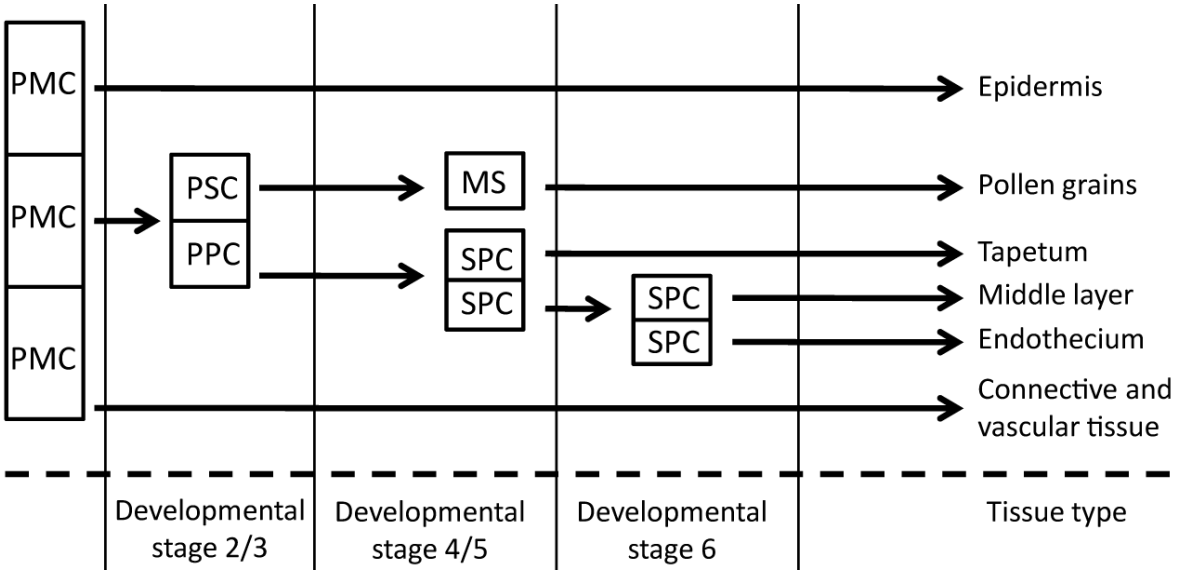

Fig.7. Schematic presentation of gametophyte development. Two layers of PMC differentiate into epidermis, connective and vascular tissue. The middle cell layer is forming during early developmental stages into PSC and PPC. The PPC differentiate into SPC, which will develop to endothetcium and middle layer. The PSC are the precursor for immature microspores leading to ripe and functional pollen grains. PMC, premature cell layer; PSC, primary sporogenous cell; PPC, primary parietal cell; MS, immature microspores; SPC, parietal cells. Developmental stages are indicated at the bottom.

1.9 Aims of this project

The central aim of this thesis was to clarify why *Arabidopsis* encode for 9 different LACS genes and how they influence the development of the plant. This is especially interesting since all 9 isoforms show similar substrate specificities and in many cases overlapping expression profiles. It was known from literature that single mutants showed no or only subtle phenotypes, therefore a systematic collection of double and triple mutants were established to analyze possible phenotypes due to overlapping functions. With this approach it was thought to develop a hypothesis for the biological relevance of specific LACS enzymes. By screening the obtained collection two double knock out lines were identified:

1.) *lacs1 lacs4* → This line showed a normal germination and development; however it had a glossy stem and was completely sterile.

2.) *lacs4 lacs9* → This line showed a significant reduced growth rate and also an affected leaf and stem morphology.

Those two lines and the corresponding single or even triple knock out lines are in the focus of this work, leading to a better understanding of plant fertility, lipid transport and general impact of specific LACS activity on plant development.

2. Material and Methods

Molecular biology experiments for basic approaches were done as described in (Ausubel 1993). During all experiments sterile pipette tips and tubes were used; for experiments involving lipid extractions plastic was avoided and only glass ware was used.

2.1 Technical Equipment

Centrifuge 5810 R	Eppendorf AG (Hamburg, Germany)
Centrifuge 5415 D	Eppendorf AG (Hamburg, Germany)
Centrifuge 5417 R	Eppendorf AG (Hamburg, Germany)
Mastercycler gradient	Eppendorf AG (Hamburg, Germany)
Mastercycler personal	Eppendorf AG (Hamburg, Germany)
Vortex-2 Genie	Scientific Industry (New York, USA)
Evaporator	Berkey
TLC Spotter	Camag (Muttenz, Switzerland)
Chromatogram immersion device III	Camag (Muttenz, Switzerland)
TLC heating plate	Camag (Muttenz, Switzerland)
TLC sray cabinet / sprayer	Camag (Muttenz, Switzerland)
TLC spray cabinet/sprayer	Camag (Muttenz, Switzerland)
6890 Series gas chromatograph	Agilent (Waldbronn, Germany)
6890 gas chromatograph coupled to 5970 mass selective detector system	Agilent (Waldbronn, Germany)
Gas chromatograph coupled to a mass selective detector system	Thermo Scientific (Waltham, USA)
1100 series HPLC system	Agilent (Waldbronn, Germany)
Gel documentation system IDA	Raytest (Straubenhardt, Germany)
Percival climate chmaber	CLF Plant Climatics (Emersacker, Germany)
Phospho- and fluorescence-imager Fuji FLA-3000	Raytest (Straubenhardt, Germany)
Light microscope BX51	Olympus (Tokyo, Japan)

2.2 Chemical Equipment

All chemicals were purchased from Amersham Bioscience (Freiburg, Germany), Duchefa (Haarlem, Netherlands), Fermentas (St. Leon-Rot, Germany), Roth (Karlsruhe, Germany), Sigma-Aldrich (Munich, Germany) unless otherwise indicated.

2.3 Lipid standards

Fatty acid methylester mix (C4 – C24)	Sigma-Aldrich, Munich, Germany
Heptadecanoic acid	Sigma-Aldrich, Munich, Germany
Pentadecanoic acid	Sigma-Aldrich, Munich, Germany
Acyl-CoA Standards	Larodan, Malmo. Sweden
Phospholipidstandards	Sigma-Aldrich, Munich, Germany
Glycolipidstandards	Sigma-Aldrich, Munich, Germany

2.4 Enzymes

TaKaRa ExTaq DNA polymerase	Cambrex BioScience (Potsdam, Germany)
RevertAid™ H Minus Reverse Transcriptase	Fermentas (St. Leon Rot, Germany)
RNase	Fermentas (St. Leon Rot, Germany)
DNaseI	Fermentas (St. Leon Rot, Germany)

2.5 Software

AIDA Raytest	Straubenhardt, Germany
Chemstation Agilent Technologies	Waldheim, Germany
Vector NTI Advanced 10	Invitrogen (Karlsruhe Germany
Photoshop 10	Adobe system (San Jose, USA)
Adobe Illustrator	Adobe system (San Jose, USA)
MS-Office 2007	Microsoft (Redmond, USA)
Xcalibur	Thermo Scientific (Waltham, USA)

2.6 Organisms

Plants: *Arabidopsis thaliana* Col-0, Wild type

Brassica napus, Wild type

T-DNA insertions lines: *lacs1* (At2g47240; SALK_127191, SALK_138782), *lacs2* (At1g49430), *lacs3* (At1g64400), *lacs4* (At4g23850; SALK_101543, SALK_126610), *lacs8* (At2g04350; SALK_136060), *lacs9* (At1g77590; SALK_111833; SALK_040810) were isolated at the SALK Institute (Alonso *et al.,* 2003) and seeds were obtained from the European Arabidopsis Stock Center (NASC).

The following double and triple mutant lines were obtained by crossing: *lacs1 lacs2, lacs1 lacs4, lacs1 lacs8, lacs1 lacs9, lacs2 lacs4, lacs1 lacs2 lacs4, lacs3 lacs4, lacs4 lacs9, lacs4 lacs8, lacs4 lacs8 lacs9, lacs8 lacs9*

2.7 Cultivation conditions

2.7.1 Surface sterilization of *Arabidopsis thaliana* seeds

For a variety of analysis plants were grown on solid media under sterile conditions, therefore seeds needed to be sterilized to prevent uncontrolled growth of microorganisms. Approx. 50 mg of seeds were washed for 1 min. in 70 % ethanol and were then incubated for 20 min. under shaking conditions in a 1 % sodium hypochlorite and 0.1 % SDS solution. Afterwards seeds were washed three times in sterile water and resuspended in sterile 0.1 % (w/v) water agar.

2.7.2 Cultivation on plates

For cultivation on agar plates surface sterilized seeds (2.7.1) were distributed on sterile Murashige Skoog (MS) medium. Additionally, Kanamycin as selection marker (40 µg/ml) and sucrose (1 %) were added if necessary. Plates were incubated under constant light (120 µmol/m² s) or under a 16 h light /8 h dark cycle (120 µmol/m²s).

MS Medium

4,3 g / l Murashige and Skoog medium

1 % (w/v) Sucrose

pH was adjusted to 5.9 with KOH

0,7 % (w / v) micro agar (Duchefa)

2.7.3 Cultivation on soil

When plants were grown under non-sterile conditions, soil (Fruehstorfer Erde Typ T25, Industrie Erdwerk Archut, Lauterbach-Wallenrod, Germany) needed to be incubated for 8 h at 80 °C to kill all kinds of insects. Seeds were directly sown with a tooth pick or carefully transferred from MS- media. Stratification to break dormancy was done for 2 – 4 days at 4 °C afterwards seeds were transferred into climate chambers with 8 h or 16 h light per day, 120 µmol/m²s light intensity, temperatures between 16°C – 22 °C and 60 % relative humidity.

2.8 Transformation of *Arabidopsis thaliana*

The flower dipping method was used for transformation of *Arabidopsis thaliana* plants. This method is described by Clough and Bent (Clough *et al.,* 1998) and is based on *A. tumefaciens* cells as a mediator for delivering DNA constructs. Transgenic seeds were selected by kanamycin and/or BASTA® screening. For further analyses the transgenic lines were characterized by genotyping and RT-PCR (see below).

The *Agrobacterium tumefacien*s cells were grown in YEB medium to an OD_{600} of 2 and subsequently washed and resuspended in sucrose solution. After addition of a detergent (Silwet L-77) to reduce the surface tension, stems from plants with non opened flower buds were incubated for 5 to 30 sec. in this suspension. The plants were kept for 4 – 8 h under a plastic coverage to avoid direct light and ensure high humidity. In the end plants were further cultivated in the greenhouse.

YEB-Medium

0,5 % (w / v) Meat extract

0,1 % (w / v) Yeast extract

0,1 % (w / v) Peptone

0,5 % (w / v) Sucrose

0,5 mM $MgSO_4 \cdot 7H_2O$

2.9 Extraction and analysis of DNA, RNA and cDNA

2.9.1 Isolation of DNA

Genomic DNA was isolated for the identification of plant genotypes. For this approach plant material (ca. 100 mg) was frozen in liquid nitrogen and grinded with a pestle to fine powder. After addition of cetyltrimethylammonium bromide (CTAB) solution samples were incubated for 30 min. at 65 °C, afterwards an equal volume of chloroform/isoamyl alcohol (24:1, v / v) was added, the samples were centrifuged and the upper phase was transferred into a new vial with CTAB / NaCl solution. After a short incubation step isopropanol was added and samples were centrifuged again. The pellet obtained was washed with 70 % ethanol and resuspended in water.

CTAB Solution

2 % (w/v) CTAB

100 mM Tris – HCl, pH 8,0

20 mM EDTA, pH 8,0

1,4 M NaCl

2.9.2 Isolation of RNA

The InviTrap® Spin Plant RNA Mini Kit was used for isolation of RNA. The principle of this kit is based on silica column purification. After a first purification step genomic DNA is removed via binding to a filter matrix. In the course of the purification process the RNA is bound to a second filter, washed and finally eluted. The eluate containing the RNA can be used for a broad spectrum of applications like Northern blotting, cDNA synthesis or RT PCR.

2.9.3 copyDNA (cDNA) Synthesis

cDNA represent the RNA pool of a cell at a given time and is therefore used for analyses of expression levels of specific genes. The reverse transcription of RNA into cDNA is catalyzed by the enzyme reverse transcriptase identified in retro-viruses 1970 (Temin *et al.,* 1970).
To obtain cDNA from *Arabidopsis* RNA was isolated and treated with DNase to avoid potential genomic DNA contamination. For reverse transcription an oligo dT / random nonamer primer mix was used with the RevertAid H Minus First Strand cDNA synthesis kit (Fermentas, St. Leon Rot, Germany). The protocol was carried out according to manufacturer's instructions; it is based on the method described by (Mullis *et al.,* 1987).

2.9.4 Amplification of specific DNA fragments by polymerase chain reaction (PCR)

PCR was first described by (Mullis *et al.,* 1987) and is a technique for the amplification of a specific DNA fragment. The technique has a broad range of applications and is extremely important for e.g. genotyping or cloning of genes. Essential components of a PCR are a heat stable polymerase, dNTPs, template DNA and primers for localizing of the starting point and direction of the PCR reaction. In the first step of a PCR (denaturation) the two DNA strands of the template are separated, in the second step (annealing) primers bind to their complementary sequences on the template DNA. In the third step (extension) the polymerase is recognizing the annealed primers and starts to synthesis a DNA strand being complementary to the template DNA. This three step cycle is repeated 30 – 35 times leading

to an exponential amplification of a specific DNA fragment. A typical PCR program is shown below:

95 °C	2 min (Denaturation)	
95 °C	30 sec. (Denaturation)	
T_M °C	30 sec. (Annealing)	
72 °C	n sec (Elongation)	Approx. 1 min. / 1kb product length
	Step 2 – 4 are repeated approx. 32 times	
72 °C	5 min.	
10 °C	hold	

Tm is the specific melting temperature of the chosen primers and can be calculated as Follows (Suggs SV 1981):

Tm [°C] = 2*(sum of A + T) + 4*(sum of G + C)

Primers were ordered from Sigma (Munich, Germany) or Invitrogen (Karlsruhe, Germany), primer sequences of utilized primers are listed in the appendix.

2.9.5 Separation of DNA fragments on agarose gels

Agarose gels were used to separate DNA fragments via electrophoresis according to their size. The gels had a concentration of 1 % agarose and were prepared with TAE buffer; as DNA standard the GeneRuler 1 kb DNA Ladder (Fermentas, St. Leon Rot, Germany) was used. Gels were run for 30 min. at 120 V and afterwards incubated in ethidium bromide solution for 10 min. The DNA bands were visualized under UV light and documented with the gel documentation system IDA.

TAE Buffer

40 mM Tris – acetate, pH 8,3

2 mM EDTA

2.10 Microscopy and histochemical techniques

2.10.1 Aniline blue staining

Aniline blue staining was used for visualization of growing pollen tubes inside stigmas and pistils. This dye is interacting with callose and is forming a fluorescent complex which can be visualized under UV light. This analysis was done for Wt, *lacs1*, *lacs4* and *lacs1 lacs4* plants. Softly squeezed samples of stigmas and pistils were incubated for up to 10 min. in aniline blue, washed in water and viewed under a fluorescent microscope.

2.10.2 Histochemical staining for β - glucuronidase (GUS) activity

With this technique it is possible to analyze the activity of specific promoters of interest in all kind of plant tissues. For this approach the β – glucuronidase (GUS) reporter gene was fused to a promoter of interest and the resulting construct was transformed into *Arabidopsis*. The promoter activity led to an expression of the GUS gene in tissues where the gene of interest is normally expressed. The expression is visualized by infiltrating the tissue with the colorless substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylammonium salt (X – Gluc).The β – glucuronidase is converting the compound into the blue dye 5′-dibromo-4,4′-dichloroindigo. Therefore intensive blue color is indicating plant tissue with high promoter activity.

Plant tissue was incubated in GUS staining solution over night at room temperature or at 30 °C. When leaf tissue was analyzed a vacuum was applied for approx. 15 min. to remove air trapped inside the tissue. After incubation the staining solution was removed and samples were incubated in an ethanol series (25 %, 50 %, 75 %, 100 %) to remove chlorophyll, each incubation step lasted 3 – 4 h. The last step was carried out over night. Staining of samples was stable for weeks and could be analyzed by a camera. However, if staining was too weak, incubation in staining solution was elongated, but not be longer then 2 -3 days, since the tissue is not fixed and therefore extensive staining could result in artifacts.

2.10.3 Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

SEM was used to analyze surface wax crystals on stems and the morphology of pollen grains. Fresh stems and mature anthers from open flowers were collected from the different mutant lines, rapidly frozen in propane - isopentane 3 / 1 at -198 °C (Jehl *et al.,* 1981), placed on stubs and coated with gold. Samples were examined using a field emission-scanning electron microscope (FE-SEM) LEO Supra 35 at 5 kV.

For TEM analysis of pollen grains, mature anthers were fixed in 2.5 % glutaraldehyde and 2 % formaldehyde in 100 mM PIPES buffer at room temperature for 2 h or overnight at 4 °C. After washing, samples were fixed in 2 % osmium tetroxide for 2 h at 4 °C, washed again several times in buffer and incubated in 1 % uranyl acetate for 4 h at room temperature. Subsequently, samples were dehydrated in graded series of ethanol and acetone and were finally embedded in epoxy resin (Spurr 1969). Ultra thin sections were prepared using an ultramicrotome (Ultracut E, Reichert-Jung, Vienna, Austria) and mounted on formvar-coated copper grids. Sections were stained with 3 % uranyl acetate in 80 % methanol for 5 min, washed with 80 % methanol and stained a second time with 0.3 % lead citrate for 5 min. Samples were viewed by Dr. Michael Hoppert with a Zeiss EM 902 transmission electron microscope at an acceleration voltage of 80 kV.

2.11 Lipid extraction, derivatization and analysis

2.11.1 Lipid extraction

For lipid extraction plant material of 6-week old plants were used. This material was frozen in liquid nitrogen and ground with a pestle. HIP solution (Hexane: Isopropanol: 2,6-Di-tert-butyl-4-methylphenol (BHT)), internal standard (heptadecanoic acid), and a small volume of K_2SO_4 were added and the suspension was shaken at 5 °C for 30 min. to obtain a good extraction rate. Afterwards samples were centrifuged, pellets obtained were discarded and supernatants were transferred into a new glass vial. K_2SO_4 was added to the suspension for removal of water soluble components. After a second centrifugation the upper lipophilic phase was transferred into a new vial and brought to dryness under a stream of nitrogen.

Lipids were resuspended in HIP solution and used for fatty acid analysis or Thin Layer Chromatography (TLC).

2.11.2 Derivatization of bound and free fatty acids

To analyze fatty acids and lipids with gas chromatography (GC) they need to be derivatized since natural lipids are not volatile enough. Therefore, (trans-) methylation was carried out with fractions of total lipid extracts or specific lipid classes from TLC plates. For this approach lipid fractions were transferred into new vials, brought to dryness under a stream of nitrogen and were resuspended in FAME solution (Methanol, sulfuric acid, Dimethoxypropane; 400:1:1). Afterwards samples were incubated for 1 h at 80 °C, so that (trans-) methylation could take place. To stop the reaction samples were cooled down to 5 °C and a 5 M NaCl solution was added. The methylated fatty acids were extracted two times with hexane and washed with water to remove traces of acid. The washed hexane phase was brought to dryness under a stream of nitrogen and was resuspended in acetonitrile for analysis with gas chromatography.

For analysis of free fatty acids it is important to avoid methylation of bound fatty acids, since they would overlay the free fatty acid profile due to their higher amount. Therefore methylation was performed without trans-esterification. Samples were resuspended in a 1.6 M methanolic 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC solution) and incubated for 2 h at room temperature (Stumpe *et al.,* 2001). The methylated fatty acids were extracted with hexane and brought to dryness under a stream of nitrogen. After resuspension in acetonitrile the samples were analyzed by GC.

2.11.3 Extraction and derivatization of surface wax

Surface wax was extracted by dipping leafs or stems for 5 seconds in pure hexane. Afterwards the hexane was filtered through prewashed cotton und brought to dryness under a stream of nitrogen. For later calculation of wax load the leaf or stem area had to be determined, therefore the plant materials were scanned and pictures were analyzed with the program

“Blattfläche”. As internal standards pentacosane, heptadecanoic acid and heptacosanol were used

For derivatization dried surface wax was resuspended in N,O-bis(trimethylsilyl) trifluoroacetamide **(**BSTFA) together with pyridine as a catalyst. BSTFA is leading to a trimethylsilylation of hydroxyl groups. Therefore, it is possible to analyze a wide range of compounds. Samples resuspended in BSTFA together with pyridine were incubated at 120 °C for 20 min. and brought to dryness under a stream of nitrogen. After resuspension in hexane samples were analyzed with gas chromatography coupled to mass spectrometer (Preuss *et al.,* 1993).

2.11.4 Extraction and derivatization of acyl – CoA (Larson *et al.*, 2001)

To extract acyl CoA 200 mg plant material was frozen in liquid nitrogen and ground to a fine powder. Extraction puffer was given to pulverized leaf material and samples were homogenized via vortexing. As an internal standard heptadecanoyl-CoA was (37.5 pmol) added. Pigments and lipids were removed from the sample with petrol ether saturated with isopropanol / water (1:1), after three washing steps saturated ammonium sulfate and methanol / chloroform (2:1) were added. Samples were incubated for 20 min. at room temperature under shaking conditions, afterwards cell debris was removed by centrifugation (16000 g; 2 min.) and the supernatant was transferred in a new vial and brought to dryness under a stream of nitrogen.

Derivatization solution was added to the dried samples and it was incubated for 20 min. at 85 °C. During this reaction the adenine group of CoA is modified via a fluorescent etheno group. The derivatized samples could be directly used for HPLC analysis. However samples could also be stored at -20 °C for weeks. It was important to centrifuge sample before analysis due to the formation of precipitate. This precipitation occurred a second time if samples were cooled down again after the first centrifugation, resulting in significant lowered signals on HPLC.

Extraction buffer:

49,85 % Isopropanol

49,85 % KH_2PO_4 (pH 7,2); 0,05 M (higher concentration lead to more increased precipitation)

0,01 % µl Acetic Acid

0,02 % BSA (50 mg/ml, defatted)

Derivatization solution:

0,5 M Chloroacetaldehyde

0,15 M Citrate buffer (pH 4,0)

0,5 % (w/v) SDS

Should be stored in the dark and not used for longer than six month due to light sensitivity.

2.11.5 Separation of lipid classes with Thin Layer Chromatography (TLC)

Thin layer chromatography was used to separate lipids, waxes and pigments. For this approach samples were loaded with a TLC robot (Camag, Muttenz, Switzerland) on a 20 x 20 TLC plate which was developed vertical in glass chambers containing specific running solvents. After development plates were taken out, dried and used for further analysis.

To separate neutral lipids hexane/diethyl ether/acetic acid (80:20:1, v/v/v) was used, the solvent system for separation of phospholipids was chloroform/methanol/acetic acid (65:25:8, v/v/v) and for glycolipids the following running solvent system has been used: chloroform / methanol (85:20, v/v).

Lipids were identified on these plates by co-migration of standard lipids. In case of analytical TLC, plates were incubated in $CuSO_4$ (10 g $CuSO_4$ x 5 H_2O, 92 ml H_2O, 8 ml H_3PO_4) solution for approx. 8 s using the Chromatogram Immersion Device III (Camag, Muttenz, Switzerland). After drying, TLC plates were heated to 170 °C to visualize the lipid bands with a TLC Heating Plate (Camag, Muttenz, Switzerland).

A preparative TLC was carried out if more detailed analyses of lipids were required. For this purpose 0.2 % (w/v) 8-anilino-1- naphthalene-sulphonic acid (ANS) were sprayed onto plates using the TLC Spray Cabinet (Camag, Muttenz, Switzerland). After drying, lipid bands were visualized under UV light (λ = 254 and 365 nm) and scraped from the plate to perform further purification or derivatization.

2.11.6 Extraction and analysis of radioactive labeled lipids

Lipids of plant material exposed to radioactive metabolites for e.g. labeling experiments were extracted according to a modified HIP protocol.

After incorporation of radioactive markers, plant material was incubated in isopropanol for 10 min at 80 °C and frozen in liquid nitrogen. The samples were ground to a fine powder and 1.5 ml HIP solution was added. Afterwards samples were shaken for 10 min. at room temperature. Cell debris was removed by centrifugation at 13000 rpm for 5 minutes; the supernatant was transferred into a new tube and washed with K_2SO_4. The remaining cell debris pellet was washed a second time with hexane. The resulting second lipophilic phase was also washed with K_2SO_4, combined with the first hexane phase and brought to dryness under a stream of nitrogen. Lipids were redissolved in 50 µl HIP solution and analyzed with a two dimensional TLC using Chloroform: Methanol: Water (65:35:4) in the first dimension and Chloroform: Methanol: Ammonia hydroxide (62:25:5) in the second dimension. This protocol is based on the study by (Bates *et al.,* 2007). The radio labeled lipids were visualized after five days of exposure using a phosphor imager (*Fuji* FLA-3000, Raytest, Straubenhardt, Germany).

2.12 HPLC, GC and GC/MS analysis

2.12.1 High-performance liquid chromatography (HPLC) analysis of acyl-etheno-CoA-Ester (Larson *et al.*, 2001)

HPLC analysis was performed by using an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany). For the separation of esters a LUNA 150 x 2,0 mm column (Phenomenex, Aschaffenburg, Germany) and a 4 x 2 mm Phenyl-Propyl-precolumn (Phenomenex, Aschaffenburg) was used. Detection was achieved using a fluorometer with an excitation wavelength of 230 nm and an emission wavelength of 420 nm. For quantification of acyl CoAs peak areas were integrated using the software *ChemStation-Software* (Rev. A. 10.02; Agilent, Waldbronn, Germany). For identification retentions times of specific peaks were compared to standard acyl-CoAs.

The gradient and the solvents for eluting the esters are given below:

Time (min)	A (%)	B(%)	C(%)	D(%)	Flow rate (ml/min)
0	90	10	0	0	0,4
5	20	80	0	0	0,4
5,1	20	0	80	0	0,4
7	0	0	97	3	0,2
10	0	0	95	5	0,2
10,1	0	0	95	5	0,2
50	0	0	55	45	0,2
51,1	0	0	0	100	0,4
52	0	0	0	100	0,4
62	0	0	0	100	0,4
62,1	90	10	0	0	0,4
65	90	10	0	0	0,4

Eluent A H_2O / acetic acid (100:1, v/v)

Eluent B Acetonitrile / H_2O / acetic acid(90:9:1, v/v/v)

Eluent C H_2O / triethylamine (100:0,25, v/v)

Eluent D Acetonitrile / H_2O (90:10, v/v)

2.12.2 Gas chromatography (GC)

For identification and quantification methylated fatty acids were analyzed with a gas chromatograph which was coupled to a flame ionization detector (FID) (6890 series GC system; Agilent, Waldbronn, Germany). For this analysis the column DB-23 (30 m x 0.25 mm, 0.25 µm coating thickness, J&W Scientific, Agilent, Waldbronn, Germany) was used with Helium as carrier gas with a flow of 1 ml / min. The detailed method as well as the temperature gradient is given below:

Chromatographer:	Agilent GC 6890 Series
Column:	DB-23, 30 m x 250 µm film thickness 0.25 µm (Agilent, Waldheim)
Split:	15:1 (for derivatives of free fatty acids)
	60:1 (for derivatives of esterified fatty acids)
Injection volume:	1 µl (for derivatives of free fatty acids)
	1 µl (for derivatives of esterified fatty acids)
Carrier gas:	Helium (1 ml / min.)
Temperature program:	150 °C 1 min
	150 °C – 160 °C, 10 °C / min.
	160 °C – 200 °C, 6 °C / min.
	200 °C – 250 °C, 25 °C / min.
	250 °C, 4 min.
Detector:	FID

Fatty acids were identified with commercially available standards; quantification was carried out using an internal standard (heptadecanoic acid).The integration of peak areas was performed by using ChemStation-software (Agilent, Waldheim).

