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DIUNSATURATED FATTY ACIDS (DUFAs) AND THEIR ROLES IN SEEDLING ESTABLISHMENT AND DEFENSE.

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DIUNSATURATED FATTY ACIDS (DUFAs) AND THEIR ROLES IN SEEDLING ESTABLISHMENT AND DEFENSE.

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

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Lausanne 2017

Cette thèse est dédiée à la mémoire de mon gramd-père

Gennadiy Sodomov

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Summary

Fatty acids (FA) are one of the major storage forms of carbon in living organisms, precursors for some plant hormones such as jasmonate (JA), and major building compounds of the membrane bilayer. In this thesis I address the question: what are the roles of diunsaturated fatty acids (DUFAs) in *Arabidopsis* seedling establishment and defence? In the first part of this work I found that fatty acid composition is different between roots and shoots of 7-day-old seedlings. The fatty acid composition of roots was constant during plant development, whereas in the shoots it changed within the first week of seedling establishment. Analysis of *Arabidopsis* mutants with low levels of polyunsaturated fatty acids (PUFAs) revealed chlorotic, dwarfish plants with delayed developmental phenotypes. Reduced levels of PUFAs in roots led to overaccumulation of reactive oxygen species (ROS) that correlated with reduced root growth and stronger susceptibility to abiotic stresses, such as salt treatment. Another interesting finding of this work was that *Arabidopsis* seedlings lacking DUFAs were highly resistant to the necrotrophic pathogen *Botrytis cinerea*. This work defines a first attempt to understand the role DUFAs in adaptation to some important abiotic and biotic stresses and their role in these processes. This research opened a lot of questions that could provide better understanding of plant development and adaptation to a changing environment.

Résumé en français

Les acides gras sont connus pour être l'une des principales formes de stockage du carbone dans les organismes vivants et les composés de construction majeurs des membranes cellulaires. Dans cette thèse, j'ai abordé la question suivante : quel est le rôle des acides gras di-insaturés (AGDI) dans l'établissement et la défense des plantules d'*Arabidopsis*? Premièrement, j'ai constaté que la composition des AG était différente entre les racines et les pousses. La composition en AG des racines était constante pendant le développement de la plante, alors que dans les pousses, elle a changé au cours de la première semaine de croissance. L'analyse de mutants avec de faibles niveaux d'acides gras polyinsaturés (AGPI) a révélé un phénotype végétal chlorotique avec un développement retardé. Les niveaux réduits d'AGPI dans les racines ont entraîné une accumulation excessive des espèces réactives de l'oxygène, ce qui corrèle avec la réduction de la croissance des racines et une forte susceptibilité aux contraintes abiotiques. Un autre résultat intéressant est que les plants dépourvus d'AGDI étaient aussi très résistants au pathogène nécrotrophique *Botrytis cinerea*. Ce travail a été une première tentative pour comprendre le rôle des AGDI dans l'adaptation à certains stress (a-) biotiques et leur rôle dans ces processus. Notre recherche ouvre beaucoup de questions concernant le développement des plantes et leur adaptation aux changements de l'environnement.

Résumé pour un large public

Acides gras di-insaturés (AGDI) et leur rôle dans l'établissement et la défense des plantules.

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Les plantes ont développé de multiples façons de s'adapter aux changements de l'environnement. Ce travail permet de mieux comprendre le rôle des acides gras di-insaturés (AGDI) et leur implication dans la résistance au stress. La première question de ce travail a été de comprendre le rôle des AGDI dans l 'adaptation au stress salin. Nous avons constaté que les plantes avec des niveaux fortement diminués d'acides gras poly-insaturés (AGPI) possèdent des racines réduites. Lorsque ces plantes sont transférées sur un milieu de croissance avec une forte concentration en sel, la longueur des racines est diminuée encore plus fortement. Cette constatation indique un rôle important des AGDI pour la croissance des plantes dans un environnement salé. La deuxième question abordée dans cette recherche était de comprendre le rôle des AGDI dans la résistance au pathogène nécrotrophique *Botrytis cinerea*. Ces résultats indiquent un rôle important des AGDI pour le développement des plantes et leur adaptation au stress et ouvrent de nouvelles questions et de nombreuses possibilités d'approfondir les recherches en lien avec cette problématique.

List of Abbreviations

2-thiobabituric acid	**TBA**
2,7-dichlorodihydrofluorescein diacetate	**H2DCFDA**
7-methylsulfinylheptyl glucosinolate	**7MSOH**
Abscisic acids	**ABA**
Allene oxide synthase mutant	***aos***
Arabidopsis Biological Resource Centre	**ABRC**
Botrytis cinerea enhanced GFP fluorescent B05 strain	***Bcgfp1***
Coenzyme A	**CoA**
Dicarboxylic acid	**DCA**
Diunsaturated fatty acids	**DUFAs**
Endoplasmic reticulum	**ER**
Ethyl methanesulfonate	**EMS**
Ethylene	**ET**
fad3-2 fad7-2 fad8	***fad trip***
Fatty acid desaturase	**FAD**
Fatty acid methyl ester	**FAME**
Fatty acids	**FA**
Gas chromatography - mass	**GCMS**

spectrometry	
Glycerol-3-phosphate acyltransferase double insertion mutant	***gpat4 gpat8***
Hydrogen peroxide	**H_2O_2**
Hydroxyl radical	**$^{\cdot}OH$**
Jasmonic acid	**JA**
Limit of quantification	**LOQ**
Malondialdehyde	**MDA**
Monogalactosyldiacylglycerol	**MGDG**
Non-enzymatic lipid oxidation	**nLPO**
Omega hydroxyl fatty acid	**ω-OH**
Pathogenesis-related protein 1	**PR1**
Phospholipase A	**PLA**
Polyunsaturated fatty acids	**PUFAs**
Potato dextrose broth	**PDB**
Principal component analysis	**PCA**
Rapid stress response elements	**RSREs**
Reactive electrophile species	**RES**
Reactive oxygen species	**ROS**
Respiratory burst oxidase homologs	**RBOH**
Salicylic acid	**SA**
Singlet oxygen	**$^1O_2$**

Superoxide	**O_2^-**
Systematic acquired resistance	**SAR**
Transmission electron microscopy	**TEM**
Triunsaturated fatty acids	**TUFAs**
Ultra-performance liquid chromatography quadrupole time of flight mass spectrometry	**UPLC–qTOF-MS/MS**
Very long chain fatty acids	**VLCFAs**
Wild type	**WT**

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Chapter I: General introduction

I.1. Polyunsaturated fatty acids in plants: structures and biosynthesis.

Fatty acids (FAs) are hydrocarbons containing a long aliphatic chain that can be either saturated (no double bonds) or unsaturated (one or more double bonds). Chain lengths of C16 (~ 30%) and C18 (~ 70%) are the most abundant in higher plants (Tjellstroem *et al.,* 2012). Depending on the level of saturation, FAs can be subdivided into the saturated (e.g. 16:0; 18:0), monounsaturated (e.g. 16:1; 18:1; MUFA), di- (e.g. 16:2; 18:2; DUFA) or tri-unsaturated (e.g. 16:3; 18:3; TUFA). Subgroups of 18:2 and 18:3 make up 55% of total fatty acids in *Arabidopsis* cell cultures (Tjellstroem *et al.,* 2012). In leaves 18:2 and 18:3 represent, 18.5% and 50% respectively of total FAs (Maatta *et al.,* 2012). In the roots the proportion of DUFAs and TUFAs is different from the shoots, 18:2 make up 37% of total FAs, whereas 18:3 only 28% (Beaudoin *et al*., 2009). Leaf and root fatty acids are, therefore, primarily polyunsaturated.

Most of the fatty acids found in cells are esterified to glycerol as glycerolipids. Their derivatives, glycolipids and phospholipids, form essential structural features of membrane bilayers. In many seeds, including *Arabidopsis thaliana*, triacylglycerols are the major energy source (Fig.1.1).

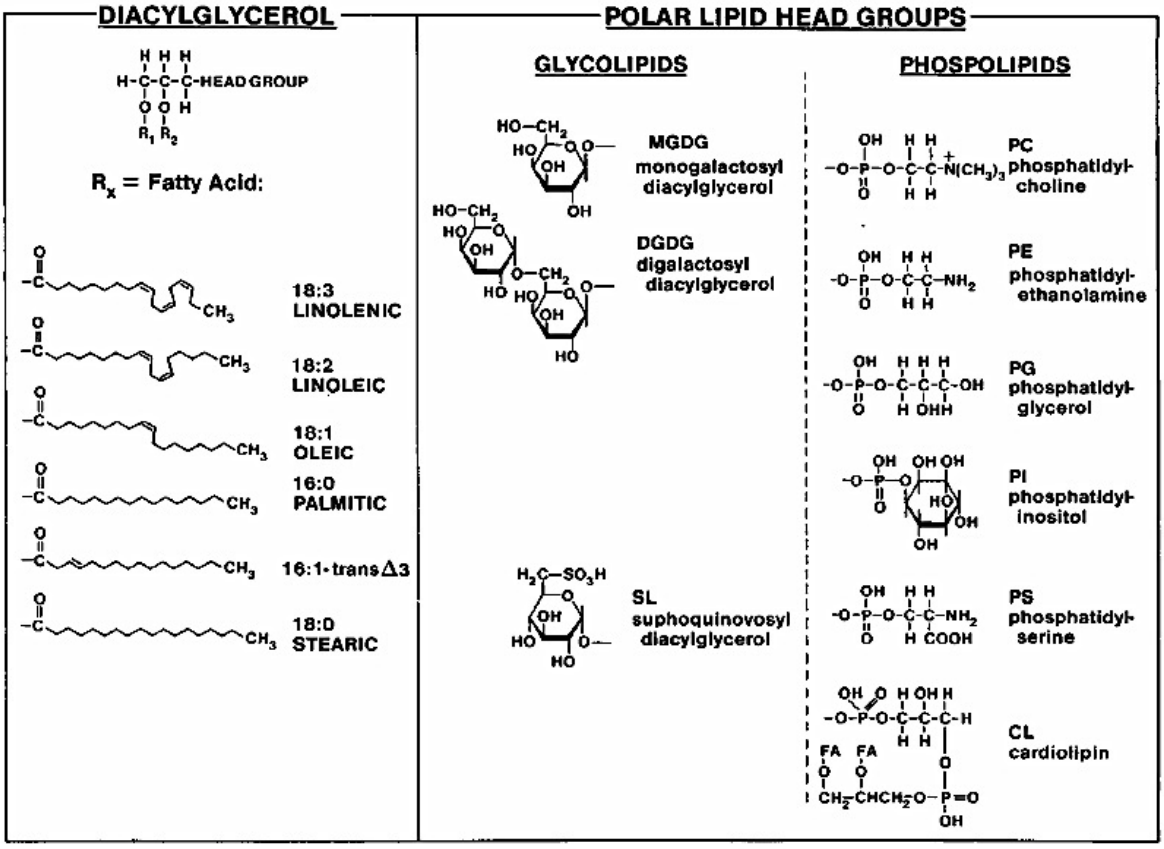


Figure 1.1. Structures of the major fatty acids and glycerolipids of plant cell membranes. From Ohlrogge and Browse (1995).

Fatty acid biosynthesis in plants is different from that in animals, fungi and some bacteria. More than 30 enzymatic reactions are required to synthetize C16 or C18 FAs from acetyl-coenzyme A (CoA) and malonyl-CoA in plants (Ohlrogge and Browse, 1995). Whereas in the other kingdoms this process requires only one multifunctional polypeptide complex located in the cytosol (Ohlrogge and Browse, 1995; Laliotis *et al.,* 2010). Synthesis of the major glycerolipids in plants is carried out on the membranes of plastids and the endoplasmic reticulum (ER), called in short the "prokaryotic" and "eukaryotic" pathways (Browse and Somerville, 1991; Heinz, 1993). 16:0 and 18:1 acyl groups are the essential substrates for polyunsaturated fatty acid synthesis in

both pathways. Fatty acid desaturase (FAD) enzymes are necessary for insertion of double bonds in newly synthetized unsaturated fatty acids (Fig.1.2).

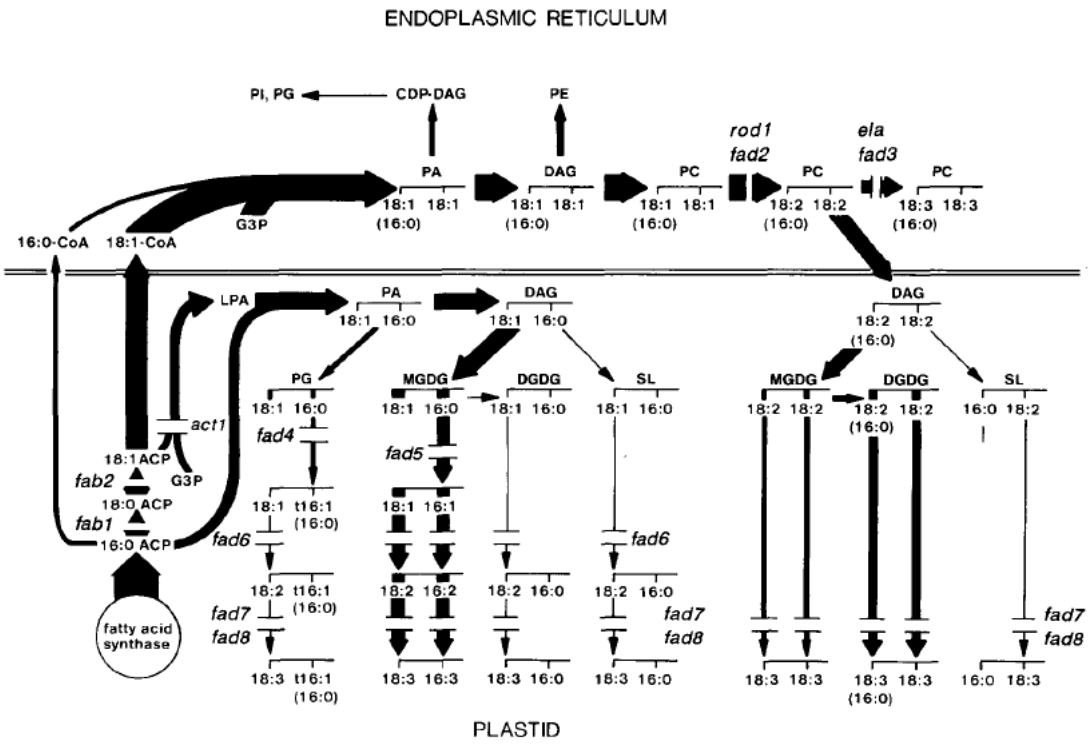


Figure 1.2. Fatty acid synthesis and glycerolipid assembly in *Arabidopsis* leaves. From Ohlrogge and Browse (1995).

As shown in Figure 1.2, desaturation of 18:1 to 18:2 and 18:3 takes places in both pathways, but it requires different FAD enzymes. In the ER 18:1 desaturation takes place through the activity of FAD2 enzyme and 18:2 is converted to 18:3 by FAD3 (Miquel and Browse, 1992; Browse *et al.,* 1993). In plastids, the synthesis of both 16:2 and 18:2 FAs is dependent on FAD6 enzyme (Browse *et al.,* 1989) and 16:3/18:3 are produced by further desaturation catalysed by FAD7 and FAD8 (Browse *et al.,* 1986; Gibson *et al.,*

1994; McConn *et al.,* 1994).

Fatty acid synthesis is tightly regulated by the light/dark cycle in the photosynthetic organs of plants; malonyl-CoA is synthetized within seconds after light exposure (Sauer and Heise, 1983). This process is triggered by pH, Mg^{2+} and redox cascade changes within the chloroplasts (Harwood, 1983; Sauer and Heiske, 1983; Eastwell and Stumpf, 1983; Sasaki *et al.,* 1997). Other external factors that can control fatty acid biosynthesis will be described in detail within the following subchapters.

I.2. Roles of PUFAs in plant development and physiology.

Fatty acids biosynthesis is a primary metabolic pathway essential for plant growth and development. *fad2-2 fad6 Arabidopsis* mutants that lack both DUFAs and TUFAs were shown to be lethal on soil, due to the important role of these FAs in the assembly and maintenance of the photosynthetic complexes (McConn and Browse, 1998). Surprisingly, plants lacking only TUFAs (*fad3-2 fad7-2 fad8*) were shown not to have a strong impact on plant growth, but on male sterility caused by deficiency in jasmonic acid (JA; McConn and Browse, 1996). Based on recent studies, the composition of galactolipids is tissue specific: the major pools of PUFAs in leaves are synthetized by FAD7, FAD8 and FAD6 enzymes (Li-Beisson *et al.,* 2013), whereas in meristematic zones,

FAD2 and FAD3 play the key role (Mei *et al.,* 2015). In fast-growing tissues (calli) and highly dividing cells (cell suspension cultures), levels of phospholipids are significantly lower than in slow-growing cells. One of the current explanations for this phenomenon is associated with low activity of FAD2 and FAD3 enzymes in these tissues that leads to over-accumulation of 18:1 in membranes (Mei *et al.,* 2015).

It has been long known that PUFAs have an important physiological role in plant adaptation to temperature changes (Pearcy, 1978; Raison *et al.,* 1982; Iba, 2002). Under cold conditions, PUFA biosynthesis increases, whereas under hot conditions it decreases. For example, the average number of double bonds per glycerolipid (diacyl lipids) in the leaves of *Arabidopsis* drops from 4.8 at 17 ^{0}C to 2.9 at 36 ^{0}C. Mutants deficient in TUFAs (*fad3-2 fad7-2 fad8*) are strongly affected in long-term exposure to 4 ^{0}C due to inability to maintain high chlorophyll levels (Fig.1.3.A). Even if TUFAs are not essential for adaptation to high temperatures, low levels of these FAs need to be maintained to promote plant survival (Fig1.3. B).

Figure 1.3. Triunsaturated fatty acids and their role in adaptation to temperature changes. (**A**) Wild type (**left**) and the *fad3-2 fad7-2 fad8* mutant (**right**) were grown for 15 d at 22 ^{0}C and transferred to 4 ^{0}C for 30 days. From Routaboul *et al*., 2000. (**B**) WT and *fad3-2 fad7-2 fad8* mutant were transferred from 22 ^{0}C to 33 ^{0}C for 15 days. From Routaboul *et al.* (2012).

Roles for DUFAs in plant physiology have been identified with the help of *fad6* and *fad2* mutants. No phenotypic differences were observed in these mutants compared to the WT at 22 ^{0}C, 4 weeks post-germination (Miquel *et al*., 1993; Maeda *et al.,* 2008). However, dramatic chilling sensitivity was detected in both mutants leading to leaf chlorosis, reduced growth rate and changes in chloroplast morphology (Hugly *et al*., 1992; Miquel *et al*., 1993). In recent years, namely an additional role of DUFAs has been identified, adaptation to salinity stress. *fad2* and *fad6* mutant plants growing on 200 to 250

mM NaCl media were shown to be more sensitive to this treatment compared to WT seedlings (shorter roots and chlorotic cotyledons) due to an altered ion homeostasis in *fad6* mutants (Zhang *et al.,* 2009) and dysfunction of membrane-embedded Na^+ / H^+ exchangers (Zhang *et al.,* 2012). These results suggest an essential role of PUFAs in plant development and physiology. Another more recently discovered role of PUFAs is in buffering of reactive oxygen species (Mene-Saffrane *et al*., 2009; Schmid-Siegert *et al.,* 2016) that are the main mediators of non-enzymatic lipid oxidation (nLPO) (Weber *et al.,* 2004). It was shown that a major by-product of lipid oxidation, malondialdehyde (MDA), is generated mainly from TUFAs (60-75%) in leaves and can promote gene expression in response to stresses (Weber *et al.,* 2004; Schmid-Siegert *et al.,* 2012). When the stress levels in plants go down, MDA is incorporated back into the chloroplast galactolipid 18:3-16:3-monogalactosyldiacylglycerol (MGDG), which is thought to act as a cell protectant (Schmid-Siegert *et al.,* 2016). The other possible sources of MDA in plants can be mono- and di-unsaturated fatty acids (Lui *et al.,* 1997), although this needs to be tested. This thesis will further investigate the role of DUFAs in plants defences.

I.3. Roles of PUFAs in defences.

PUFAs play important direct and indirect roles in defence. When plants are exposed to biotic stresses, PUFAs present in the membrane bilayer are oxidized leading to the formation of oxylipins and various reactive electrophile species (RES; Chehab *et al.,* 2008). Oxylipins are oxygenated fatty acids that contain one or more oxygen atoms in addition to one in the carboxylic acid group. Oxygenation of 18:3 and 16:3 FAs generates oxylipins known as jasmonates (Wasternack and Kombrink, 2010; McDowell and Dangl, 2000). These molecules play important roles in plant defence against herbivores and necrotrophic pathogens (Walling, 2000; Kachroo and Kachroo, 2009). Plants subjected to herbivore attack were shown to produce more trichomes on the new emerging leaves and smaller petioles as response to this stress, features that are controlled by jasmonic acid pathway (Yan and Chen 2007; Zhang and Turner, 2008; Yoshida *et al.,* 2009). Three cellular compartments (chloroplasts, peroxisomes and the cytosol) and multiple biosynthetic enzymes are required for JA biosynthesis (Fig.1.4).