2.12.3 Gas Chromatography coupled to Mass Spectrometry (GC/MS)

GC/MS analysis was performed to study the chemical composition of surface wax from stems and leafs but also for the analysis of unknown substances seen on GC chromatograms. The analysis was carried out using a Thermo Finnigan (Austin, Texas, USA) Polaris Q mass

selective detector connected to Thermo Finnigan Trace gas chromatograph equipped with a capillary Rtx-5MS column (20 m x 0.25 mm, 0.25 µm coating thickness; Restek, Bad Homburg, Germany) and helium as carrier gas (1 ml min^{-1}). An electron energy of 70 eV, an ion source temperature of 200 °C and a temperature of 300 °C for the transfer line was used. The samples were measured in the electron impact ionization mode and the splitless injection mode (opened after 1 min) with an injector temperature of 220 °C. The programmed temperature gradient for the GC is given below:

Standard temperature program:	100 °C 3 min.
	100 °C – 320 °C, 15 °C / min.
	320 °C 10 min.
Wax ester temperature program:	60 °C 2 min.
	60 °C – 200 °C, 40 °C / min.
	200 °C, 2 min.
	200 °C – 320 °C, 3 °C / min.
	320 °C 16 min.

The different wax compounds were identified by commercially available standards, synthesized standards, or GC/MS databank information.

2.13 Radioactive labeling experiments

The export of lipids from plastids to ER was analyzed by an *in vivo* labeling experiment according to (Bates *et al.,* 2007).

^{14}C acetate [50 mCi / mmol] was dissolved in 5 ml of media containing 20 mM MES pH 5.5, 0.1 x MS salts and 0.01% Tween 20 to a final concentration of 250 µCi. 0.2 g of freshly

detached leafs were incubated in this buffer for 1 min, 5 min. and 10 min. under intensive illumination. The incorporation was terminated by washing leafs in buffer containing no radioactivity for a few seconds. Leafs were then incubated in isopropyl alcohol for 10 min. at 80 °C and frozen in liquid nitrogen. After lipid extraction a 2D-TLC was performed as described above.

3. Results

The impact of LACS enzymes on plant lipid metabolism has been discussed for a long time, with only few examples where loss of specific LACS activities resulted in visible phenotypes (Fulda *et al.,* 2004; Lu *et al.,* 2009; Schnurr *et al.,* 2004; Weng *et al.,* 2010). However, this class of enzyme is thought to play an essential role in lipid metabolism being responsible for the reactivation of fatty acids after their release from the chloroplast (Benning 2009; Schnurr *et al.,* 2002). Furthermore, recent reports suggested that LACS enzymes are also important for secondary metabolism like surface wax and storage lipid synthesis but the biological relevance of most of the nine LACS enzymes remained unknown. To solve this problem a large number of double and triple knock out lines were generated and screened for potential phenotypes. The results of this screen are schematically shown in Fig. 8. The scheme illustrates the observed influences of LACS activities on different aspects of the plant life cycle. In the following identification and analysis of LACS mutant lines is presented, showing the influence of this enzyme family on developmental stages in detail.

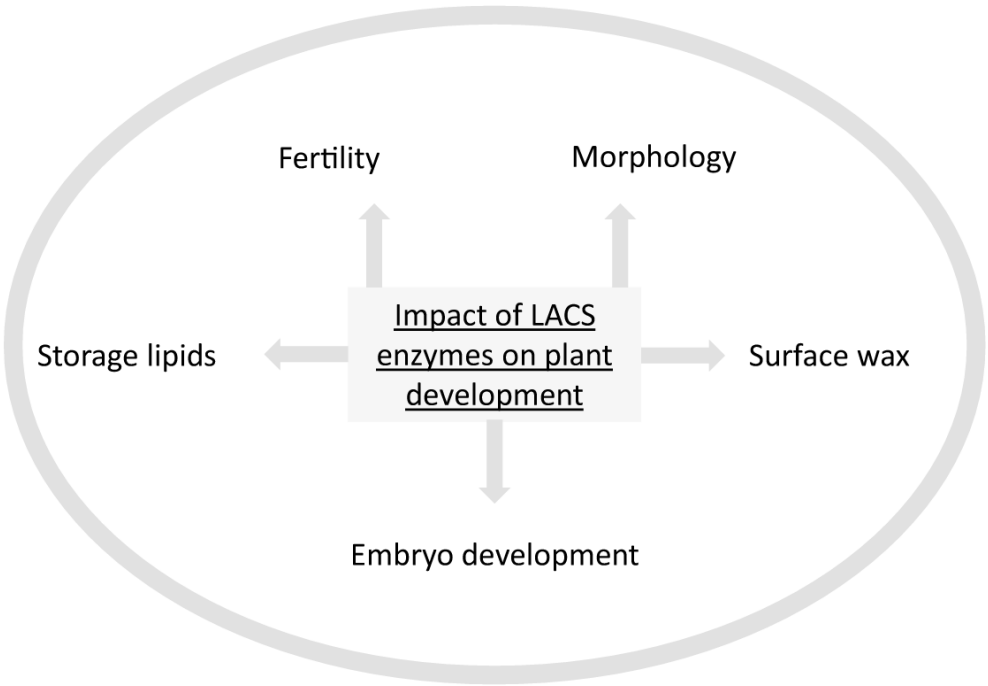

Fig.8: Impact of LACS enzymes on plant development. Developmental processes during the whole life cycle of *Arabidopsis* are affected by inactivation of specific LACS enzymes. Fertility as well as embryo development and storage lipid synthesis are LACS dependent, however established plants are also affected by LACS inactivation, since it influences morphology and surface wax synthesis.

3.1 Identification of mutants

A mutant collection harboring about three dozen different genotypes was established in order to identify LACS proteins with essential influence on lipid metabolism. The single knock out mutants were identified from the T-DNA Express database (http://signal.salk.edu/cgibin/tdnaexpress) and obtained from The Nottingham Arabidopsis Stock Centre (NASC). The individual single mutant lines were systematically crossed to produce double and triple mutants. Six genotypes have been identified by screening this collection for visible phenotypes (Tab.2.). Four out of these six genotypes were verified by independent allele combinations.

Tab.2. Identified LACS mutant lines with observed phenotypes and allele combination.

Genotype	Phenotype	Allele
lacs1	Reduced fertility and surface wax layer	SALK_127191 (*lacs1-1*)
		SALK_138782 (*lacs1-2*)
lacs4	Affected pollen morphology	SALK_101543 (*lacs4-1*)
		SALK_126610 (*lacs4-2*)
lacs1 lacs4	Conditional sterility and reduced surface wax layer	SALK_127191 / SALK_101543 (*lacs1-1 lacs4-1*);
		SALK_138782 **/** SALK_126610 (*lacs1-2 lacs4-2*)
lacs4 lacs9	Reduced growth rate and affected leaf morphology	SALK_101543 / SALK_111833 (*lacs4-1 lacs9-1*);
		SALK_126610 / SALK_040810 (*lacs4-2 lacs9-2*)
lacs1 lacs4 lacs9	Conditional sterility, reduced growth rate and reduced surface wax layer	SALK_127191 / SALK_101543 / SALK_111833
lacs4 lacs8 lacs9	Gene dosage effect; Heterozygosity of *LACS4* results in mild growth phenotype, Heterozygosity of *LACS8* results in phenotype comparable to *lacs4 lacs9*, Heterozygosity of *LACS9* results in phenotype with massive morphological changes.	SALK_101543 / SALK_136060 / SALK_111833

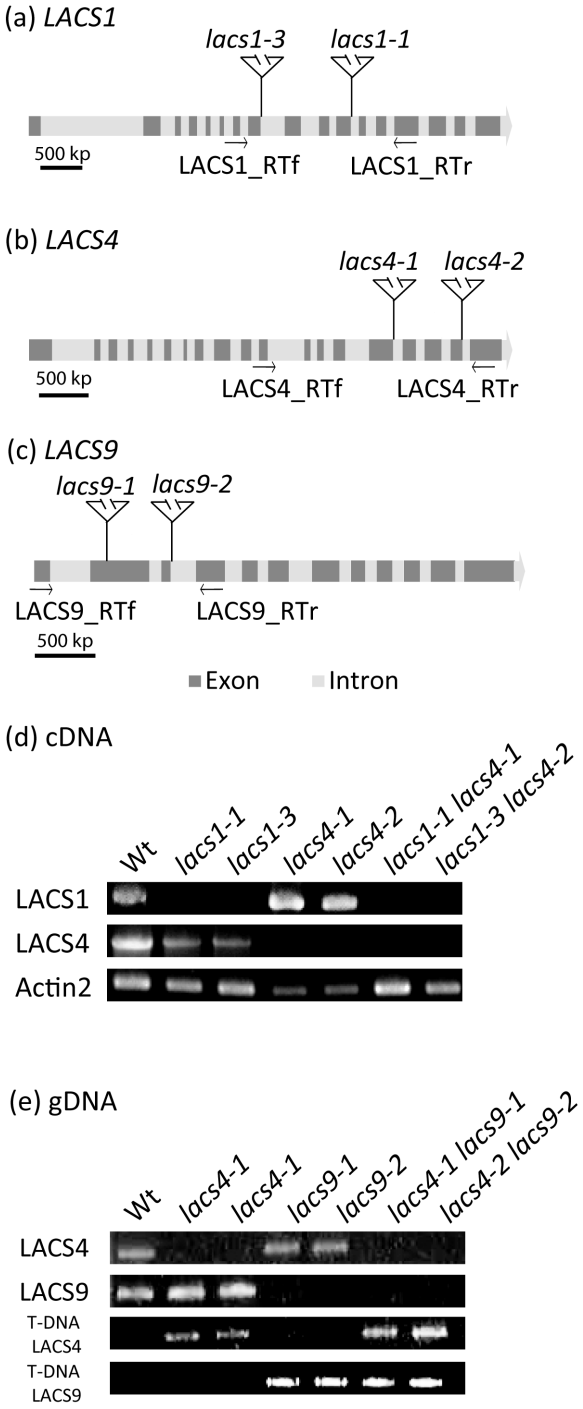

Fig.9. Identification of *lacs1*, *lacs4* and *lacs9* mutant lines. The schemes show the gene structure of *LACS1* (a), *LACS4* (b) and *LACS9* (c) depicting exons in dark grey and introns in light grey. For all three genes the locations of two independent T-DNA insertions are indicated. Arrows indicate positions of the primers used for (RT-) PCR. (d) The RT-PCR detected transcript of *LACS1* in wild-type (Wt) and in the *lacs4* single mutant lines but not in *lacs1-1* or *lacs1-3* mutants. *LACS4* transcript was found in wild type and in the *lacs1* single mutant lines but not in *lacs4-1* or *lacs4-2* mutants. In the two *lacs1 lacs4* double mutant lines both *LACS* transcripts were absent. *Actin2* served as control. (e) The PCR detected T-DNA insertions of *LACS9* in *lacs9* and *lacs4 lacs9* but not in wild-type (Wt) or *lacs4*. The genomic version of *LACS9* was only found in the wild-type and *lacs4*.

To verify that plants displaying a visible phenotype are indeed null mutants, gene expression was analyzed by RT-PCR or PCR on genomic DNA (Fig. 9). For this approach RNA/DNA was extracted from leafs and individual primer pairs flanking the T-DNA insertions were designed, gene models as well as localization of primers are shown in Fig.9 a, b and c. The high sequence homology within the LACS family had an enormous influence on the analysis potentially leading to wrong positive results. Therefore primer pairs were designed and tested with Invitrogens VectorNTI® on all sequences of the whole family (Appendix. 1).

By RT-PCR it could be demonstrated that in *lacs1*, *lacs4*, and *lacs1 lacs4* corresponding *LACS* transcripts are completely absent (Fig.9 d). Therefore it can be assumed that these plants are in fact null mutants. Possible genomic contaminations of cDNA could be excluded, since all samples were tested with a primer pair specific for *LACS5* as control. *LACS5* is exclusively expressed in flowers (Shockey *et al.,* 2002); therefore transcript detection in leafs is not possible and indeed no *LACS5* transcript signal has been detected proofing the absence of genomic DNA.

Identification of *lacs4 lacs9* (Fig.9 e) was based on genomic DNA. As shown in Fig. 9 e for all mutant alleles tested T-DNA insertions have been detected while wild type alleles were absent. Therefore, the complete inactivation of the corresponding LACS genes can be assumed. This view is further supported by the fact that both allele combinations of *lacs4 lacs9* developed a clear and similar phenotype.

3.2 Phenotypes of LACS mutants

As indicated in Tab.2 and Fig.8 the loss of specific LACS activities led to severe phenotypes with an impact on all parts of the plant life cycle. The physiological consequences will be presented in the following in more detail focusing on fertility (Chap. 3.2.1), surface wax synthesis (Chap. 3.2.2), morphology (Chap. 3.2.3), storage lipid synthesis (Chap. 3.2.4) and embryo development (Chap. 3.3.1).

3.2.1 Fertility

The mutant lines *lacs1* and *lacs1 lacs4* showed normal vegetative growth and development compared to wild type but were characterized by a significantly reduced fertility in case of *lacs1* and even complete sterility in case of *lacs1 lacs4* (Fig.10). The phenotype could be rescued by applying high humidity indicating that the reproductive organs are functional in general.

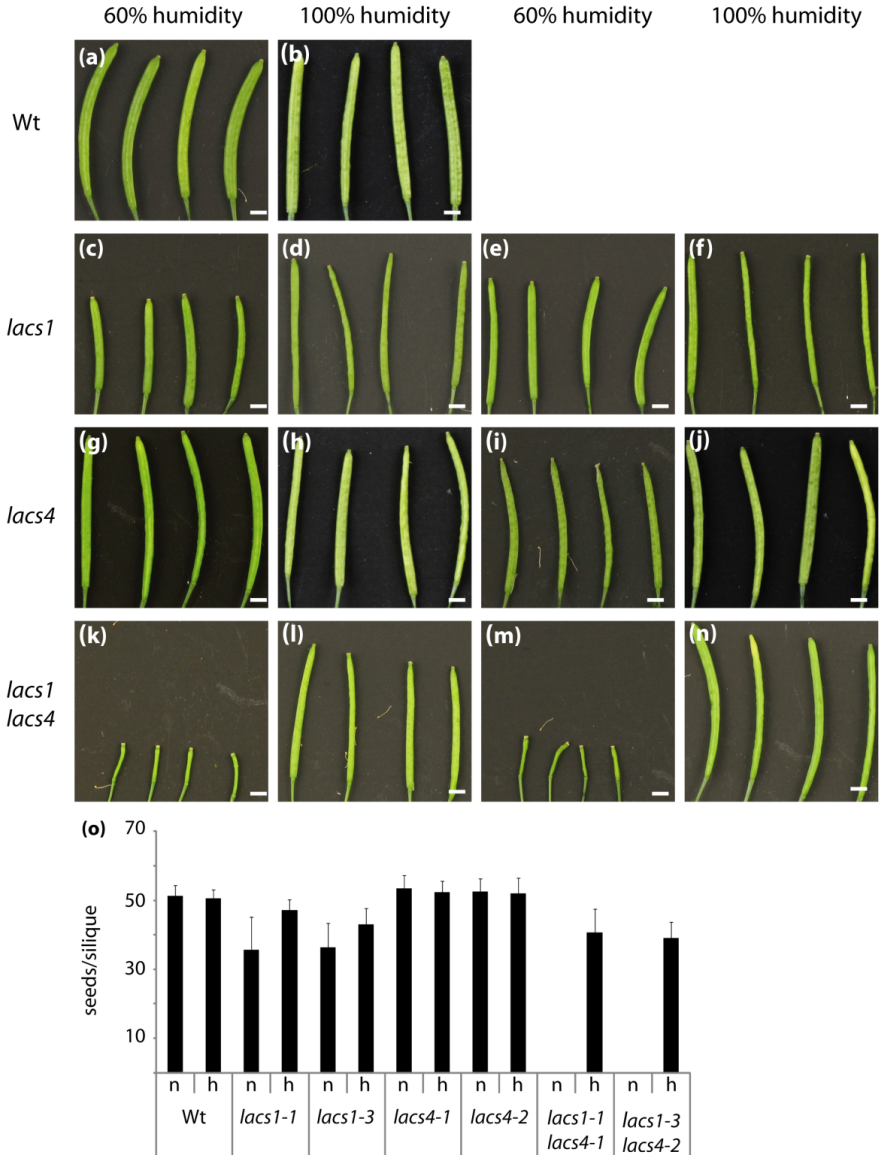

Fig.10. Fertility assessment of *lacs1*, *lacs4* and *lacs1 lacs4*. The different lines were compared with respect to the size of their siliques 10 days after flowering and to the seed yield per silique. The four siliques of each line were randomly chosen from four different plants. Plants were grown in 60 % relative humidity (a, c, e, g, i, k, m) or 100 % relative humidity (b, d, f, h, j, l, n). Shown are siliques of wild type (a, b), *lacs1-1* (c, d), *lacs1-3* (e, f), *lacs4-1* (g, h), *lacs4-2* (i, j), *lacs1-1 lacs4-1* (k, l), *lacs1-3 lacs4-2* (m, n). Scale bar = 0,5 mm.(o) The number of seeds per silique was determined in all different mutant lines under normal relative humidity (n) and high relative humidity (h). Each value represents the mean of five randomly selected siliques and the error bars indicate SD.

The impact of LACS on fertility has been roughly estimated by comparing siliques ten days after flowering (Fig.10 a - n). The siliques of *lacs1* were clearly reduced in size but still contained seeds having normal germination rates (Fig. 10 c, e). The single knock outs of *lacs4* produced siliques comparable to wild type (Fig. 10 g, i). In contrast, siliques of *lacs1 lacs4* had an even more reduced size than *lacs1* and contained no seeds when grown under standard conditions (Fig. 10k, m). However the observed phenotypes of *lacs1* and *lacs1 lacs4* could be rescued by high humidity, as shown in Fig.10 d, f, l and n. Plants grown under this condition reached a normal silique length with a significantly increased number of seeds (Fig.10 o).

3.2.1.1 Pollen stigma interactions

The reduced or in case of the double knock out even absent fertility could be rescued by high humidity suggesting that the early communication between pollen grain and stigma might be affected. This assumption was supported by staining growing pollen tubes inside stigma and pistell with aniline blue (Fig.11). That dye is interacting with callose of pollen tube cell walls and the formed complex is fluorescent under UV light. A high number of growing pollen tubes could be detected inside the wild type (Fig. 11 a). In contrast, no pollen tube growth was visible in *lacs1 lacs4* (Fig.11 b). This observation was supported by light microscopy studies identifying almost no hydrated pollen grains on the *lacs1 lacs4* stigma, indicating that pollen grains did not germinate. However, already 24 h after applying high humidity first pollen tubes were detected in the *lacs1 lacs4* stigma (Fig. 11 c).

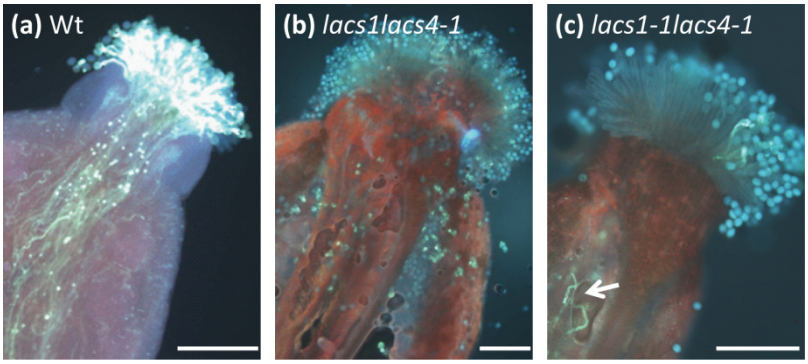

Fig.11. Pollen tube growth in wild type and in *lacs1-1 lacs4-1*. Fluorescence micrographs were taken of softly squeezed and stained wild-type and *lacs1-1 lacs4-1* stigmas. Aniline blue was used a fluorescent dye. Germinating pollen tubes penetrated stigma tissue of wild-type (a) but not the stigma of *lacs1-1 lacs4-1* (b). In flowers of *lacs1-1 lacs4-1* exposed to high relative humidity for 24h pollen tube growth (arrow) was partially restored (c). Scale bar = 200 µm.

Interestingly, *lacs1* and *lacs1 lacs4* pollen grains had normal germination rates on solid pollen culture media. It can be speculated that artificial media and humidity in the air act in a similar way in complementing the defects of the *lacs1* and *lacs1 lacs4* pollen grains. Together the results are supporting the assumption that only early communication between pollen grain and stigma is effected whereas all following steps function normally.

For deeper analysis the contribution of the male (pollen grain) and female (stigma) organs to the observed sterility was evaluated. As a first step a crossing experiment was performed schematically shown in Fig.12. The transfer of wild type pollen on non pollinated *lacs1 lacs4* stigmas resulted in normal seed development. In contrast, *lacs1 lacs4* pollen grains were not able to fertilize wild type stigma. To avoid the risk of wrong positive or negative results the sterile *opr3* mutant plant line (Stintzi *et al.,* 2000) was included into the crossings. This line is completely sterile due to reduced filament growth and deficient pollen dehiscence; therefore no pollination of the stigma can take place.

When wild type pollen grains were placed on the *opr3* stigma normal seed development was observed. However the pollination with *lacs1 lacs4* pollen resulted in empty siliques with no seeds. The results indicated that the sterility is most probably due to defects of the pollen grains of *lacs1 lacs4*.

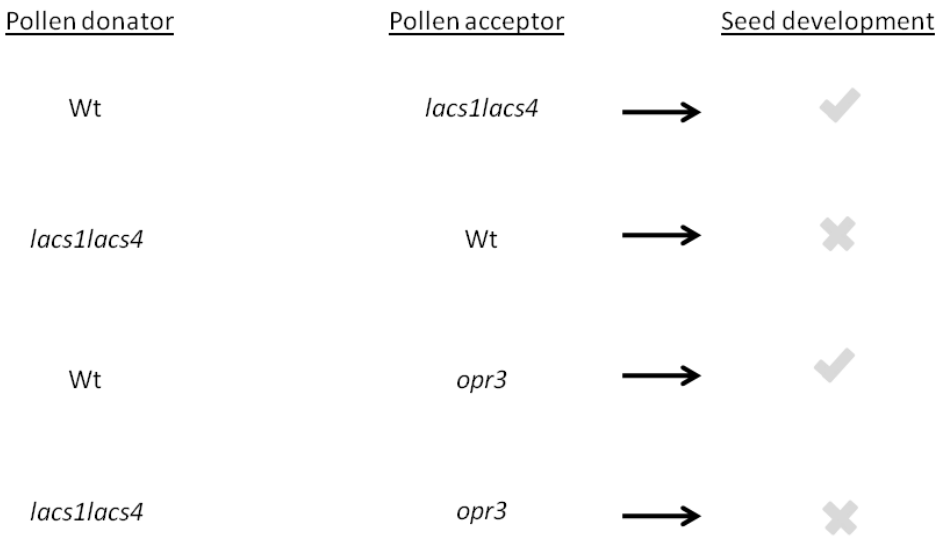

Fig. 12. Schematic pattern of crossings between Wt, *lacs1 lacs4* and *opr3*. Wt pollen grains lead to normal seed development when placed on non pollinated *lacs1 lacs4* stigmas, indicating that pollen grain recognition, acceptance and support is not affected in the double knock out line. In contrast, *lacs1 lacs4* pollen grains were unable to fertilize non pollinated Wt stigmas. Comparable results were obtained when *opr3* plants were used as pollen acceptor. The results indicated that the sterility of *lacs1 lacs4* is due to the defects of the pollen grains.

3.2.1.2 Pollen coat and its impact on fertility

The identification of pollen grains as the main reason for sterility in *lacs1 lacs4* led to focused further analysis on the male gametophyte. The interaction of pollen and stigma is rather complex, however, a central step is the hydration with water from the stigma. Since the phenotype of *lacs1 lacs4* could be complemented by increased levels of relative humidity it can be speculated that pollen hydration might be compromised in the mutant. It was reported earlier that reduced levels of lipophilic components on the pollen surface are responsible for conditional sterility, a sterility which can be complemented by increased relative humidity (Preuss *et al.,* 1993). These lipophilic components are found in an extracellular layer called tryphine covering the whole pollen grain. The extraction of tryphine lipids is relatively simple and can be performed with any lipophilic solvent. Best results were reported for the use of hexane (Piffanelli *et al.,* 1997), since it has been shown by electron microscopy studies that the tryphine was almost completely extracted after a short washing step.

To obtain sufficient amounts of pollen grains for lipid analysis approx. 25 trays per line were grown and the pollen was collected by a self made harvester consisting of a three layer filter system and a vacuum cleaner (Johnson-Brousseau *et al.,* 2004). With this approach it was possible to obtain up to 90 mg of highly pure pollen. The amount was sufficient for up to six extractions since approx. 15 mg pollen was required for one extraction and the analysis by GC/MS.

The results of the analysis are presented in Fig.13 a and b. The tryphine of *Arabidopsis* contained a mixture of different lipids, mainly alkanes, ketones and alkenes; other components like fatty acids or primary alcohols were also detectable but in significantly lower amounts. The analysis revealed that plants with a reduced fertility (*lacs1* and *lacs1 lacs4)* showed also reduced tryphine levels (Fig.13 a, b; Fig. 14 b, d). Compared to wild type the tryphine lipids were reduced to 61 % in case of *lacs1* and 22 % in case of *lacs1 lacs4.* Interestingly, the major lipid classes of the tryphine were affected in different ways (Fig. 13 b; Fig. 14 b, d). The amount of ketones did not change in *lacs1* whereas *lacs1 lacs4* showed a reduction by 54 %. Alkanes, being the most prominent compound class, displayed in *lacs1* a moderate reduction by 20 % whereas they were reduced by over 70 % in *lacs1 lacs4.* The values for alkenes were also severely reduced showing a loss of about 76 % in the single knock out lines and

94 % in *lacs1 lacs4*. These outcomes suggested overlapping functions of both LACS enzymes, since reductions were more pronounced in *lacs1 lacs4* compared to *lacs1*. Surprisingly, we detected remarkable increases of specific lipid classes in pollen of the *lacs4* single mutant (Fig. 13 a, b; Fig 14 c). In detail, pollen of *lacs4* contained 40 % more alkanes, 30 % more ketones and 10 % more alkenes compared to the wild type (Fig.13 b).