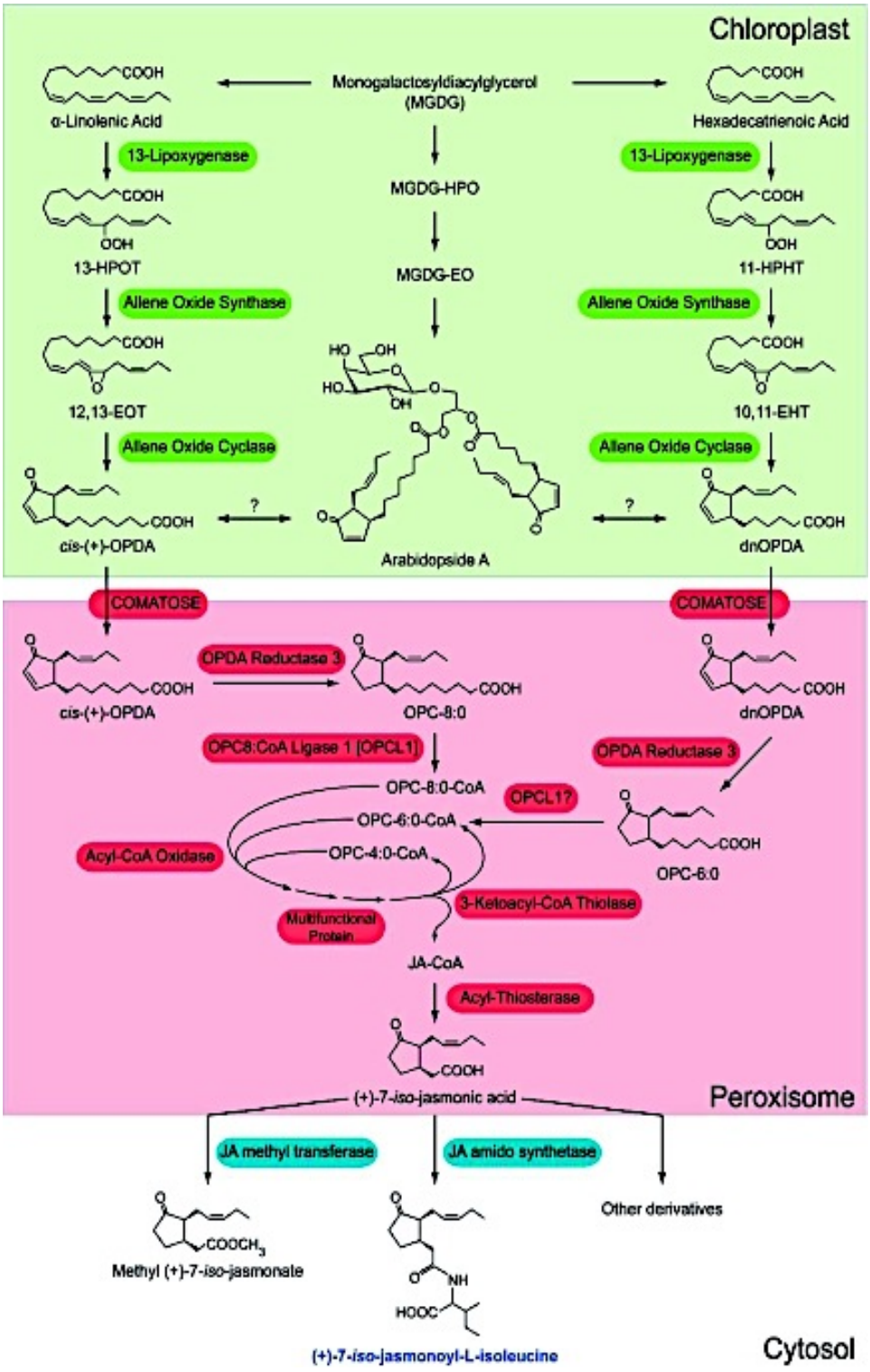


Figure 1.4. Jasmonic acid biosynthesis and metabolism in *Arabidopsis thaliana*. From Acosta and Farmer (2010).

Another compound synthtetized from PUFAs during nLPO is azelaic acid (one of the reactive electrophile species (RES)). This reactive electrofile species

(RES) was shown to induce systemic acquired resistance in *Arabidopsis* plants upon stress conditions (Jung *et al.,* 2009).

In the last years, a new role of PUFAs was identified, namely mediation of transcriptional responses. Phospholipase A (PLA) was shown to activate promoters containing rapid stress response elements (RSREs) *via* cleavage of 18:2 and 18:3 FAs from plant membranes upon pathogen attack. RSREs induce activation of defence genes upon multiple abiotic and biotic stresses providing plant protection on the early stages (Savchenko *et al.,* 2010). Another by-product of lipid oxidation involved in defence is phytoprostane (an oxylipin) that was shown to activate TGA motifs. TGA2, TGA5 and TGA6 factors induce expression of defence genes upon treatment with cyclopentenone oxylipins (Mueller *et al.,* 2008).

I.4. PUFAs and fungal pathogens including *B. cinerea.*

The cuticle is an extracellular hydrophobic layer that covers aerial parts of the plant epidermis and works as a barrier between internal parts of the plant and the environment (Yeats and Rose, 2013). It consists of highly water-repellent thin wax layer (procuticle) in young leaves that develops to cutin and cuticular wax in mature leaves (Jeffree, 1996). C16 and C18 diacids, ω - and mid-chain hydroxy FAs are the major structural components of cutin in *Arabidopsis*

leaves (Molina *et al.,* 2006). Surprisingly, the main cutin monomer in leaf and stem cuticle is 18:2 dicarboxylic acid (Bonaventure *et al.,* 2004; Franke *et al.,* 2005). Very long chain fatty acids (VLCFAs) obtained by elongation of plastidial FAs are the major source for wax (Fig. 1.5; Jenks *et al.,* 1995; Samuels *et al.,* 2008).

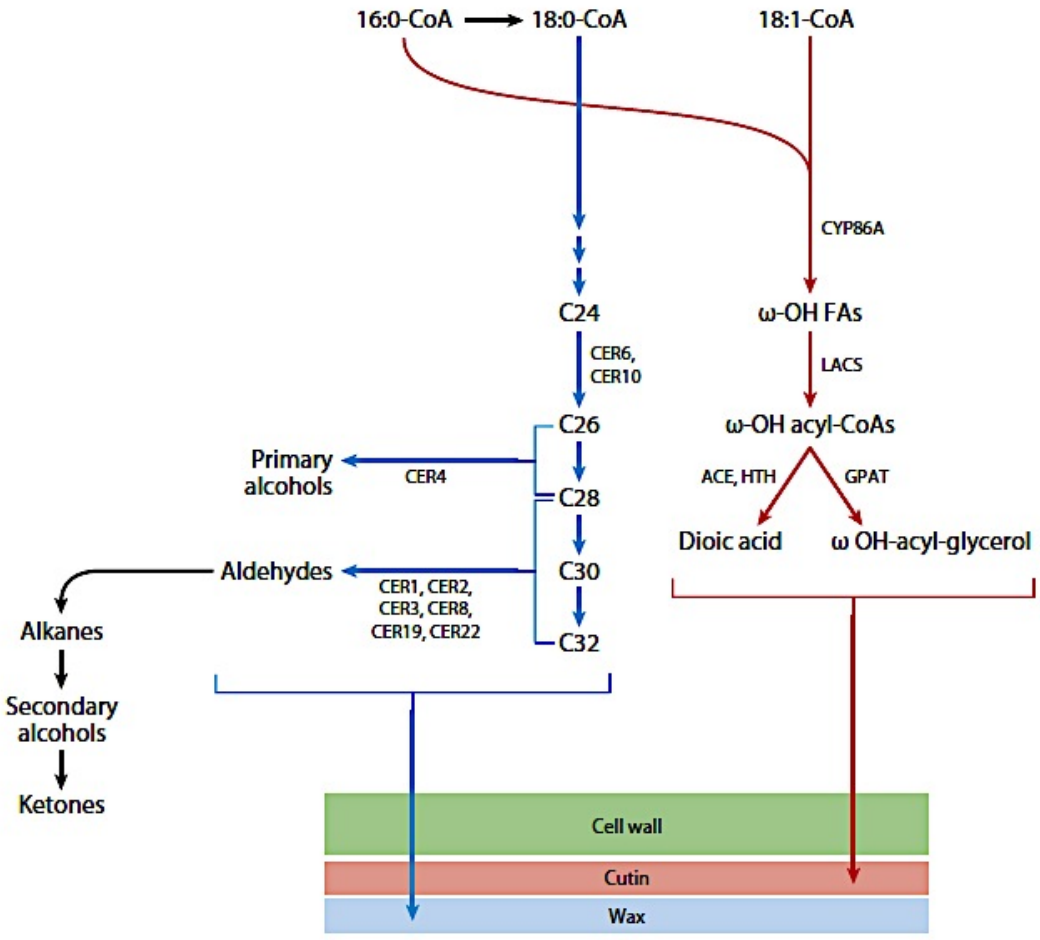


Figure 1.5. Cuticle biosynthesis in *Arabidopsis thaliana* plants. From Kachroo and Kachroo (2009).

The cuticle is an important barrier that interacts with the surrounding environment. It provides the first layer of defence against pathogens and herbivores in addition to prevention of water, gas and solute loss (Eigenbroade

et al., 1995; Kolattukudy, 1996; Koteyeva, 2005). For cuticle-degrading pathogens, such as *Fusarium solani pisi, Botrytis cinerea*, *Magnaporthe oryzae* etc. cuticle and waxes play an important role in initiating pathogen development on the plant surface (Lin and Kolattukudy, 1978; Chassot *et al.,* 2008; Gilbert *et al.,* 1996). Changes in cuticle composition can affect fungal development and prevent penetration into the plant (Chassot *et al.,* 2007). Additionally, FA levels can change during pathogenesis. Increased levels of 18:3 and reduced levels of 18:2 were seen 12 hours after *Phytophthora sojae* application to parsley cells. Surprisingly, no significant changes were detected in C16 fatty acids (Kirsch *et al.,* 1997). Changes in the levels of linolenic acid (18:3) were also observed in other species, such as *Phaseolus vulgaris*, *Poa pratensis* and *Eschscholtza californica* upon attack by diverse rust species (*Puccinia spp.*; Hope and Heitefuss, 1974; Losel, 1978; Muller *et al,* 1993).

Jasmonate plays an important role in resistance to necrotrophic fungi; plants lacking TUFAs were shown to have strong susceptibility to these pathogens (Kachroo *et al.,* 2001; Yaeno *et al.,* 2004). The role of DUFAs in pathogenesis has been described only in a few studies. It was shown that 18:2 can be used as a precursor for antifungal dienes such as Z,Z-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene that is induced in avocado fruits upon *Collectotrichum gloeosporioides* infection (Madi *et al.,* 2003). Another role of linoleic acid (18:2) is to promote morphological differentiation in *Aspergillus nidulans*, *A.*

flavus and *A. parasiticus* (Calvo *et al.,* 1999), as well as sporogenesis in *Aspergillus tomato*, *Sclerotinia fructicola* and *Neurospora crassa* (Katayama and Marumo, 1978; Nukima *et al.,* 1981). In summary, PUFAs have diverse roles in pathogenesis. Additional discoveries in this area are likely.

I.5 Main goals of this thesis

The purpose of this research was to examine the role of DUFAs in defence and development at the seedling stage, using *Arabidopsis fad2* and *fad6* mutants. Early in this thesis, I characterized *fad* mutants at different developmental stages from 3-day-old seedlings to 3-week-old rosettes. This was followed by analysis of PUFAs on development in *fad2* mutants and its involvement in the adaptation to salt stress. In the last chapter of this thesis, I try to understand the role of DUFAs in resistance to the necrotrophic pathogen *Botrytis cinerea.* Application of this pathogen on wild type and *fad* seedlings showed resistance of *fad* mutants compared to WT plants. The next and the biggest goal of this work was to understand what leads to *B. cinerea* resistance. For this purpose multiple experimental approaches were used, such as atomic force microscopy, transmission electron microscopy, biochemical and molecular biological approaches.

II.6. Literature.

Acosta IF and Farmer EE. 2010. Jasmonates. *Arabidopsis book*. 8:e0129.

Beaudoin F, Wu X, Li F, Haslam RP, Markham JE, Zheng H, Napier JA, Kunst L. 2009. Functional characterization of the *Arabidopsis* beta-ketoacyl-coenzyme a reductase candidates of the fatty acid elongase. *Plant Physiol*. 150(3):1174-1191.

Bonaventure G, Beisson F, Ohlrogge J, Pollard M. 2004. Analysis of the aliphatic monomer composition of polyesters associated with *Arabidopsis* epidermis: occurrence of octadeca-cis-6,cis-9-diene-1,18-dioate as the major component. *Plant J.* 40:920–930.

Browse J and Somervllle C. 1991. Glycerolipid synthesis: Biochemistry and regulation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*42:467-506.

Browse J, Kunst L, Anderson S, Hugly S, Samerville C. 1989. A mutant of *Arabidopsis* deficient in the chloroplast 16:1/18:1 desaturase. *Plant Physiol*. 90(2):522-529.

Browse J, McConn M, James D and Mlquel M. 1993. Mutants of *Arabidopsis* deficient in the synthesis of a-linolenate. Biochemical and genetic characterization of the endoplasmic reticulum linoleoyl desaturase. *J. Biol. Chem.* 268:16345-16351.

Browse J, McCourt P, Samorville C. 1986. A mutant of *Arabidopsis* deficient in C(18:3) and C(16:3) leaf lipids. *Plant Physiol*. 81(3):859-864.

C, Schreiber L (2005) Apoplastic polyesters in *Arabidopsis* surface tissues: a typical suberin and a particular cutin. *Phytochemistry.* 66:2643–2658.

Calvo AM, Hinze LL, Gardner HW, Keller NP. 1999. Sporogenic effect of polyunsaturated fatty acids on development of *Aspergillus spp*. *Appl. Environ. Microbiol*. 65:3668–73.

Chassot C, Buchala A, Schoonbeek HJ, Metraux, JP, Lamotte O. 2008. Wounding of *Arabidopsis* leaves causes a powerful but transient protection against *B. cinerea* infection. *Plant J.* 55:555–567.

Chassot C, Nawrath C, Métraux JP. 2007. Cuticular defects lead to full immunity to a major plant pathogen. *Plant J*. 49(6):972-980.

Chehab EW, Kaspi R, Savchenko T, Rowe H, Negre-Zakharov F, Kliebenstein D, Dehesh K. 2008. Distinct roles of jasmonates and aldehydes in plant-defense responses. *PLoS ONE*. 3:e1904.

Eastwell KC and Stumpf PK. 1983. Regulation of plant acytil-CoA carboxylase by adenylate nucleotides. *Plant Physiol*. 72:50-55.

Eigenbrode SD and Espelie KE. 1995. Effects of plant epicuticlar lipids on insect herbivores. *Ann. Rev. Entoml.* 40:171-194.

Franke R, Briesen I, Wojciechowski T, Faust A, Yephremov A, Nawrath C, Gibson S, Arondel V, Iba K and Somerville C. 1994. Cloning of a temperature-regulated gene encoding a chloroplast ω-3 desaturase from *Arabidopsis thaliana*. *Plant Physiol.* 106:1615-1621.

Gilbert RD, Johnson AM, Dean RA. 1996. Chemical signals responsible for appressorium formation in the rice blast fungus *Magnaporthe grisea. Physiol Mol Plant Pathol.* 48:335–346.

Harwood, J.L. 1980. Plant acyl lipids: Structure, distribution and analysis. *In The Biochemistry of Plants.* 4:2-56.

Helnz E. 1993. Biosynthesis of polyunsaturated fatty acids. *In Lipid Metabolism in Plants*. pp. 33-89.

Hoppe HH, and Heitefuss R. 1974. Permeability and membrane lipid metabolism of *Phaseolus vulgaris* infected with *Uromyces phaseoli*. Changes in efflux of cell constitution. *Physiol. Plant Pathol.*4:11–24.

Hugly S, Somerville C. 1992. A role for membrane lipid polyunsaturation in chloroplast biogenesis at low temperature. *Plant Physiology.* 99:197–202.

Iba K. 2002. Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. *Ann. Rev. Plant Biol.* 53:225–245.

Jeffree CE. 1996. Structure and ontogeny of plant cuticles. *BIOS scientific publishers,* Oxford. pp. 33-82.

Jenks MA, Tuttle HA, Eigenbrode SD, Feldmann KA. 1995. Leaf epicuticular waxes of the eceriferum mutants in *Arabidopsis*. *Plant Physiol*. 108:369–77.

Jung HW, Tschaplinski TJ, Wang L, Glazebrook J, Greenberg JT. 2009. Priming in systemic plant immunity. *Science.* 324:89–91.

Kachroo A and Kachroo P. 2009. Fatty acid-derived signals in plant defense. *Annu. Rev. Phytopayhol*. 47:153-176.

Kachroo P, Shanklin J, Shah J, Whittle EJ, Klessig DF. 2001. A fatty acid desaturase modulates the activation of defense signalling pathways in plants. *Proc Natl Acad Sci USA*. 98(16):9448-53.

Katayama M and Marumo S. 1978. Isolation of sclerosporin, a sporogenic substance, from *Sclerotinia fructicola*, *Agr. and Biol. Chem*. 42:505-506.

Kirsch C, Takamiya-Wik M, Reinold S, Hahlbrock K and Somssich IE. 1997. Rapid, transient, and highly localized induction of plastidial x-3 fatty acid desaturase mRNA at fungal infection sites in *Petroselinum crispum*. *Proc. Natl Acad. Sci. USA*. 94:2079–2084.

Koteeva NK. 2005. A novel structural type of plant cuticle. *Doki. Biol. Sci.* 403:272-274.

Laliotis Gp, Bizelis I, Rogdakis E. 2010. Comparitive Approach of the de novo fatty acid synthesis (lipogenesis) between Ruminant and non ruminant Mammalian species: from Biochemical level of the main regulatory lipogenic genes. *Curr. Genomics*. 11(3):168-183.

Li-Beisson Y, Shorrosh B, Beisson F, Andersson MX, Arondel V, Bates PD, Baud S, Bird D, Debono A, Durrett TP, Franke RB, Graham IA, Katayama K, Kelly AA, Larson T, Markham JE, Miquel M, Molina I, Nishida I, Rowland O, Samuels L, Schmid KM, Wada H, Welti R, Xu C, Zallot R, Ohlrogge J. 2013. Acyl-lipid metabolism. *Arabidopsis book*. 11:e0161.

Lin TS and Kolattukudy PE. 1978. Induction of a biopolyester hydrolase (cutinase) by low levels of cutin monomers in *Fusarium solani f.sp. pisi*. *J bacterial.* 133(2):942-951.

Liu J, Yeo HC, Doniger SJ, Ames BN. 1997. Assay of aldehydes from lipid peroxidation: gas chromatography-mass spectrometry compared to thiobarbituric acid. *Anal. Biochem.* 245:161–166.

Losel DM. 1978. Lipid metabolism of leaves of *Poa pratensis* during infection by *Puccinia poarum*. *New Phytol.* 80:167–174.

Maatta S, Scheu B, Roth MR, Tamura P, Li M, Williams TD, Wang X, Welti R. 2012. Levels of Arabidopsis thaliana Leaf Phosphatidic Acids, Phosphatidylserines, and Most Trienoate-Containing Polar Lipid Molecular Species Increase during the Dark Period of the Diurnal Cycle. *Front. Plant Sci.* 14:3-49.

Madi L, Wang X, Kobiler I, Lichter A, Prusky D. 2003. Stress on avocado fruits regulates 9-stearoyl ACP desaturase expression, fatty acid composition, antifungal diene level and resistance to *Colletotrichum gleosporiodes* attack. *Physiol. Mol. Plant Pathol*. 62:277–83.

Maeda H, Sage LT, Isaac G, Welti R, DellaPenna D. 2008. Tocopherols modulate extraplastidic polyunsaturated fatty acid metabolism in *Arabidopsis* at low temperature. *Plant Cell.* 20(2):452-470.

McConn M and Browse J. 1996. The critical requirement for linolenic acid is pollen development, not photosynthesis, in *Arabidopsis* mutant. *Plant Cell*.

8(3):403-416.

McConn M and Browse J. 1998. Polyunsaturated membranes are required for photosynthetic competence in a mutant of *Arabidopsis*. *Plant J.* 15(4):521-530.

McConn M, Hugly S, Somerville C and Browse J. 1994. A mutation at the *fad8* locus of *Arabidopsis* identifies a second chloroplast ω-3 desaturase. *Plant Physiol.* 106:1609-1614.

McDowell JM and Dangl JL. 2000. Signal transduction in the plant immune response. *Trends Biochem Sci*. 25(2):79-82.

Meï C, Michaud M, Cussac M, Albrieux C, Gros V, Maréchal E, Block MA, Jouhet J, Rébeillé F. 2015. Levels of polyunsaturated fatty acids correlate with growth rate in plant cell cultures. *Sci. Rep.* 5:15207.

Mène-Saffrané L, Dubugnon L, Chételat A, Stolz S, Gouhier-Darimont C, Farmer EE. 2009. Nonenzymatic oxidation of trienoic fatty acids contributes to reactive oxygen species management in *Arabidopsis*. *J. Biol. Chem*. 284(3):1702-1708.

Miquel M and Browse J. 1992. *Arabidopsis* mutants deficient in polyunsaturated fatty acid synthesis. Biochemical and genetic characterization of a plant oleoyl-phosphatidylcholine desaturase. *J Biol Chem.* 267:1502-1509.

Miquel M, James D Jr, Dooner H, Browse J. 1993. *Arabidopsis* requires polyunsaturated lipids for low-temperature survival. *Proc. Natl. Acad. Sci. USA*. 90(13):6208-6212.

Molina I, Bonaventure G, Ohlrogge J, Pollard M. 2006. The lipid polyester composition of *Arabidopsis thaliana* and *Brassica napus* seeds. *Phytochemistry*. 67:2597–610.

Mueller MJ, Brodschelm W, Spannagl E and Zenk MH. 1993. Signaling in the elicitation process is mediated through the octadecanoid pathway leading to jasmonic acid. *Proc. Natl. Acad. Sci. USA*. 90:7490–7494.

Mueller S, Hilbert B, Dueckershoff K, Roitsch T, Krischke M, Mueller MJ, Berger S. 2008. General detoxification and stress responses are mediated by oxidized lipids through TGA transcription factors in Arabidopsis. *Plant Cell.* 20:768-785.

Nikuma MT, Sassa T, Ikeda M and Takahashi K. 1981. Linoleic acid enhances perithecial production in *Neurospora crassa*. *Agric. Biol.Chem*. 45:2371-2373.

Ohlrogge J and Browse J. 1995. Lipid biosynthesis. *Plant Cell*. 7:957–970.

Pearcy R. 1978. Effect of growth temperature on the fatty acid composition of the leaf lipids *in Atriplex lentiformis (Torr.) Wats*. *Plant Physiol*. 61:484–486.

Raison JK, Roberts JKM, Berry JA. 1982. Correlations between the thermal-stability of chloroplast (thylakoid) membranes and the composition and fluidity of their polar lipids upon acclimation of the higher plant, *Nerium oleander*, to growth temperature. *Biochimica et Biophysica Acta.* 688:218–228.

Routaboul JM, Fischer SF, Browse J. 2000. Trienoic fatty acids are required to maintain chloroplast function at low temperatures. *Plant Physiol.* 124:1697–1705.

Routaboul JM, Skidmore C, Wallis JG, Browse J. 2012. *Arabidopsis* mutants reveal that short- and long-term thermotolerance have different requirements for trienoic fatty acids. *J. Exp. Bot*. 63(3):1435-1443.

Samuels L, Kunst L, Jetter R. 2008. Sealing plant surfaces: cuticular wax formation by epidermal cells. *Annu. Rev. Plant Biol.* 59:683–707.

Sasaki Y, Kozaki A, Hatano M. 1997. Link between light and fatty acid synthesis: thioredoxin-linked reductive activation of plastidic acetyl-CoA carboxylase. *Proc Natl Acad Sci USA*. 94(20):11096-11101.

Sauer A and Heise KP. 1983. On the light dependence of fatty acid synthesis in spinach chloroplasts. *Plant Physiol.* 73(1):11-15.

Savchenko T, Walley JW, Chehab EW, Xiao Y, Kaspi R, Pye MF, Mohamed ME, Lazarus CM, Bostock RM, Dehesh K. 2010. Arachidonic acid: An evolutionarily conserved signaling molecule modulates plant stress signaling networks. *Plant Cell*. 22: 3193–3205.

Schmid-Siegert E, Loscos J. 2012. Inducible malondialdehyde pools in zones of cell proliferation and developing tissues in *Arabidopsis*. *J. Biol. Chem.* 287:8954-8962.

Schmid-Siegert E, Stepushchenko O, Glauser G, Farmer E.E. 2016. Membranes as structural antioxidants: Recycling of malondialdehyde to its source in oxidation-sensitive chloroplast fatty acids. *J. Biol. Chem.* 291:13005-13013.

Tjellstroem H, Yang Z, Allen DK and Ohlrogge JB. 2012. Rapid kinetic

labeling of *Arabidopsis* cell suspension cultures: implications for models of lipid export from plastids. *Plant Physiology.* 158:01–611.

Walling LL. 2000. The myriad plant responses to herbivores. *J Plant Growth Regul*. 19(2):195-216.

Wasternack C and Kombrink E. 2010. Jasmonates: structural requirements for lipid-derived signals active in plant stress responses and development. *ACS Chem Biol*. 5(1):63-77.

Weber H, Chételat A, Reymond P, Farmer EE. 2004. Selective and powerful stress gene expression in *Arabidopsis* in response to malondialdehyde. *Plant J.* 37: 877–888.

Yaeno T, Matsuda O, Iba K. 2004. Role of chloroplast trienoic fatty acids in plant disease defence responses. *Plant J*. 40:931-941.

Yan X and Chen S. 2007. Regulation of plant glucosinolate metabolism. *Planta*. 226:1343-1352.

Yeats TH and Rose JKC. 2013. The formation and function of plant cuticles. *Plant Physiol*. 163:5–20.

Yoshida Y, Sano R, Wada T, Takabayashi J, Okada K. 2009. Jasmonic acid control of *GLABRA3* links inducible defense and trichome patterning in *Arabidopsis*. *Development.* 136(6):1039-1048.

Zhang J, Liu H, Sun J, Li B, Zhu Q, Chen S, Zhang H. 2012. *Arabidopsis* fatty acid desaturase *FAD2* is require for salt tolerance during seed germination and early seedling growth. *PLoS One.* 7:e30355.

Zhang JT, Zhu JQ, Zhu Q, Liu H, Gao XS, Zhang HX. 2009. Fatty acid desaturase-6 (*FAD6*) is required for salt tolerance in *Arabidopsis thailana. Biochem. Biophys. Res. Commun.* 390:469-474.

Zhang Y and Turner JG. 2008. Wound-induced endogenous jasmonates stunt plant growth by inhibiting mitosis. *PLoS One*. 3(11):e3699.

Chapter II: Characterization of *Arabidopsis* PUFA-deficient *fad* mutants

II.1. General characterization of *fad* mutants.

Fatty acids are one of the major storage forms of carbon in living organisms, precursors for some plant hormones such as jasmonate, and major components of membrane bilayers. Polyunsaturated fatty acids are highly abundant in plants: diunsaturated and triunsaturated fatty acids account for more than 70% of fatty acids in leaf cells and 50 to 70% in roots (Harwood, 1996). Fatty acid biosynthesis starts in the plastids with synthesis of 16:0 and 18:1 acyl groups. Higher unsaturated forms are made by fatty acid desaturases localized on the endoplasmic reticulum or in plastids (Browse *et al.,* 1991; Heinz, 1993, Lou *et al.,* 2014). Synthesis of linoleic acid (18:2) from oleic acid (18:1) requires two enzymes: FAD2 localized on the ER, and FAD6 in plastids (Miquel *et al.,* 1993; Browse *et al.,* 1989). At the seedling stage, *fad2* and *fad6* single mutants have a similar phenotype as wild type (WT).

The conversion of linoleic acid (18:2) to linolenic acid (18:3) requires 3 enzymes: FAD3 localized on the ER, FAD7 and FAD8 in plastids. The *fad3-2 fad7-2 fad8* (*fad trip*) mutants have lighter leaf coloration and cannot produce seeds. These plants are able to grow in low-stress conditions but cannot be maintained at low temperatures (Routaboul *et al.,* 2000). Remarkably, TUFAs do not play a crucial role in photosynthesis (McConn *et al.,* 1996). Plant that is almost fully deficient in both di- and triunsaturated FA (*fad2-2 fad6*) cannot

grow on soil, but can be propagated on 2% sucrose media (McConn *et al.,* 1998). The *fad2-2 fad6* double mutants have a chlorotic and dwarfed phenotype, but are able to produce fertile seeds under these conditions. The aim of this chapter is to understand the role of DUFAs at different stages of plant development.

To obtain a mutant capable of growth on soil we investigated three mutant alleles of *FAD2* and one allele of the *FAD6* gene. The *fad2-1* mutant was produced by ethyl methanesulfonate (EMS) mutagenesis and has a point mutation in the second exon, leading to an amino acid change from alanine to threonine (A->T). This is one amino acid before the first conserved His-Box (Zhang *et al.,* 2012) that is involved in iron coordination complex at the active site together with two other His-Boxes (Shanklin *et al*, 1994). The *fad2-2* allele (also obtained by EMS mutagenesis (James *et al.,* 1990)) has a point mutation in the second exon, leading to an amino acid change from serine to phenyalanine (S->F), five amino acids before the second conserved region. This mutation was identified in this work. The *fad2-3* mutant has a T-DNA insertion in the second exon, obtained from *Arabidopsis* Biological Resource Centre (SK18137). The *fad6* mutant has a missense mutation in the fourth exon, leading to an amino acid change from glycine to alanine (G->A) in a membrane trans region (Zhang *et al.,* 2009). All the mutations were confirmed by DNA sequencing and checked for homozygosity by PCR.

RT-PCR revealed similar to wild type levels of *FAD2* and *FAD6* gene expression in *fad2-1*, *fad2-2* and *fad6* mutants, whereas no expression of *FAD2* was detected of the *fad2-3* allele, consistent with T-DNA insertion. The *UBC21* (At5g25760) transcript was used as a control (Fig. 2.1). Presence of three different *FAD2* alleles allowed us to exclude the *fad2-2* mutant from further work, due to its intermediate phenotype revealed in crosses of *fad2-1 fad6*; *fad2-2 fad6* and *fad2-3 fad6* double mutants and focus on *fad2-1* and *fad2-3* alleles of *FAD2* gene.

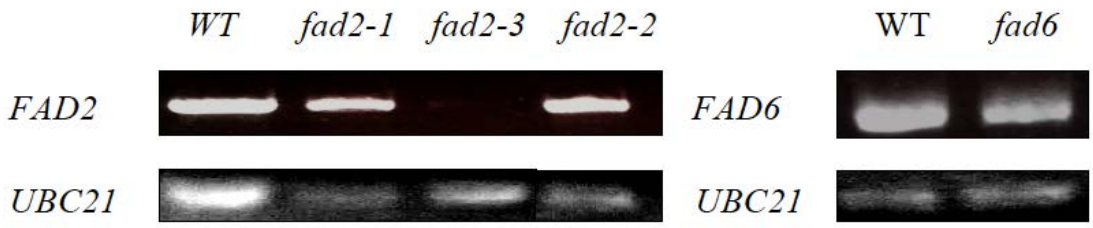


Figure 2.1. *FAD2* and *FAD6* transcript expression in 3-day-old seedlings. *UBC21* was used as a loading control.

PUFAs are one of the major components of membrane bilayers; deficiency in them might therefore strongly affect cell viability during plant development. To see if reduced levels of DUFAs affect plant morphology we compared 4-week-old mutant rosettes under two different light conditions. This revealed phenotypic difference between WT and *fad2* mutants (Fig.2.2).

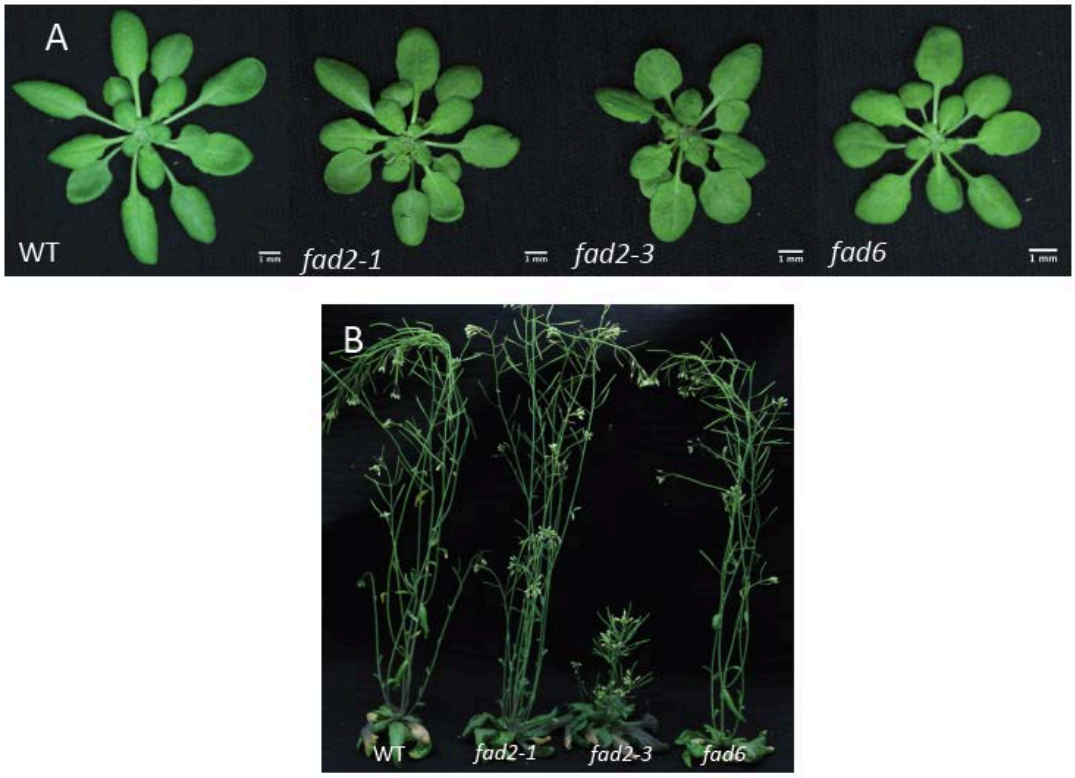


Figure 2.2. Developmental phenotype of diunsaturated fatty acid mutants. (**A**) 4-week-old rosettes grown in 9 h light / 15 h dark photoperiod. Scale bar 1 mm. (**B**) 6-week-old flowering plants growing in continuous light.

Figure 2.2, A shows that *fad2* mutants grown under short day conditions were found to have shorter petioles than WT with darker and rounded leaves. *fad6* mutants more resemble the WT phenotype with an exception of lighter leaf color and smaller rosette size. During flowering the main difference was observed in *fad2-3* mutants that have a bushy phenotype, most probably due to lack of apical dominance. The remaining *fad* mutants were morphologically similar to the WT (Fig. 2.2, B).

To eliminate all detectable DUFAs and TUFAs from plants, two double mutants: *fad2-1 fad6* and *fad2-3 fad6* were generated in this work. We obtained similar results to *fad2-2 fad6* plants (McConn *et al.,* 1998). The *fad2-3 fad6* double mutants had a dwarf phenotype, chlorotic leaves and did not produce seeds (Fig.2.3, A). The double mutants with the weaker allele (*fad2-1*) were able to growth on soil, seed production was delayed to 2 extra months (Fig.2.3, B). The size of these plants was significantly smaller compared to the WT and the double mutant had chlorotic leaves. Under short day conditions the *fad2-1 fad6* double mutants produced larger rosettes compared to those grown in 24 h light conditions.

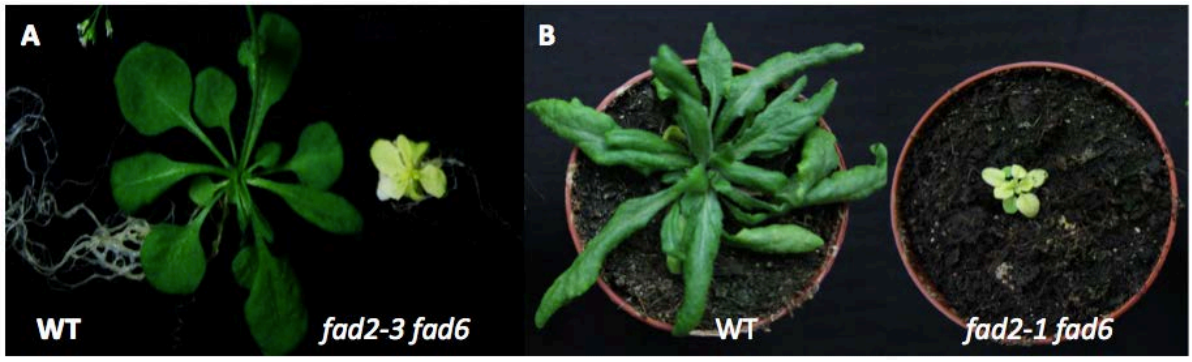


Figure 2.3. Monounsaturated plants. 4-week-old plants grown on (**A**) 2% sucrose media in Magenta boxes and (**B**) on soil. In both cases the same 14 h light, 10 h dark conditions were used.

The *fad2-2 fad6* and *fad2-1 fad6* double mutants are strongly affected in development (McConn *et al.,* 1998; this work), whereas the single mutants have morphologies similar to WT (Zhang *et al.,* 2009; 2012; this work). That brought us to the idea of compensatory role between *FAD2* and *FAD6* enzymes. The aim was to understand if this mechanism was controlled at the

translational or transcriptional levels. For this purpose, we conducted qPCR analysis of *FAD2* and *FAD6* transcript levels on 7-day-old seedlings grown on agar media (Fig.2.4).

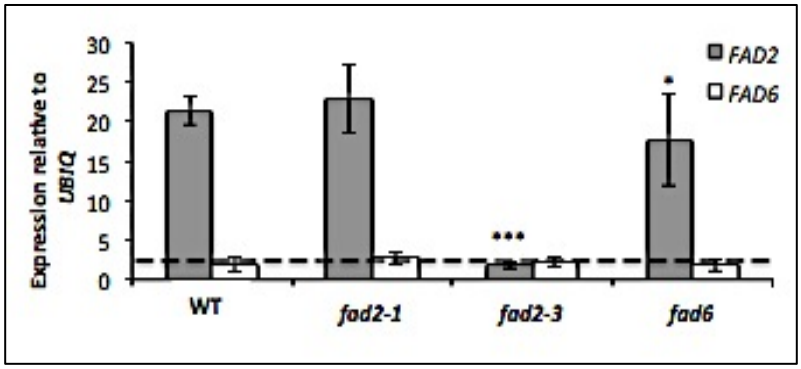


Figure 2.4. Relative expression of *FAD2* and *FAD6* genes in 7-day-old *fad* single mutants. *FAD2* and *FAD6* transcriptional levels were normalized to those of *UBC21* in WT seedlings. Error bars indicate a mean of ± SD. Bars represent the means of 4 biological replicates; each contains a pool of ~ 40 seedlings. Statistical significance in pair-wise comparison was evaluated by Student's test, where * $p \leq 0.05$, *** $p \leq 0.001$.

The results obtained did not show significant upregulation of the *FAD6* gene in *fad2* mutants or of the *FAD2* gene in the *fad6* background. Suggesting that, compensatory mechanisms make take place at the post-transcriptional levels.

II.2. Changes in fatty acid composition during development.

Fatty acid profiling *via* gas chromatography - mass spectrometry (GCMS) was performed to monitor changes in the levels of PUFAs in *fad* mutants at early developmental stages. The time course from 3- to 7-day-old was chosen due to the known difference in the expression of *FAD2* and *FAD6* genes in this period

(Zhang *et al.,* 2009; Zhang *et al.,* 2012). Our results revealed similar FA profiling for seeds and 3-day-old seedlings (Fig.2.5) and further on changes during the first week of seedling establishment in the WT and mutants (Fig.2.6).