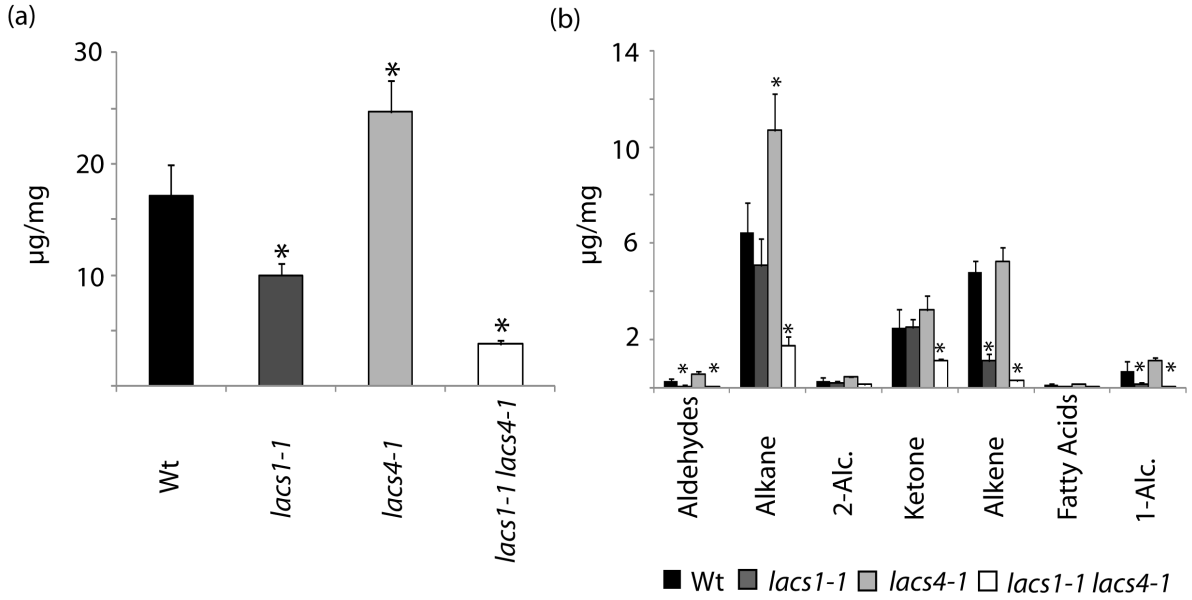

Fig 13. Lipid composition of the tryphine of wild-type, *lacs1-1*, *lacs4-1,* and *lacs1-1 lacs4-1*. (a) The total amount of tryphine lipids is given in µg per mg of pollen. (b) Values for the individual lipid compounds of tryphine lipids are given per mg of pollen. Each value represents the mean of three replicates and the error bars indicate SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

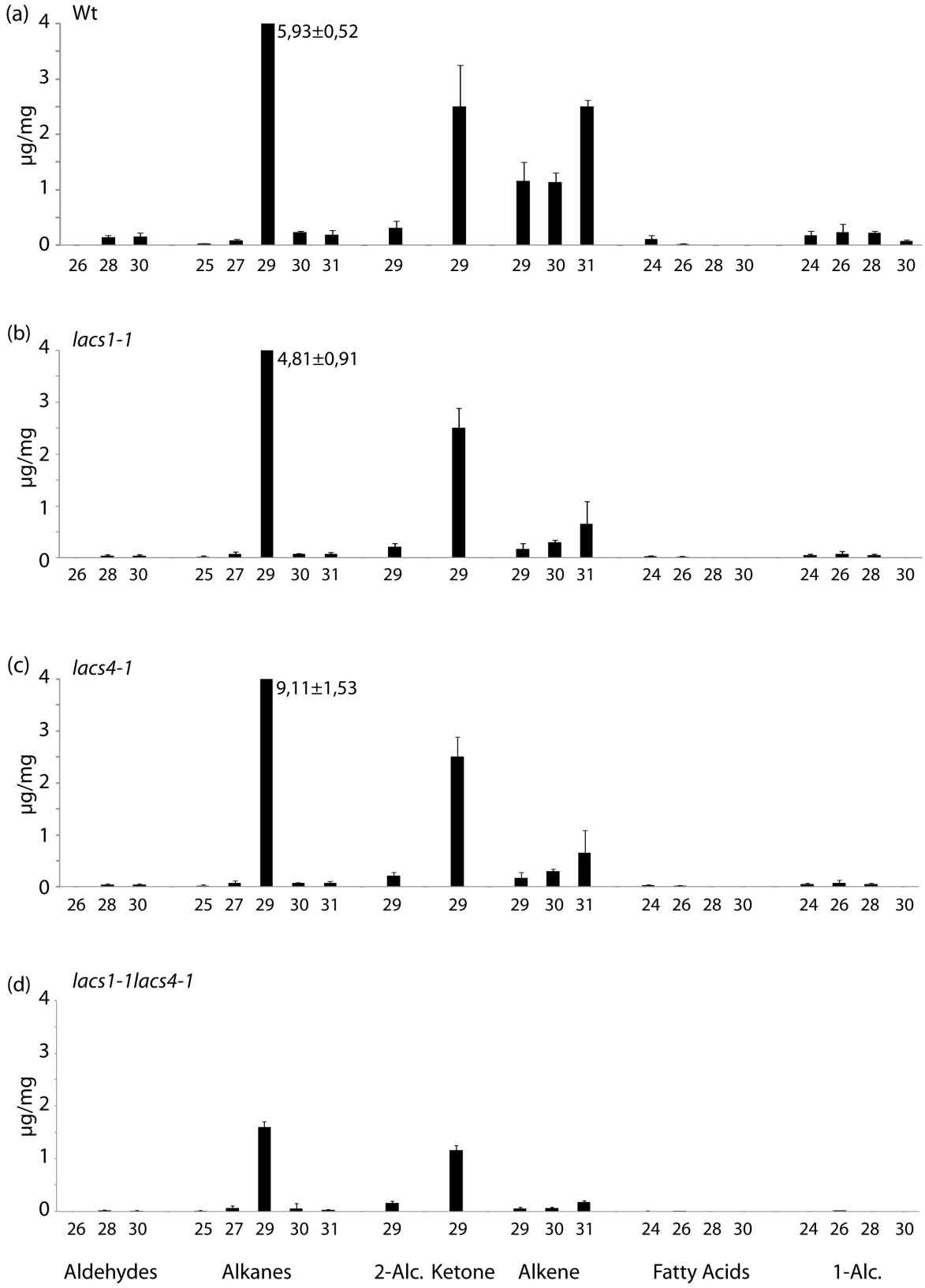

Fig 14. Detailed tryphine wax composition of wild-type (a), *lacs1-1* (b), *lacs4-1* (c) and *lacs1-1 lacs4-1* (d). The numbers on the *x*-axis indicate the carbon chain length of the respective compound and the chemical class is given at the bottom of the diagram. Values exceeding the maximum of the *y*-axis are indicated by numbers next to the respective bar. Each value represents the mean of 5 independent replicates. Error bars = SD.

3.2.1.3 Wax components inside the pollen grain

Due to the unexpected increase of tryphine components in *lacs4*, its impact on pollen lipid biosynthesis was analyzed in more detail. The aim of the following experiment was to distinguish between intra- and extracellular lipophilic components. Therefore, pollen grains were mechanically disrupted and lipids were extracted. The obtained total lipid profile of the pollen lysate allowed a comparison with the tryphine profile and especially a calculation of lipid amounts inside pollen grains. The results are presented in three diagrams: 1. Total lipid profile of pollen lysates (Fig. 15 a); 2. Tryphine lipid profile (Fig. 15 b); 3. Calculated internal pollen lipid profile (Fig. 15 c). The first interesting observation was the detection of high amounts of secondary alcohol C29 inside the pollen grains of Wt and *lacs4* (Fig. 15 c). In contrast to that only trace amounts had been detected in the tryphine (Fig. 15 b). Similar results were already reported by (Fiebig *et al.,* 2000; Preuss *et al.,* 1993), but they did not comment on this aspect. Similar data was also obtained from our analysis of *Brassica napus* pollen grains indicating that the special distribution of secondary alcohol C29 is not limited to *Arabidopsis*. Also in *Brassica napus* the secondary alcohol C29 was detected mainly inside pollen grains whereas ketone C29, being directly synthesized from secondary alcohol C29, was detected in high amounts in the tryphine.

The comparison between Wt and *lacs4* regarding external and internal data sets was even more surprising. Whereas the analysis of pollen coat indicated that *lacs4* contained higher amounts of very long chain lipids, it turned out that the concentration inside the pollen grains were reduced. The result suggested a modified distribution in the in pollen of the *lacs4* mutant line rather than increased biosynthesis (Fig. 15 c). In *lacs4* the concentration of specific very long chain lipids showed a 40 % increase in the tryphine, whereas total amount of very long chain lipids were reduced by about 20 % inside the pollen grains compared to wild type. The clearest effect was observed for alkane C29 being the most prominent wax component in the tryphine. This compound was detected in almost similar total amounts in wild type and *lacs4*. However, in *lacs4* pollen grains almost 100 % of alkane C29 was detected in the tryphine and only trace amounts were found inside the pollen grain. In contrast, in wild type pollen grains 67 % of alkane C29 was distributed to the tryphine and 33 % to

domains inside pollen grains. Furthermore, the amount of secondary alcohol C29 was significantly higher inside wild type pollen grains, whereas alkenes showed slightly higher concentrations in internal domains of *lacs4* pollen.

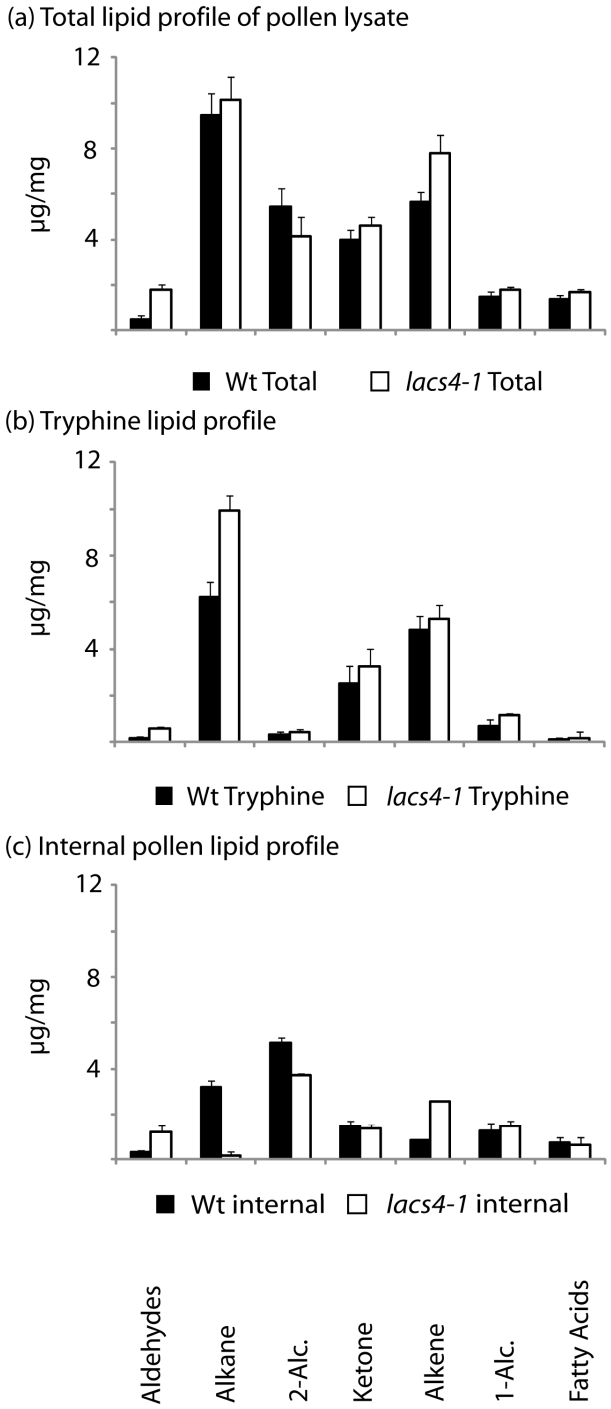

Fig 15. Localization of very long chain lipids in different domains of *lacs4-1* and wild-type pollen grains. (a) Comparison of very long chain lipids of pollen lysates of wild-type and *lacs4-1*. (b) Comparison of tryphine lipids of wild-type and *lacs4-1*. (c) Comparison of very long chain lipids present in internal domains of the pollen grains of wild type and *lacs4-1*. The values were obtained by calculating the difference between total content and the tryphine lipids. Each value represents the mean of four replicates and the error bars indicate SD.

Altogether 53 % of very long chain lipids were found in the pollen coat of wild type pollen grains whereas 65 % of these components were found in the pollen coat of *lacs4* supporting the hypothesis of a modified distribution of very long chain lipids in the mutant pollen.

3.2.1.4 Structural analysis of pollen grains

Besides biochemical alterations of the tryphine also morphological modifications of pollen could affect its fertility. Therefore, the pollen structure of the different mutant lines was analyzed in more detail. For the analyses we used Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) to investigate the overall and the intracellular architecture. The results from the TEM supported the biochemical data. Tryphine of *lacs4* was almost indistinguishable from the wild type in its form and shape; however in some cases unusual structures looking almost like cellular or membranous fragments were detected (Fig. 16 c). This disordered matrix might be a consequent of the modified very long chain lipid distribution within the *lacs4* pollen grains. The reaming mutant lines *lacs1* and *lacs1 lacs4* showed identical results regarding the extracellular membrane structure (Fig. 16 b, d), since exine as well as intine were not affected. However, the tryphine showed in both cases a reduced thickness but the matrix was homogenous and had no structural components.

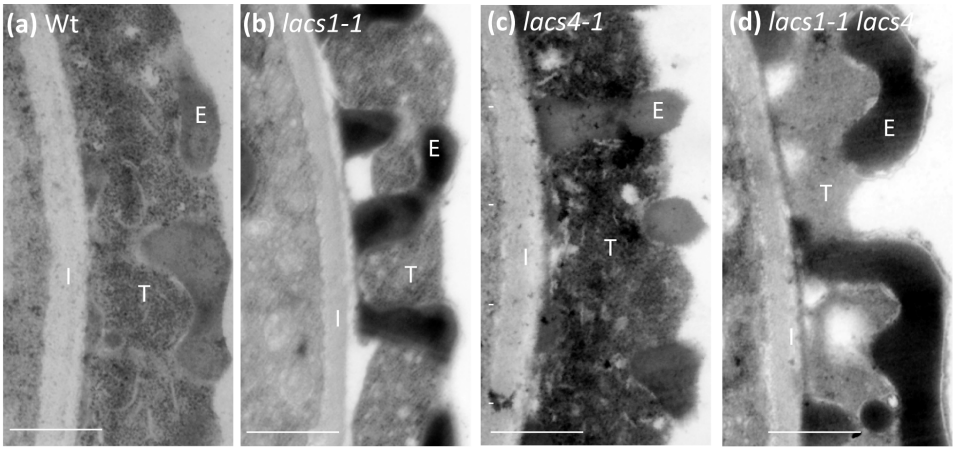

Fig. 16. Analysis of pollen wall structure by transmission electron microscopy. The ultra structure of the tryphine of *lacs1-1* (b) showed only subtle differences compared to wild-type (a). In *lacs4-1* (c) the matrix of the tryphine appeared to be severely disordered. The tryphine of *lacs1-1 lacs4-1* (d) appeared to be highly homogenous showing almost no internal structures. Intine (I), Exine (E), Tryphine (T). Bar = 500 nm. Pictures are taken by Dr. Michael Hoppert.

SEM analysis revealed that the pollen morphology of both T-DNA insertion lines of *lacs1* (Fig.17 b, c) and also of both variants of *lacs1 lacs4* (Fig. 17 f, g) was indistinguishable from wild type, indicating that conditional sterility was not caused by any structural changes. Interestingly morphological defects were detected in pollen grains of both *lacs4* lines (Fig. 16 d, e). 60 % to 80 % of all investigated *lacs4* pollen grains showed severe morphological abnormalities without having a clear pattern as it is the case in other established mutant lines (Enns *et al.,* 2005). The morphological distorted pollen grains of *lacs4* rather looked as if they were collapsed. The dramatically changed morphology is remarkable, since fertility of this plant line is completely unaffected. Even more surprising was the fact, that the additional inactivation of *LACS1* was rescuing the pollen phenotype, since pollen grains from *lacs1 lacs4* looked normal again, as mentioned above.

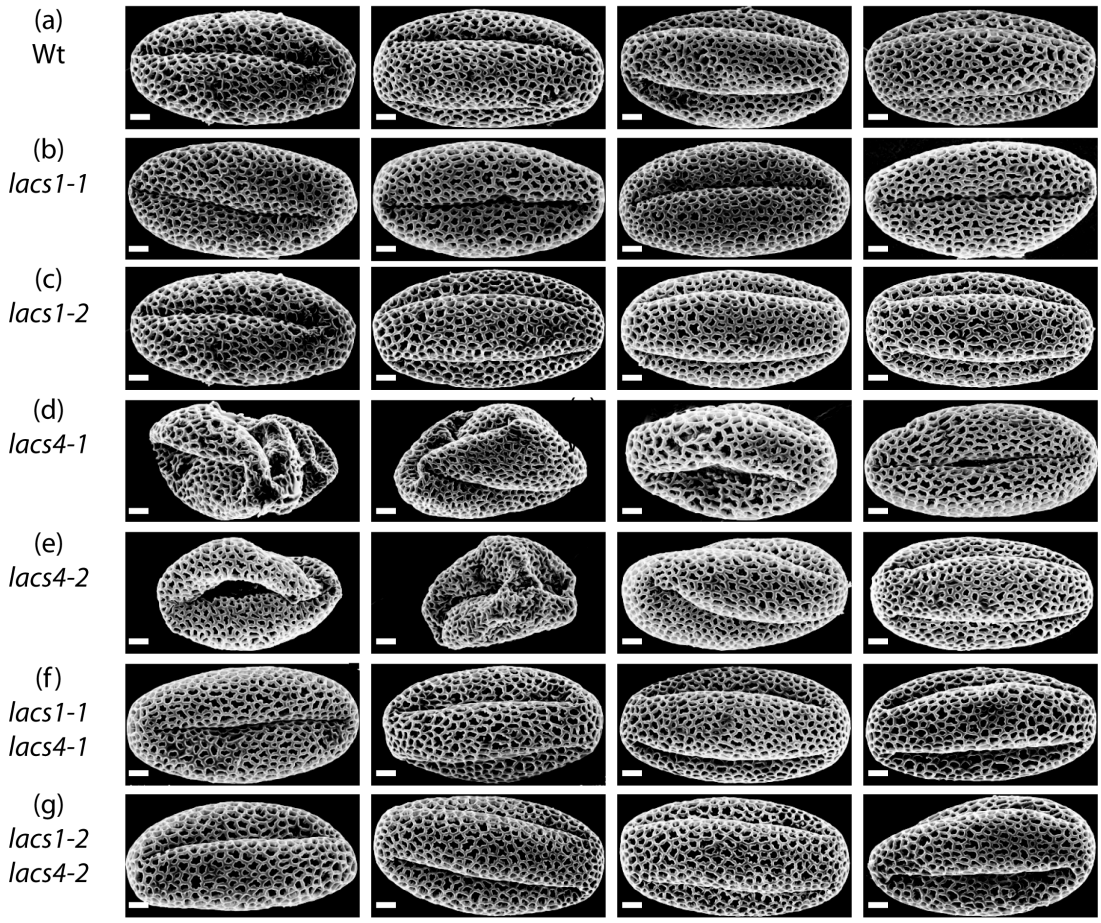

Fig.17 Pollen grain morphology. Scanning electron micrographs showing pollen grains from Wt (a), *lacs1-1* (b), *lacs1-3* (c), *lacs4-1* (d), *lacs4-2* (e), *lacs1-1 lacs4-1* (f), *lacs1-3 lacs4-2* (g). Pollen grains were harvested from 10 independent plants and images of four representative pollen grains are shown for each line. Bar = 1 µm

3.2.1.5 Expression pattern of *LACS1* and *LACS4* in reproductive organs

Conditional sterility was only observed when both genes were inactivated and therefore an overlapping expression pattern in reproductive organs was assumed. To investigate the expression of both genes in detail histochemical staining of *promoter:GUS* lines were employed (Fig 18). For this approach the transgenic plant lines *LACS1 promoter:GUS* and *LACS4 promoter:GUS* were generated. As positive and negative control for expression inside pollen grains *PIPK3 promoter:GUS* and *PIPK11 promoter:GUS* were used. Careful inspection by light microscopy revealed that *LACS1* and *LACS4* showed in fact no overlapping expression pattern in the reproductive organs (Fig. 18 c, d, g, h). The experiment clearly demonstrated that *LACS1* is expressed inside the pollen-sac tissue (Fig. 18 c). *LACS4* expression on the other hand was exclusively found in the pollen grains itself (Fig. 18 h). This highly specific expression pattern is very interesting since *lacs4* pollen grains showed severe deformations. The results suggested an important role of LACS4 in gametophyte development. Furthermore the pronounced biochemical phenotype of *lacs1 lacs4* on one hand and the rescue of the *lacs4* pollen phenotype by additional inactivation of *LACS1* on the other hand were providing evidence for a coordinated interaction of both proteins during pollen maturation.

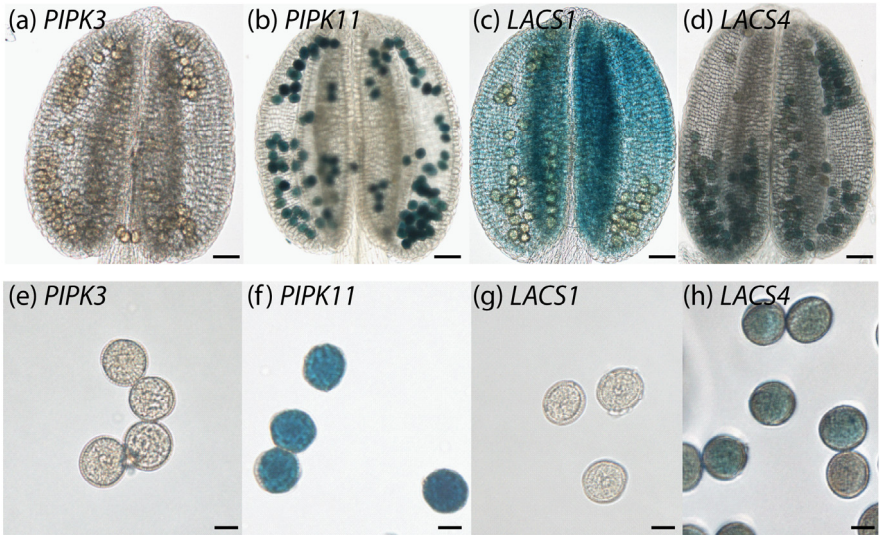

Fig 18. Expression of *LACS1* and *LACS4* in anthers and pollen grains. The histochemical staining of anthers and pollen grains were performed on transgenic plants expressing the *GUS* reporter gene under control of *LACS1* promoter (c, g) or *LACS4* promoter (d, h). As negative control the expression of the *GUS* reporter gene regulated by the *PIPK3* promoter was analyzed (a, e), and as positive control the pollen specific expression driven by the *PIPK11* promoter was used (b, f). Scale bar = 500 µm (a – d); Scale bar = 100 µm (e – h).

3.2.2 Surface wax

Plants with compromised fertility due to reduced levels of tryphine lipids often suffer from defects in the biosynthesis of very long chain lipids in general (Preuss *et al.,* 1993). Consequently, theses plants produce only reduced levels of surface wax, and in severe cases this can be directly recognized by an intensive green color of stems due to lower numbers of wax crystals on the epidermis. Closer inspection of *lacs1* and *lacs1 lacs4* plants indeed revealed intensive green and glossy stems, suggesting defects in the biosynthesis of very long chain lipids. SEM studies supported this assumption (Fig. 19). A reduced number of crystals were detected on the stems of *lacs1* (Fig 19 b, c) and *lacs1 lacs4* (Fig. 19 f, g) suggesting reduced levels of surface wax. Stems of *lacs4* (Fig. 19 d, e) were almost indistinguishable from wild type regarding the amount of wax crystals.

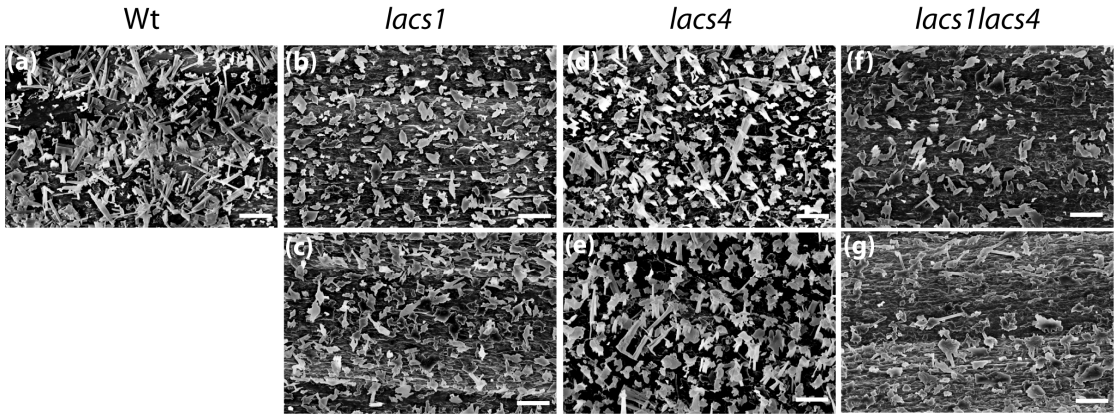

Fig. 19. Scanning electron micrographs of epicuticular wax crystals on stems. Shapes as well as densities of wax crystals are clearly modified in *lacs1-1* (b) and *lacs1-3* (c) compared to wild-type (a). Even more severe reductions in wax crystal number can be observed in the double mutant lines *lacs1-1 lacs4-1* (f) and *lacs1-3 lacs4-2* (g) whereas the single mutant lines *lacs4-1* (d) and *lacs4-2* (e) are not significantly different to wild-type. Scale bar = 3 µm.

These observations were verified in more detail by biochemical analysis, showing very consistent results for both mutant alleles of the respective loci (Fig 20). As already indicated by SEM analysis the total wax load on *lacs1* stems was significantly reduced having only 46 % of surface wax compared to the wild type (Fig. 20 a). In the double knock out lines this reduction was even more pronounced and only 29 % of surface wax was detected on the stem (Fig. 20 a). The *lacs4* lines showed only moderate reduction of surface wax by about 27 % (Fig. 20 a). Interestingly, the results for stems of *lacs4* are contrary to the results for the tryphine where an increase of very long chain lipids was detected.

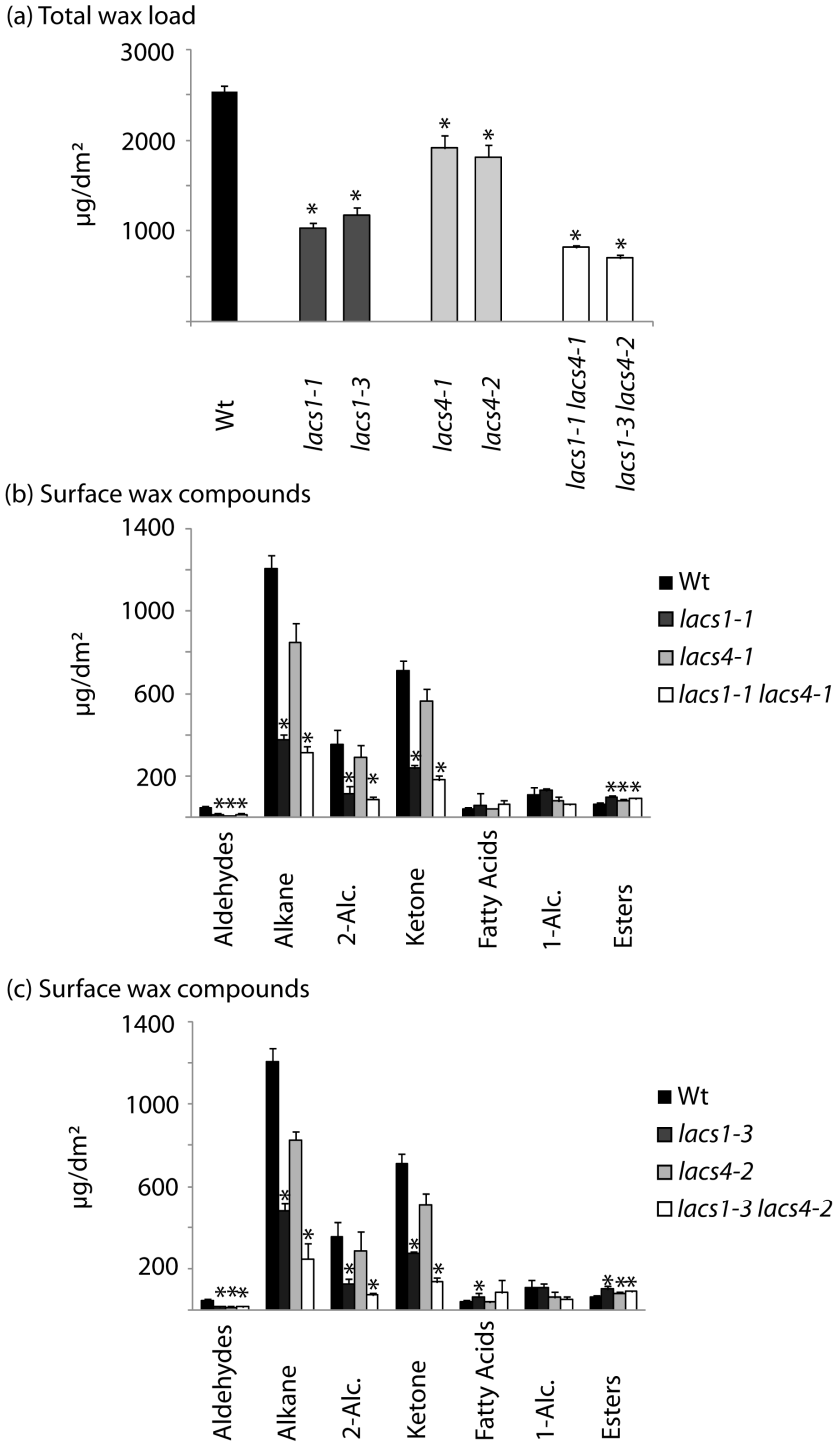

Fig. 20. Surface wax composition on stems. (a) Total wax load on stems of the different plant lines investigated. (b) Comparison of amounts of surface wax compounds in one set of mutant alleles. (c) Comparison of amounts of surface wax compounds in the second set of mutant alleles. Each value represents the mean of 5 independent replicates. Error bars = SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

As already noticed in the analysis of the pollen coat not all components of very long chain lipids were affected in the same way. This was also the case for surface wax since specific components showed no changes or even an increase whereas other components displayed severe reductions (Fig 20 b, c).