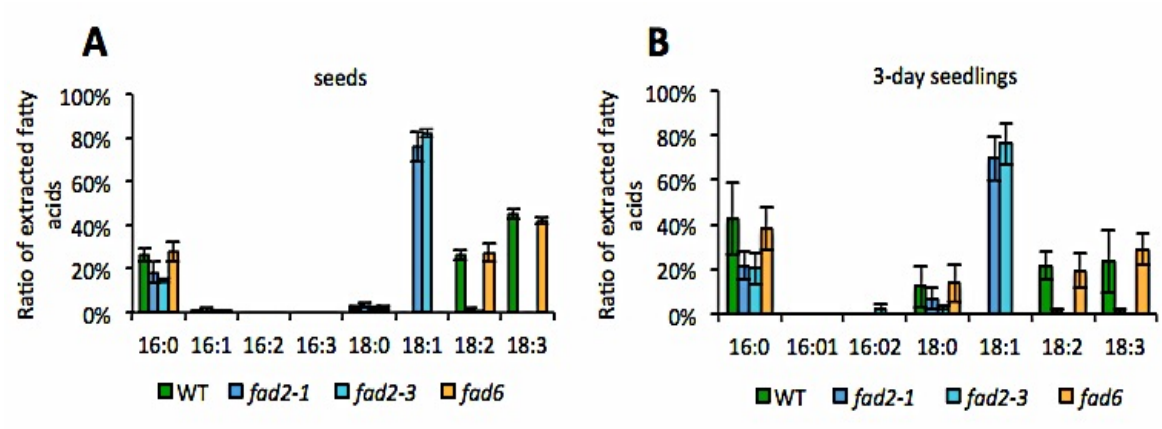


Figure 2.5. Fatty acid composition in *fad* mutants. *Arabidopsis* (**A**), seeds; (**B**), 3-day-old seedlings were used to perform fatty acid profiling. Error bars indicate a mean of ± SD. Bars represent the means of 3 - 6 biological replicates; each contains a pool of ~300 seeds or ~120 seedlings.

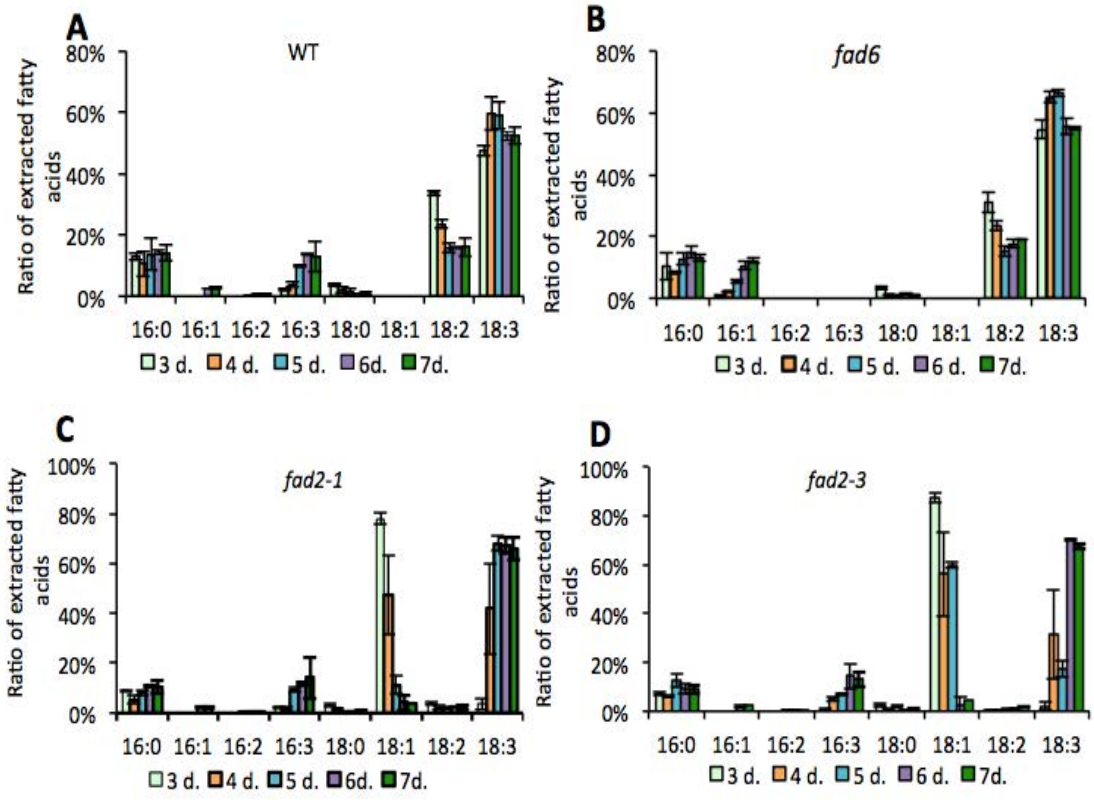


Figure 2.6. Fatty acid composition during the first week of seedling establishment. (**A**), WT; (**B**), *fad6*; (**C**), *fad2-1*; (**D**), *fad2-3* seedlings were used for this analysis. Error bars indicate a mean of ± SD, bars represent the means of 4 - 6 biological replicates; each contained a pool of ≈ 40 seedlings.

Significant changes in FA composition occurred in the WT seedlings between the third and sixth day after germination. There was an increase in the levels of 16:3 from 2% to 13.5% and a decrease in 18:2 levels from 34% to 15% (Fig.2.6, A). The *fad6* mutants showed similar changes in the levels of 18:2. However, *fad6* plant reveales changes in C16 fatty acid composition, this mutant has no 16:2 FA, and it is associated with the over accumulation of 16:1 which increases from 1% to 10% of extracted fatty acids by the end of the 6th day (Fig.2.5, B). The *fad2* single mutants have a similar to WT composition of

C16 FAs, but differ to WT C18 FA compositions due to strongly reduced levels of 18:2. This correlated with over - accumulation of 18:1 and 10% higher levels of 18:3 compared to the WT. Surprisingly, in *fad2-3* mutants 18:3 levels stabilized on the 6th day after germination and in the *fad2-1* on the 5th day (Fig.2.5, C-D). Unexpectedly, FA composition did not change strongly during the 7-day to 4-week stage in any of the genotypes analyzed (Fig 2.5, E). The *fad2-1 fad6* double mutant had a consistent FA profile at all analyzed stages. The major FA components in *fad2-1 fad6* mutants are 18:1 and 16:1 with an almost complete absence of 16:2, 18:2 and 16:3,18:3 FAs. Based on these results, *fad2-1 fad6* can be considered as a monounsaturated plant.

II.3. PUFAs as major precursors of malondialdehyde.

Reactive oxygen species are the main source of non-enzymatic lipid oxidation, a process where polyunsaturated fatty acids (PUFAs) present in membrane lipid bilayers get oxidized and that leads to aldehyde formation (Esterbauer *et al.,* 1991). By-products of lipid oxidation are of great interest, because of their association with mammalian diseases such as cancer, aging, arthritis, Alzheimer's disease, atherosclerosis and alcoholic liver disease (Ramos *et al.,* 1995; Esterbauer *et al.*, 1991; Spiteller, 1996; Montine *et al*., 2002; Levonen *et al.,* 2014; Han *et al.,* 2016). According to a recent hypothesis, PUFAs work as

a buffering system for ROS and the by-products of lipid oxidation induce gene expression in response to stresses, leading to cell protection (Mene-Saffrane *et al.,* 2007, Schmid-Siegert *et al*, 2016).

Malondialdehyde is one of the most studied products of lipid oxidation that is commonly believed to be a genotoxic and proteotoxic molecule (Marnett, 1999) generated from oxidative degradation of PUFAs with more than two methylene-interrupted double bonds (Pryor and Stanley, 1975). MDA can be present in two forms, depending on its pH: enolate, with a low chemical reactivity at cytosolic pH and a highly reactive protonated form at acidic pH that can lead to cell damage (Sawicki *et al.,* 1963). MDA pools in *Arabidopsis* seedling can be visualized by 2-thiobarbituric acid (TBA) staining, which forms $MDA(TBA)_2$ adducts that can be detected by their fluorescence at 555±15 nm (Mène-Saffrané et al., 2007) and quantified *via* GC/MS using the cognate MDA internal standard D_2-MDA (Yeo *et al.,* 1994; Liu *et al.,* 1997, Weber *et al.,* 2004).

The main sources of MDA in mammalian tissues are arachidonic and docosahexaenoic PUFAs, oleic and elaidic acids produce much lower amounts of MDA (Pryor and Stanley, 1975; Liu et al., 1997). In rodents the highest concentration of MDA was found in kidney and heart (Kelley *et al.,* 2006; Kumasaka *et al.,* 2007). This tightly correlates with the ratio of PUFAs in

kidney and heart (around 45-50%) compared to 25-30% in liver and brain (Spector, 2001).

Due to the availability of lipid biosynthesis mutants, *Arabidopsis* is a widely used model organism in lipid oxidation studies. The highest concentration of MDA in *Arabidopsis* was found in the leaves (Muckenschnabel et al., 2002), where the major pool (76% of total MDA) originates from α-linolenic fatty acid (18:3; Weber *et al.,* 2004). The sources of MDA in seedlings differ from those in adult plants and remain unknown (Schmid-Siegert *et al.,* 2012). In this case di- and monounsaturated FAs could be the most likely source for MDA generation, since their abundance in shoots of *Arabidopsis* seedlings.

The main question of this part of the thesis was to find the major source of MDA in *Arabidopsis* seedlings, its localization and potential role in defense responses. To answer these questions we used 3- and 7-day-old *Arabidopsis* seedlings and tested how changes in fatty acid levels affect MDA pools.

According to earlier work (Schmid-Siegert *et al.,* 2012), pools of MDA are localized in the meristematic zones of *Arabidopsis* seedlings. However, we found this tissue unsuitable, due to its small size, for biochemical analysis. To check this hypothesis, we used 3-day-old roots of *Brassica oleracea* (that is known to be closest relative to *Arabidopsis*) to conduct quantitative MDA

measurements of the tip and remaining part of the root (Fig.2.7, B). As a control we made fatty acid measurements from the root tip and remaining parts of the seedlings' roots (Fig.2.7, A).

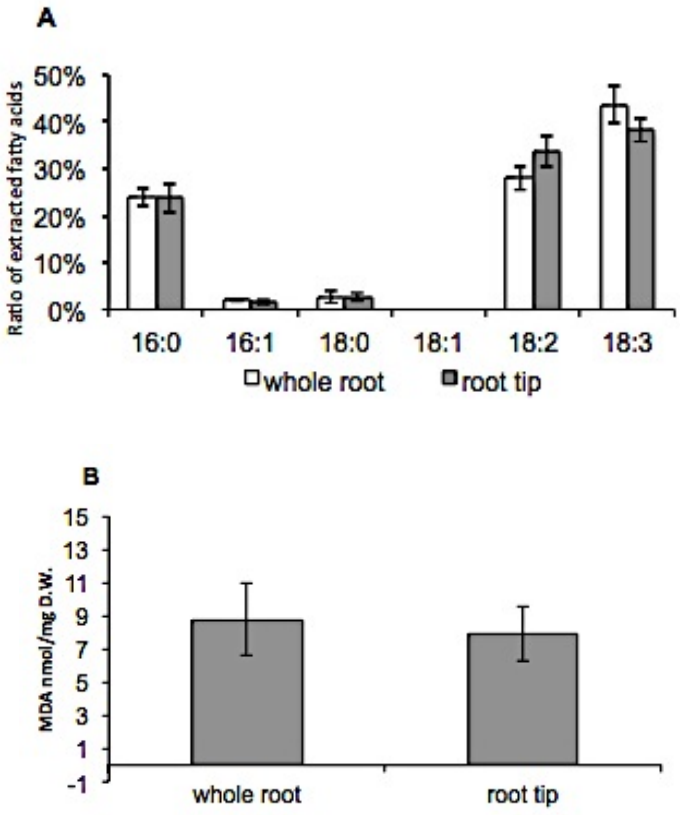


Figure 2.7. Biochemical characterization of *Brassica oleracea* roots. (**A**) Fatty acid composition of 3-day-old entire roots and separated root tips. This experiment includes 6 independent replicates. (**B**) Total MDA levels in 3-day-old tips and remaining part of the root. Error bars indicate a mean of ± SD, bars represent the means of 4-6 biological replicates; each containing a pool of ≈ 60 roots and 120 root rips**.** All tissue material was collected in liquid nitrogen to prevent oxidation of FAs.

The results obtained did not confirm the hypothesis of high MDA concentration in the meristematic regions. On the contrary, we found no difference in FA composition and MDA levels of the tip and entire *Brassica*'s root. These results suggest that high concentration of MDA detected in the

meristematic regions of *Arabidopsis* seedlings previously, could be caused by defects in root tip permeability.

fad mutants were used to estimate which polyunsaturated fatty acids can act as MDA precursors. To visualize MDA pools within the plant 2 - thiobarbituric acid solution (35 mM TBA, for 60 min) was used (Mene-Saffrance *et al.,* 2007). Signal intensity was shown to correlate with MDA levels; the more MDA the higher fluorescence. TBA staining of the 3-day-old *Arabidopsis* roots revealed stronger fluorescence in the root tips of WT and *fad6* seedlings compared to *fad2* mutants (Fig.2.8).

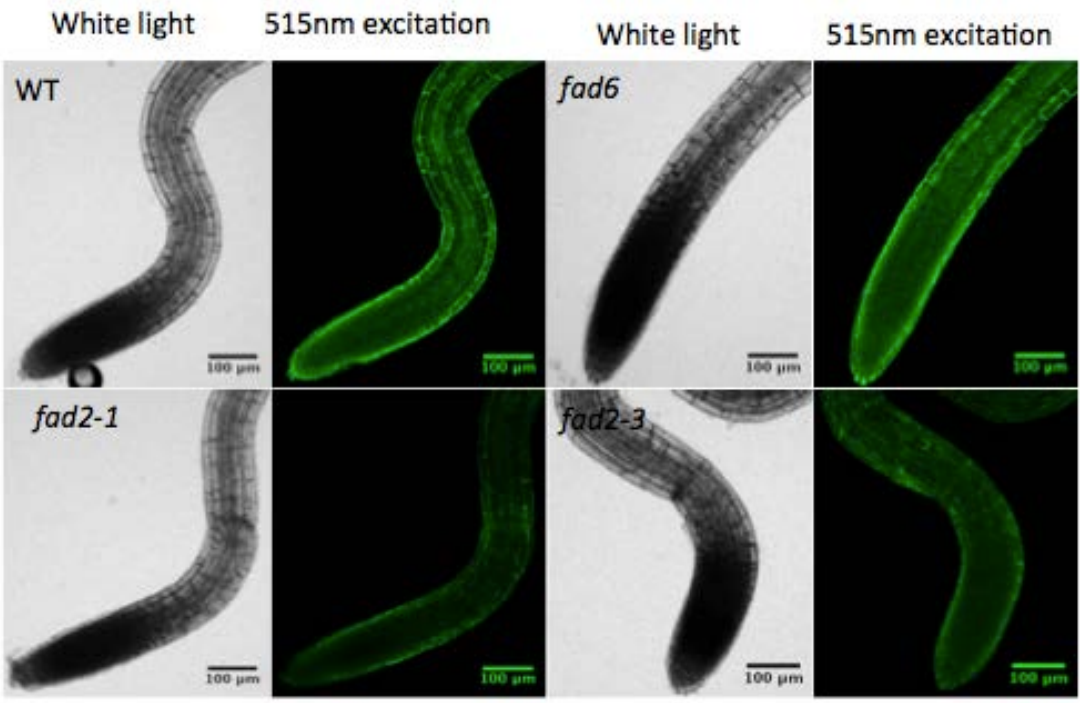
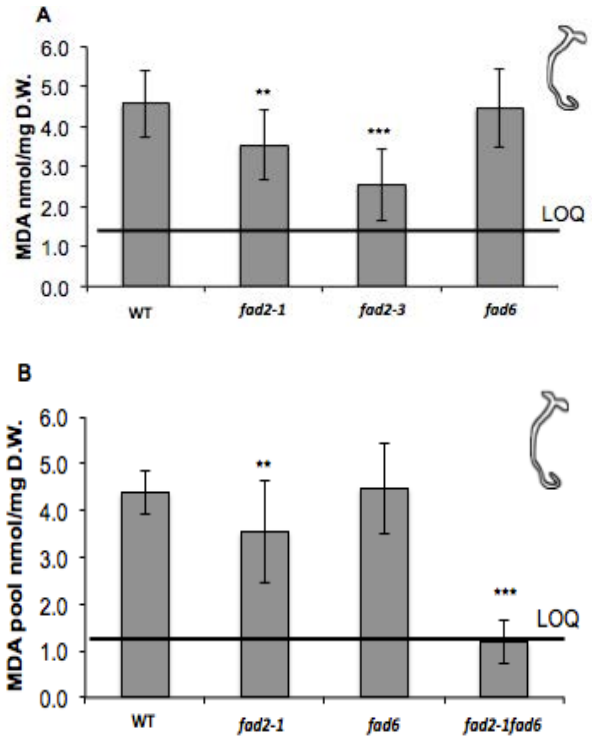


Figure 2.8. *In situ* malondialdehyde (MDA) detection. 3-day-old *Arabidopsis* roots were incubated in 35 mM TBA solution for 60 min and visualized by confocal microscopy with excitation at 555 ± 15 nm.

To confirm these results we conducted quantitative MDA measurements *via* GCMS. This revealed a decrease in MDA levels of *fad2* mutants from 4.5 to 3 ng/mg dry weight (D.W.) in whole 3-day-old seedlings. We found no differences between *fad6* and WT seedlings (Fig.2.9, A) and a strong MDA decrease in the *fad2-1 fad6* double mutant – 1.19 ng/mg D.W. (below limit of quantification (LOQ)) relative to 4.5 ng/mg D.W. in the WT (Fig.2.9, B). MDA measurements in 3-day old roots showed stronger decreases in *fad2* mutants that could be explained by overall lover levels of DUFAs, due to the absence of *FAD6* activity in this tissue (Fig.2.9, C).



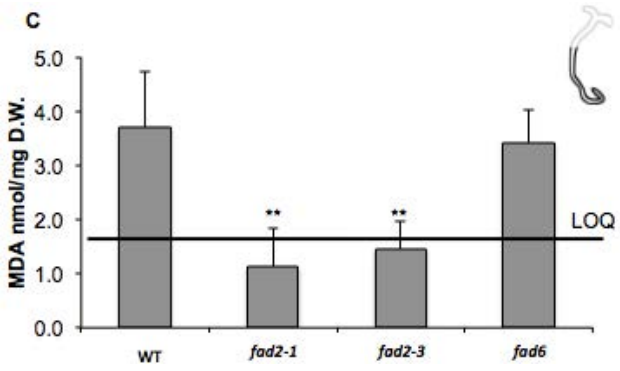


Figure 2.9. Total MDA levels in 3-day-old seedlings. MDA measurements were conducted on (**A**-**B**) whole seedlings and (**C**) roots of WT, *fad2* and *fad6* mutants. Each experiment was conducted 3 times with 4 independent replicates per each genotype. The limit of quantification (LOQ) is shown as the balck line. Statistical significance in pair-wise comparison was evaluated by Student's test, where ** $p \geq 0.01$; *** $p \leq 0.001$.

Due to massive changes in fatty acid composition during seedling establishment, we decided to make MDA measurements at the stage of 7-day-old seedlings (Fig. 2.10)

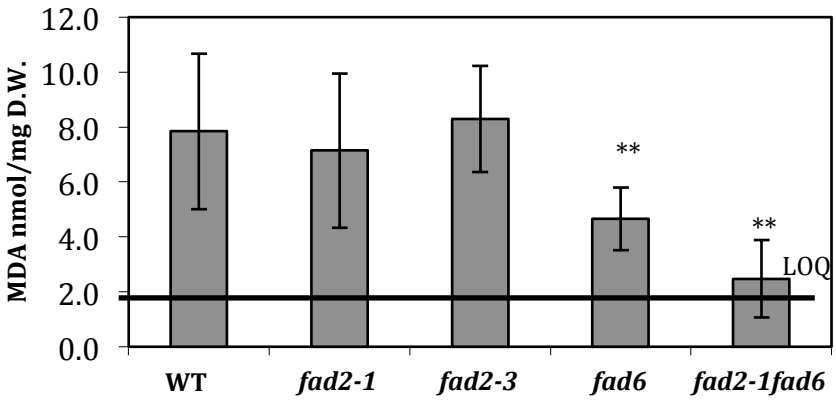


Figure 2.10. Total MDA levels in 7-day-old seedlings. Each experiment was conducted 3 times with 4 independent replicates per each genotype. The limit of quantification (LOQ) is shown as the balck line. Statistical significance in pair-wise comparison of mutant *vs* WT was evaluated by Student's test, where ** $p \geq 0.01$.

Exogenous MDA is known to activate defence and stress-related genes upon stresses (Weber *et al.,* 2004). MDA induction can be caused by jasmonic and salicylic acid (SA) treatment in *Arabidopsis* seedlings (Schmid-Siegert *et. al.,* 2012). JA was also shown to increase degradation of Rubisco that leads to the rapid chlorophyll loss and oxidative stress in barley leaves (Jung, 2004). In this work we extended the results of Schmid-Siegert *et. al.* (2012) on MDA induction upon external hormonal treatment. JA application on 3-day-old seedlings leads to MDA induction in WT and mutants. There was a tight correlation between the amount of PUFAs and MDA increase upon JA treatment (Fig.2.11).