Generally the surface wax synthesis can be separated into decarbonylation and primary alcohol forming pathway. The components of the decarbonylation pathway are aldehydes, alkanes, secondary alcohols and ketones making up more than 90 % of total wax load on stems. In *lacs1* these components were reduced to 35 % of wild type level (Fig. 20 b, c), in *lacs1 lacs4* the reductions were even more pronounced showing only 23 % of wild type level (Fig. 20 b, c).

At the same time only minor changes were found for the components of the primary alcohol forming pathway (fatty acids, primary alcohol and wax esters). Individual components were slightly reduced, not changed at all or showed even slight increases in *lacs1* and *lacs1 lacs4* lines (Fig. 20 b, c). Similar results were already published for *lacs1* by (Lu *et al.,* 2009). More detailed analysis of surface wax revealed that the observed decreases of the decarbonylation pathway in *lacs1* and *lacs1 lacs4* are not due to defects in the biosynthesis of specific components, but caused by a uniform reduction of all components (Fig 21). Interestingly, in *lacs1* the main components (alkane C29, ketone C29 and secondary alcohol C29) were all reduced by 65 % (Fig. 21 b), suggesting that no specific step inside the decarbonylation pathway is affected but the overall flux through the pathway.

The results of *lacs1 lacs4* were not so homogenous. Alkane C29 showed a decrease by 78 %, being in the range of the overall wax reduction of the double mutant. However, the reduction of secondary alcohol C29 was more pronounced with 89 % whereas the ketone C29 showed a relatively mild reduction by 35 % being in the range of *lacs1* (Fig. 21 d). In *lacs4* alkane C29 and secondary alcohol C29 both showed a reduction by 30 % whereas ketone C29 displayed a less severe reduction of 18 % (Fig. 21 c).

The data supported the potential impact of LACS enzymes on the decarbonylation pathway, but suggested also an interesting role of ketone C29 for the surface wax, since it had in *lacs1 lacs4* and *lacs4* less pronounced reductions compared to its precursor's alkane C29 and secondary alcohol C29.

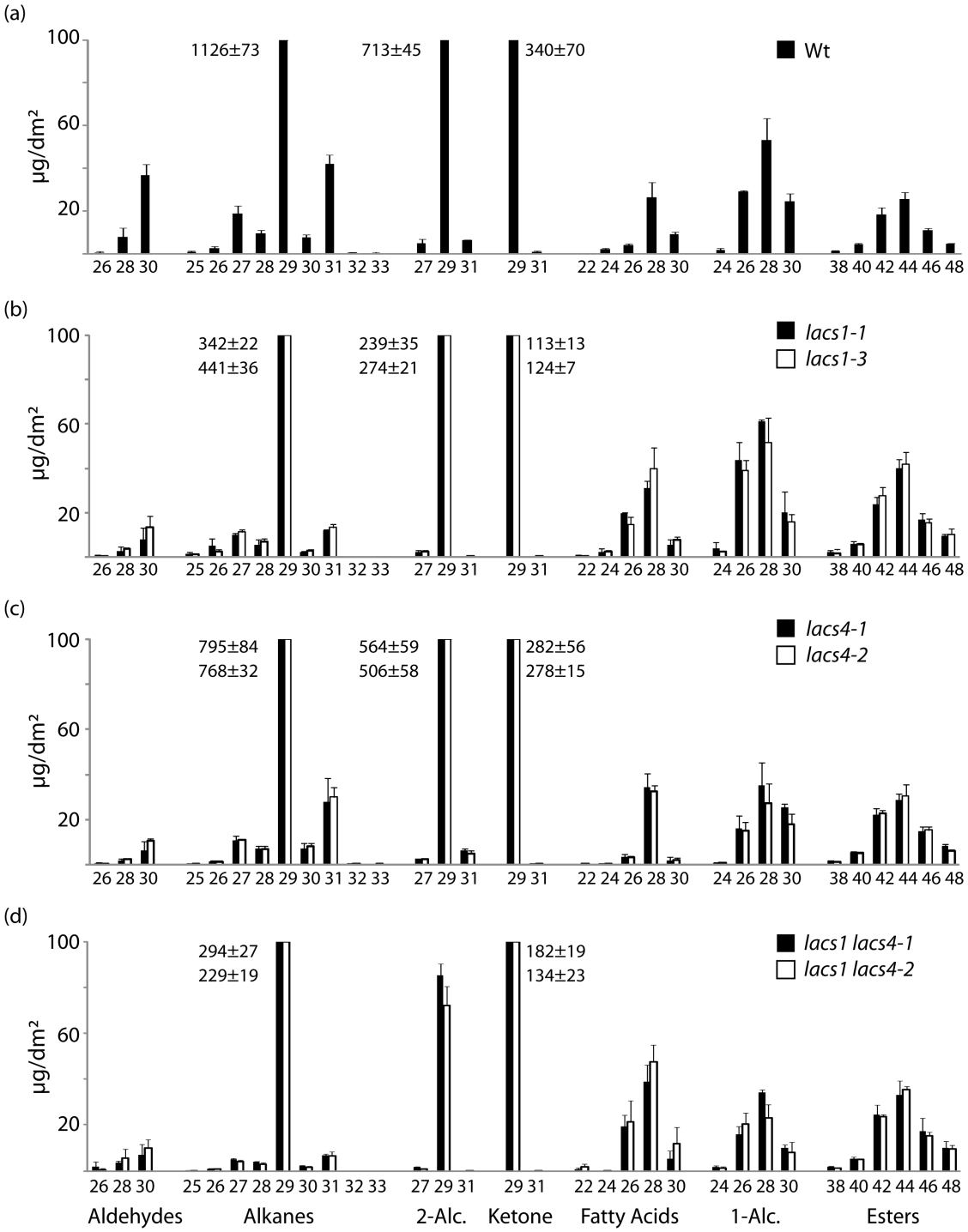

Fig 21. Detailed surface wax composition of stems. Data is given for wild-type (a), *lacs1-1* and *lacs1-3* (b), *lacs4-1* and *lacs4-2* (c), and *lacs1-1 lacs4-1* and *lacs1-3 lacs4-2* (d). The numbers on the *x*-axis indicate the carbon chain length of the respective compound and the chemical class is given at the bottom of the diagram. Values exceeding the maximum of the *y*-axis are indicated by numbers next to the respective bar. Each value represents the mean of 5 independent replicates. Error bars = SD.

The wax load on leafs showed similar results as described for stems. Compared to wild type *lacs4* had only moderate reductions with a approx. 94 % wax load, *lacs1* leafs contained 73 % and *lacs1 lacs4* approx. 46 % remaining surface wax. Hence the reductions in the mutant lines were not as intensive as on stems (Fig. 22 a). Alkanes were the most prominent compound class also in leaf wax making up 76 % of the wax load. Therefore reductions in this class had significant impact on the total wax load. In *lacs4* 84 % remaining alkanes were detected whereas *lacs1* had 60 % and *lacs1 lacs4* 34 % alkanes (Fig. 22 b, c) compared to the wild type. In contrast to these outcomes free fatty acids were significantly increased in all mutant lines (Fig 22 b, c). These results support the hypothesis that the decarbonylation pathway is significantly affected by the inactivation of *LACS1* and *LACS4.*

The analysis of very long chain lipids showed that reduction in surface wax of leafs were almost exclusively due to alkanes, being the only compound class which had decreased (Fig. 23). Interestingly, alkanes with longer chain length were more strongly reduced than those with shorter chains. In *lacs1* alkane C29 was reduced by 27 %, alkane C31 by 41 % and alkane C33 by 67 % (Fig. 23 b). In *lacs4* alkane C29 was not affected, but alkane C31 showed a decrease of 22 % and alkane C33 of 30 % (Fig. 23 c). The alkanes of *lacs1 lacs4* lines showed severely reduced amounts with 50 % loss of alkane C29, 65 % decrease of alkane C31 and a 90 % reduction of alkane C33 (Fig 23. D). Furthermore *lacs1 lacs4* was the only line were also ketone C29 (50 %) and secondary alcohol C29 (45 %) showed significant reductions in the surface wax of leafs.

Free fatty acids and to a certain extent also primary alcohols showed increased levels for almost all considered chain lengths (Fig. 23 a, b, c, d). These results were again indicating that the decarbonylation pathway is affected by the inactivation of *LACS1* and *LACS4*, whereas the primary alcohol way seems to be independent of both enzymatic activities.

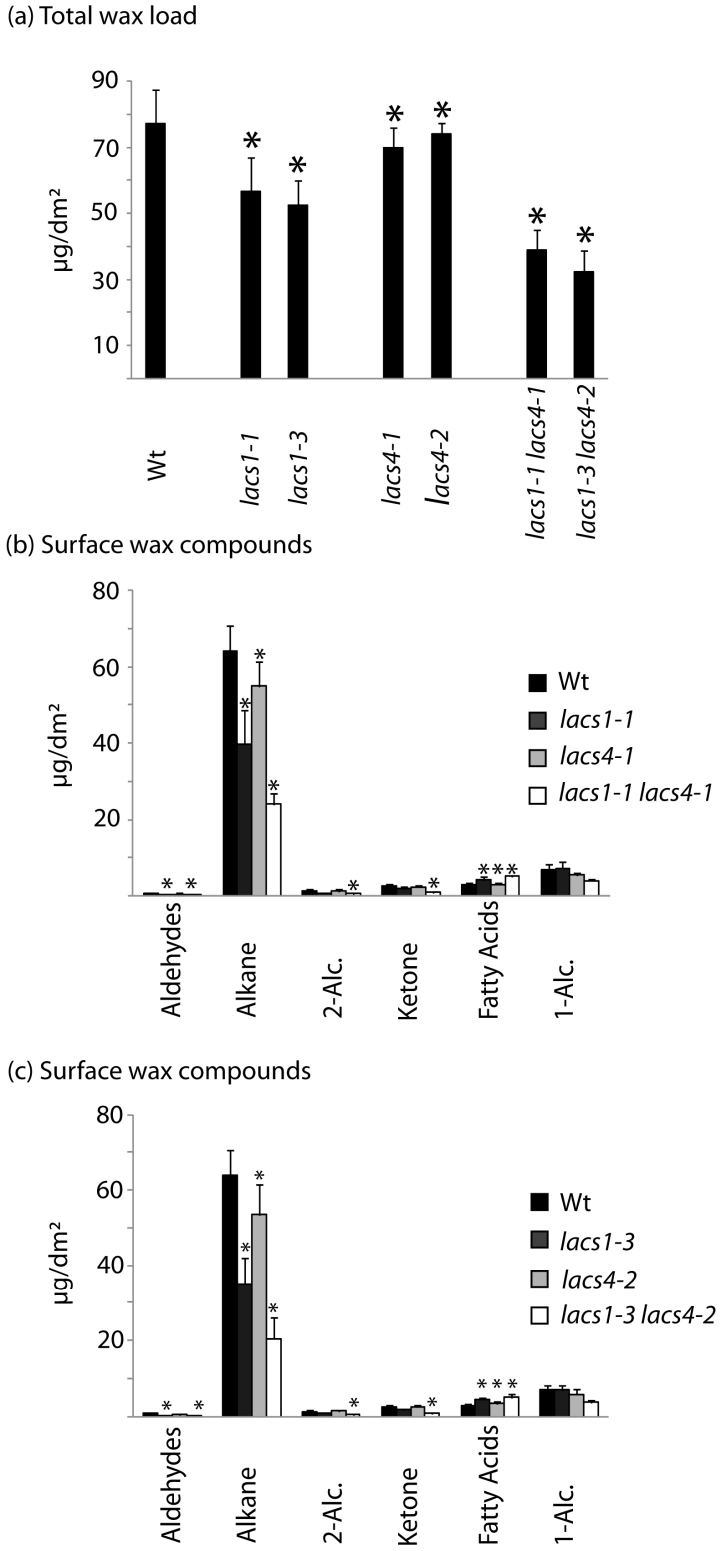

Fig 22. Surface wax composition of leafs. (a) Total wax load of the individual mutant lines. (b) Comparison of surface wax compounds with respect to their chemical classes for one set of mutant lines. (c) Comparison of surface wax compounds for the second set of mutant lines. The results for the two alleles of a respective gene proofed to be almost identical. Each value represents the mean of 5 independent replicates and the error bars indicate SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

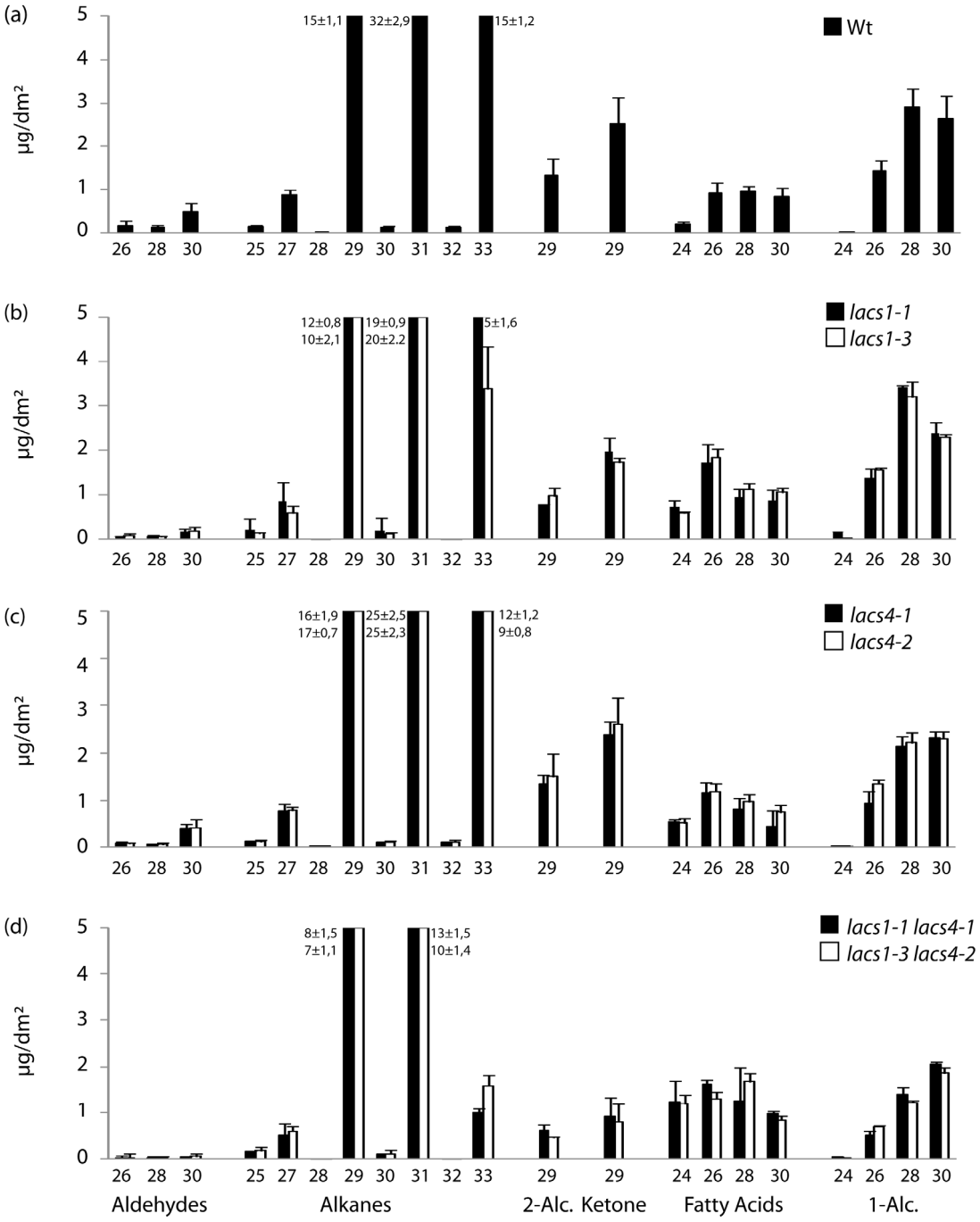

Fig. 23. Detailed surface wax composition of leafs. Data is given for wild-type (a), *lacs1-1* and *lacs1-3* (b), *lacs4-1* and *lacs4-2* (c), and *lacs1-1 lacs4-1* and *lacs1-3 lacs4-2* (d). The numbers on the *x*-axis indicate the carbon chain length of the respective compound and the chemical class is given at the bottom of the diagram. Values exceeding the maximum of the *y*-axis are indicated by numbers next to the respective bar. Each value represents the mean of 5 independent replicates. Error bars = SD.

In an additional experiment the impact of changes in surface wax composition on the physiological role of the cuticle was investigated. One role of the cuticle is the protection against non-stomatal water loss. Consequently, a reduced wax layer is resulting in higher sensitivity against drought stress and to an increased demand of water under normal conditions. This aspect was analyzed with detached leafs of wild type, *lacs1*, *lacs4* and *lacs1 lacs4*. The weight of these leafs was determined at different times over a period of 100 min. Since the loss of weight correlates directly with the loss of water, the results can be used for estimating the functionality of the cuticula as a barrier against non-stomatal water loss.

The results, shown in Fig. 24, supported the outcomes of the electron microscopic and biochemical analysis. *lacs4* showed the lowest water loss rate whereas *lacs1 lacs4* had clearly the most pronounced loss indicating an additive effect of LACS1 and LACS4.

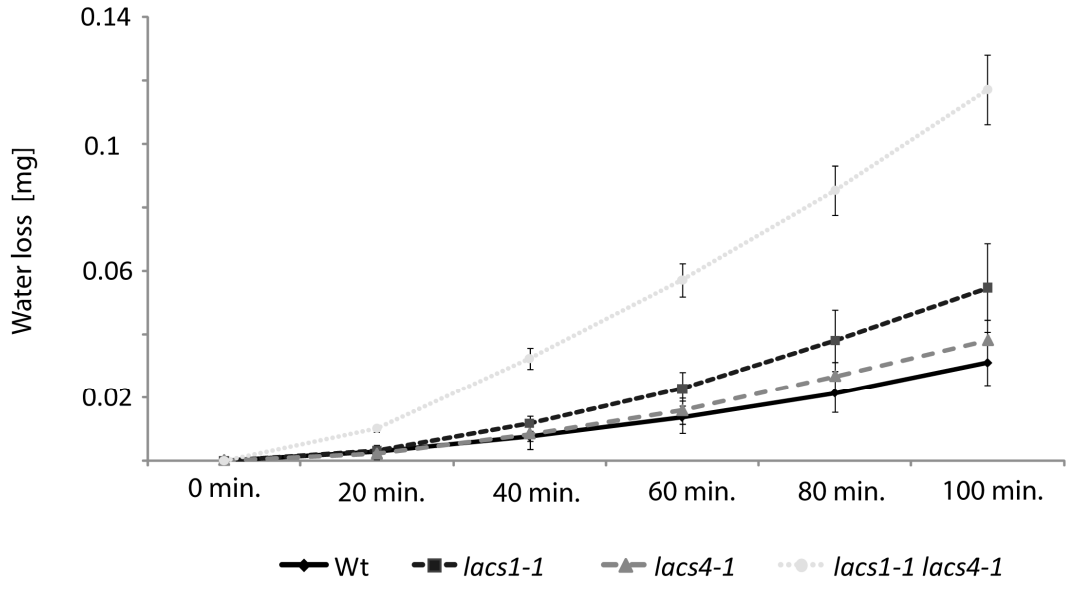

Fig 24. Water loss of detached rosette leafs. To quantify water loss rates, five rosette leaves of 4-week-old plants were detached and each cutting site was sealed with modeling material. The leaves were maintained at room temperature in the light and the weight was determined every 20 min. The results were obtained from two experiments performed with leaves from different plants. The data show a direct correlation between reductions in surface lipid levels and water loss of the individual mutant lines indicated by increasing levels of water loss in the order *lacs4-1*, *lacs1-1*, and *lacs1-1 lacs4-1*. Each value represents the mean of five independent replicates. Error bars = SD.

3.2.2.1 Expression of *LACS1* and *LACS4* in wax synthesizing tissue

The expression pattern of *LACS1* and *LACS4* was analyzed similar to reproductive organs by histochemical staining. An overlapping pattern was expected in epidermal cells of stems and leafs, since wax synthesis takes place predominantly in this tissue type. The results of *LACS1 promoter:GUS* and *LACS4 promoter:GUS* lines revealed that *LACS1* is intensively expressed in epidermal cells of the stem (Fig. 25 a, c), as it has been shown for other wax related genes (Li *et al.,* 2008). However for *LACS4* no signal was observed in stems although incubation times were increased to 48 h.

In leafs the expression level of *LACS4* was also relatively low, but interestingly vascular tissue showed substantial *LACS4* promoter activity (Fig. 25 d). This was surprising since wax synthesis is thought to take place inside epidermal cells. *LACS1* showed also a weak but visible overall staining of leaf veins and tissue (Fig. 25 b). Consequently, overlapping expression has only been detected in vascular and connective tissue of leafs but not in stem epidermis.

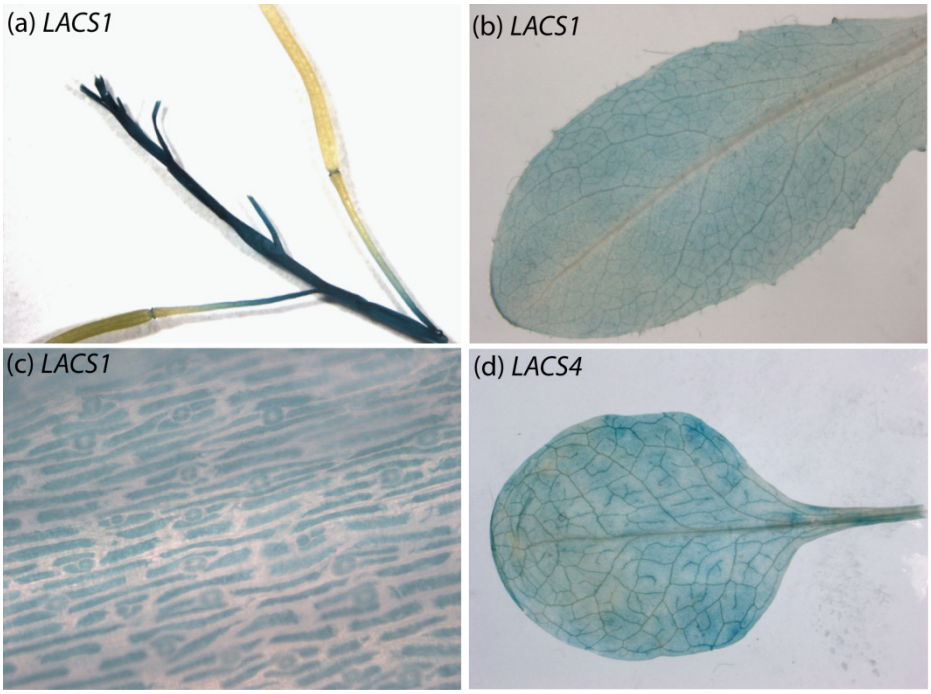

Fig 25. Expression of *LACS1* and *LACS4* in stem epidermis and leafs. The histochemical staining of stem and leafs were performed on transgenic plants expressing the *GUS* reporter gene under control of *LACS1* promoter (a, b, c) or *LACS4* promoter (d).

3.2.3 Plant morphology

The morphology of the adult plant of the mutant lines presented so far were comparable to wild type. However, in our collection of multiple LACS mutants also lines with defects in growth and with morphological abnormalities were identified. All these mutants had in common the combined inactivation of *LACS4* and *LACS9*. The additional inactivation of other LACS genes resulted in various modifications of the observed phenotypes. In this work the double mutant *lacs4 lacs9* will be described in detail and in chapter 3.3 some preliminary data on the triple mutant *lacs4 lacs8 lacs9* will be presented.

3.2.3.1 Morphological phenotype of *lacs4 lacs9*

Seeds of both allele combinations of *lacs4 lacs9* showed a normal germination rate. However already during the first days after germination a clearly reduced growth rate was detectable, which remained slow also during later development. Six weeks after germination *lacs4 lacs9* plants had only about 50 % of leaf area (Fig 26). The growth of the corresponding single knock out lines did not differ significantly from wild type. Only the combined inactivation of *LACS4* and *LACS9* was resulting in the severe leaf growth phenotype.

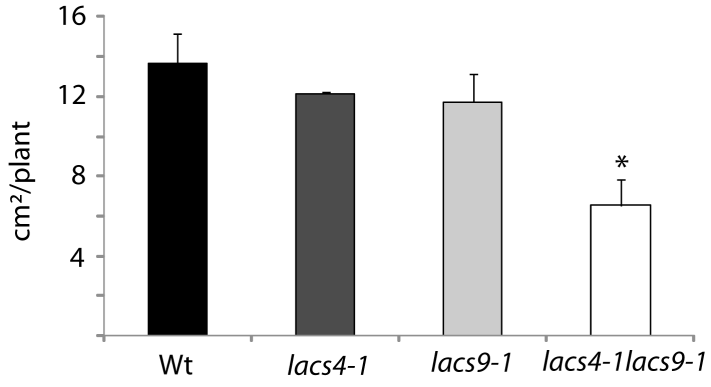

Fig. 26. Leaf area per plant of wild-type, *lacs4-1*, *lacs9-1,* and *lacs4-1 lacs9-1*.Leaf area is expressed in cm² / plant. Each value represents the mean of ten replicates and the error bars indicate SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

Beside the general growth rate, *lacs4 lacs9* plants also showed significantly altered morphology of leaves and shoots. The leaves of the double knock out were stiffer and looked curly and the lengths of the petioles were reduced. Furthermore anthocyanins were accumulating at the lower leaf site as well as at the bottom of the stem. Although mutant leaves had a more intensive green color than those of wild type changes in the pigment composition were not detected. Interestingly, these morphological changes developed after approx. 4 weeks under long day conditions. Before this time mutant plants grew slower but regarding their morphology they were indistinguishable from wild type plants. After about four weeks the phenotypes developed rapidly in young but also in established leafs. Furthermore, *lacs4 lacs9* leaves had a higher susceptibility for lesions especially those having direct contact with the soil.

Beside the described leaf phenotype of the mutant, also the development of roots and stems were altered. The root length of *lacs4 lacs9* was significantly reduced. This data was obtained from plants grown on vertical MS – plates by Bettina Gumbrecht (Gumprecht 2010). The stem phenotype resulted in a reduced apical dominance, a reduced thickness of the stem and the accumulation of anthocyanins in the lower part of the stems

Especially the loss of apical dominance suggested influences on the hormone level; however, changes could not be verified by analytical quantifications.

3.2.3.2 Influence of light on the *lacs4 lacs9* phenotype

The phenotype of *lacs4 lacs9* described in chap. 3.2.3.1 was only observed in plants grown under long day conditions (16 h light / 8 h darkness) (Fig. 27). When the mutant plants were cultivated under short day conditions (8h light / 16h darkness) (Fig 28) they were almost indistinguishable from wild type plants. The pronounced morphological abnormalities were completely absent and only very subtle deformations could be detected. Also the intensive reduction in the size of leaves as well as the accumulation of anthocyanins at basal parts was not detected. Single knock outs of *lacs4* and *lacs9* had the same appearance as wild type under both light regimes. Based on this observation it can be assumed that the length of day has a significant impact on the development of the described phenotypes.

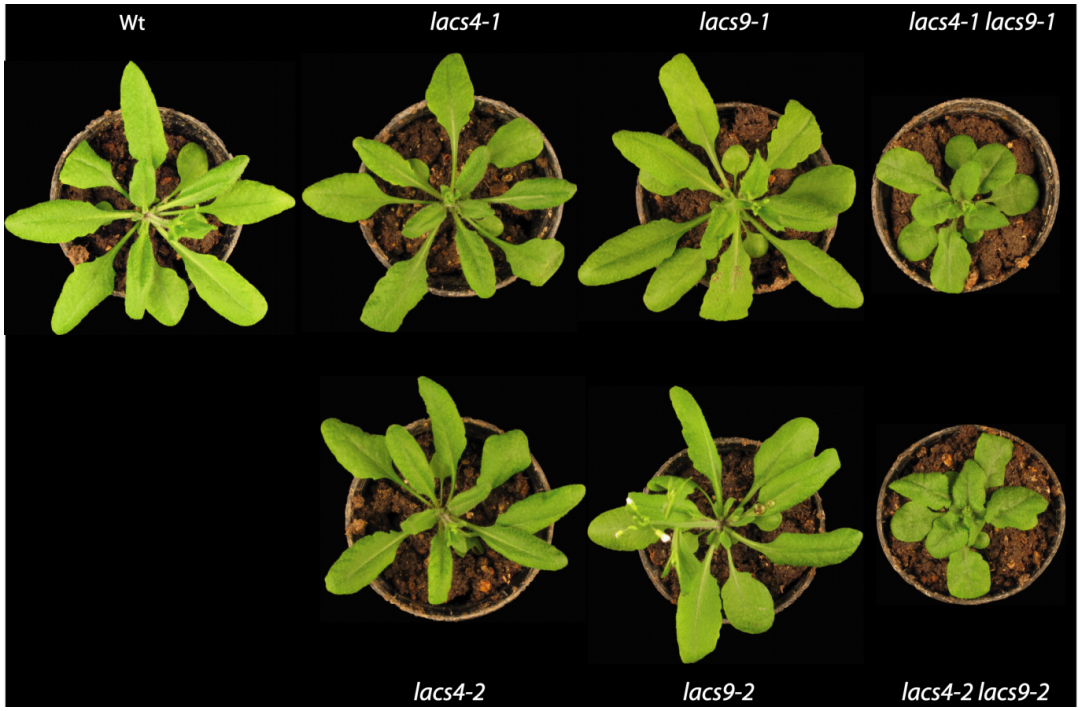

Fig 27. Wt, *lacs4-1, lacs4-2, lacs9-1, lacs9-2, lacs4-1 lacs9-1* and *lacs4-2 lacs9-2* plants grown under long day conditions. All four single knock out lines were indistinguishable from wild type, however both *lacs4 lacs9* lines had severe growth as well as morphological changes. Photos were taken from six week old plants. Presented plants were randomly chosen from trays with approx. 30 plants.

Fig. 28. Wt, *lacs4-1, lacs4-2, lacs9-1, lacs9-2, lacs4-1 lacs9-1* and *lacs4-2 lacs9-2* plants grown under short day conditions. All single knock out lines were indistinguishable from Wt, however the changes in both *lacs4 lacs9* lines were relatively small. Only a very light growth phenotype were visible. Photos were taken from six week old plants. Presented plants were randomly chosen from trays with approx. 30 plants.

3.2.3.3 Expression pattern of *LACS4* and *LACS9*

The expression of *LACS4* and *LACS9* was analyzed by histochemical staining, as described before for reproductive organs and wax synthesizing tissue. Due to the intracellular localization of *LACS9* at the outer envelope of the chloroplast an intensive expression was expected in leafs. This has been verified by histochemical staining, as shown in Fig. 29 b. In contrast, *LACS4::GUS* leafs showed only a very faint staining in six weeks old leafs (Fig. 29 a), as already shown in Fig. 25. Moreover, Sarah Kramer could show that *LACS9* is expressed already one day post germination, whereas first *LACS4::GUS* signals were detected 7 days post germination (Kramer S., 2011). However, for both genes substantial expression was detected in young plants (Fig. 29 c, d).

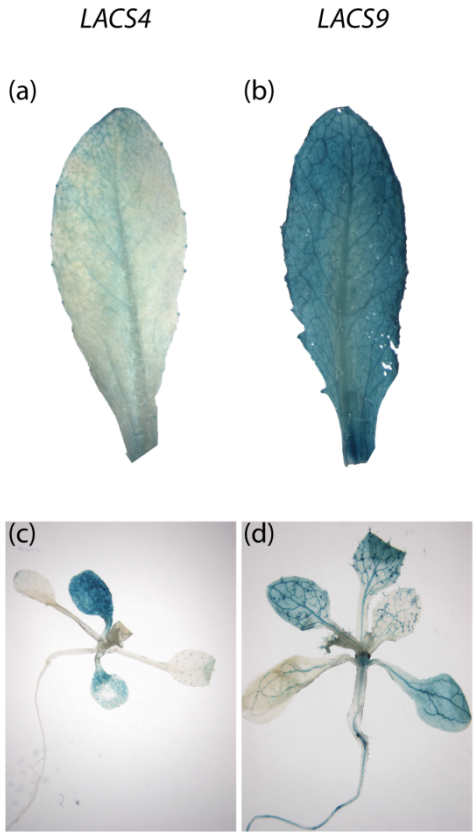

Fig. 29. Expression of *LACS4* and *LACS9* in leaves (approx. six weeks after germination) and young plants (approx. 10 days after germination). The histochemical staining were performed on transgenic plants expressing the *GUS* reporter gene under control of *LACS4* promoter (a, c) or *LACS9* promoter (b, d).

3.2.3.4 Biochemical phenotype of *lacs4 lacs9*

To investigate the impact of the combined inactivation of LACS4 and LACS9 on the lipid metabolism of the plant fatty acids, lipids and acyl-CoA profiles were established. During the next chapters the results of membrane lipid composition will be presented with a focus on total fatty acid profile, lipid profile and fatty acid profiles of different lipid classes.

3.2.3.5 Total fatty acid profiles of *lacs4*, *lacs9* and *lacs4 lacs9*

Total fatty acid profiles of plants grown under long day conditions were obtained directly from lipid extracts and represented the sum of all free and lipid bound fatty acids of the tissue. The results of this analysis are shown in Fig.30, including two independent alleles for each gene. Single knock out lines of *lacs4* and *lacs9* displayed no or only minor changes compared to wild type. For *lacs4 lacs9* no or only minor changes were found for C16 – fatty acids but considerable different concentrations of C18 fatty acids were detected. 18:1 and 18:2 showed increases of 133 % and 50 %, respectively, whereas 18:3 was reduced by 16 % (Fig 30). These results were obtained from plants grown under long day conditions showing an intensive phenotype.

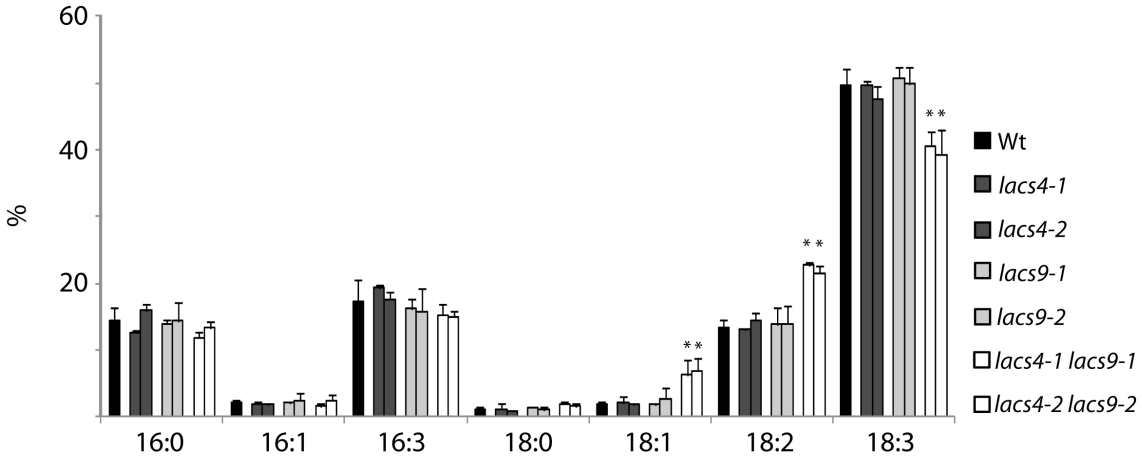

Fig. 30. Fatty acid profile of Wt, *lacs4-1, lacs4-2, lacs9-1, lacs9-2, lacs4-1 lacs9-1* and *lacs4-2 lacs9-2* grown under long day conditions. The numbers on the *x*-axis indicate the carbon chain length of the respective fatty acid. Each value represents the mean of 3 independent replicates. Error bars = SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

To determine the impact of light on the total fatty acid profiles the analysis was repeated with plants grown under short day conditions showing no obvious morphological phenotype. As depicted in Fig. 31 the two single knock out lines showed, as already seen under long day conditions no or only very minor changes. However, in *lacs4 lacs9* an increase of 16:3 by 13 % was detected. Also 18:1 and 18:2 were significantly increased by 83 % and 13 %, respectively. Interestingly, in both cases the changes were smaller than under long day conditions. The only fatty acid showing a decrease was again 18:3 (26 % reduction).

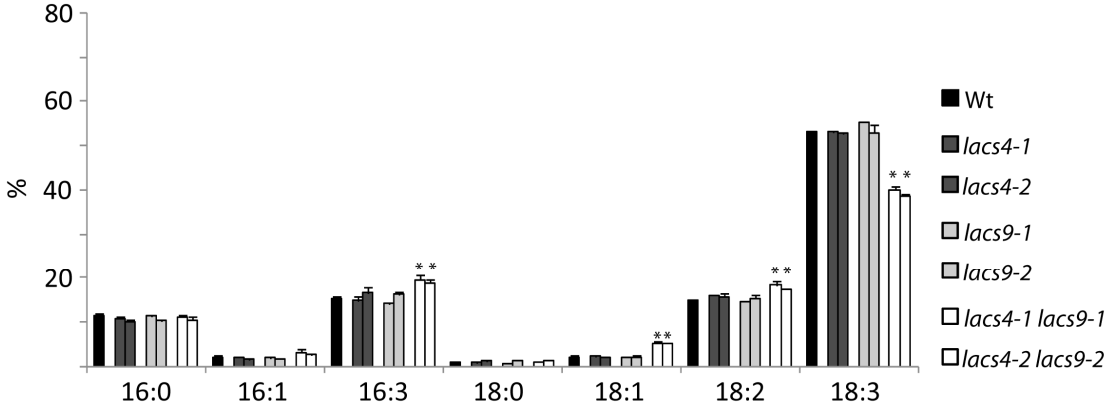

Fig 31. Fatty acid profile of Wt, *lacs4-1, lacs4-2, lacs9-1, lacs9-2, lacs4-1 lacs9*-1 and *lacs4-2 lacs9-2* grown under short day conditions. The numbers on the *x*-axis indicate the carbon chain length of the respective fatty acid. Each value represents the mean of 3 independent replicates. Error bars = SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

In contrast to the expectation significant changes in total fatty acid profiles were not only detected in plant material grown in long day conditions but also in plants grown in the short day. Beside this general aspect it became clear that mainly C18 - fatty acids were affected with an increase of 18:1 and 18:2 and a decrease of 18:3. These results could suggest that the lipids of the eukaryotic pathway are preferentially affected in the double mutant. To verify this aspect different lipid classes were analyzed in the next step.

3.2.3.6 Lipid profiles of *lacs4, lacs9, lacs4 lacs9*

Lipid profiles of the mutant plants were analyzed to investigate if ER derived lipids were preferentially affected by the mutations. Moreover knowledge on changes of

certain membrane lipids could help to understand the morphological phenotypes. The lipid analysis of plants grown under long day conditions revealed no or only minor changes between wild type and the mutant plants as shown in Fig. 32 a. Wild type, *lacs4*, *lacs9,* and *lacs4 lacs9* lines had almost identical amounts of all lipid classes. Also under short day conditions no changes in lipid composition were detectable (Fig. 32 b). Potential mistakes or problems with the analysis, leading to wrong data, can be excluded since results were comparable to data in the literature (Welti *et al.,* 2002).

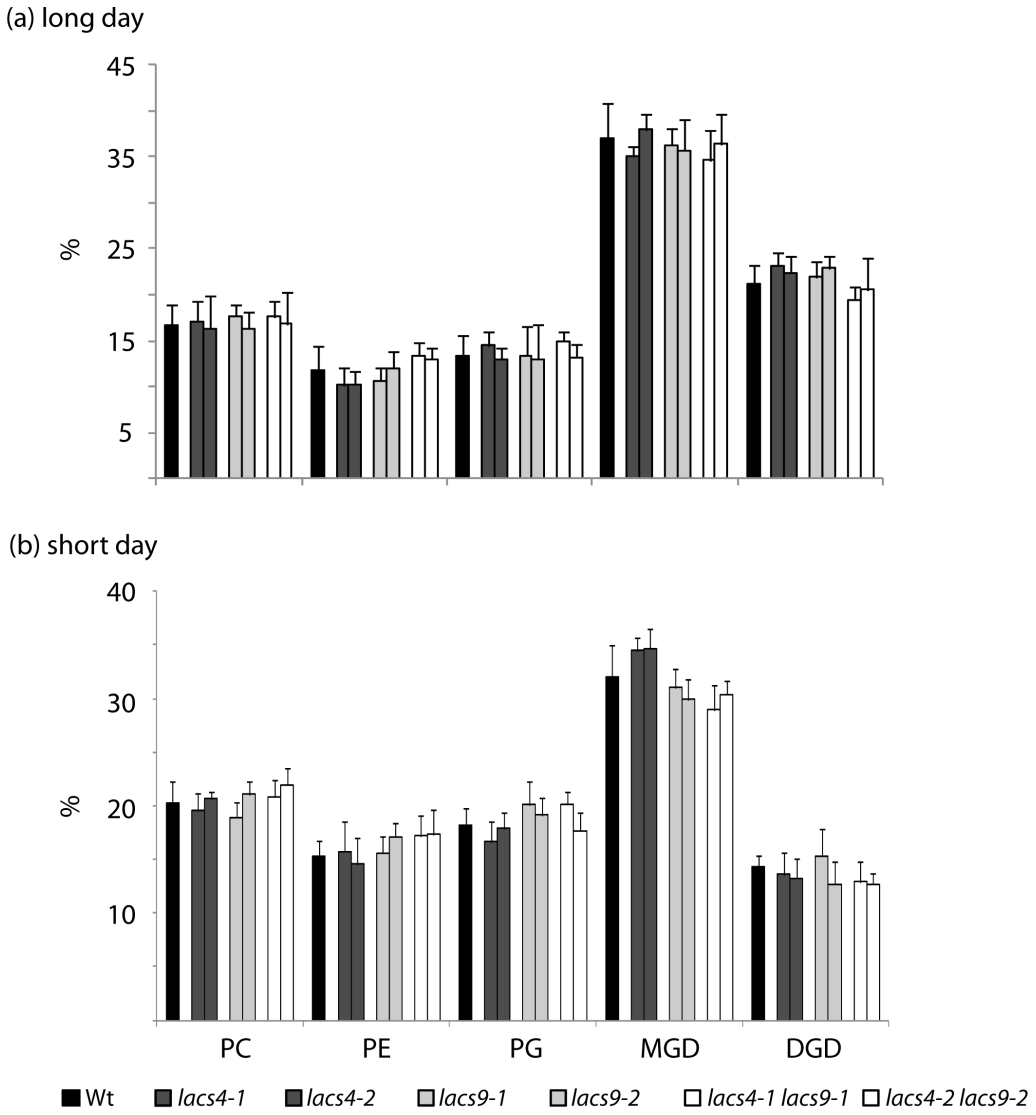

Fig 32. Lipid profile of Wt, *lacs4-1, lacs4-2, lacs9-1, lacs9-2, lacs4-1 lacs9-1* and *lacs4-2 lacs9-2* grown under (a) long day and (b) short day conditions. The different lipid classes are indicated at the bottom of the diagram on the *x*-axis of (b). Each value represents the mean of 3 independent replicates. Error bars = SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

As described in chap. 3.2.3.2 *lacs4 lacs9* had a reduced growth and an affected morphology with curly leafs. A similar phenotype is reported for the *dgd1* mutant (Hartel *et al.,* 1997). Since this line has severely reduced amounts of DGD it is thought that biophysical changes in the membranes are causing the observed physiological alterations. However although DGD amounts were not affected in *lacs4 lacs9* under both light regimes, plant were grown on phosphate free media to investigate if glycolipid amounts are affected according to intensive membrane remodeling.

Plants need phosphate for many different processes (e.g. DNA synthesis), therefore growth and cell division are dependent on the availability of phosphate. If the supply of phosphate is limiting the plant is using its own phosphate pool in form of phospholipids. When these lipids are shuttled out of the membrane they are substituted by the bi-layer forming DGD, leading to increased amounts of glycolipids. Due to this increase potential changes in the synthesis of glycolipids might be detected being masked before when plants were grown under normal conditions.

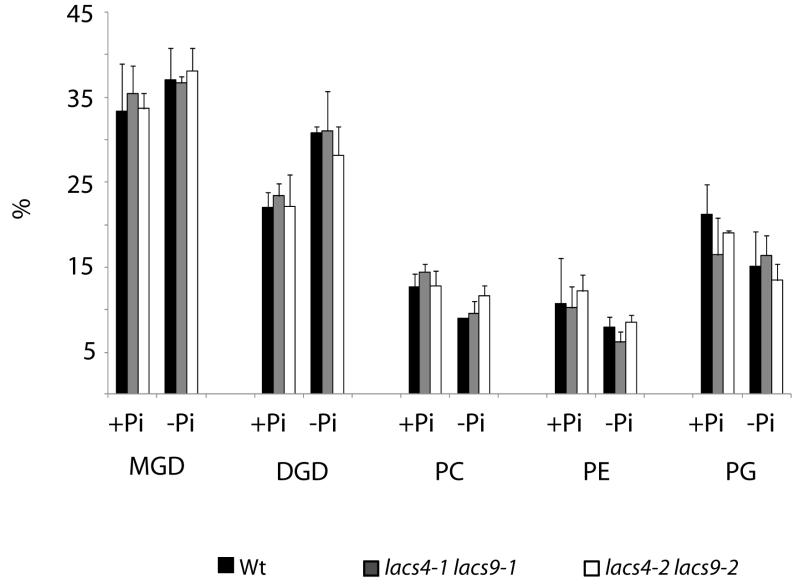

Fig 33. Lipid profile of Wt, *lacs4-1 lacs9-1* and *lacs4-2 lacs9-2* grown with and without phosphate. The different lipid classes are indicated at the bottom of the diagram, on the *x*-axis growth conditions are shown indicating whether plants were grown with or without phosphate. Each value represents the mean of 3 independent replicates. Error bars = SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

As expected from the literature a decrease of phospholipids and an increase of glycolipids have been observed when plants were grown without phosphate (Fig.33). However, as already shown for long- and short day conditions, no or only minor changes in the lipid profiles of Wt and *lacs4 lacs9* have been detected. The results

were again comparable to data from literature indicating that experimental procedure and analysis were performed correctly.

3.2.3.7 Fatty acid profiles of lipid classes under different light conditions

The analysis of fatty acid profiles from all lipid classes detectable by TLC was performed to investigate if the observed differences in the total fatty acid profiles are restricted to certain lipid classes. In addition, further potential changes in fatty acid composition of specific lipids could be revealed which are masked when total fatty acid profiles were analyzed.

First data of PG as lipid deriving from the prokaryotic pathway will be presented, followed by the presentation of lipids based on the prokaryotic as well as the eukaryotic pathway (MGD, DGD) and finally fatty acid profiles of lipids exclusively synthesized via the eukaryotic pathway are shown.

3.2.3.7.1 Phosphatidyl glycerol (PG)

PG is a phospholipid mainly found in the chloroplasts. It is characterized by the presence of the PG specific fatty acid Δ3-trans-hexadecenoate (16:1 carrying an unusual trans double bond in position Δ3). In plants grown under long day conditions the fatty acid profile of PG showed no or only minor changes for both alleles of the single mutants *lacs4* and *lacs9*. In contrast, the fatty acid profile of the double mutant showed elevated levels of 16:0 (12 %) and 18:0 (150 %) whereas 18:3 was decreased by 30 % (Fig. 34a).

Under short day conditions both alleles of the single knock out line showed again no or only minor changes. Interestingly, also *lacs4 lacs9* exhibited only minimal and non significant differences compared to the wild type (Fig 34b).

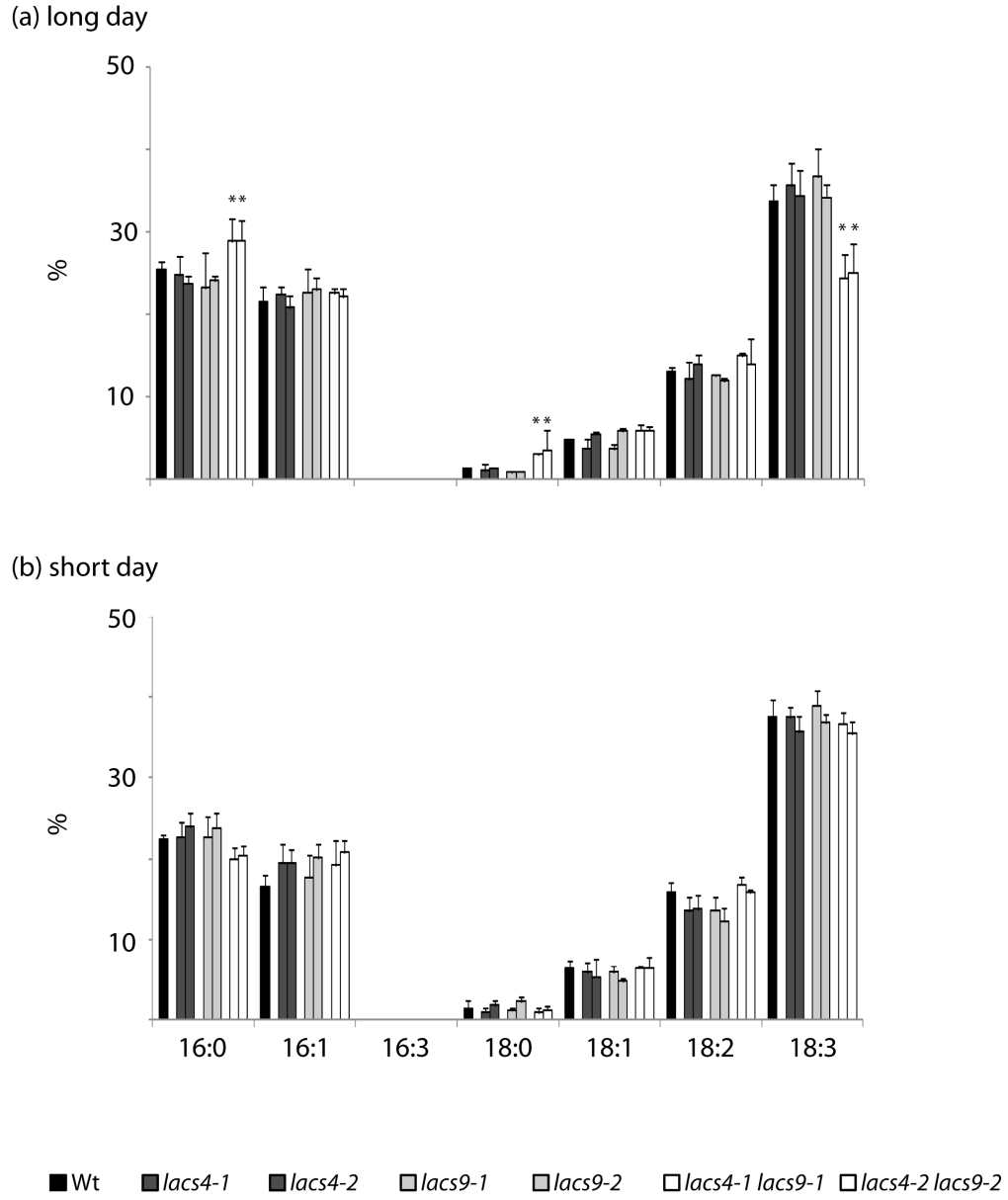

Fig 34. Fatty acid profile of PG form Wt, *lacs4-1, lacs4-2, lacs9-1, lacs9-2, lacs4-1 lacs9-*1 and *lacs4-2 lacs9-2* grown under (a) long day and (b) short day conditions. The fatty acids are indicated at the bottom of the diagram on the *x*-axis of (b). Each value represents the mean of 3 independent replicates. Error bars = SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

3.2.3.7.2 Monogalactosyl diacylglycerol (MGD)

MGD is the most prominent lipid in *Arabidopsis*; it is exclusively present in the chloroplast but does not belong to the bilayer-forming lipids due to its structure. The fatty acid profile of MGD is characterized by 16:3 and 18:3 as the most prominent

components. The fatty acid profiles of both independent alleles of *lacs4* and *lacs9* were almost completely identical compared to the wild type under long and short day conditions (Fig. 35 a, b). However, the *lacs4 lacs9* lines showed under long day conditions a 12 % increase of 16:3 and a 10 % decrease of 18:3 (Fig. 35 a). Under short day conditions a 14 % increase of 16:3 as well as a 5 % reduction of 18:3 was measured.

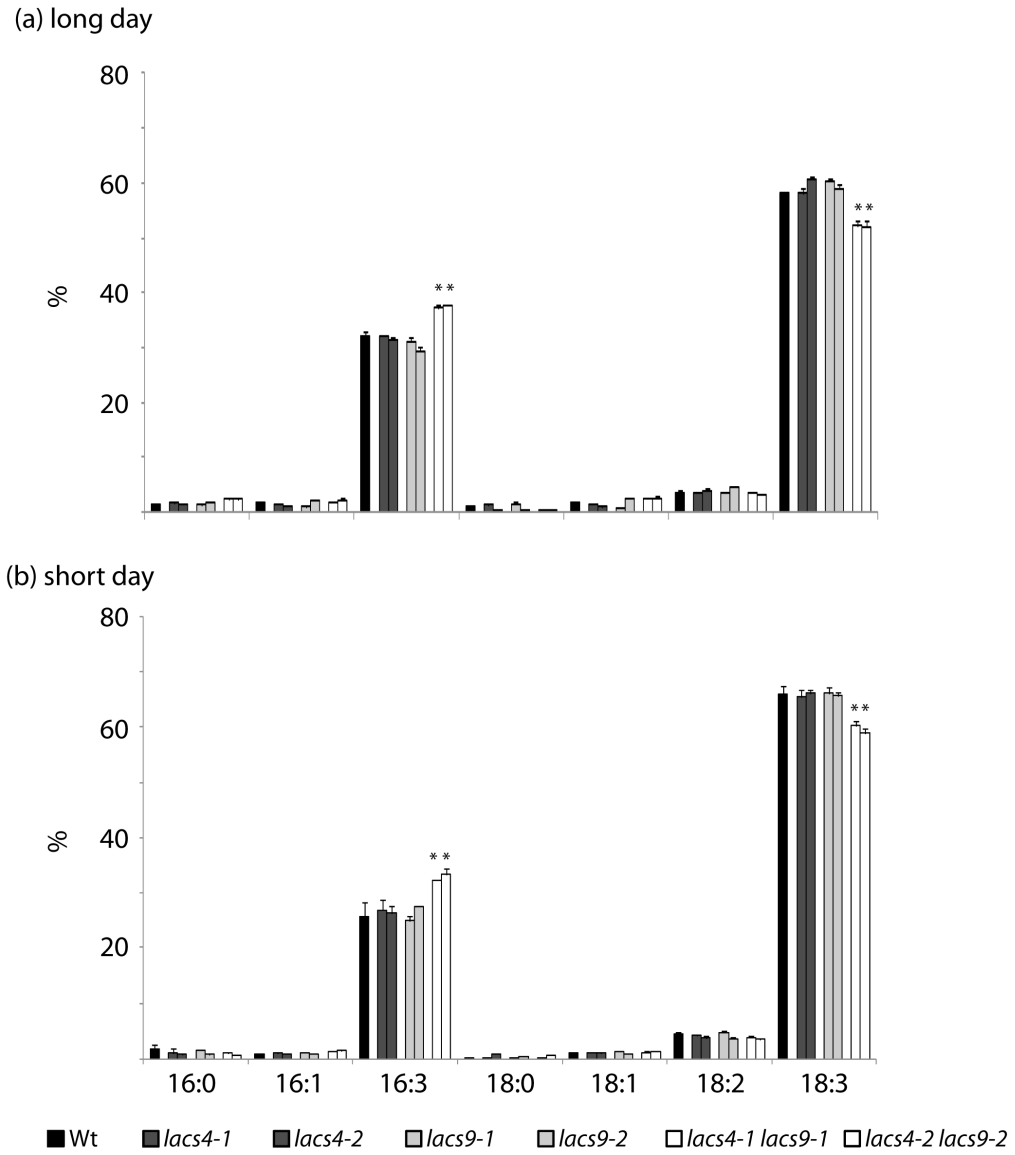

Fig 35. Fatty acid profile of MGD form Wt, *lacs4-1, lacs4-2, lacs9-1, lacs9-2, lacs4-1 lacs9*-1 and *lacs4-2 lacs9-2* grown under (a) long day and (b) short day conditions. The fatty acids are indicated at the bottom of the diagram on the *x*-axis of (b). Each value represents the mean of 3 independent replicates. Error bars = SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

3.2.3.7.3 Digalactosyl diacylglycerol (DGD)

DGD is a glycolipid normally occurring in the plastids, but under certain stress conditions it can be exported to other membrane system. Moreover, the main difference to MGD is its bilayer-forming character. Therefore it is supposed to be an important component of membranes.