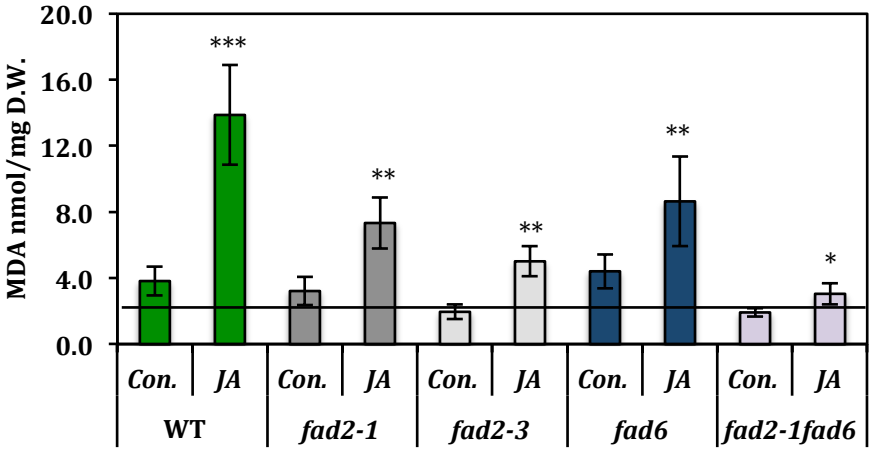


Figure 2.11. MDA pools in 3-day-old seedlings after 50 μM JA treatment, 20 hours post-application. Error bars indicate a mean of ± SD. Each experiment was conducted 3 times with 4 independent replicates per each genotype. One biological replicate consisted of a pool of ≈ 300 -400 seedlings. The limit of quantification is shown as the balck line. Statistical significance in pair-wise comparison was evaluated by Student's test, where * $p \geq 0.05$; ** $p \geq 0.01$; *** $p \leq 0.001$.

II.4. Discussion.

PUFAs are major components of lipid bilayers and, in plants; the lack of both di- and triunsaturated FAs is lethal (McConn *et al.,* 1998). However, plants with only one type of PUFAs (di- or triunsaturated FAs) can grow under laboratory conditions. This leads to the conclusion that DUFAs and TUFAs may have similar overall roles in development. Nevertheless, TUFAs have at least one unique role that cannot be fulfilled by DUFAs. TUFAS are the only precursors for JA synthesis (Schaller *et al.,* 2009). One of the questions addressed in this work was to look for specific roles of DUFAs in plant development by analysing single and double mutants of *FAD2* and *FAD6* genes.

The rosette phenotypes of 4-week old *fad2* and *fad6* mutants differed from that of the WT. The *fad2* mutants were slightly smaller, with shorter petioles and rounded leaves, whereas *fad6* mutants are similar in size to the WT, but with lighter leaf coloration, due to the dysfunction of photoinhibitory machinery (Perumal *et al.,* 2002). The *fad2-3* mutants had bushy morphology during flowering period. Generation of *fad2-1 fad6* double mutants, confirmed the overall importance of DUFA and TUFAs in plant development. However, since these plants remained fertile they must produce very low levels of TUFAs that are involved in promotion of male fertility in *Arabidopsis* plants.

That is why we assume that the *fad2-1* allele is likely to be hypomorphic. Even a small amount of PUFA can allow the plant to survive under low stress conditions. Chlorotic leaves are the result of strong inhibition of chlorophyll accumulation. This was already described in the *fad2-2 fad6* mutant, where the leaf chlorophyll content was 11% of WT levels. Surprisingly, such a strong reduction did not affect the entire photosynthetic complex, but mainly the PSII quantum yield - parameter that best characterizes photosynthetic efficiency due to its link with downstream process (PSI and CO_2 assimilation), was 50% less efficient in the *fad2-2 fad6* mutant compared to the WT (McConn *et al.,* 1998). Such a stong defect in photosynthetic activity of *fad2-2 fad6* mutant could be explained by the deficiency in PUFAs that work as buffers for ROS, molecules that are generated during the photosynthesis process and lead to oxidative stress.

Fatty acid composition changes during the early steps of plant development. Strong changes in fatty acid profiles were observed during the transition phase from germination to the appearance of the first true leaves. Prior to this, during the first 3-days after germination FA composition remained relatively stable but changed within the following days. To summarize, the 3-day-old *fad2* seedlings display an over accumulation of 18:1 FA whereas at the 7-day old stage levels of 18:1 decreased from approximately 80 to 5%, but the levels of 18:3 increased approximately from 10 to 65%.

Changes in FA composition could be explained by seedling transition from semi-autotrophic growth at 3-day-old stage to autotrophic that takes place at the end of the first week. Due to the absence of fully established photosynthetic machinery and the use of reserves in the form of oil bodies plants obtain major source of the energy, such as triacyl glycerols from the seed. However, at the end of the first week, seedlings have fully expendent cotyledons that can perform photosynthesis and generate needed amount of energy to promote plant growth and *de novo* fatty acid synthesis (Frey-Wyssling *et al.,* 1963; Murphy, 1990).

Diunsaturated FAs are second major source of MDA in *Arabidopsis* seedlings. *In vitro* studies suggest that polyunsaturated fatty acids are the major source of MDA and recent discoveries revealed role of TUFAs as a major MDA source in the shoots of 3-day-old seedlings, but not in the roots (Schmid-Siegert *et al.,* 2012). This can be explained by abundance of PUFAs in different *Arabidopsis* tissues: in roots DUFAs and TUFAs are equally abundant, however in shoots TUFAs are the dominating FAs. Quantitative MDA measurements in seedlings support correlation in the amount of di- and triunsaturated fatty acids and MDA levels (Fig.2.6; 2.9; 2.10).

To estimate the sensitivity to oxidation *in vivo* of different PUFAs in 3-day-old seedlings, we combined data obtained from *fad2* and *fad2-1 fa6* mutants with

data from the *fad3 fad7-2 fad8* mutant that was published previously (Mene-Saffrane *et al.,* 2007; Schmid-Siegert *et al.*, 2012). Involvment of different PUFAs in MDA generation was estimated by the following principle: percentage of MDA generated in seedlings of *fad2-1 fad6* and roots of *fad2-3* mutants minus percentage of MDA generated in roots and whole seedlings of *fad trip* mutants (Schmid-Siegert *et al.*, 2012) (Fig.2.12).

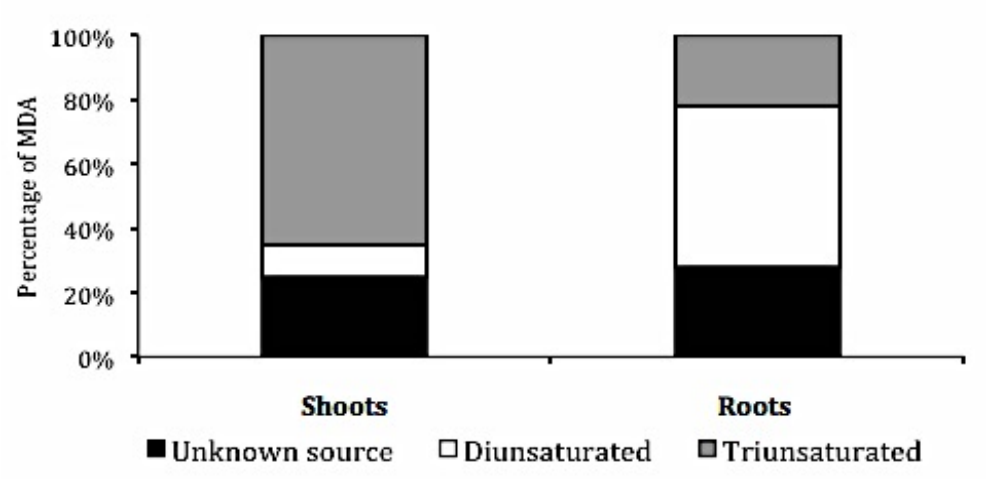


Figure 2.12. Estimation of the contribution of different PUFAs to MDA generation in *Arabidopsis* seedlings.

According to our results, PUFAs are the major source of MDA in *Arabidopsis* seedlings. Surprisingly the effect of different PUFAs on MDA generation appears to be tissue specific. Despite the fact that shoots and roots contain similarly high levels of 18:2 and 18:3 the main source of MDA in the shoots are triunsaturated fatty acids (16:3, 18:3), whereas in roots it is diunsaturated fatty acids (16:2, 18:2).

MDA induction in seedlings can be caused by stresses such as JA, SA treatment and wounding (Schmid-Siegert *et al.*, 2012; 2016). To investigate if the basal and induced MDA pools originate from the same source, oleate desturase mutants were treated with jasmonic acid. This treatment is known to stimulate MDA production in roots (Schmid-Siegert *et al.,* 2012). The results obtained confirmed that PUFAs are the major source of basal and induced MDA; the lower amount of PUFAs the less MDA can be generated under control and stress conditions. Reduced levels of PUFAs in plant can lead to over accumulation of ROS and permanent oxidative stress that will diminish plant growth.

II.5. Experimental procedures.

Plant growth conditions, genotypes and chemicals - Wild-type *Arabidopsis* (WT *Columbia* background) was used. The following alleles in the *Columbia* background were received from the Arabidopsis Biological Resource Centre (ABRC): *fad2-1* (CS8041), *fad2-3* (SK18137), *fad6* (CS207), J. Browse kindly provided *fad2-2* and *fad2-2 fad6* alleles and *fad2-1 fad6* and *fad2-3 fad6* double mutants obtained in this work, *Brassica oleracea* var. Mezzo Nano was from Samen Mauser (Winterthur, Switzerland). Seeds were stratified for 2 days at 4 ^{0}C and then grown at 21 ^{0}C under 100 µE m^{-2} s^{-1} of light with photoperiod depending on the application (seedlings: 14 h light, 10 h dark (long days); on

soil for seed propagation: 24 h light (continuous days) or experiments with adult plants at: 9 h light, 15 h dark (short day)). Seedlings were grown on half-strength Murashige and Skoog solid media (half-strength MS, 2.15 g/L, pH 5.7; Duhefa Biochemie, Haarlem, The Nederlands) supplemented with 0.5 g/L of MES hydrate (Sigma, Buchs, Switzerland) and 0.7% agar.

Gene expression in fad2-1, fad2-2, fad2-3- and fad6 mutants – Each biological replicate consisted of ≈ 40 seedlings. Collected tissue was immediately frozen in liquid N_2. RNA isolation and RT-PCR of *UBC21* (At5g25760) were conduct as described (Park *et al.,* 2002). For the *FAD2* gene the following primer pair was used: forward - GCCATTCCCCATCTGACCACC and reverse - CCAACAAAACAGCGATGAGA for *FAD6*: forward - GAGGTGAGGGCTCTTCACAG and reverse - AGAAGCTCATCGCTTGGAAA. RT-PCR was performed with GoTaq DNA polymerase (Promega, Dubendorf, Switzerland) as indicated by the manufacturer. For the *FAD2* gene the following qPCR primer pairs were used: forward - GCTCCATCTCCAGAAACATGG and reverse - TTGAGCGTTTGAAACAATGC for *FAD6*: forward - GAGGTGAGGGCTCTTCACAG and reverse – TCACAAAGAACCCGGTAATTG.

Fatty acid composition analysis – 3-day-old seedlings and roots were collected

(≈ 30 mg) and placed in 15 ml Pyrex SVL capped test tubes (Milian, Switzerland). 1ml of 2.5 % H_2SO_4 solution, in methanol was added to the samples. Samples were incubated for 90 min at 80 ^{0}C, 1.5 ml 0.9% NaCl and 1 ml of hexane (≥97.0%, Sigma-Aldrich, Buchs, Switzerland) were added. The solution was vortexed briefly (2 sec) then centrifuged for 5 min at 1000 rpm. 250 µl of the upper phase containing fatty acid methyl esters were collected. 1µl of a 1:5 dilution was analyzed by GCMS (initial T = 150^{0}C for 3 min, with a following increase from 150^{0}C to 200^{0}C).

Gas chromatography/Mass Spectrometry – 3-day-old seedlings or roots (≈ 150 mg) were harvested in liquid nitrogen and ground for 15 sec at 3000 rpm/sec in TissueLyser II (Quiagen, Hombrechtikon, Switzerland) MDA levels were measured by a gas chromatography/ mass spectrometry with D_2-MDA internal standard generated from ($2D_2$)-1,1,3,3-tetraethoxypropan as described (Weber *et al.,* 2004)

2-Thiobarbituric acid staining – 3-day-old seedlings were transferred in 35 mM TBA or in 35 mM trichloroacetic acid solution as a negative control and incubated for 60 min at 37 ^{0}C. MDA-TBA adducts were detected by excitation 515nm and emission: 555±15 nm.

Jasmonic acid seedling treatment – At the end of day three, seedlings were

transferred in 2 different plates with: control (half-strength Murashige and Skoog solid media with 0.5 g/L of MES hydrate dissolved in double distillated H_2O) and jasmonic acid (50 μM) dissolved in control solution. Plates were incubated for 18 hours (long days conditions) before the tissue collection and following GC/MS analysis.

Statistical analysis - Statistical significance in pair-wise comparison was evaluated by Student's test, where * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

II.6. Literature.

Browse J, Kunst L, Anderson S, Hugly S, Somerville C. 1989. A mutant of *Arabidopsis* deficient in the chloroplast 16:1/18:1 desaturase. *Plant Physiol.* 90:522-529.

Browse J, Somerville C. 1991. Plant lipids: metabolism, mutants, and membranes. *Science.* 252:80-87.

Esterbauer H, Schaur R J, Zollner H. 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic. Biol. Med.* 11:81–128.

Frey-Wyssling A, Grieshaber E, Muhlethaler K. 1963. Origin of spherosomes in plant cells. *J Ultrastruct Res.* 8:506-516.

Han K H, Hashimato N, Fukushima M. 2016. Relationships among alcoholic liver disease, antioxidants, and antioxidant enzymes. *World J Gastoenterol*. 22(1):37-49.

Harwood J.L. 1996. Recent advances in the biosynthesis of plant fatty acids, *Biochem. Biophys. Acta.* 1301:7-56.

Heinz E. 1993. Biosynthesis of polyunsaturated fatty acids. *Lipid Methabolism in Plants,* Boca Raton, FL:CRC press. pp.33-89.

James D W and Dooner H K. 1990. Isolation of EMS-induced mutants in *Arabidopsis* altered in seed fatty acid composition. *Theor. Appl. Genet.* 80:241-245.

Jung S. 2004. Effect of chlorophyll reduction in *Arabidopsis thaliana* by methyl jasmonate or norflurazon on antioxidant systems. *Plant Physiol. Biochem*. 42:225–231.

Kelley GA, Kelley KS. 2006. Aerobic exercise and lipids and lipoproteins in men: a meta-analysis of randomized controlled trials. *J. Mens. Health. Gend.* 3:61-70.

Kumasaka R, Nakamura N, Yamabe H, Osawa H, Shirato K, Schmada M, Murakami R, Fujita T, Okumura K, Hamazaki K, Hamazaki T. 2007. Fatty acid composition of plasma and kidney in rats with anti-THY1.1 nephritis. *In Vivo.* 21:77-79.

Levonen A L, Hill B G, Kansanen E, Zhang J, darley-Usmar V M. 2014. Redox regulation of antioxidants, autophagy, and the response to stress: implication for electrophile therapeutics. *Free Radic Biol Med.* 71:196-207.

Liu J, Yeo HC, Doniger SJ, Ames BN. 1997. Assay of aldehydes from lipid peroxidation: gas chromatography-mass spectrometry compared to thiobarbituric acid. *Anal. Biochem.* 245:161–166.

Lou Y, Schwender J, Shanklin J. 2014. *FAD*2 and *FAD3* desaturases from heterodimers that facilitate metabolic channeling *in vivo*. *J Biol Chem.* 289(26):17996-8007.

Marnett L J. 1999. Lipid peroxidation-DNA damage by malondialdehyde. *Mutat. Res*. 424:83-95.

Mène-Saffrané L, Dubugnon L, Chételat A, Stolz S, Gouhier-Darimont C, Farmer EE. 2007. Nonenzymatic oxidation of trienoic fatty acids contributes to reactive oxygen species management in *Arabidopsis*. *J Biol Chem.* 284:1702–1708.

McConn M, Browse J. 1996. The critical requirement of linolenic acid is pollen development, not photosynthesis, in an *Arabidopsis* mutant. *Plant Cell.* 8:403-416.

McConn M, Browse J. 1998. Polyunsaturated membranes are required for photosynthetic competence in a mutant of *Arabidopsis*. *Plant J.* 15:521-530.

Miquel M, James D. J, Dooner H, Browse J. 1993. Arabidopsis requires polyunsaturated lipids for low-temperature survival. *Proc. Natl. Acad. Sci. USA*. 90:6208-6212.

Montine TJ, Neely MD, Quinn JF, Beal MF, Markesbery WR, Roberts LJ, Marrow JD. 2002. Lipid peroxidation in aging brain and Alzheimer's disease. *Free Radic. Biol. Med.* 33:620-626.

Muckenschnabel I, Goodman BA, Williamson B, Lyon GD, Deighton N. 2002. Infection of leaves of *Arabidopsis thaliana* by *B. cinerea*: changes in ascorbic acid, free radicals and lipid peroxidation products. *J. Exp. Bot.* 53:207-214.

Murphy D. J. 1990. Storage lipid bodies in plants and other organisms. *Prog. Lipid Res.* 29:299-324.

Park JY, Kim HJ, Kim J. 2002. Mutation in domain II of IAA1 confers diverse auxin-related phenotypes and represses auxin-activated expression of *Aux/IAA* genes in steroid regulator - inducible system. *Plant J.* 32:669-683.

Pryor WA, Stanley JP. 1975. Letter: A suggestion mechanism for the production of malondialdehyde during the autoxidation of polyunsaturated fatty acids. Nonenzymatic production of prostaglandin endoperoxides during autooxadation. *J. Org. Chem.* 40:3615-3617.

Ramos P, Gieseg SP, Schuster B, Esterbauer H. 1995. Effect of temperature and phase transition on oxidation resistance of low density lipoprotein. *J Lipid Res*. 36(10):2113-2128.

Routaboul J M, Fischer S F, Browse J. 2000. Trienoic fatty acids are required to maintain chloroplast function at low temperatures. *Plant Physiol*. 124(4):1697-1705.

Sawicki E, Stanley T.W, Johnson H. 1963. Comparison of spectrophotometric and spectrofluorometric methods for the determination of malondialdehyde. *Anal. Chem.* 35:199-205.

Schaller A, Stintzi A. 2009. Enzymes in jasmonic acid biosynthesis – Structure, function, regulation. *Phytochemistry*. 70(13-14):1532- 1538.

Schmid-Siegert E, Loscos J. 2012. Inducible malondialdehyde pools in zones of cell proliferation and developing tissues in *Arabidopsis*. *J. Biol. Chem.* 287:8954-8962.

Schmid-Siegert E, Stepushchenko O, Glauser G, Farmer E.E. 2016. Membranes as structural antioxidants: Recycling of malondialdehyde to its source in oxidation-sensitive chloroplast fatty acids. *J. Biol. Chem.* 291:13005-13013.

Shanklin J, Whittle E, Fox BG. 1994. Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase. *Biochemistry.* 33:2787-12794.

Spector A A. 2001. Plasma free fatty acid and lipoproteins as sources of polyunsaturated fatty acid for the brain. *J. Mol. Neurosci.* 16 (2-3):159-165.

Spiteller G. 1996. Enzymic lipid peroxidation – a consequence of cell injury? *Free Radic Biol Med*. 21(7):1003-1009.

Tjellstroem H, Yang Z, Allen D, Ohlrogge JB. Rapid kinetic labeling of Arabidopsis cell suspension cultures: implications for models of lipid export from plastids. *Plant Physiol*. 158:601–611.

Weber H, Chételat A, Reymond P, Farmer EE. 2004. Selective and powerful stress gene expression in *Arabidopsis* in response to malondialdehyde. *Plant J.* 37:877–888.

Yeo HC, Helbock HJ, Chyu DW, Ames BN. 1994. Assay of malondialdehyde in biological fluids by gas chromatography-mass spectrometry. *Anal. Biochem.* 220:391–396.

Zhang JT, Zhu JQ, Zhu Q, Liu H, Gao XS, Zhang HX. 2009. Fatty acid desaturase-6 (*FAD6*) is required for salt tolerance in *Arabidopsis thailana. Biochem. Biophys. Res. Commun.* 390:469-474.

Zhang J, Liu H, Sun J, Li B, Zhu Q, Chen S, Zhang H. 2012. *Arabidopsis* fatty acid desaturase *FAD2* is require for salt tolerance during seed germination and early seedling growth. *PLoS One.* 7:e30355.