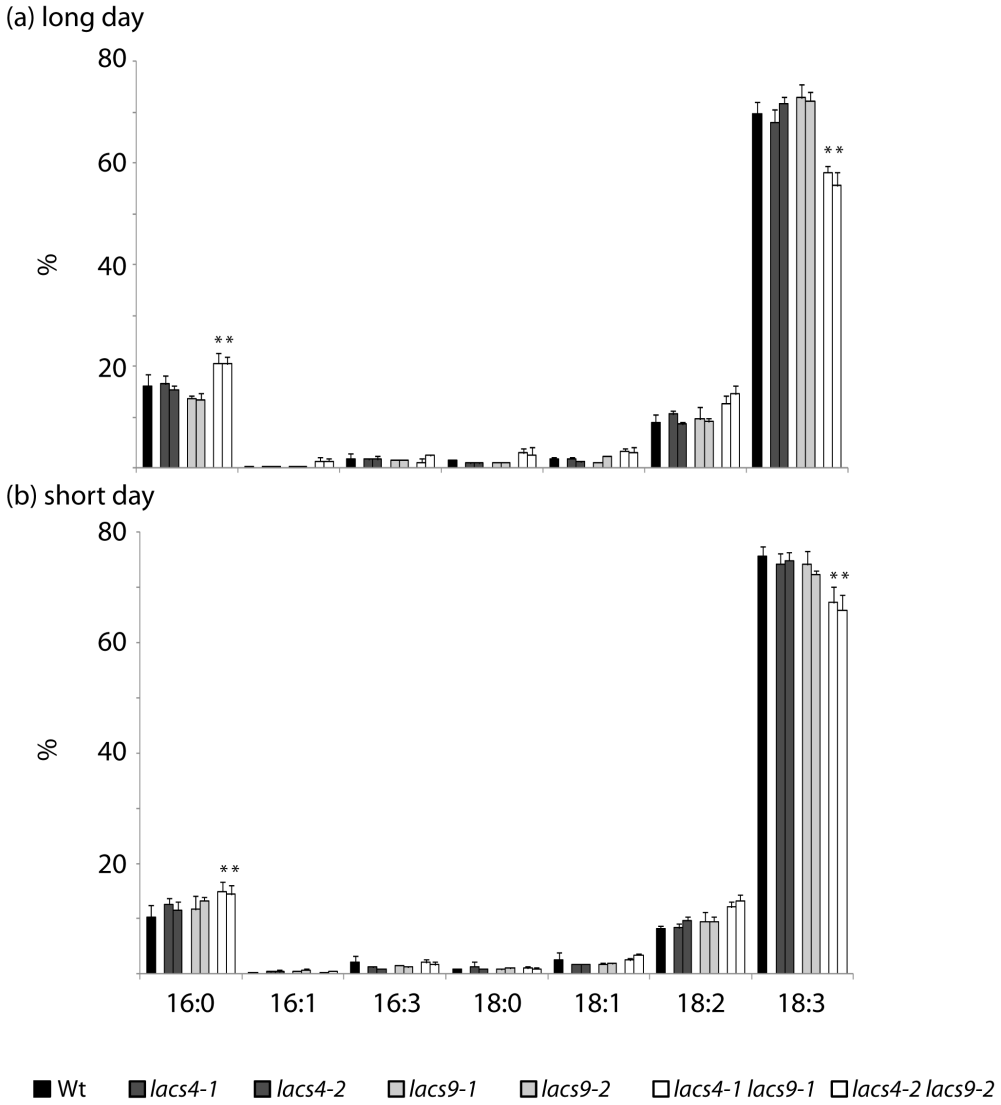

Fig. 36. Fatty acid profile of DGD form Wt, *lacs4-1, lacs4-2, lacs9-1, lacs9-2, lacs4-1 lacs9*-1 and *lacs4-2 lacs9-2* grown under (a) long day and (b) short day conditions. The fatty acids are indicated at the bottom of the diagram on the *x*-axis of (b). Each value represents the mean of 3 independent replicates. Error bars = SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

The fatty acid profile of DGD is characterized by the highest amounts of 18:3 fatty acids among all endogen lipids of *Arabidopsis*, but it contains also significant amounts of 16:0 and 18:2. As for MGD both alleles of the single knock out lines *lacs4* and *lacs9* showed no or only minor changes under short as well as long day conditions (Fig 36. a, b). However, the double knock-out *lacs4 lacs9* exhibited a 17 % increase of 16:0 and a 15 % decrease of 18:3 under long day conditions (Fig. 36 a).
Under short day conditions *lacs4 lacs9* displayed an increase of approx. 20 % for 16:0 and an 8 % decrease of 18:3.

3.2.3.7.4 Phosphatidyl choline (PC)

PC is one of the most prominent phospholipids in *Arabidopsis*. It is synthesized at the ER but can also be found in all other membrane systems of the cell. The fatty acid profile of PC is characterized by almost similar amount of 16:0, 18:2 and 18:3. Both alleles of *lacs4* and *lacs9* showed the same fatty acid composition as wild type under short and long day conditions (Fig. 37 a, b). The double mutant *lacs4 lacs9* displayed under long day conditions a 20 % reduction of 16:0 and a 30 % reduction of 18:3. In contrast, 18:1 and 18:2 were elevated by 66 % and 50 %, respectively (Fig. 37 a). Under short day conditions the biochemical phenotype of *lacs4 lacs9* was significantly milder, with 20 % reduction in 16:0 and a 13 % decrease in 18:3, whereas 18:2 was elevated by 28 % (Fig. 37 b).

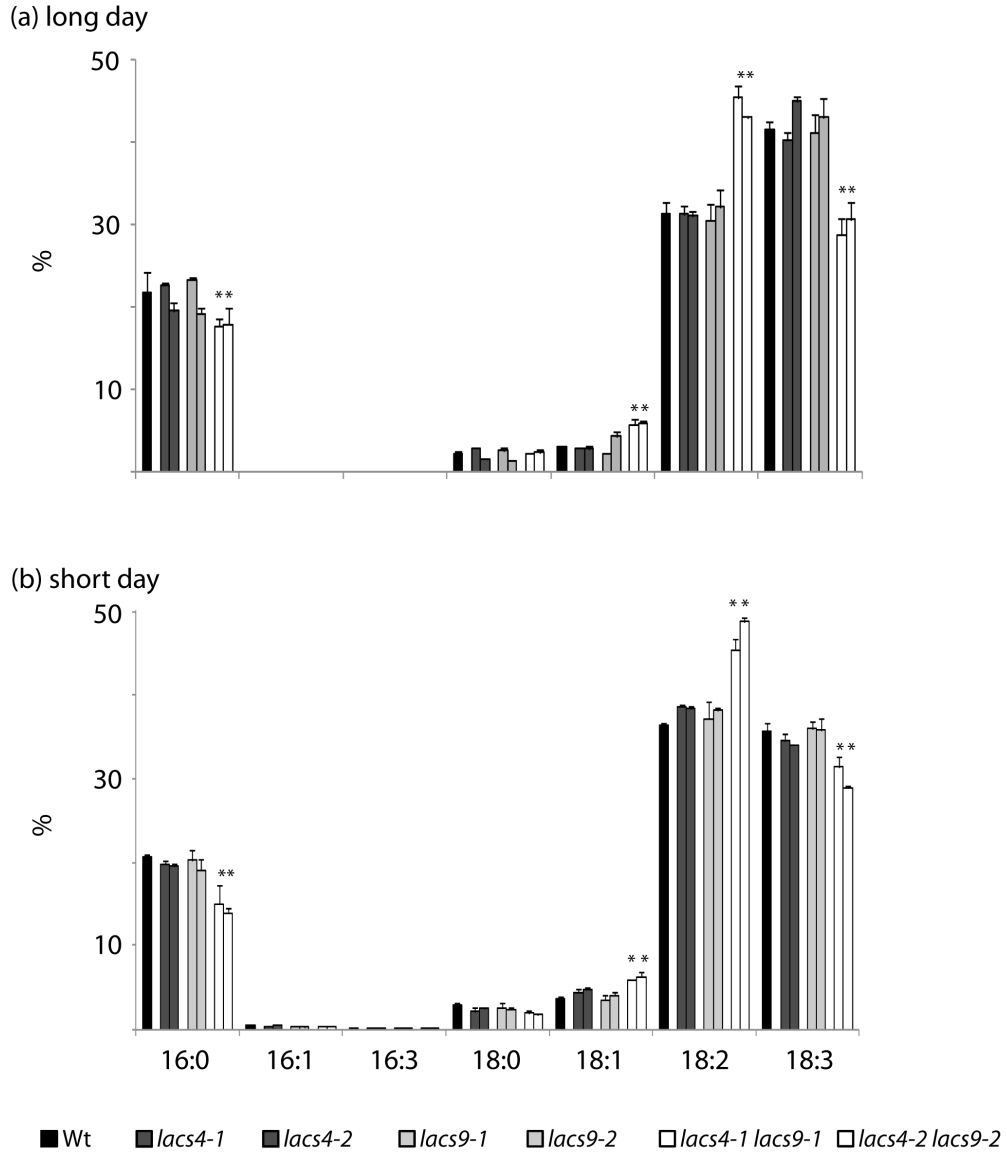

Fig. 37. Fatty acid profile of PC form Wt, *lacs4-1, lacs4-2, lacs9-1, lacs9-2, lacs4-1 lacs9*-1 and *lacs4-2 lacs9-2* grown under (a) long day and (b) short day conditions. The fatty acids are indicated at the bottom of the diagram on the *x*-axis of (b). Each value represents the mean of 3 independent replicates. Error bars = SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

3.2.3.7.5 Phosphatidyl ethanolamine (PE)

PE belongs, together with PC, to the most important phospholipids in *Arabidopsis.* It is also synthesized at the ER but does not have such a central position in metabolism as PC does. However it is a very important component of all cellular membrane systems. The fatty acid profile of PE is very similar to PC with 16:0, 18:2 and 18:3 as the most

prominent fatty acids. However, PE contains more 18:2 than 18:3 in contrast to PC containing more 18:3 than 18:2.

As already seen for the lipids described before both alleles of the single knock out lines *lacs4* and *lacs9* showed no or only minor changes in comparison to wild type (Fig. 38 a, b). In contrast, significant changes were detected in the *lacs4 lacs9* double mutant under both light regimes. When grown under long day conditions 16:0 and 18:3 were decreased by 20 % and 16 %, respectively, whereas 18:2 was increased by 40 % (Fig. 38 a). When grown under short day conditions 16:0 and 18:3 showed a reduction by 15 % and 22 %, respectively whereas 18:2 was increased by about 15 % (Fig.38 b).

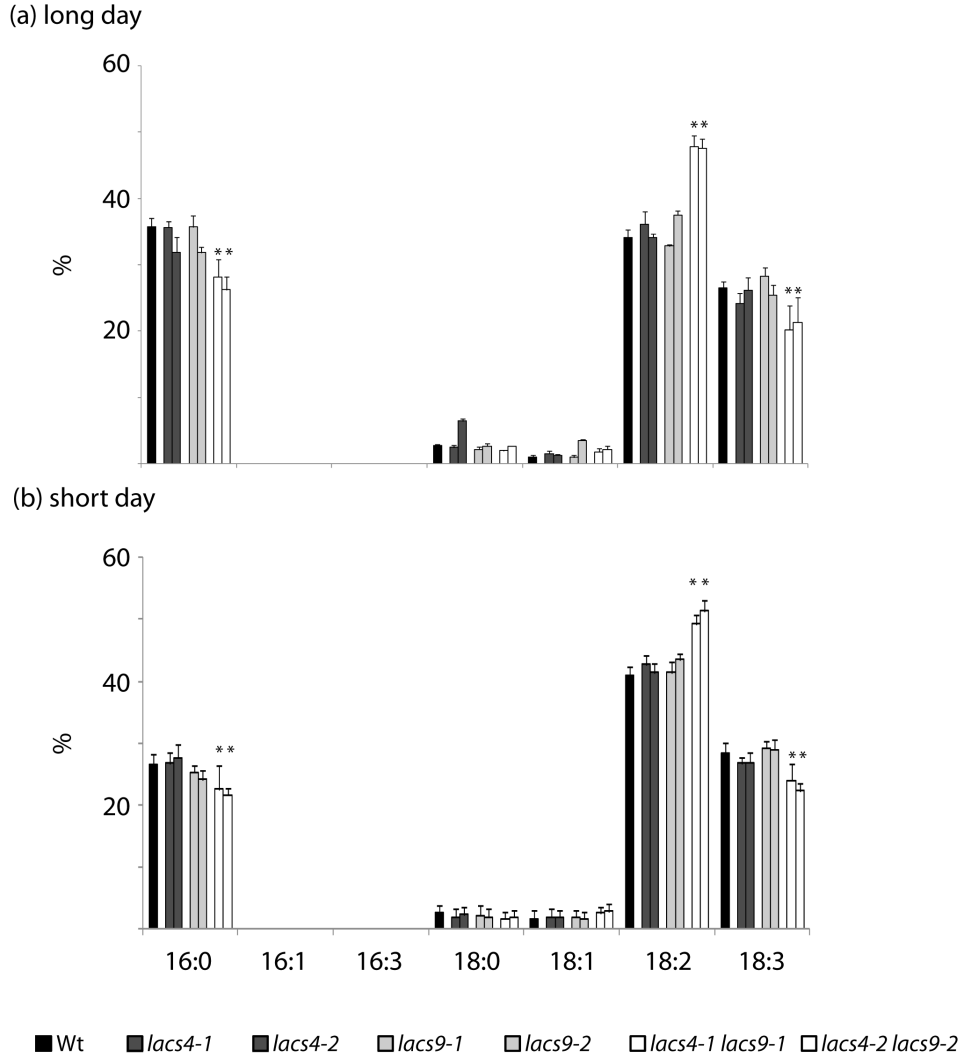

Fig. 38. Fatty acid profile of PE form Wt, *lacs4-1, lacs4-2, lacs9-1, lacs9-2, lacs4-1 lacs9-1* and *lacs4-2 lacs9-2* grown under (a) long day and (b) short day conditions. The fatty acids are indicated at the bottom of the diagram on the *x*-axis of (b). Each value represents the mean of 3 independent replicates. Error bars = SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

3.2.3.7.6 Comparison of fatty acid profiles of all lipid classes

The obtained fatty acid profiles showed that both single knock out lines showed indeed no changes in the investigated part of lipid metabolism. However, data for *lacs4 lacs9* revealed the complex impact of LACS activity on lipid metabolism as summarized in two main points below:

1.) The total fatty acid profile of plants grown under long day conditions showed mainly an increase of 18:1 as well as 18:2 and a decrease of 18:3. However, specific lipid class data suggests that the investigated lipids are affected in different ways, e.g. DGD and PG of *lacs4 lacs9* showed an increase of 16:0, the 16:0 levels of MGD remained constant whereas 16:0 of PE and PC displayed significant reductions. This result was suggesting that effects of *LACS4* and *LACS9* inactivation are much more complex than expected, influencing many different aspects of the lipid metabolism.

2.) Although a shift in 18:2 / 18:3 ratio was also detectable under short day conditions, lipids were significantly less affected.
Furthermore it was remarkable that the effects on 16 fatty acids were relative constant under both light regimes. However, 18 fatty acids displayed most often smaller changes under short day conditions in comparison to the wild type as shown in Tab. 3.

Tab. 3. Comparison between changes in *lacs4 lacs9* plants grown under short and long day conditions. (=) is indicating that changes of a fatty acids were similar under both light regimes; variations of up to 3% were tolerated. (LD) depicts that changes under long day conditions are more pronounced and (SD) indicate that changes were more intensive under short day conditions.

	16:0	**16:1**	**16:3**	**18:0**	**18:1**	**18:2**	**18:3**
MGD	=	=	=	=	LD	LD	LD
DGD	=	=	=	=	=	=	LD
PG	LD	=	=	LD	=	=	LD
PC	=	=	=	=	=	=	LD
PE	=	=	=	=	=	=	LD

3.2.3.8 Acyl CoA synthesis

The direct product of LACS activity are acyl-CoAs, therefore it is thought that an inactivation of one or more LACS genes is leading to changes in the total acyl-CoA pool. Furthermore, acyl-CoAs are the transport form of fatty acids when shuttled from plastids to ER. Hence this compound class is essential for lipid metabolism and changes in this pool might cause severe physiological consequences.

The acyl-CoA pool of *lacs4 lacs9* was analyzed from plants grown under short and long day conditions and compared to wild type. Interestingly, reductions in the total amount of acyl-CoA were detected under both light regimes. *lacs4 lacs9* plants grown in the short day showed a reduction of total acyl-CoAs by 39 % whereas plants grown in the long day exhibited a reduction by 30 % (Fig. 39). It is remarkable that the decrease was more pronounced in plants from the short day which showed only minor changes in morphology.

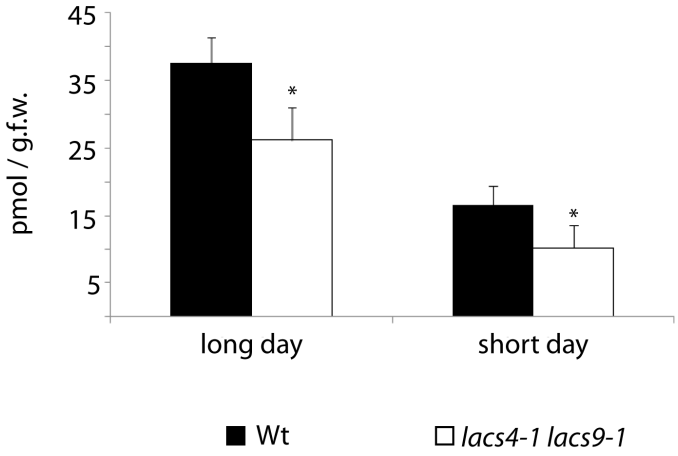

Fig. 39. Comparison of acyl-CoA amounts from Wt and *lacs4 lacs*9 grown under long and short day conditions. Each value represents the mean of 3 independent replicates. Error bars = SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

The analysis of the acyl-CoA profiles verified the data shown in Fig. 40. *lacs4 lacs9* plants grown under short day conditions showed a reduction of 16:0-CoA by 66 %, of 18:0-CoA by 65 %, and of 18:1-CoA by 6 % (Fig. 40 b). Hence all three acyl-CoAs based on fatty acids exported from the chloroplasts were negatively affected.

18:2-CoA showed no changes whereas 18:3-CoA was decreased by 25 % (Fig. 40 b). When *lacs4 lacs9* plants were grown under long day conditions 16:0-CoA and 18:0-CoA showed an almost similar reduction of approx. 40 % as under short day conditions, whereas the reduction of 18:1-CoA was even more pronounced exhibiting a decrease by 50 % (Fig. 40 a). 18:2-CoA was reduced by 39 % whereas 18:3-CoA remained on wild type level (Fig. 40 a).

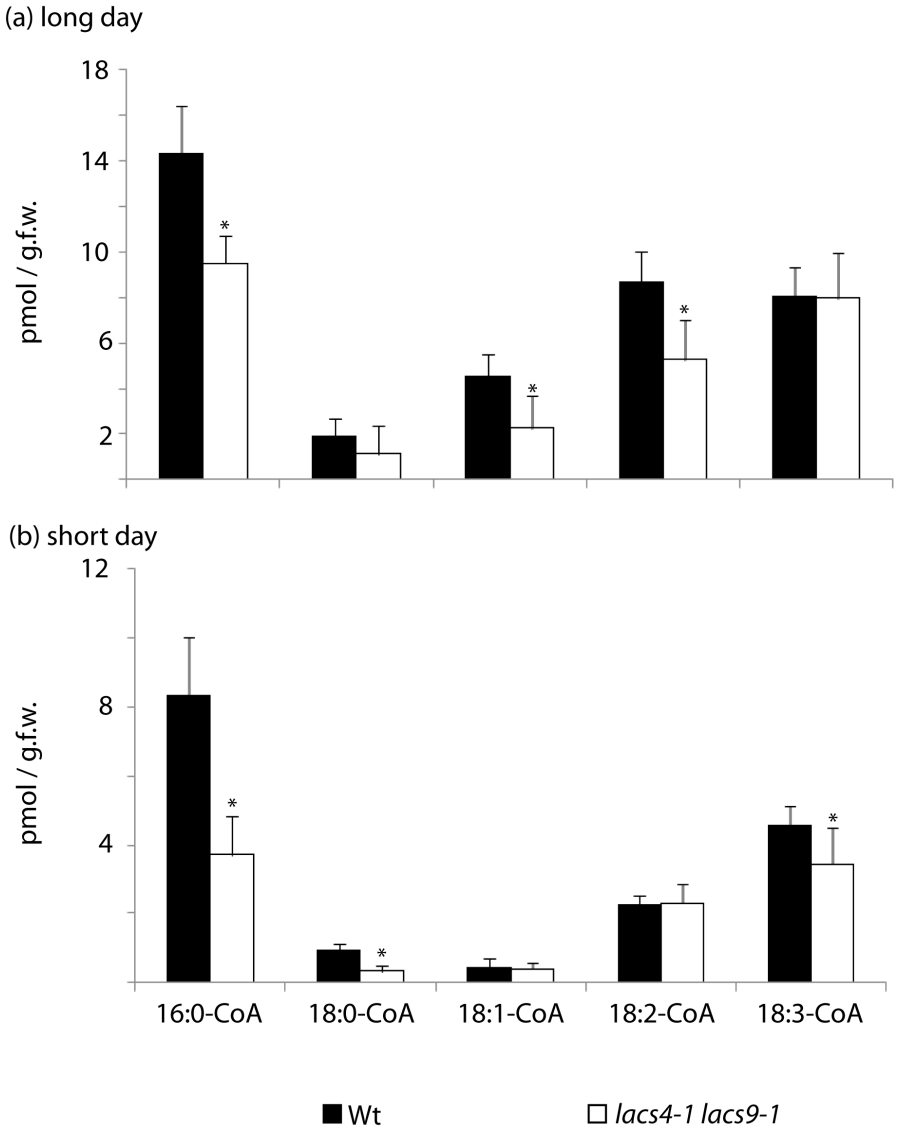

Fig. 40. Comparison of acyl-CoA profiles from Wt and *lacs4 lacs*9 grown under long (a) and short day (b) conditions. The specific acyl-CoAs are indicated at the bottom of the diagram on the *x*-axis of (b).Each value represents the mean of 3 independent replicates. Error bars = SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

3.2.3.9 Free fatty acids

Free fatty acids are the direct educt of LACS enzymes, therefore their amounts are thought to be affected by inactivation of specific LACS genes. Furthermore they are an important intermediate in the utilization of lipids as well as energy metabolism. However, due to their chemical character they can act as a detergent and therefore might introduce membrane damage. It was reported several times (Scharnewski *et al.,* 2008; Wu *et al.,* 2006) that such an effect causes physiological changes; and hence free fatty acids might be the factor leading to the morphological *lacs4 lacs9* phenotype. Consequently, they have been analyzed in double knock out lines grown under short and long day light regimes.

The total amount of free fatty acids from *lacs4 lacs9* plants grown under short day conditions showed a 14 % increase compared to the wild type (Fig. 41 a). Interestingly, the overall fatty acid profile revealed just minor differences with the exception of 18:2 which showed an increase by 54 % (Fig. 41 c). When plants were grown under long day conditions an increase of free fatty acids by 65 % compared to wild type was measured (Fig. 41 a). This increase was again mainly due to the elevated amounts of 18:2, showing an outstanding increase of 462 % compared to wild type (Fig. 41 b). This observation might help to initiate further studies to understand the morphological phenotype of *lacs4 lacs9*, since it might be caused by membrane damage due to increased amounts of free fatty acids.

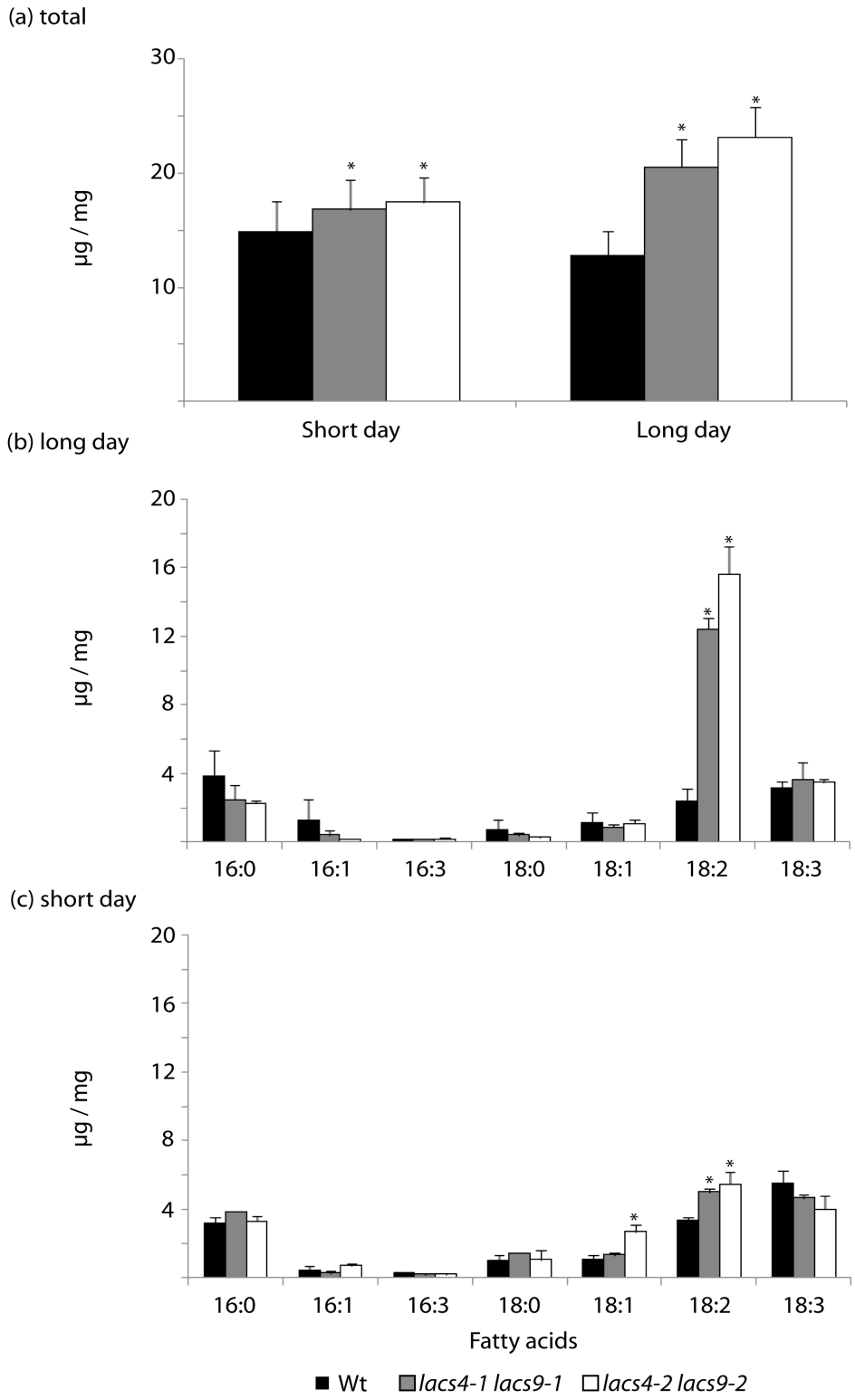

Fig. 41. Comparison of free fatty acids in Wt, *lacs4-1 lacs9-1* and *lacs4-2 lacs9-2*. (a) Total amounts of free fatty acids detected in lipid extracts. (b) Comparison of free fatty acid profiles from plants grown under long day conditions. (c) Comparison of free fatty acid profiles from plants grown under short day conditions. Specific free fatty acids are indicated on the *x*-axis. Each value represents the mean of 5 independent replicates. Error bars = SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

3.2.3.10 Incorporation of ^{14}C labeled acetate

As described in Chap 1.4.2 LACS9 is localized at the outer envelope of the plastidial membrane and is thought to be essential for the activation of free fatty acids leaving the chloroplast. In case of inactivation of LACS9 the flux of plastidial derived components could be reduced. This hypothesis was analyzed by radioactive labeling of lipids. ^{14}C-acetate was chosen as a marker for this approach, because it can directly enter fatty acid synthesis as shown in Fig. 2a. After conversion into a fatty acid it is used for the prokaryotic as well as the eukaryotic lipid synthesis. If transport between plastid and ER is affected by the inactivation of LACS activities more label should be directed to prokaryotic glycolipid and less label should be detectable in the eukaryotic lipids.