Chapter III: The effect of *Arabidopsis* DUFA-deficient *fad2* mutants on seedling growth

III.1. Characterization of root growth in *fad2* mutants.

An effect of *fad2* mutants on *Arabidopsis* root growth in the presence of sodium chloride has already been reported (Zhang *et al.,* 2012). Here we focussed further on the effect of *fad2* mutation on root development. We were able to extend the results of Zhang *et al.* (2012) and to provide insights on the growth differences of wild type and *fad2* roots.

Roots are important not only for providing nutrients and water to shoots, but also for detecting and adapting to environmental changes (Tsukagoshi, 2016). The *Arabidopsis* root (which is typical for dicotyledonous plants) can be divided longitudinally into four regions: apical and basal meristem, elongation zone and differentiation zone (Fig. 3.1).

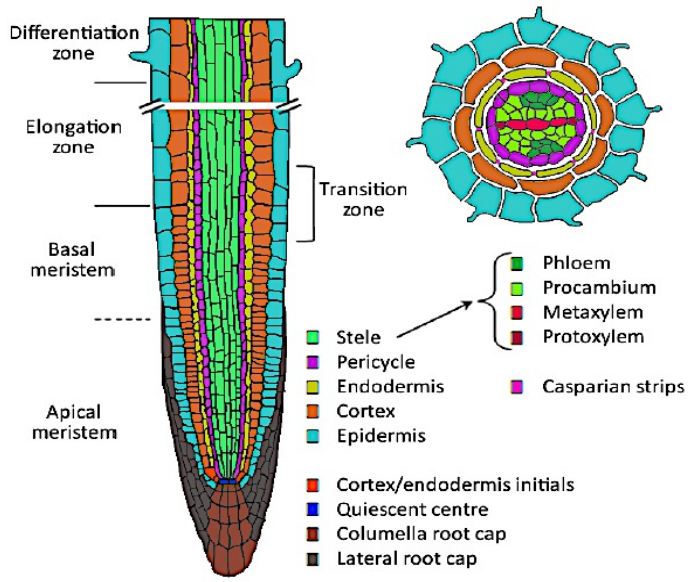


Figure 3.1. Organization of the *Arabidopsis* root. **Left**: Longitudinal cross section. **Right**: Transversal section. Adapted from De Smet *et al.* (2015).

Apical and basal meristematic zones undergo active division, whereas cellular elongation and partial differentiation strictly take place in the elongation zone (Beemster *et al.,* 1998). The final step of root tissue establishment together with lateral root formation takes place in the differentition zone (Mendrinna and Persson, 2015; Cano-Delgado *et al.,* 2010).

Root development is tightly regulated by plant hormones such as auxin, cytokinin, ethylene, gibberellins, jasmonate, brassinosteroids and abscisic acid that can induce changes in the reactive oxygen species (ROS) levels (Vanstraelen and Benkova, 2012; Yoshimitsu *et al.,* 2011; Bu *et al.,* 2008; Guzman *et al.,* 1990; Wang *et al.,* 2011; Claeys *et al.,* 2013; Laplaze *et al.,* 2007; De Smet *et al.,* 2015). ROS that are strongly abundant in plants were also shown to play an important role in regulation of the root growth (O'Brien *et al.,* 2012). Hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and superoxide (O_2^{-}) are the major ROS in plant that are known to be by-products of oxygen metabolism (Wojciechowski, 2014; Fridovich, 1986). During photosynthesis, respiration and pathogen attack oxygen undergoes a series of univalent reductions that requires acception of one electron at a time by the oxygen atom. The outcome of four univalent reductions is synthesis of two water molecules and multiple ROS (Fig.3.2; Triantaphylides and Havaux, 2009; Triantaphylides *et al*., 2008, Halliwell, 2006; Krieger-Liszkay, 2005, Fukai and Ushio-Fukai, 2011).

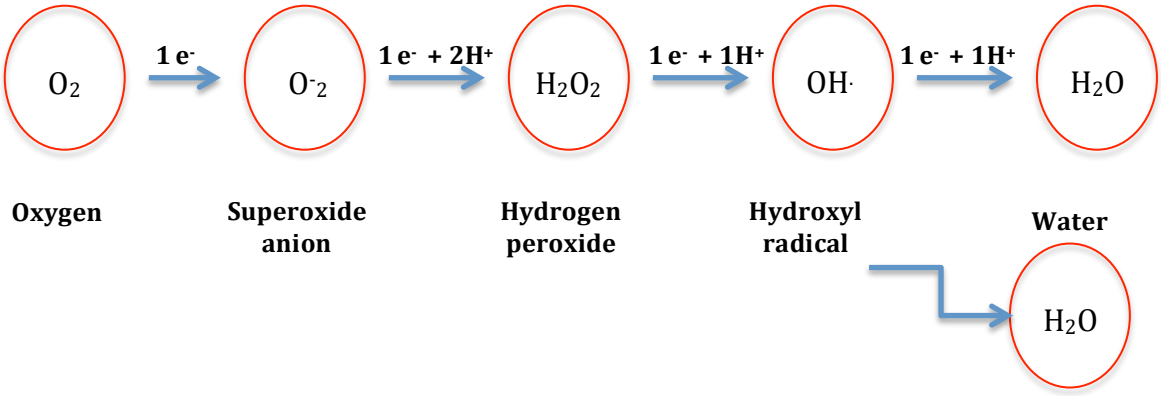


Figure 3.2. Reduction of oxygen to water. Adapted from Wojciechowski (2014). This scheme does not include singlet oxygen ($^{\cdot}O_2$).

In general, ROS in low concentration work as regulatory compounds, whereas at high concentration they lead to arrested growth and become toxic for the plant (Swanson *et al.,* 2010; Choudhury *et al.,* 2013). In *Arabidopsis* roots, O_2^- is generally required for cellular proliferation, whereas H_2O_2 plays role in cell differentiation and in the lignification process (Tsukagoshi *et al.,* 2010). $^{\cdot}OH$ is thought to be involved in cell wall loosening and cell expansion (Liszkay *et al.,* 2004).

At the molecular level, ROS such as $^{\cdot}OH$ or $^1O_2$ (singlet oxygen) are the main mediators of non-enzymatic lipid oxidation that leads to generation of fatty acid–derived aldehydes (Esterbauer *et al.,* 1991). The end-products of lipid oxidation are of great interest due to their association with diseases such as cancer, arthritis, Alzheimer's disease and atherosclerosis (Spiteller, 1996; Montine *et al*., 2002). Recent studies on plants have led to the hypothesis that

polyunsaturated fatty acids may work as a buffering system for ROS (Mene-Saffrane *et al.,* 2009, Farmer and Mueller, 2013; Schmid-Siegert, *et al.,* 2016). Another role of fatty acid fragments produced as by-products of lipid oxidation is modulation of gene expression in response to stresses (Weber *et al.,* 2004). The current findings are mainly based on the aerial tissues and so far, only a small amount of work has been conducted on the role of PUFAs in root stress responses. For example, there is evidence for the involvement of diunsaturated fatty acids in adaptation to salinity stress (Zhang *et al.,* 2009; Zhang *et al.,* 2012). External PUFAs application was shown to decrease ROS production in stimulated 264.7 human macrophages that leads to protection of innate lymphoid cells (Ambrozova *et al.,* 2010). These examples confirm the potentially important role of DUFAs in stress responses. The purpose of this work was to understand the role of DUFAs in *Arabidopsis* roots during control growth conditions and under stresses.

In order to adress these questions we used *Arabidopsis* WT, oleate desaturase mutants and seedlings deficient in triunsaturated fatty acids (*fad3-2 fad7-2 fad8*). Root growth was the first parameter investigated. The length of *Arabidopsis* roots was measured at 7-days post germination (Fig.3.3).

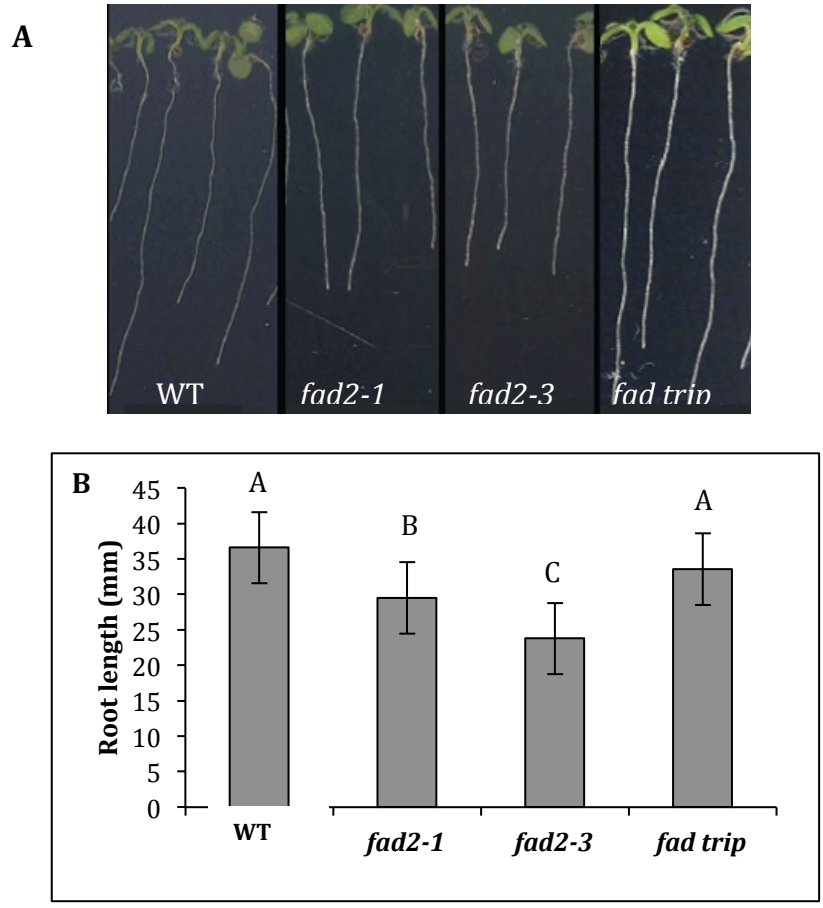


Figure 3.3. Root length analysis of 7-day-old *Arabidopsis* seedlings. (**A**) Image of the WT and *fad* mutants growing vertically on half MS plates. (**B**) Quantitative analysis of root length. Error bars indicate a mean of ± SD, bars represent the means of three independent experiments; each contains a pool of ~ 60 seedlings. Letters indicate statistically significant differences between pairs as determined by Tukey's HSD test ($p < 0.05$).

Both *fad2* mutants caused significant reduction in root length (Fig. 3.3). This phenotype is not caused by deficiency in triunsaturated fatty acids or jasmonate, since no significant difference was observed between WT and *fad trip* roots.

To understand what affects development of *fad2* mutants, we conducted fatty acid analysis on 7-day-old seedling roots (Fig.3.4).

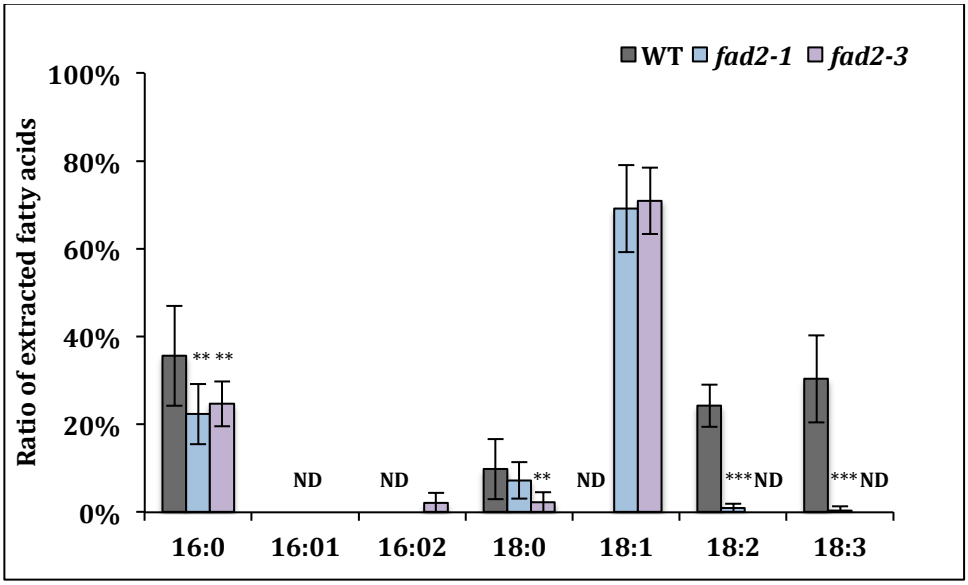


Figure 3.4. Fatty acid composition of 7-day-old roots. Bars represent the means of six biological replicates; each contains a pool of ~ 120 seedlings roots. Statistical significance in pair-wise comparison WT *vs* mutant was evaluated by Student's test, where ** $p \leq 0.01$; *** $p \leq 0.001$. ND – not detected.

These results showed changes in the levels of C18 FAs in *fad2* mutants, revealing strong reduction in the levels of 18:2 and 18:3. This led to the idea that *fad2* plants may be under constant oxidative stress, due to the absence of PUFAs (Mene-Saffrane *et al.,* 2009, Schmid-Siegert, *et al.,* 2016). To test this hypothesis, we performed malondialdehyde measurements using GCMS (Fig. 3.5). MDA is a marker of singlet oxygen or free radical-catalysed peroxidation in *Arabidopsis* that is synthetized mainly from PUFAs (Janero *et al.,* 1990, Schmid-Siegert, *et al.,* 2012; 2016). Earlier studies showed that reduced levels of MDA in *fad trip* mutant leaves correlated with higher levels of ROS in these

tissues (Mene-Saffrane et al., 2007; Schmid-Siegert, *et al.,* 2012). Based on these studies we can predict that reduced levels of PUFAs can lead to ROS accumulation.

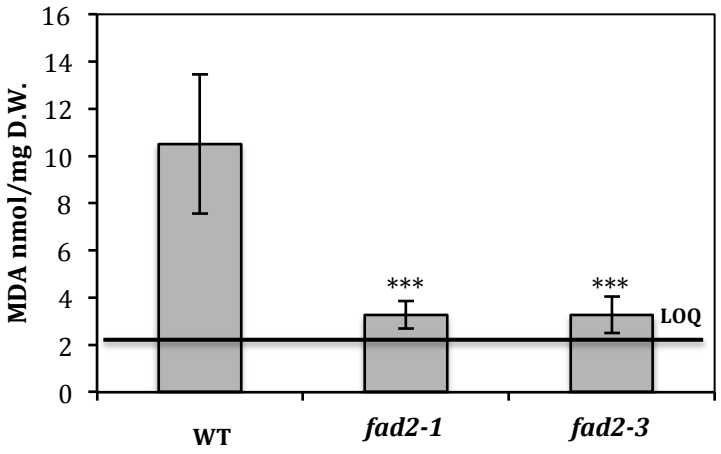


Figure 3.5. MDA levels in 7-day-old *Arabidopsis* roots. Bars represent the means of 4 biological replicates; each contains a pool of ~ 480 seedlings roots. The limit of quantification (LOQ) is shown as a blck line. Statistical significance in pair-wise comparison was evaluated by Student's test, where *** $p \leq 0.001$.

In 7-day old roots of WT plants, MDA levels are almost three times higher than in *fad2* mutants. These results are consistent with the hypothesis that PUFAs (and in this case, DUFAs) are the major source of MDA in the root. For the next step, we tested ROS levels in the roots, hypothesizing that reduced MDA levels lead to a more oxidative environment in the cells (Fig.3.6). The ROS-ID total ROS/Superoxide detection kit from Enzo (Lausanne, Switzerland; Fig.3.6, A) and 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) overall ROS detection solutions (Fig.3.6, B) were used to perform root staining.

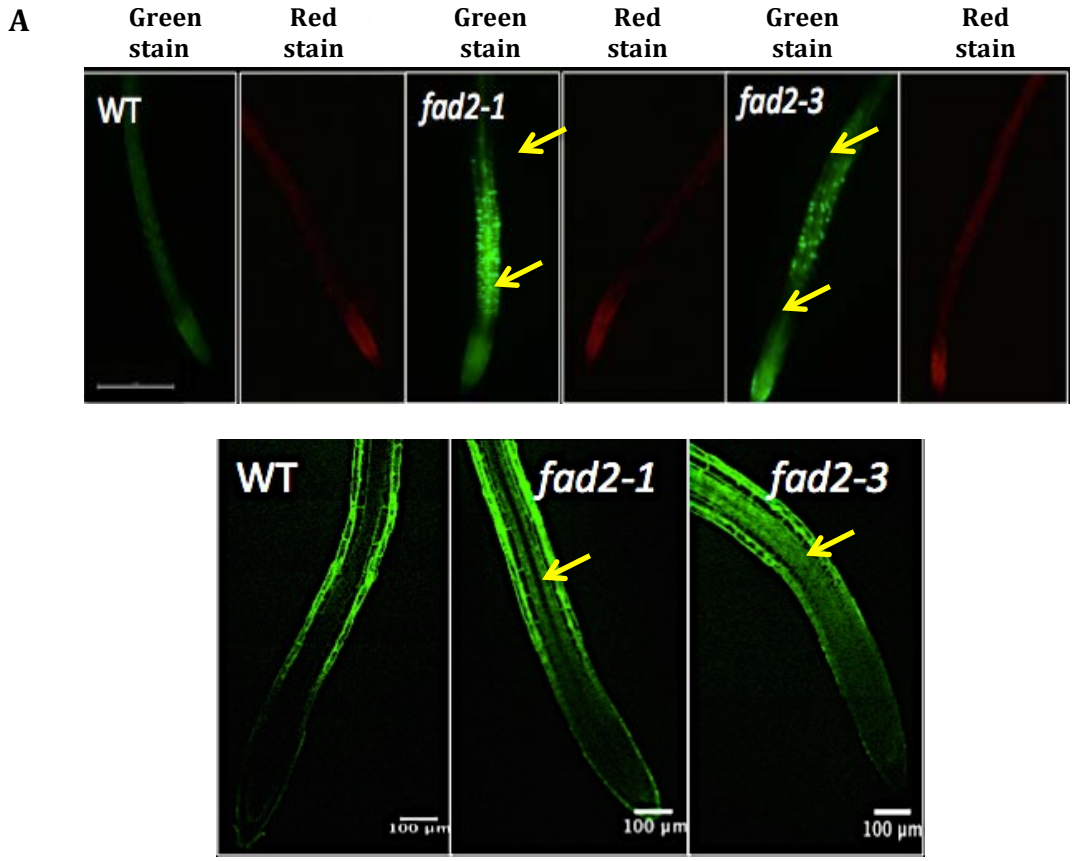


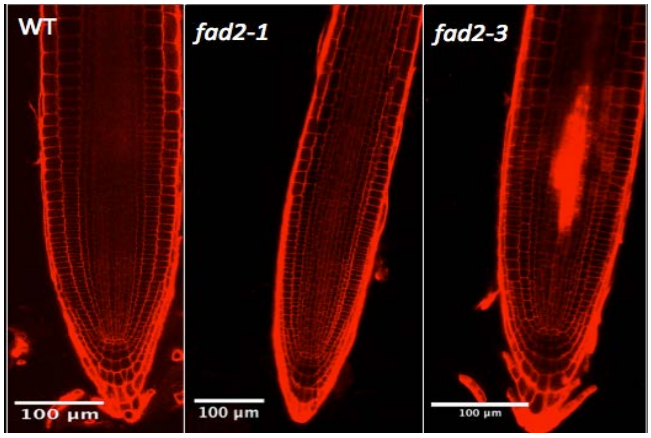
Figure 3.6. ROS staining of 7-day-old roots. (**A**) Seedlings treated with ROS-ID total ROS/Superoxide detection kit were vacuum infiltrated for 15 min. Hydrogen peroxide, peroxynitrite and hydroxyl radicals were visualized at wavelengths 490 nm /525 nm excitation/emission (Ex/Em) and labelled 'green stain', superoxide '$\cdot O^-_2$' at 550 nm /620 nm, 'red stain' (Ex/Em). Each photo is representative of the six biological replicates. Scale bar 1 mm. (**B**) 15 µM of H2DCFDA ROS detection solution was used in living roots of *Arabidopsis* seedlings visualized at wavelengths 495 nm /527 nm (Ex/Em). Arrows indicate differences in fluorescence between WT and f*ad2* mutants.

Figure 3.6, **A**. highlights the difference between ROS fluorescence in WT and *fad2* mutants. Strong green fluorescence was observed in meristematic and differentiation zones of *fad2* mutants, but a difference in superoxide (red staining) was only observed in case of *fad2-3* mutants relative to the WT. The

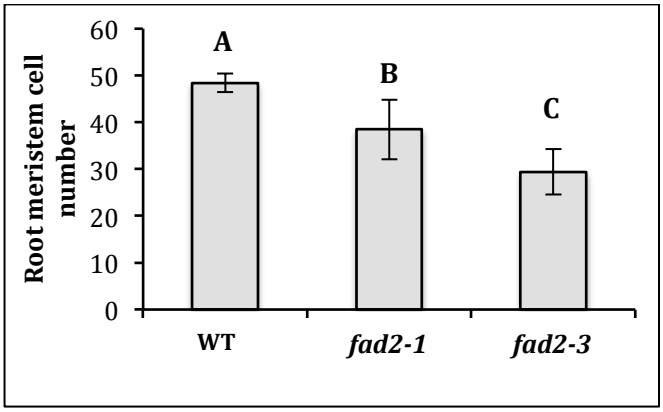
total ROS-ID solution also indicated increased fluorescence in diffrentiation zone of both *fad2* mutants' roots (Fig.3.6, **B)**.

The next aim of this work was to identify which cell types were affected by low DUFA levels in the roots of *fad2* mutants. For this purpose, we measured root meristem cell number (cells from quiescent center to the elongational zone) and cortex cells (Fig.3.7).

A



B



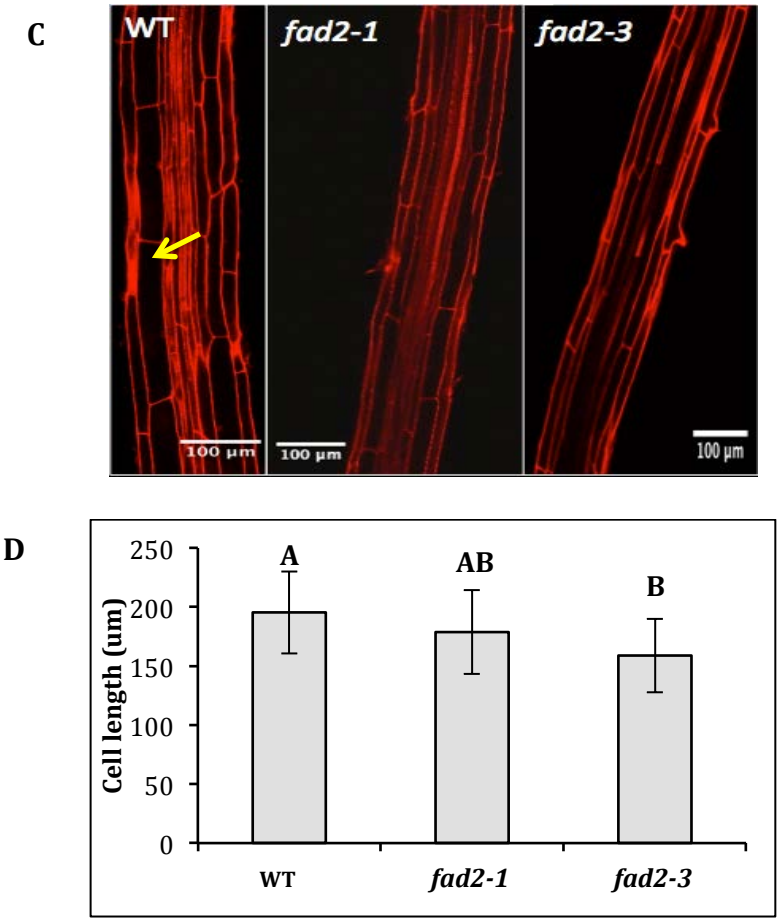


Figure 3.7. Morphological analysis of 7-day-old WT and *fad2* roots. (**A-B**) Primary root meristem visualization and cell number quantification. (**C**) Cortex cell visualization, the arrow indicates cells of interest. (**D**) Measurements of cortex cell lengths. Bars represent the means of 10 roots (± SD). Letters indicate statistically significant differences between pairs as determined by Tukey's HSD test ($p < 0.05$).

Analysis of the meristematic and elongation zones of WT and *fad2* mutants revealed significant changes in both regions. Interestingly, stronger differences were observed in meristematic zone where cell number was reduced by 20% in *fad2-1* and 39% in *fad2-3* mutants. The elongation zone was not significantly affected in *fad2-1* mutants compared to the WT plants, but showed 18% reduction in *fad2-3* seedlings. In conclusion, reduced root length in *fad2-1*

mutants is caused primarily by a reduced number of meristematic cells. The *fad2-3* mutants had stronger decrease in root length compared to WT and *fad2-1* seedlings. This phenotype could be explained by reduction in both the number of meristematic cells and a reduction of cortex cell lengt that is caused by deficiency in PUFAs.

III.2. DUFAs and their role in root growth in stress conditions.

Arid and semi-arid territories are increasing yearly and amount to more than 40% of the land surface (Shanon, 1986). Fertilizers and climate change are two of the main factors leading to such modifications. Aridification is often associated to an over-accumulation of salt and minerals in the soil (Jiang *et al*., 2016), which can be even more challenging for plants. However, some plants are able to partly cope with increased salinity. For example, it was shown, that *Arabidopsis* seedlings can grow at relatively mild salt levels (<100 mM; Jiang *et al*., 2016), but once the salinity levels increase the plant cannot function normally. For example, high salt levels lead to reduced *Arabidopsis* root lengths through decreases in cell elongation (Potters *et al.,* 2007; Bernstein, 2013), reduced number of meristematic cells (West *et al*., 2004), as well as changes in root architecture (Julkowska *et al.,* 2014) and gravity responses (Sun *et al.,* 2008).

Root development during salt treatment depends on multiple factors including hormonal changes, such as upregulation of abscisic acid (ABA) and down regulation auxin (IAA) (Hernandez *et al.,* 2010), but one general mechanism has been described for all of them – induction of ROS (Bernstein, 2010; Hernandez *et al.,* 2010). Due to the high potential toxicity of ROS, plants have developed defence mechanisms, e. g. the production of ROS-detoxifying enzymes (Dat *et al*., 2000; Apel and Hirt, 2004). Recently, it has been shown that salt treatment down-regulates auxin signalling in roots by stabilization of Aux/IAA repressors (Iglesias *et al.,* 2014). The aim of this part was to deduce the role of DUFAs in root adaptation to salinity stress, by growing WT and *fad2* seedlings in 100 mM NaCl media or exposing them to salt for brief periods (1 hour).

For the first part of this research we grew WT, *fad2-1* and *fad2-3 Arabidopsis* seedlings on 100 mM NaCl media and then analysed their root length (Fig.3.8).

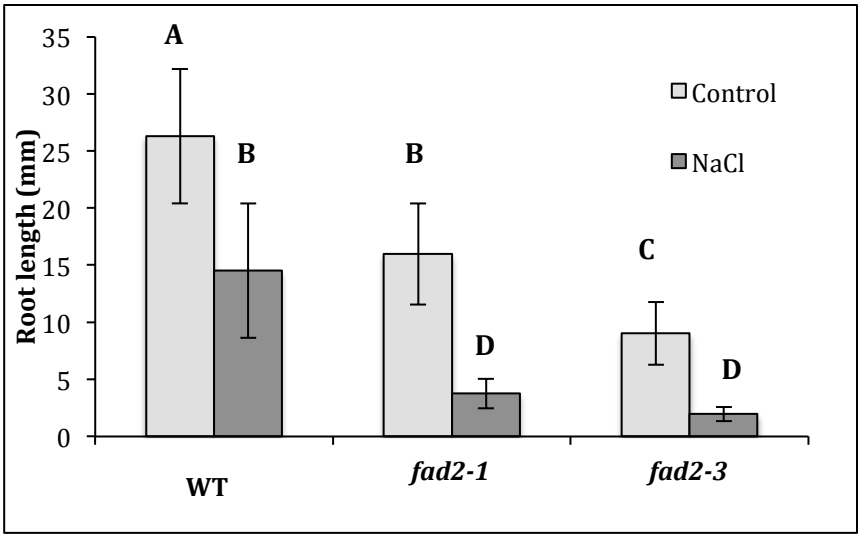
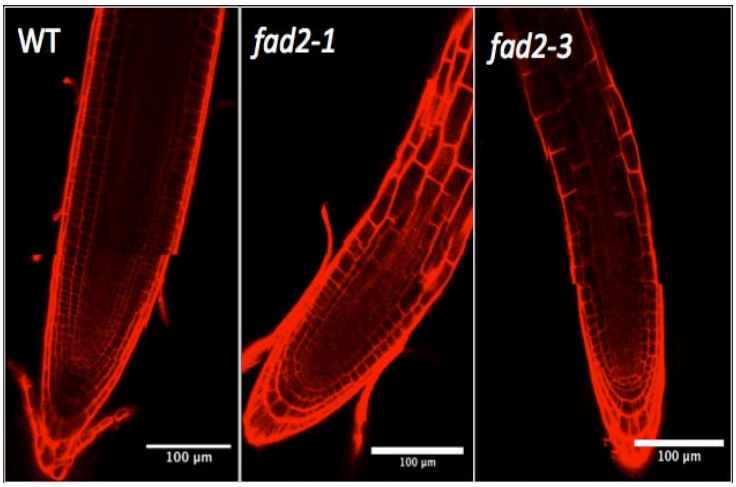


Figure 3.8. Root length analysis of 7-day-old *Arabidopsis* seedlings growing in control and salt-rich media. Bars represent the means of 3 independent experiments; each contains a pool of ~ 60 seedlings. Letters indicate statistically significant differences between pairs as determined by Tukey's HSD test ($p < 0.05$).

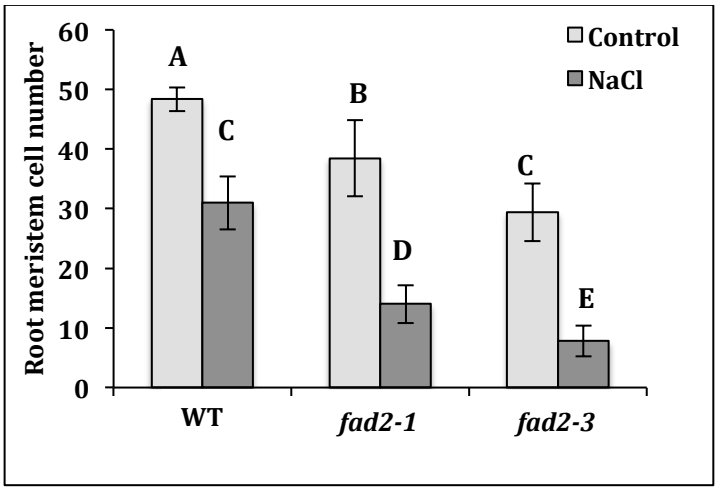
Constant exposure to salt led to a strong reduction in the growth of WT and *fad2* roots. In the case of WT plants, salt treatment led to a 45% reduced root length compared to the control conditions. In *fad2* mutants this effect was even stronger, leading to respectively 86 and 94% reduced root length in *fad2-1* and *fad2-3* mutants, hence supporting the earlier findings on strong susceptibility to salt treatment in *fad2* mutants (Zhang *et al.,* 2012).

The next part of this project was to localize the root zone associated with length reduction upon salt treatment. For this purpose plants were grown for 7-days on media containing 100 mM NaCl. Meristematic and elongation zones were analysed (Fig. 3.9).

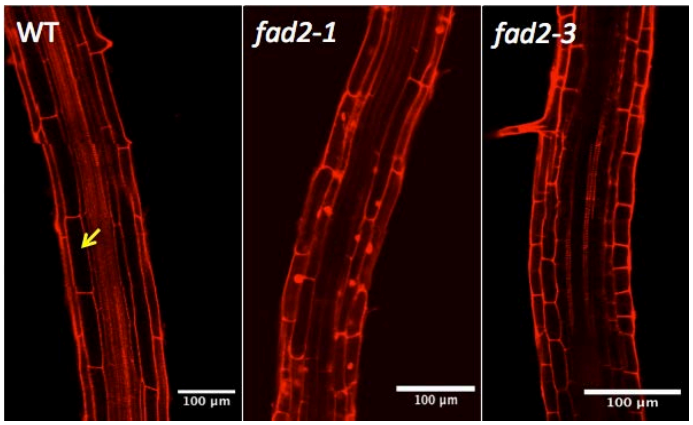
A


WT
fad2-1
fad2-3
100 µm
100 µm
100 µm

B


Root meristem cell number
60
50
40
30
20
10
0
A
C
B
D
C
E
Control
NaCl
WT
fad2-1
fad2-3

C


WT
fad2-1
fad2-3
100 µm
100 µm
100 µm

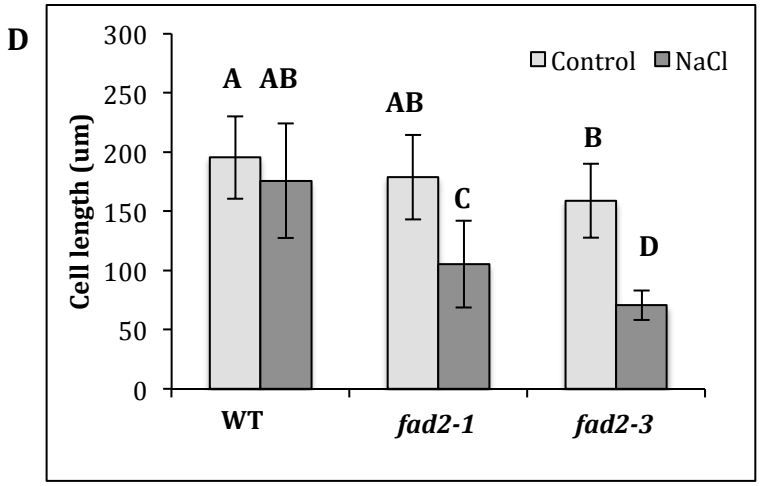


Figure 3.9. Morphological analysis of 7-day-old roots of WT and *fad2* seedlings grown in control and salt media were visualized with propidium iodide (PI) staining. (**A**-**B**) Primary root meristem visualization and cell number quantification and comparison with control conditions. (**C**) Cortex cell visualization, the arrows indicate cells of interest. (**A, C**) Scale bar 100 μm.(**D**) Measurements of the cortex cells grown in salt media. Bars represent the means of 10 roots (±SD). Letters indicate statistically significant differences between pairs as determined by Tukey's HSD test ($p < 0.05$).

The results obtained revealed that root length in the WT plants was reduced upon salt treatment, due to a 37% reduction in the number of meristematic cells. Changes in cell sizes in elongation zones were not significant. In *fad2* mutants, root growth was affected in both meristematic and elongation zones. Relative to the WT, *fad2-1* mutants showed decreases in the number of meristematic cells from 79% in control to 29% in salt conditions and in cortex cell length from 91 to 53%. Similarly to the changes observed in the *fad2-1* mutant, in *fad2-3* roots the number of meristematic cells was reduced from 60% in control to 16% in salt - treated conditions. The strongest reduction in

cortex cell length was observed in *fad2-3* mutants, showing a decrease from 81 to 36% in treated conditions relative to the WT.

For the next step of this work, we decided to use the ROS-ID total ROS/Superoxide detection kit to compare WT and *fad2-1* seedlings in three different conditions: control, transfer to salt media for 1 h followed by visualization, and growth on salt media (Fig. 3.10). We performed a time course assay to find highest ROS induction in WT seedlings upon salt treatment. 1 h treatment showed higher fluorescence in roots. Hence, it was used to detect early root responses to salt stress in combination with other treatments.

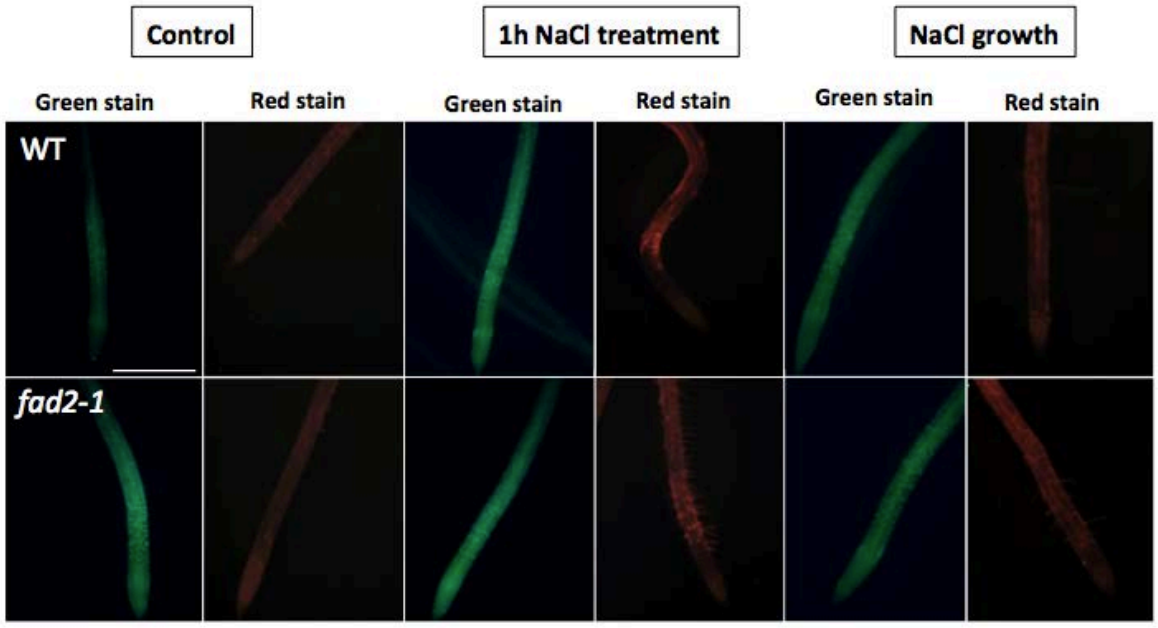


Figure 3.10. ROS staining of 7-day old *Arabidopsis* roots. WT and *fad2-1* roots were grown in 3 different conditions, followed by ROS staining. Hydrogen peroxide, peroxynitrite and hydroxyl radicals were visualized at wavelengths 490 nm /525 nm (Ex/Em) and labelled 'green stain', superoxide 'red stain' was

detected at 550 nm /620 nm (Ex/Em). Each photo is representative of the six biological replicates. Scale bar – 0.5 mm.

The difference between ROS-associated fluorescence in WT control and salt treatment is shown in Figure 3.9. In the control, green associated fluorescence is mainly localized in elongation zone and superoxide is mildly induced all along the root. ROS-associated fluorescence in the WT plants grown on salt media was equally spread along the root with stronger fluorescence than in the control conditions. A one-hour salt treatment lead to the strongest ROS induction compared to the other treatments in both WT and *fad2-1* mutant, with no differences in ROS localization. Green fluorescence was induced over the whole root, whereas red was mainly induced in the elongation root zone. MDA measurenments and ROS staining showed that roots of *fad2-1* mutants have higher overall ROS levels in control conditions, compare to the WT plants. The *fad2-1* seedlings grown on salt-rich media displayed no difference to the control conditions for green stain, but the *fad2-1* mutant had higher superoxide levels. These results led us to the idea to test the expression of the detoxification genes. For this reason, we used genes that were induced upon 75 μM phytoprostane A_1 treatment that is reported to inhibit root growth and cell division (Mueller *et al.,* 2008). Two detoxification genes that showed more than 100 fold induction in that publication were chosen for this work: cytochrome P450 family protein (*CYP81D11*) and UDP-glucoronosyl/ UDP-

glucosyl transferase family (*UGT73B3*). Their expression was checked in three different conditions in WT and *fad2* mutants (Figure 3.11).

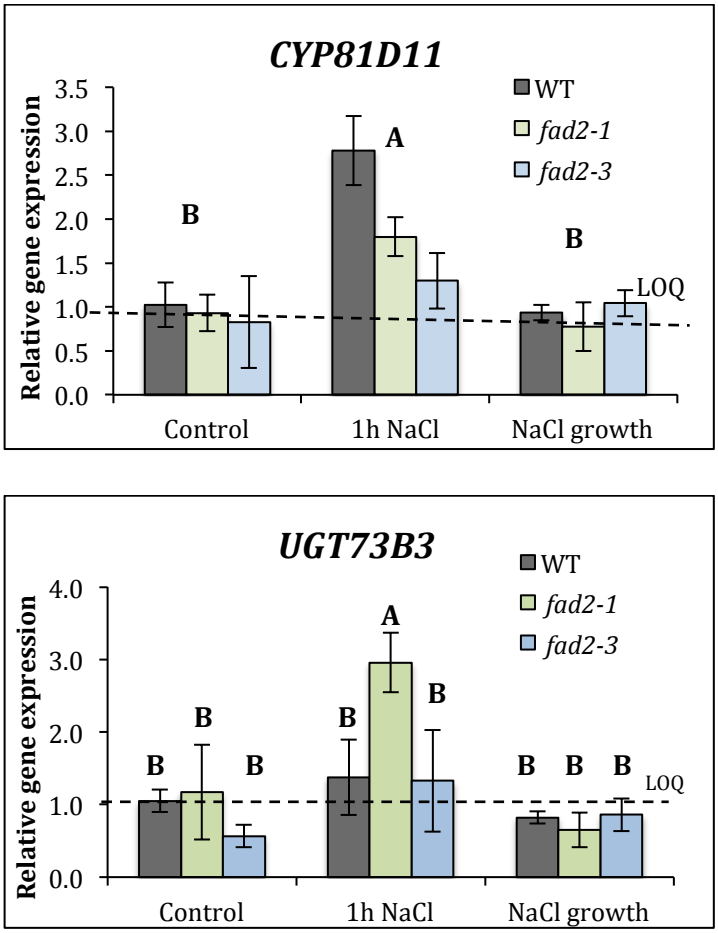


Figure 3.11. Relative expression of *CYP81D11* and *UGT73B3* genes in 7-day-old seedlings. Transcriptional levels of detoxification genes were normalized to those of *UBC21*. Bars represent the means of 4 biological replicates; each contains a pool of ≈ 30 (control and 1h salt treatment) to 120 (salt grown) seedlings. Letters indicate statistically significant differences between pairs as determined by Tukey's HSD test ($p < 0.05$).