Plant samples of wild type and both *lacs4 lacs9* lines were incubated for 1 min., 5 min. and 10 min. in buffer containing ^{14}C-acetate. After 1 min. radioactivity was only detectable in low amounts in MGD for all plants tested. However after 5 min. and 10 min. clear signals for many different lipid classes were obtained. As shown in Fig. 42 after 5 min. of incubation the incorporation of radioactivity in *lacs4 lacs9* were increased by 6 % in MGD and decreased by 40 % in PG in comparison to wild type. In DGD label was not detectable. For the eukaryotic lipids PE and PC the incorporation of label in *lacs4 lacs9* was reduced by 60 % and 55 %, respectively in comparison to wild type. Interestingly, the incorporation of label in free fatty acids was significantly increased by 342 % in *lacs4 lacs9* (Fig 42 a).

After 10 min. the preferred incorporation of label into MGD of *lacs4 lacs9* was more pronounced showing 12 % more label than in wild type. In contrast, the incorporation into PG of the double mutant was reduced by 42 % whereas DGD was detectable only in trace amounts. The eukaryotic components PE and PC of *lacs4 lacs9* showed a reduction of incorporation by 51 % and 37 %, respectively whereas labeling of free fatty acids was again increased by 388 % (Fig. 42 b).
These results supported the idea that the incorporation into glycolipids might benefit from the inactivation of both LACS activities whereas the incorporation into phospholipids was penalized.

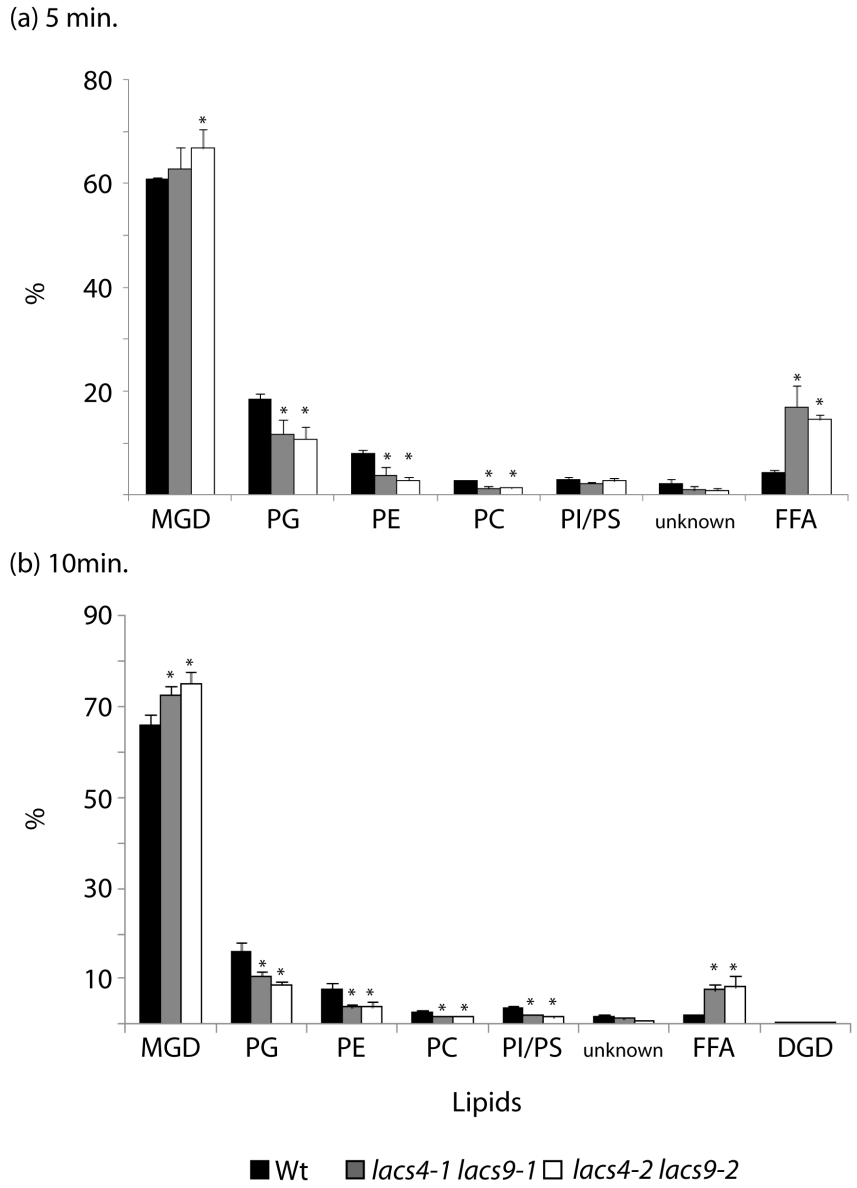

Fig. 42. Comparison of radioactivity in different lipid classes from Wt, *lacs4-1 lacs9-1* and *lacs4-2 lacs9-2*. (a) Relative amounts of radioactivity in lipid classes after 5 min. of incubation in ^{14}C acetate. (b) Relative amounts of radioactivity in lipid classes after 10 min. of incubation in ^{14}C acetate. Specific lipid classes are indicated on the *x*-axis. Each value represents the mean of 3 independent replicates. Error bars = SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

3.2.4 Seed development

It has been shown in the data presented so far that fertility, surface wax synthesis as well as morphology and membrane lipid synthesis are affected by LACS inactivation. However, also a very late aspect of *Arabidopsis* life cycle is negatively influenced as

shown in Fig. 8, since the absence of *LACS4* and *LACS9* activity resulted in reduced levels of storage lipids in the seeds.

Seeds of *lacs4* and *lacs9* looked like wild type when being viewed under the light microscope or binocular, however *lacs4 lacs9* displayed a clear reduced size and thickness. This morphology resembled of *wrinkled 1* (Focks *et al.,* 1998), a mutant having significant reduced lipid content in seeds. Furthermore, seed batches of *lacs4 lacs9* had a more intensive brown color, suggesting a modified ratio between seed shell and seed content.

The analysis of the total lipid content of *lacs4 lacs9* seeds revealed that this line had approx. 30 % - 40 % less lipids per milligram seeds than wild type or the two corresponding single knock outs (Fig. 43).

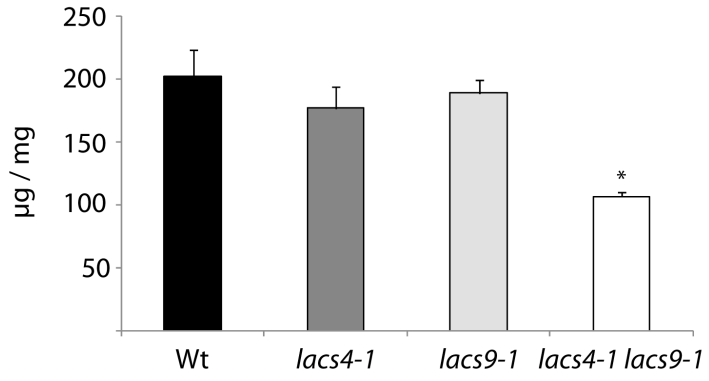

Fig. 43. Comparison of total lipid amounts in seeds of Wt, *lacs4-1, lacs9-1* and *lacs4-1 lacs9-1*. Each value represents the mean of 5 independent replicates. Error bars = SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

Besides total lipid content also the fatty acid profile was analyzed (Fig. 44). The fatty acid profile of the *lacs4 lacs9* double mutant showed a decrease of all fatty acids. However, this reduction was most pronounced for 18:1 (65 % decrease) and 18:2 (54 % decrease) whereas 18:3 showed only a moderate reduction by 22 % (Fig. 44). When relative data was calculated the level of 18:3 was even increased. The result was remarkable, since it was opposite to the fatty acid profile of leaf extracts, were a significant decrease of 18:3 and an increase of 18:1 and 18:2 were detected.

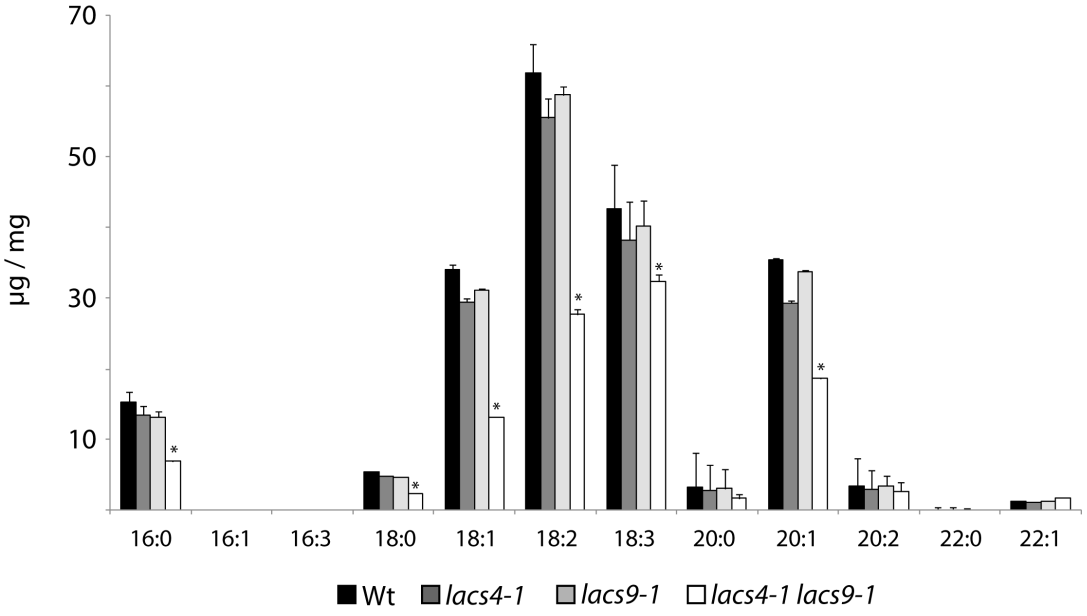

Fig. 44. Comparison of seed fatty acid profiles from Wt, *lacs4-1, lacs9-1* and *lacs4-1 lacs9-1*. Specific fatty acids are indicated on the *x*-axis. Each value represents the mean of 5 independent replicates. Error bars = SD. Asterisks indicate significantly different values between wild-type and the respective mutant line ($P \leq 0.05$).

3.3. Triple knock outs

As already mentioned in chap. 3.1 the obtained LACS mutant collection also contained triple knock out lines. These lines showed new symptoms or developed more pronounced versions of the already known and described phenotypes (fertility, morphology, lipid metabolism). One of the triple mutants, having the most severe phenotype, is presented shortly in the following.

3.3.1. *lacs4 lacs8 lacs9*

The triple knock out combination of *lacs4 lacs8 lacs9* introduced another LACS inactivation into the characterized *lacs4 lacs9* line. During the process of generating the triple mutant it turned out that although several hundred of plants were screened no complete triple knock out was obtained. Only plants being homozygous for two mutant alleles but being heterozygous for the remaining gene were identified, as shown in Tab.4. These plant lines were nevertheless interesting since they showed different

phenotypes depending on which gene remained in heterozygous state. Plants with the genotype *lacs4* lacs8 lacs9* (the asterisk is indicating heterozygosity) looked similar to wild type but were slightly reduced in size. *lacs4 lacs8* lacs9* plants showed a phenotype like *lacs4 lacs9*. Plants with the genotype *lacs4 lacs8 lacs9** developed also a phenotype similar to *lacs4 lacs9* but the symptoms were much more pronounced. *lacs4 lacs8 lacs9** plants had intensive green leafs of reduced size and with a severely distorted morphology. Furthermore, the stems were smaller and their thickness as well as their number was reduced in comparison to *lacs4 lacs9*. The fertility seemed to be not directly affected in *lacs4 lacs8 lacs9**, however seed development was compromised and only a relative low number of seeds could be harvested per plant. To understand the reasons for the low number of seeds immature siliques were carefully opened and the seed development was examined. It became obvious that a high number of seeds did not develop properly. Quantification revealed that up to 50 % of the embryos in the siliques of *lacs4 lacs8 lacs9** plants aborted (Fig.45 a, b). This rate was twice as high as it would be expected if only triple homozygous embryos would be unable to develop properly. One possible explanation could be that in addition also some embryos with the genotype *lacs4 lacs8 lacs9** failed to establish normal seeds.

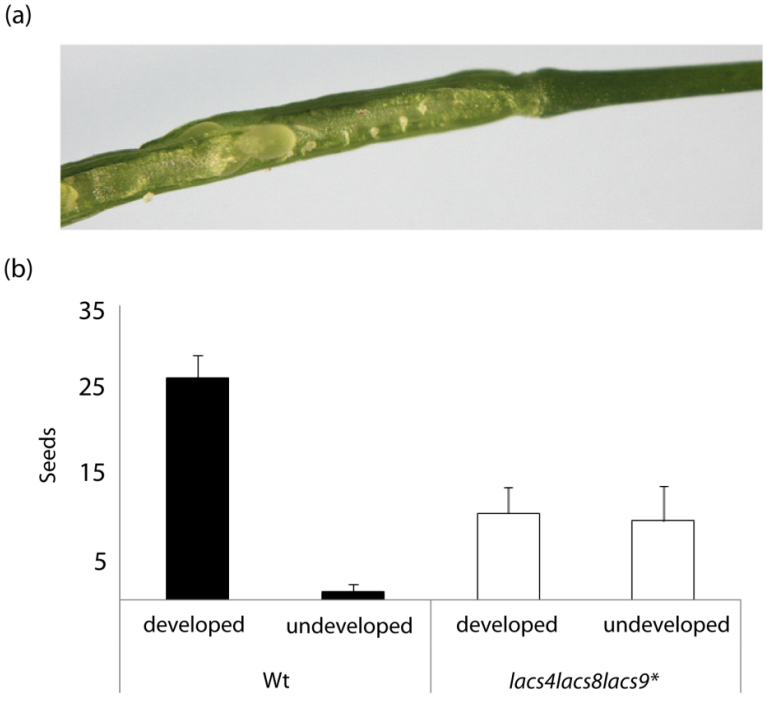

Fig 45. Defective embryo development in *lacs4 lacs8 lacs9**. (a) The silique of a plant with the genotype *lacs4 lacs8 lacs9** was carefully opened showing developed and non developed embryos. (b) Comparison of seed development between Wt and *lacs4 lacs8 lacs9**. Indicated are the numbers of developed and undeveloped seeds per silique. Each value represents the mean of 5 independent replicates. Error bars = SD.

In order to test this hypothesis the segregation pattern of the mutant alleles was determined by genotyping 150 progeny plants of two triple mutants with the genotypes *lacs4 lacs8 lacs9** and *lacs4* lacs8 lacs9*. The gene indicated by asterisk was in heterozygous state and according to Mendel's laws 50 % of the offspring should be again heterozygous for this allele. If we consider that triple homozygous seeds were not produced due to embryo lethality the correct value for heterozygous offspring is indeed 66 % whereas 33 % would be expected to contain the wild type allele. The results of the genotyping revealed that the recovery rate was dramatically lower as expected with only 47 % of heterozygous plants in case of *lacs4* lacs8 lacs9* and 29 % in case of *lacs4 lacs8 lacs9** (Tab. 4). This result indicated that the transmission of the mutant alleles in the described mutant background was severely compromised. This data is in nice agreement with the observed rate of undeveloped seeds in the siliques of the *lacs4 lacs8 lacs9** triple mutant.

Tab. 4. Segregation of mutant alleles in *lacs4 lacs8 lacs9* related plant lines. As expectation the probability to receive in F1 generation the genotype of the parental line is given. The measured recovery rate of the parental genotype is based on the analysis of 150 F1 plants repeated three times. ho = homozygote knock out; he = heterozygote, mutant line contains wild type as well as T-DNA insertion allele; n.d. = non detected

Genotype	Allele	Phenotype	Expectation	Measured
4ho 9ho	aa cc	Growth, Morphology		
4ho 8ho	aa bb	-		
8ho 9ho	bb cc	-		
4ho 8ho 9he	aa bb Cc	Growth, Morphology	66 %*	29 %
4he 8ho 9ho	Aa bb cc	Growth	66 %*	47 %
4ho 8ho 9ho	aa bb cc	Lethal	n.d.	n.d.

*: based on the assumption that 4ho 8ho 9ho is lethal

The reduced transmission of mutant alleles seemed to be due to embryo lethality but also diminished pollen grain viability could contribute to the observed results. In order

to evaluate the performance of the mutant pollen crossings were performed with *lacs4 lacs8 lacs9** as pollen donor and wild type plants as pollen acceptor. If this crossing would produce plants heterozygous for *LACS9*, this would proof that triple knock out pollen grains are viable and are able to fertilize the wild type. Offspring obtained from this crossing contained wild type as well as the mutant allele of *LACS9*. This result proofed that *lacs4 lacs8 lacs9* triple knock out pollen grains are viable and able to fertilize other plants. However, the transmission of the mutant allele was expected to be 50 % according to Mendel's laws, but only 10 % were actually detectable indicating that the fitness of the *lacs4 lacs8 lacs9* triple knock out pollen was indeed severely reduced.

4. Discussion

Plant lipid metabolism has become very important for biotechnology during the last years holding new promising opportunities for chemical, energy, and food industries. However, many aspects of lipid metabolism are still poorly understood which is impeding a faster and more successful development in this area. One of the poorly understood chapters are LACS enzymes, which are catalyzing the activation of free fatty acids to acyl-CoA. The chemical mechanism of this reaction is well understood, but their biological impact remained mainly unknown.

Nine LACS enzymes have been identified in *Arabidopsis*, but their analysis is complicated by the fact that corresponding single knock outs have no or only weakly developed phenotypes. Furthermore, only minor differences in substrate specificity or expression pattern have been detected and deeper molecular characterizations are complicated due to the membrane association of the enzymes. Based on this knowledge a reversed genetic approach has been chosen to reveal the impact of specific LACS enzymes on plant development and lipid metabolism. The available single knock outs have been crossed systematically resulting in a large mutant collection with about three dozen of double and triple mutants. This collection has been screened for visible phenotypes with fascinating results, identifying mutants affected in fertility, morphology, and embryo development. These results were highlighting the importance of LACS enzymes for specific developmental processes in plant.

4.1. Impact of LACS enzymes on fertility

The double knock out of *LACS1* and *LACS4* was leading to conditional sterility, which was rescued by incubation in high relative humidity. A similar phenotype was already described for *cer* mutants () which are affected in the biosynthesis of very long chain lipids. These special lipid molecules are important constituents of the tryphine a lipophilic layer on the pollen surface essential for proper pollen – stigma interaction. Based on this data it was speculated that also LACS1 and LACS4 could be involved in the synthesis of very long chain lipids for the establishment of a proper tryphine layer. Analysis revealed that the inactivation of LACS1 was indeed resulting in the reduction of pollen coat lipids, correlating with the reduced fertility of this line. The reduction was much more severe in both *lacs1 lacs4* double mutants supporting the hypothesis that defective very long chain lipid biosynthesis is responsible for the observed conditional sterility. Understanding the precise role of both

LACS enzymes for tryphine lipid biosynthesis was complicated by the fact that pollen of *lacs4* showed significant increases in specific tryphine lipids. From this data it could be concluded that the contribution of both proteins was non-additive, indicating more complex impact on the synthesis of pollen coat lipids. This complexity is depicted by the biochemical data of *lacs4* pollen grains. On the one hand increases in specific tryphine components have been found as mentioned above, on the the other hand significant reductions of lipid concentration in internal domains were detetced. This modified distribution of specific long chain lipids might be explained with a compromised conversion of tapetum derived precursors caused by the inactivation of LACS4. However, the increased levels of tryphine components are more difficult to explain. It might be assumed that compromised lipid synthesis of *lacs4* pollen led to a reduced transfer of lipid precursor from tapetum to pollen grains at early developmental stages. The idea is based on the highly regulated interaction of gametophyte and tapetal cells. This lower transfer rate could cause an accumulation of lipid precursors inside the tapetum leading to higher amounts of very long chain lipids. These in turn are transferred at later time to become tryphine components causing increased tryphine amounts on *lacs4* pollen (Fig. 46). Since this model is highly speculative it requires further verifications by additional experiments in the future. In tryphine of *lacs1* significant reduced levels of very long chain lipids were detected probably being responsible for the reduced fertility. This phenotype is most likely caused by a decreased acyl-CoA pool in anther tissue as consequence of LACS1 inactivation; leading to lower amounts of very long chain lipids transferred to the developing pollen grains (Fig. 46 b). Further important information to better understand the cooperation of LACS1 and LACS4 in pollen lipid biosynthesis was obtained from promoter GUS studies. The analysis in anthers revealed distinct expression patterns for both genes. These results indicated that *LACS1* is expressed in anther tissue whereas *LACS4* is specifically expressed in pollen grains; suggesting an interdependent connection between LACS1 and LACS4 and not an overlapping function. In conclusion, we propose a modified model for the biosynthesis of very long chain lipids found in pollen. The model is based on two spatially separated pathways for the biosynthesis of very long chain lipids in anthers. The scheme of synthesis is supported by uneven distribution of very long chain lipids on the one hand and distinct expression patterns of *LACS1* and *LACS4* on the other hand. In the model the tapetum would still synthesize tryphine components being released after disintegration of this nursing tissue; however during earlier gametophyte development other precursors would be secreted, absorbed by developing microspores and used for very-long-chain lipid synthesis

inside pollen grains (Fig. 46). It had been shown before that precursors are released by tapetal cells according to different developmental stages of microspores (Owen *et al.,* 1995). However, the intracellular localization of very long chain lipids as well as their functions are unknown and have to be clarified by microscopic and biochemical approaches. Furthermore, the analysis of intracellular lipid composition of *lacs1* as well as of *lacs1 lacs4* pollen grains could provide insight into role of the corresponding proteins in this pathway.

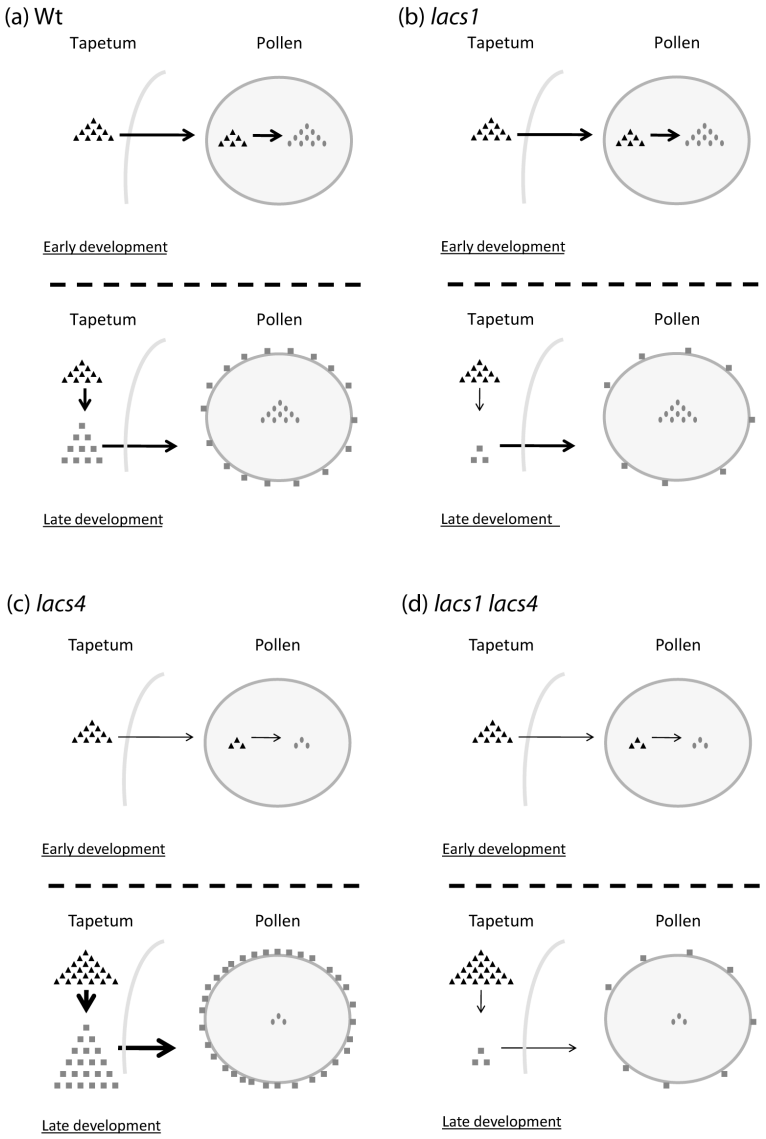

Fig. 46. Synthesis of very long chain lipids during pollen ripening. Symbols illustrate the distribution of very long chain lipids between pollen surface (tryphine lipids) and internal domains. The process is depicted for wild type in (a), for *lacs1* in (b), for *lacs4* in, (c), and for *lacs1 lacs4* in (d). The upper part of each section is visualizing in a simplified way the flux of lipids during early pollen grain development; the lower part of each section is depicting the developing processes in later developmental stages. (▲) Tapetum derived lipophilic precursors, (●) very long chain lipids synthesized based on tapetum derived precursors by LACS4 activity, (■) tryphine wax components synthesized in tapetal cells and transported to pollen grains upon tapetum disintegration. Amount of symbols correlates with the concentration of the compounds. The thickness of the arrows is indicating the capacity of the flux of metabolites through the given pathway.

Another interesting observation was the uneven distribution of secondary alcohol within pollen grains. Almost no secondary alcohol C_{29} was found in the tryphine of all lines whereas alkane C_{29} and ketone C_{29} were detected in significant amounts. Comparable results were also obtained by other groups before (Preuss *et al.*, 1993). This is surprising and rather unexpected, since the currently established model for the alkane pathway of surface wax describes the secondary alcohol as a central intermediate in the conversion of alkane into ketone. This two step reaction with a hydroxylation of alkane C_{29} and an oxidation of secondary alcohol C_{29} is catalyzed by a mid-chain alkane hydroxylase (MAH1) (Greer *et al.*, 2007). Whereas no secondary alcohol C_{29} was detected in tryphine lipids the compound was found in high amounts in total extracts of pollen grains from all lines investigated (this work and (Fiebig *et al.*, 2000). The data strongly suggested the presence of secondary alcohol in internal domains of pollen grains. In these internal domains the secondary alcohol seemed to be protected from being extracted by hexane washes of the intact pollen and only mechanical disruption of the pollen allowed its extraction.

The current model is assigning the biosynthesis of very long chain lipids in anthers exclusively to the tapetum. To reconcile this model with the observed discrepancies in the distribution of secondary alcohol very specific transport mechanism discriminating between alkanes, secondary alcohols and ketones need to be assumed. Such specific transport could result in the presence of alkane and ketone in the tryphine and the complete delivery of secondary alcohol into inner domains of the pollen grain. However, the data obtained might be explained more easily by assuming two independent pathways for very long chain lipids one inside the pollen grain and one in the tapetum. MAH1-like P450 enzymes could be involved in these independent pathways. In this case products of pollen specific wax synthesis would remain inside the gametophytes whereas tryphine components are exclusively delivered by tapetum cells. This model would also consider the presumably high impermeability of the pollen exine.

Another possibility could be a so far unidentified enzymatic activity. If the conversion of alkanes into ketones is catalyzed by two different enzymes, spatial separation and specific transport processes would be a more realistic option. Evidence for this idea is provided by microarray data indicating very low expression of MAH1 in pollen grains and closed flower buds (Gene investigator). Therefore other P450 enzymes might be responsible for the formation of secondary alcohol C_{29} and ketone C_{29} in reproductive organs.

Beside the biochemical phenotype also the morphology of *lacs4* pollen was affected resulting in 60 % to 80 % of deformed pollen grains. Interestingly the fertility of *lacs4* plants was still comparable to wild type. However, it was not possible to reveal whether morphological affected pollen grains were still fertile or if the amount of normal shaped pollen grains is sufficient for maintaining fertility.

Similar pollen phenotypes have been observed in callose mutants (Enns *et al.*, 2005), but those plants were completely sterile. Furthermore, aspects of callose deficiency have not been detected in *lacs4* by aniline blue staining or any other experiment. Hence it is assumed that rather the disturbed lipid metabolism has a direct impact on pollen morphology. However, so far it has not been proven whether the increased tryphine amounts, the reduced internal concentration or the imbalanced distribution of very long chain lipids is leading to this morphological phenotype. In addition, it cannot be ruled out that other so far not detected metabolites are causing this effect. It has been shown in pollen grains that lyso-phospholipids and free fatty acids are inducing membrane damage and causing premature aging potentially leading to an affected morphology. However, this data has not been obtained in annual plants like Arabidopsis.

Even more surprising was the finding that the disturbed pollen morphology of *lacs4* could be rescued by the additional inactivation of *LACS1*. In the resulting *lacs1 lacs4* line the tryphine lipids were reduced by about 80 % whereas *lacs4* pollen showed a 40 % increase of tryphine components in comparison to the wild type. This data is suggesting a direct correleation between tryphine amounts and pollen morphology. However, also other scenarios could explain these observations; e.g. the loss of LACS1 activity in tapetal cells is probably leading to a reduced flux of lipophilic tryphine components towards the developing pollen grain. Furthermore the dysfunctional metabolism of *lacs4* might convert tapetum derived incoming metabolites into compounds introducing cellular damage and hence also affected morphology. Consequently, inactivation of LACS1 in the background of *lacs4* could result in fewer amounts of potentially harmful metabolic products and therefore the morphology of *lacs1 lacs4* pollen could be restored.

4.2. Impact of LACS enzymes on surface wax synthesis

Besides the described conditional sterility *lacs1 lacs4* displayed also a glossy stem indicating reduced levels of surface wax. This visual symptom was investigated closer by electron microscopy studies revealing that wax crystal amounts were indeed reduced in *lacs4*, *lacs1* and *lacs1 lacs4*. Biochemical analysis verified that the level of different wax components was moderately reduced in *lacs4*, showed a more pronounced reduction in *lacs1* and were severely decreased in *lacs1 lacs4*. All reductions were due to significantly lower amounts of components generated by the alkane pathway of wax synthesis. All metabolites of the acyl reduction pathway like VLCFAs, wax esters, and primary alcohols, on the other hand, were detected at wild type or even higher levels in the investigated LACS mutants. A similar increase of VLCFAs was reported recently for the *lacs1* single mutant and for the *lacs1 lacs2* double mutant. (Lü *et al.*, 2009; Weng *et al.*, 2010).

The branching point in the synthesis of these different components is C16:0-CoA. It can be directly used as a precursor for wax ester biosynthesis or, alternatively, elongated to up to C32-CoA. The elongated VLC-acyl-CoAs are substrate for the alkane forming pathway or for the synthesis of primary alcohols. Since increased amounts of VLCFA and fatty alcohols were detected in the mutants it can be assumed that the elongation of acyl-CoAs is not affected in *lacs1*, *lacs4* and *lacs1lacs4*. Consequently, it has to be the flux into the alkane pathway which is reduced in the mutants. This is remarkable since it was believed that the elongation process provides acyl-CoAs which could serve directly as substrate for the alkane pathway. If the removal of LACS activity is hampering the flux into the alkane pathway it must be assumed that elongated VLC-acyl-CoAs get hydrolyzed and require subsequently reactivation by LACS activities. Potential acyl-CoA thioesterases had been tentatively identified at the ER (AtACH4 and AtACH5) but so far their physiological role remained unknown. Even though such a process seems to be contra productive, it would allow a more direct control of specific acyl-CoA levels. Similar processes involving deactivation and subsequent reactivation of fatty acids have been already suggested for other LACS enzymes in the peroxisomes (Fulda *et al.,* 2004; Schnurr *et al.,* 2002). Furthermore, it would indicate the existence of several acyl-CoA pools for specific metabolic pathways.

Another interesting observation was the different disturbance of the synthesis of individual alkanes in leaf wax of *lacs1 lacs4*. Alkanes were the only compound class being reduced in the leaf cuticle, however the longer chain length were decreased to much higher degree than

the shorter ones. This phenomenon could be interpreted as effect of impaired elongation. Another explanation would be different substrate specificity of LACS enzymes potentially substituting the inactivation of *LACS1* and *LACS4*. To investigate this aspect in more detail expression studies by RT-PCR should be performed, analyzing the expression pattern of other LACS genes in the mutant background considering stem and leaf epidermis.

The biochemical data of *lacs1*, *lacs4* and *lacs1 lacs4* was indicating that both LACS enzymes are important for the alkane pathway. Therefore, an overlapping expression pattern in wax synthesizing tissue would be expected. Such result was indeed obtained for the expression in leaves, although the detected expression level was relatively low. In contrast to that, in stem epidermis only intensive expression of *LACS1* had been detected but no expression of *LACS4*. This result could be due to a very low expression level, which cannot be detected by GUS promoter analysis; but similar results were also obtained earlier by.more sensitive RT-PCR (Shockey *et al.,* 2002). However, in a recent study relatively high amounts of *LACS4* expression were reported in stem epidermis. To resolve these contradictions further analyses will be required to obtain conclusive data.

4.3 Impact of LACS enzymes on plant Morphology

The double knock out of *LACS4* and *LACS9* caused severe defects in plant morphology, such as reduced growth, curly leaves and reduced apical dominance. Interestingly, the development of these symptoms seemed to be light dependent. When plants were grown with 8h illumination per day (short day) only a slight growth reduction was observable whereas when grown with 16h illumination per day (long day) all the described symptoms became established. A similar morphological phenotype was reported for *dgd1* (Froehlich *et al.,* 2001), a mutant containing only minor amounts of DGD. For this line the morphological alterations were explained by modified biophysical properties of the plastidial membranes due to the absence of DGD. Consequently, plastid architecture was severely affected and the rate of photosynthesis was partially reduced. The analyses of *lacs4 lacs9* mutant plants revealed that the observed phenotype is most likely caused by different reasons since DGD levels and photosynthesis rates were unaffected.

To elucidate the biochemical background of the observed phenotypes lipid extracts of *lacs4 lacs9* mutant plants were subjected to detailed analyses. The total fatty acid profile revealed a significant increase for fatty acid 18:2 and a decrease of fatty acid 18:3. Surprisingly, mutant plants of both light regimes developed this alteration in their total fatty acid profile indicating that this change in 18:2 / 18:3 ratio is not causative for the observed phenotypes. This view is supported by the fact that different mutant lines defective in fatty acid desaturases (fad) are established with more severe changes in 18:2 / 18:3 ratio but without showing symptoms as described for *lacs4 lacs9*.The shift in the ration could be nevertheless interpreted as a desaturase phenotype, maybe caused by modified membrane properties leading to a weaker activity of specific FAD enzymes. The analysis of such membrane anchored enzymes is very complicated and ambitious; hence RT-PCR was chosen for analyzing the transcript levels of the corresponding genes. Preliminary expression studies indicated no changes or even an increase in FAD expression, therefore the reduced 18:3 amounts are supposable not due to a reduced FAD transcription.

The analysis of fatty acid profiles from different lipid classes revealed very complex changes not easily to integrate into a unifying model. Most obvious were under long day conditions the decrease of 18:3 in all lipid classes in *lacs4 lacs9* and the increase of 18:2 in PC and PE. Moreover, in MGD and DGD a replacement of prokaryotic lipid species by eukaryotic species were observed. The results seemed to indicate a subtle shift from glycolipids synthesized by the eukaryotic pathway towards those generated by the prokaryotic pathway.

One possible explanation for this effect would be a reduced flux of plastid derived fatty acids towards the ER. Support for this hypothesis was obtained by labeling studies using ^{14}C-acetate. At early times MGD of *lacs4 lacs9* accumulated higher levels of label compared to wild type suggesting that more glycolipids are synthesized via the prokaryotic way in the double mutant. In parallel, smaller levels of label were identified in the phospholipids of *lacs4 lacs9* supporting the idea of a reduced flux from the plastids to the ER. One reason for the diminished flux is most likely the almost complete absence of LACS activity at the outer envelope. In *lacs9* only 5 % remaining LACS activity was detected here (Schnurr *et al.,* 2002). Since LACS activity at the outer envelope is considered to be important for the reactivation of de-novo synthesized fatty acids leaving the plastid it came as a surprise that the lacs9 single mutant did not show any detectable phenotype. One possible reason could be that the remaining 5 % activity is sufficient for a normal development. Another explanation

would be transport and reactivation of free fatty acids at the ER. ER localization of LACS enzymes have already been reported (Lu *et al.,* 2009; Zhao *et al.,* 2010), but they were so far not thought to be involved in the flux of fatty acids from plastids to ER. Assuming that also LACS4 might be localized at the ER its absence in the *lacs4 lacs9* double mutant could severely compromise the supply of fatty acids for lipid biosynthesis at the ER. A reduction in de novo acyl-CoA transport from plastids to ER could explain the shift in glycolipid fatty acid profiles towards rather prokaryotic components. Although it does not explain the affected morphology it might deliver a model for the reduced growth of *lacs4 lacs9*.

The probably most interesting modification of the lipid metabolism in *lacs4 lacs9* was observed in the profile of the free fatty acids. Already the level of total free fatty acids were promising since it was strongly elevated only in plants grown in long day conditions. The analysis of the fatty acid profile revealed that the increase of free fatty acids is surprisingly due to the drastically elevated level of only one specific fatty acid which is 18:2. The detected concentration of free 18:2 is about 4 - 500 % higher than in wild type and is accompanied by elevated levels of 18:2 also in PC and PE. On the other hand, the concentration of 18:2-CoA was significantly reduced in the double mutant. The acyl-CoA pool of the double mutant was decreased compared to wild type under both light regimes. But under long day conditions especially 16:0-CoA, 18:0-CoA, and 18:2-CoA were reduced significantly. The comparison of the fatty acid profiles of membrane lipids, free fatty acids, and acyl-CoA clearly point out 18:2 to play a key role in the modified lipid metabolism of the *lacs4 lacs9* double mutant. 18:2 is generated in the cytoplasm by Fad2 on the basis of PC. Interestingly, 18:2 is specifc for transport of lipid precursor from cytoplasm to the chloroplast. The nature of this precursor is not finally defined but there is strong evidence that die-18:2-PA generated at the ER is imported by the plastid (Benning 2009). The lipid transfer is mediated by the TGD proteins as detailed in the introduction (Benning 2009). In this process di-18:2-PC is generated at the ER, an unknown phospholipase D is releasing choline and the generated PA is delivered to the plastid. There are several reasons to believe that the increased levels of 18:2 in the *lacs4 lacs9* double mutant could be connected to modifications in the lipid transport from ER to plastids. First, the fatty acid profiles of DGD and MGD indicate a reduced flow of lipids from ER to plastids. If the transfer is reduced why do the fatty acids of the transport molecule accumulate in the pool of free fatty acids? As stated before, for the currently accepted model it is assumed

that PC (1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine) is converted to PA by head group remodeling and used for transport to the plastids (Benning 2009) (Fig. 47). However, another possibility would be a *de novo* synthesis of PA using 18:2 released by phospholipases from PC. In such scenario the freed fatty acids would require activation by LACS to be used for *de novo* lipid synthesis leading to the formation of di-18:2-PA / DAG (Fig. 47). The inactivation of specific LACS activities would cause an accumulation of free 18:2, since fatty acids are hydrolyzed but not reactivated. Since we observed the specific accumulation of 18:2 such scenario could perfectly explain the metabolic situation in the *lacs4 lacs9* double mutant. The accompanying elevated concentration of 18:2 in PC might be due to inhibition of the phospholipase by the high levels of free 18:2. The compromised reactivation of free 18:2 could also explain the reduced levels of 18:2-CoA in a metabolic setting with increased levels of 18:2 in lipids as well as in the free fatty acid pool. If the increase in 18:2 would be due to diminished activity of desaturase Fad3 it would be expected that 18:2 would also increase in the acyl-CoA pool.

The proposed model for the generation of PA as transport molecule for the transfer to the plastid is currently still highly speculative and will require thorough examination by additional experiments. Analyses by LC/ MS-MS should give insight into pool size and fatty acid composition of PA, DAG, and lyso–PC. If the model would be correct, less di-18:2 species of PA or DAG should be detected. However, in the light of the low concentrations and the temporary character of these lipids it might be problematic to detect significant differences. Moreover, it is possible to analyze the transport of lipids from ER to plastids by a pulse chase labeling experiment with acetate (according to (Xu *et al.,* 2005)). With this approach the potentially reduced flow of eukaryotic components into the glycolipid pool could be evaluated. A relatively high amount of label will occur early in MGD or DGD synthesized via the prokaryotic pathway. However, this intensive label will decrease over time due to remodeling and *de novo* synthesis of new and unlabeled fatty acids. Labeled fatty acids will be transported also to the ER and will return with some delay to the plastid resulting in a second increase of label in the glycolipids. If the back transport is affected in *lacs4 lacs9*, this second peak of label in the glycolipids should be smaller, delayed or even absent.

Another approach would be labeling of the head group (^{13}C-choline). If the new theory would be correct substantial amounts of labeled lyso-PC or glycerol-phosphocholine should be

detectable. However, if the current model is correct substantial amounts of labeled free choline should be detectable upon release by phospholipase D catalyzing the hydrolysis of PC into PA and choline.

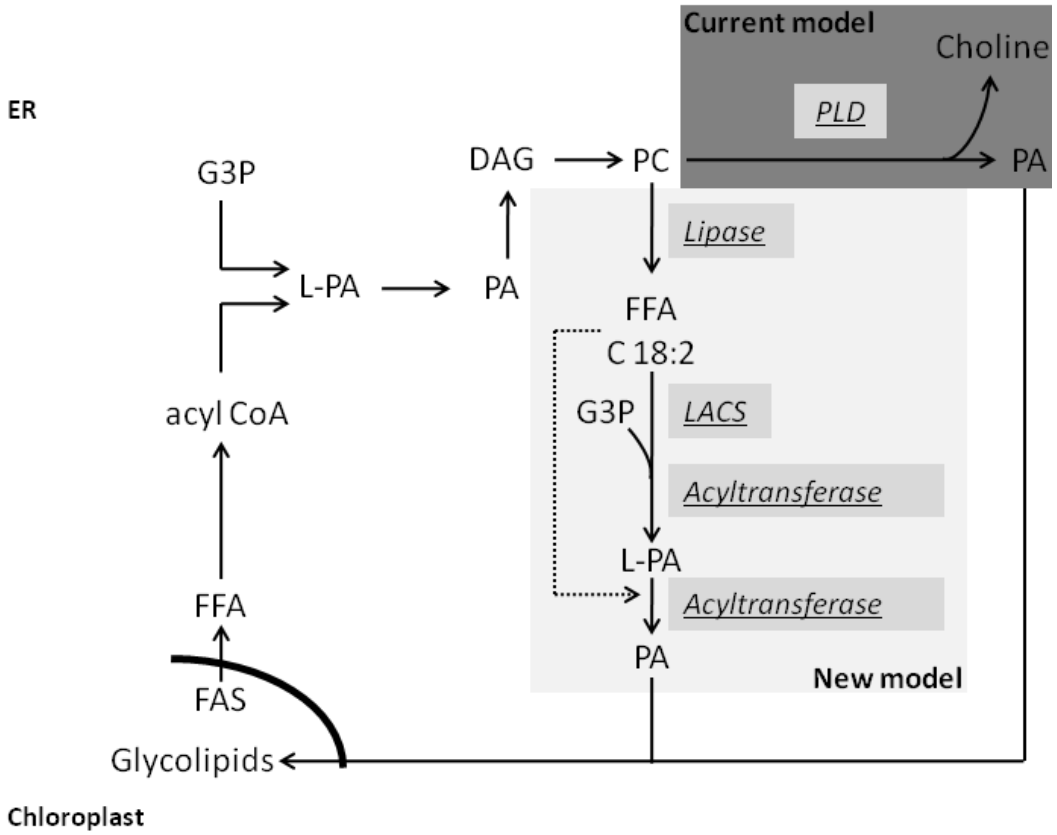

Fig.47. Simplified model of eukaryotic lipid synthesis. PC as eukaryotic lipid is synthesized at the ER. Besides being an important membrane lipid by itself PC serves presumably also as precursor for di-18:2-PA. This special PA species is believed to be the lipid transport form being transferred to the plastids. In the plastid the delivered PA serves as precursor for glycolipid biosynthesis. In dark grey the current model is shown with PC being converted to PA catalyzed by a predicted phospholipase D. The resulting PA is potentially used for the transport into plastids. The suggested new model is depicted in light grey. Free fatty acids (FFA) are cleaved from PC by a lipase and will be reactivated by a LACS enzyme. The resulting acyl-CoA and glycerol-3-phosphate are used for *de novo* synthesis of PA catalyzed by acyltransferases; however this is one potential scenario, it might be also possible that the acyl CoA is used for acylation of lyso-PA. The generated di-18:2-PA will be delivered to the plastids. Abbreviation: FAS – Fatty acis synthase, FFA – Free fatty acid, G3P – Glycerol-3-phosphate, PA – Phosphatidic acid, DAG – Diacylglycerol, PC – Phosphatidylcholine, L-PA – Lyso phosphatidic acid.

The elevated concentration of 18:2 in the *lacs4 lacs9* offered also a possible explanation for the morphological phenotype of the double mutant plants. Free fatty acids are known to be

toxic for cells. They have the potential to induce membrane damage and to inhibit enzyme activities. Interestingly, *lacs4 lacs9* plants grown under short day conditions showed only a very moderate increase in free fatty acids whereas plants grown under long day conditions showed the pronounced accumulation. In both cases the increase was due to elevated levels of 18:2. This data might suggest that the accumulation of a specific fatty acid could be the so far unidentified factor causing the morphological phenotype of *lacs4 lacs9*.

4.4 Impact of LACS enzymes on seed oils

Seed oils consist almost exclusively of triacylglycerols (TAG) which is used as source of carbon skeletons as well as energy for the developing seedling. The synthesis of this lipid takes place at the ER and one important route of synthesis is involving three acylation steps of glycerol-3-phosphate. It is believed that LACS enzymes are a key player in this process, since acyl-CoAs are the relevant substrate for TAG biosynthesis. This assumption was supported by a recent study showing that LACS1 and LACS9 have overlapping function regarding TAG synthesis. The authors demonstrated that the TAG level in a *lacs1 lacs9* double mutant was reduced by 11 %. Remarkably, the level of TAG specific 20:1 was unchanged compared to wild type, whereas 16:0, 18:0, 18:1 and 18:2 showed pronounced reductions.

In *lacs4 lacs9* the TAG content of seeds was decreased by about 40 % and 20:1 was decreased by up to 30 %. Furthermore, the morphology of the seeds was modified, matching most closely the *wrinkled 1* phenotype (Focks *et al.,* 1998). This latter mutant line is characterized by an 80 % reduction in seed lipid content and is severely affected in seed morphology after drying, due to relatively high water content. It could be argued that the reduction of TAG in *lacs4 lacs9* is might be due to pleiotropic effects caused by the severe changes in lipid metabolism but there are also facts suggesting that these observations are specifically caused by the inactivation of *LACS4* and *LACS9*. The shift of the 18:2 / 18:3 ratio detected in leaf lipids was not observed in *lacs4 lacs9* seeds. Although 18:3 showed a moderate decrease, probably due to the overall reduction, 18:2 was significantly more reduced. The absence of elevated 18:2 amounts and a less reduced 18:3 levels suggested that other processes than in leafs were leading to the alterations in biochemical composition. Furthermore the pronounced reduction of 20:1 indicated that indeed the TAG content is

decreased. In contrast, in *lacs1 lacs9* the 20:1 content was rather unchanged suggesting that maybe non storage lipids were reduced.

The specific reduction of TAG in *lacs4 lacs9* would be remarkable since LACS enzymes involved in TAG synthesis were not identified yet. To investigate this point in more detail more seeds harvested from individual plants need to be tested. Moreover, neutral and phospholipid pools should be separated by solid phase extraction and analyzed independently to verify if neutral lipids are indeed more strongly affected than phospholipids. Furthermore, over expression lines expressing elevated levels of LACS4 and LACS9 could be interesting, since such lines might produce increased amounts of TAG.

A critical point to be addressed in the future is the intracellular localization of both enzymes. LACS9 is known to be localized at the outer envelope of plastids whereas the localization of LACS4 is unknown but is assumed to be localized at the ER.

Based on the fatty acid profile found in TAG molecules the acyl-CoA pool for TAG synthesis could be supplied in part directly by plastidial fatty acid export from the plastids and in addition by acyl-CoAs deriving from the PC pool. Based on the current knowledge it can be assumed that LACS9 is responsible for acyl-CoA deriving from the plastid. Upon inactivation of LACS9, LACS4 might be able to substitute this function despite having most likely a different subcellular localization. In *lacs9* mutant cells the free fatty acids released by the plastid might reach the ER via fatty acid binding proteins or by contact sites between plastids and the ER. The native task of LACS4 might be nevertheless the reactivation of fatty acids hydrolyzed from PC or other membrane lipids.

If LACS4 is inactivated on the other hand, acyl-CoA flux deriving from plastids is unaffected however the flux of PC derived components might be reduced. Hence a more detailed analysis of fatty acid composition from *lacs4* seeds could shed some light on this process. Furthermore, the acyl-CoA composition in *lacs4* developing seeds might also provide valuable information helpful for the development of more detailed model. Ideally, an increase of plastidial fatty acids could be detected proofing the idea that LACS4 is essential for the supply of TAG synthesis by PC derived acyl-CoAs. In addition further expression analysis of developing seeds could result in the identification of other LACS proteins with overlapping activities being responsible for the residual TAG content of *lacs4 lacs9* seeds.

4.4 Impact of LACS enzymes on embryo development

A closely related aspect to seed oil synthesis is embryo development and according to the results of this project LACS enzymes have also impact on this process. During the screen of the mutant collection the *lacs4 lacs8 lacs9** was identified, being a homozygous knock-out for *LACS4* and *LACS8* and heterozygous for *LACS9*. This line had a phenotype mostly comparable to *lacs4 lacs9* but the distinctive features were much more pronounced. However, it was observed that in addition to the described characteristics up to 50 % of the embryos inside a silique were not developed. This defect is most likely the reason for the unavailability of a homozygous triple knock-out line. How the triple knock-out translates into embryo lethality need to be answered by future experiments. First hints might be obtained by the analysis of seeds of different developmental stages to define the block in embryogenesis more precisely.

Summary

It has been shown in this project, that LACS enzymes have a significant impact on several developmental aspects of the plant life cycle. LACS mutant lines were identified showing defects in fertility, surface wax synthesis, morphology and seed development. The phenotypes were mainly observed in double or triple knock-out lines indicating overlapping function of the corresponding LACS enzymes. This functional redundancy did not correlate with phylogenetic comparisons, since especially distantly related enzymes seemed to have overlapping functions.

Our studies revealed the special role of LACS4 in the enzyme family. The gene is characterized by the highest expression levels and a widespread activity in almost all tissues. Already the single knock-out results in severe morphological defects of the pollen grains, although no impact on fertility was detected. More interestingly, LACS4 activity is contributing in cooperation with other LACS activities to functional distinguishable acyl-CoA pools. In combination with LACS1 precursors for the metabolism of very long chain lipids are provided feeding into wax and tryphine lipid biosynthesis. LACS4 and LACS9 are involved in delivering precursors for the biosynthesis of membrane lipids and triacylglycerols.

The results support the idea of independent acyl-CoA pools within the plant cell. The detection of alterations in individual pools remains technically impossible. However, mutant lines with physiological phenotypes and the analysis of down-stream products of lipid metabolism allow for conclusions about the contribution of individual LACS enzymes to specific acyl-CoA pools.

The deeper analysis of LACS enzymes and their impact on plant development will improve our understanding of plant lipid metabolism. Furthermore, it has the potential to provide further stimuli for green biotechnology. The appropriate use of LACS enzymes could potentially result in specific modifications of flux in pathways of interest.

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Appendix

The following table gives the sequences of primers used for identification of LACS mutants. Primer sequences are given in the 5' to 3' orientation.

Appendix 1. LACS Primer sequences

Name	Sequence
LACS1_RTf	AGGCGTTTAACATATGCACCATAATGTAC
LACS1_RTr	TCGATGATCTTGAGTACTCCGTTTGGAAG
LACS4_RTf	CAACATCAGAAGAGCCTATCACTCTTCGT
LACS4_RTr	AAATGAGAGACCCAAGGACCGAGAGTGA
LACS9_RTf	GACGTCTCCGCTAAAAGACCATTAT
LACS9_RTr	CAATCAGGACATAAACAACCGCAGAGCAT

Curriculum vitae

Name	Dirk Jessen
Date of birth	27.04.1984
Place of birth	Husum, Germany

Education and Qualifications

since 05/2008	**Ph.D. program Molecular Biology** International Max Planck Research School Ph.D. thesis with Dr. Martin Fulda and Prof. Dr. Ivo Feußner at the Albrecht von Haller Institue of Plant Science, Georg August University, Goettingen Germany
10/2006 – 03/2008	**M.Sc. program Molecular Biology** International Max Planck Research School MSc thesis with Dr. Martin Fulda and Prof. Dr. Ivo Feussner at the Albrecht von - Haller-Institute of Plant Sciences, Georg August University Göttingen **Degree:** Master of Science
10/2003 – 09/2006	**Studies in Biology** Georg August University Goettingen, Germany **Degree:** Vordiplom
08/1994 – 06/2003	Hermann Tast Gymnasium, Husum, Germany

Presentations at International Conferences (selection)

2011	Poster at 5th European Symposium on Plant Lipids; Gdansk, Poland
	Oral presentation at 24th Symposiun on plant molecular biology; Dabringhausen Germany
2010	Oral presentation at the 19th International Symposium on Plant Lipids; Carins, Australia
2009	Poster at the 22rd Symposiun on plant molecular biology; Dabringhausen Germany

Stipends and awards

2011	Reinhold-von-Sengbusch-Vortragspreis
2011	GGNB Bridging Fund
10/2008 – 03/2011	"Promotionsstipendium" from the evang. Studienwerk Villigst e.V.
05/2008 – 09/2008	Georg-Christoph-Lichtenberg-Stipend from the state Lower Saxony
03/2004 – 03/2008	Stipend from the evang. Studienwerk Villigst e.V.