These results indicate induction of both detoxification genes upon 1h salt treatment in WT and *fad2* mutants. Surprisingly, they showed different expression rates: *CYP81D11* had higher levels upon 1h salt treatment in WT,

whereas *UGT73B3* was expressed most highly in *fad2-1* after one hour exposure to salt. Interestingly, expression of the *UGT73B3* gene under control conditions was lower in *fad2-3* mutants compared to the WT seedlings. No difference in gene expression was found between WT and *fad2* mutants grown on salt for 7-days. These findings suggest that adaptation to salt stress is a very complicated process that requires multiple levels of plant adaptation.

III.3. Discussion.

PUFAs play an important role in plant adaptation to stresses, by forming membrane bilayers and working as substrates for hormone production and buffering ROS levels (Vick and Zimmerman, 1984; Routaboul *et al.,* 2000; Falcone *et al.,* 2004, Zhang *et al*., 2009; Zhang *et al*., 2012; Schmid-Siegert *et al*., 2012; 2016). Plants deficient in TUFAs showed no major changes in growth and development, other than a male sterility phenotype due to the absence of jasmonic acid (McConn and Browse, 1996). Currently, there is almost no information available on the role of DUFAs in plant development. This is was why we decided to base our research on the root growth of *fad2* mutants.

***fad2* mutants have shorter root lengths than the WT in control conditions**. Plants lacking both di- and tri-unsaturated fatty acids (*fad2-2 fad6* double

mutants) were shown to be unviable on soil, whereas if only one PUFA is missing they can grow normally (McConn and Browse, 1998). In this work we analysed the fatty acid composition analyses of *fad2* roots. This revealed significant differences to the WT plants. 2% of PUFAs and 68% of MUFAs from the overall extracted fatty acids were detected in both *fad2* roots, compared to 55% of PUFAs and no detected levels of MUFAs in WT plants. These findings lead us to the assumption that overall ROS levels might be higher in *fad2* mutants, due to their deficiency in PUFAs, which may prevent them from buffering ROS (Mene-Saffrane *et al.,* 2009, Schmid-Siegert, *et al.,* 2016). ROS staining assays and malondialdehyde measurements provided some support for this hypothesis. Our results are also consistent with the hypothesis that high levels of H_2O_2 and O^-_2 can lead to reduced root growth in *Arabidopsis* (Swanson and Gilroy, 2010; Choudhury *et al.,* 2013).

***fad2* mutants are more susceptible to salt treatment compared to WT plants.** An earlier study has shown an important role of the *FAD2* enzyme in the function of Na^+/H^+ exchangers. Roots of *fad2* mutants were susceptible to salt treatment and had high cytoplasmic levels of Na^+ (Zhang *et al*., 2012). Similarly, our study confirmed that *fad2* mutants are sensitive to salt and provided further insights into the effect of salt and growth.

Based on our findings, the short root length of *fad2* mutants growing on salt media was caused by a reduced number of meristematic cells and reduced cortical cell lengths. This effect could be related to changes in redox status and hormone signalling.

Salt treatment induces ROS accumulation in *Arabidopsis* roots, leading to a more oxidized redox status, which in turn may cause the reduction in meristem cell number (Jiang *et al.,* 2016). Based on the results of root staining, *fad2* mutants grown on salt media have overall higher ROS levels and a stronger reduction in the number of meristematic cells than in WT plants.

Reduced root cortex cell length is observed in *Arabidopsis* plants exposed to salt treatment (Jiang *et al.,* 2016). This correlated with high levels of abscisic acid (ABA) in the elongation zone (Duan *et al.*, 2013; Ondzighi-Assoume *et al.,* 2016). Unfortunately, we did not perform a quantitative analysis of ABA levels. However, our germination assay on WT and *fad2* mutants (Fig.3.12) is consistent with the fact that plants with upregulated levels of ABA are known to have delayed germination rates compared to the WT (Kermode, 2005; Bentsink and Soppe, 2008).

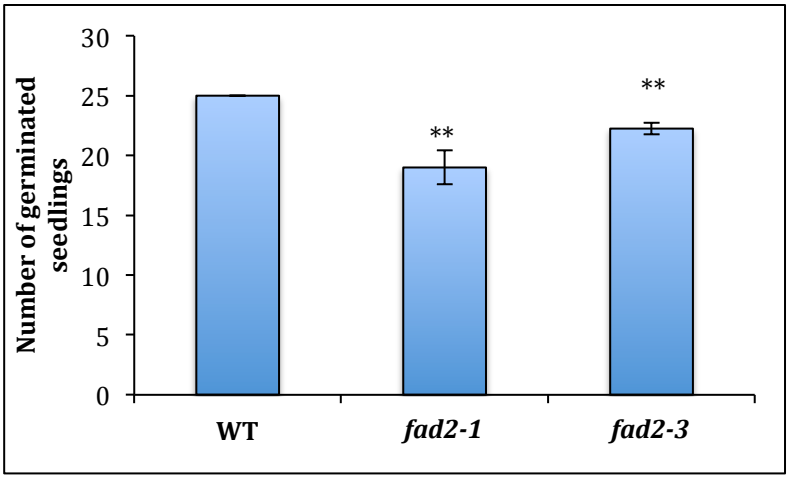


Figure 3.12. Germination of *Arabidopsis* seeds (assessed 1 day post-germination). Bars represent the means of 3 biological replicates; each contains a pool of 25 seeds. Statistical significance in pair-wise comparison was evaluated by Student's test, where ** $p \leq 0.01$.

Indeed, we found that seed germination rate was reduced respectively by 24% and 11% in *fad2-1* and *fad2-3* mutants relative to WT. These results might be related to changes in ABA levels of *fad2* seedlings. However, this hypothesis would require further testing.

III.4. Experimental procedures.

Plant growth conditions, genotypes and chemicals - Wild-type *Arabidopsis* (WT *Columbia* background) was used. The following alleles in the *Columbia* background were received from the *Arabidopsis* Biological Resource Centre: *fad2-1* (CS8041), *fad2-3* (SK18137) and *fad3-2 fad7-2 fad8* mutant seeds from McConn and Browse (1996). Seeds were stratified for 2 days at 4 ^{0}C and then

grown at 21 ^{0}C under 100 µE m^{-2} s^{-1} of light with photoperiod depending on the application (seedlings: 14 h light, 10 h dark (long days); on soil for seed propagation: 24 h light (continuous days) or experiments with adult plants at: 9 h light, 15 h dark (short day)). Seedlings were grown on half-strength Murashige and Skoog solid media (½ MS, 2.15 g/L, pH 5.7; Duhefa Biochemie, Haarlem, The Nederlands) supplemented with 0.5 g/L of MES hydrate (Sigma, Buchs, Switzerland) and 0.7 % agar.

Root phenotypic analysis – plants were grown vertically for 7-days on half-strength Murashige and Skoog solid media (½ MS) or 100 mM NaCl in ½ MS media. Photos were taken and analysed by ImageJ (https://imagej.nih.gov), 60 seedlings were used for one biological replicate. Root cell measurements were performed as described in Acosta *et al.* (2013).

Fatty acid composition analysis – roots of 7-day-old *Arabidopsis* seedlings (≈ 30 mg) were placed in 15ml Pyrex SVL capped test tubes (Milian, Switzerland). 1ml of 2.5% (v/v) H_2SO_4 in methanol was added to the samples. Samples were incubated for 90 min at 80 ^{0}C, 1.5 ml 0.9 % NaCl and 1 ml of hexane (≥97.0% (GC), Sigma-Aldrich, Buchs, Switzerland) were added. The solution was vortexed briefly (2 sec) then centrifuged for 5 min at 1000 rpm. 250 µl of the upper phase containing fatty acid methyl esters were collected. 1 µl of a 1:5 dilution in hexane was analyzed by GCMS (initial T = 150 ^{0}C for 3

min, followed by a ramp 150 ^{0}C to 200 ^{0}C at 10 ^{0}C per minute).

Gas chromatography/Mass Spectrometry – 7-day-old roots (≈ 150 mg) were harvested in liquid nitrogen and ground for 15 sec at 3000 rpm/sec in TissueLyser II (Quiagen, Hombrechtikon, Switzerland). MDA levels were measured by a gas chromatography/ mass spectrometry with D_2-MDA internal standard generated from ($2D_2$)-1,1,3,3-tetraethoxypropan as described (Weber *et al.,* 2004).

Salt assay – 7-day-old *Arabidopsis* seedlings were subjected to salt stress. In one case, seedlings were permanently grown in ½ MS media complimented with 100 mM NaCl (Shi *et al.,* 2000). In the second case, to detect early changes upon salt stresses, plants were grown for 7-days on ½ MS followed by transfer and 1h incubation on ½ MS media containing 100 mM NaCl.

Germination assay – Arabidopsis seeds were planted on ½ MS media and left in a long day conditions for 24 h, followed by quantification of the emerged roots.

Gene expression analysis – 7-day-old *Arabidopsis* roots were collected after 3 different treatments: control (Growing on half-strength MS plates), 1 h 100 mM NaCl treatment and permanent growth on ½ MS plates containing 100

mM salt. Finally, seedlings were collected and stored in liquid nitrogen. 30 to 50 seedlings were used per one biological replicate. RNA isolation and quantitative RT-PCR was performed as described earlier (Gfeller *et al.*, 2011). *CYP81D11* and *UGT73B3* transcripts were quantified as described earlier (Köster *et al*., 2012; Langlois-Meurinne *et al.*, 2005).

ROS detection assay – 7-day-old *Arabidopsis* roots were stained with two different ROS indicators: ROS-ID™ Total ROS/Superoxide Detection Kit (Enzo Life Sciences AG, Lausanne, Switzerland) and 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA; Sigma-Aldrich, Buchs, Switzerland). For the first solution, roots were vacuum infiltrated with ROS-ID™ Total ROS/Superoxide Detection Kit for 15 min under dark conditions followed by visualization with Leica MZ16A stereomicroscope. Signals from hydrogen peroxide, peroxynitrite and/or hydroxyl radicals were visualized at wavelengths 490 nm /525 nm (Ex/Em) and labelled 'green stain'. Superoxide 'red stain' was detected at 550 nm /620 nm (Ex/Em). Second solution - 15 μM H2DCFDA in water was used to visualize living roots (Oyama *et al.,* 1994) at wavelengths 495 nm /527 nm (Ex/Em) using Zeiss 200 BIOP confocal microscope.

Statistical analysis - Statistical significance in pair-wise comparison was evaluated by Student's test, where * $p \leq 0.05$; ** $p \leq 0.01$; ***$p \leq 0.001$,

multiple comparison analysis of variances (ANOVA) followed by Turkey's HSD test was performed using JMP – statistical analysis software.

III.5. Literature.

Acosta IF, Gasperini D, Chetelat A, Stolz S, Santuari L, Farmer EE. 2013. Role of *NINJA* in root jasmonate signaling. *Proc Natl Acad Sci USA*. 110(38):1547-1578.

Ambrozova G, Pekarova M, Lojek A. 2010. Effect of polyunsaturated fatty acids on the reactive oxygen and nitrogen species production by raw 264.7 macrophages. *Eur J Nutr*. 49(3) :133-139.

Apel K and Hirt H. 2004. Reactive oxygen species: metabolism, oxidative stress and signal transduction. *Annu. Rev. Plant Biol.* 55:373-399.

Beemster GT, Baskin TI. 1998. Analysis of cell division and elongation underlying the developmental acceleration of root growth in *Arabidopsis thaliana*. *Plant Physiol*. 116:1515–1526.

Bentsink L and Soppe WJJ. 2008. Molecular networks regulating *Arabidopsis* seed maturation, after-ripening, dormancy and germination. *New Phytologist.* 179:33–54.

Bernstein N. 2010. Involvement of the plant antioxidative response in the differential growth sensitivity to salinity of leaves vs. roots during call development. *Free Radic. Biol. Med.* 49:1161-1171.

Bernstein N. 2013. Effects of salinity on root growth plant roots. *Plant Roots: the hidden Half, 4th Edn.* 36:1-18.

Bu Q, Jiang H, Li CB, Zhai Q, Zhang J, Wu X, Sun J, Xie Q, Li C. 2008. Role of the *Arabidopsis thaliana* NAC transcription factors *ANAC019* and *ANAC055* in regulating jasmonic acid-signaled defense responses. *Cell Res.* 18:756–767.

Cano-Delgado A, Lee JY, Demura T. 2010. Regulatory mechanisms for specification and patterning of plant vascular tissues. *Annu Rev Cell Dev Biol*. 26:605-637.

Choudhury S, Panda P, Sahoo L, Panda SK. 2013. Reactive oxygen species signaling in plants under abiotic stress. *Plant Signal Behav*. 8:e2368.

Choudhury S, Panda P, Sahoo L, Panda SK. Reactive oxygen species signaling in plants under abiotic stress. *Plant Signal Behav*. 8: e23681.

Claeys, H.; De Bodt, S.; Inzé, D. 2013. Gibberellins and DELLAs: Central nodes in growth regulatory networks. *Trends Plant Sci.* 19:231–239.

Dat J, Vandenabeele S, Vranova E, Van Montagu M, Inze D, Van Breusegen F. 2000. Dual action of the active oxygen species during plant stress responses. *Cell Mol. Life Sci.* 57:779-995.

De Smet S, Cuypers A, Vangronsveld J, Remans T. 2015. Gene networks involved in hormonal control of root development in *Arabidopsis thaliana*: a framework for studying its disturbance by metal stress. *Int. J. Mol. Sci.* 16:19195-19224.

Duan L, Dietrich D, Ng CH, Chan MY, Bhalerao R, Bennett MJ, Dinneny JR. 2013. Endodermal ABA Signaling Promotes Lateral Root Quiescence during Salt Stress in *Arabidopsis* Seedlings. *The Plant Cell*. 25: 324–341.

Esterbauer H, Schaur R J, Zollner H. 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic. Biol. Med.* 11:81–128.

Falcone DL, Ogas JP, Somerville CR. 2004. Regulation of membrane fatty acid composition by temperature in mutants of *Arabidopsis* with alterations in membrane lipid composition. *BMC Plant Biol*. 4:17.

Farmer EE and Mueller MJ. 2013. ROS-mediated lipid peroxidation and RES-activated signalling. *Annu. Rev. Plant Biol.* 64:429-450.

Fridovich I. 1986. Superoxide dismutases. A*dv.in Enzymol.* 58:61-97.

Fukai T and Ushio-Fukai M. 2011. Superoxide dismutases: role in redox signaling, vascular formation, and diseases. *Antioxid Redox Signal*. 15(6) :1583-1606.

Gfeller A, Baerenfaller K, Loscos J, Chetelat A, Baginsky S, Farmer EE. 2011. Jasmonate controls polypeptide patterning in undamaged tissue in wounded *Arabidopsis* leaves. *Plant Physiol.* 156(4):1797-1807.

Guzman, P.; Ecker, J. 1990. Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. *Plant Cell*. 2:513–523.

Halliwell B. 2006. Reactive species and antioxidants. Redox biology is a

fundamental theme of aerobic life. *Plant Physiol.* 141:312–322.

Hernandez M, Fernandez-Garcia N, Diaz-Vivancos P, Olmos E. 2010. A different role for hydrogen peroxide and the antioxidative system under short and long salt stress in *Brassica oleracea* roots. *J. Exp. Bot.* 61:521-535.

Iglesias MJ, Terrile MC, Windels D, Lombardo MC, Bartoli CG, Vazquez F, Estelle M, Casalongue CA. 2014. MiR393 regulation of auxin signaling and redox-related components during acclimation to salinity in *Arabidopsis*. *PloS One.* 15;9(9):e107678.

Jiang K, Moe-Lange J, Hennet L, Feldman LJ. 2016. Salt stress affects the redox status of *Arabidopis* root meristems. *Front Plant Sci.* 7:1-10.

Julkowska MM, Hoefsloot HCJ, Mol S, Feron R, de Boer GJ, Haring MA, Testerink C. 2014. Capturing *Arabidopsis* root architecture dynamics with ROOT-FIT reveals diversity in responses to salinity. *Plant Physiol.* 166(3):1378-1402.

Kermode AR. 2005. Role of abscisic acid in seed dormancy. *J. Plant Growth Regul.* 24:319-344.

Köster J, Thurow C, Kruse K, Meier A, Iven T, Feussner I, Gatz C. 2012. Xenobiotic- and jasmonic acid-inducible signal transduction pathways have become interdependent at the Arabidopsis *CYP81D11* promoter. *Plant Physiol.* 159(1):391-402.

Krieger-Liszkay A. 2005. Singlet oxygen production in photosynthesis. *J. Exp. Bot.* 56:337–346.

Langlois-Meurinne M, Gachon CMM, Saindrenan P. 2005. Pathogen-responsive expression of glycosyltransferase genes *UGT73B3-and UGT73B5* is necessary for resistance to *Pseudomonas syringae* pv tomato in *Arabidopsis*. *Plant Physiol.* 139(4):1890-1901.

Laplaze L, Benkova E, Casimiro I, Maes L, Vanneste S, Swarup R, Weijers D, Calvo V, Parizot B, Herrera-Rodriguez MB, Offringa R, Graham N, Doumas P, Frimi J, Bousz D, Beeckman T, Bennet M. 2007. Cytokinins act directly on lateral root founder cells to inhibit root initiation. *Plant Cell*. 19:3889–3900.

Liszkay A, van der Zalm E, Schopfer P. 2004. Production of reactive oxygen intermediates ($O^{2\bullet-}$, H_2O_2 and OH) by maize roots and their role in wall loosening and elongation growth. *Plant Physiol.* 136:1-10.

McConn M and Browse J. 1996. The critical requirement for linolenic acid is pollen development, not photosynthesis, in *Arabidopsis* mutant. *Plant Cell*. 8(3):403-416.

McConn M and Browse J. 1998. Polyunsaturated membranes are required for photosynthetic competence in a mutant of *Arabidopsis*. *Plant J.* 15(4): 521-530.

Mendrinna A and Persson S. 2015. Root hair growth: it's a one way street. *F1000Prime Rep*. 7:23.

Mène-Saffrané L, Dubugnon L, Chételat A, Stolz S, Gouhier-Darimont C, Farmer E. E. 2009. Nonenzymatic oxidation of trienoic fatty acids contributes to reactive oxygen species management in Arabidopsis. *J. Biol. Chem.* 284:1702–1708.

Mène-Saffrané L, Dubugnon L, Chételat A, Stolz S, Gouhier-Darimont C, Farmer EE. 2009. Nonenzymatic oxidation of trienoic fatty acids contributes to reactive oxygen species management in *Arabidopsis*. *J. Biol. Chem*. 284(3):1702-1708.

Montine TJ, Neely MD, Quinn JF, Beal MF, Markesbery WR, Roberts LJ, Marrow JD. 2002. Lipid peroxidation in aging brain and Alzheimer's disease. *Free Radic. Biol. Med.* 33:620-626.

Mueller S, Hilbert B, Dueckershoff K, Roitsch T, Krischke M, Mueller MJ, Berger S. 2008. General detoxification and stress responses are mediated by oxidized lipids through TGA transcription factors in Arabidopsis. *The Plant Cell*. 20:768-785.

O'Brien JA, Daudi A, Butt VS, Bolwell GP. 2012. Reactive oxygen species and their role in plant defence and cell wall metabolism. *Planta*. 236:765-779.

Ondzighi-Assoume CA, Chakraborty S, Harris MJ. 2016. Environmental nitrate stimulates abscisic acid accumulation in *Arabidopsis* root tips by releasing it from inactive stores. *The Plant Cell*. 28:729:745.

Oyama Y, Hayashi A, Ueha T, Maekawa K. 1994. Characterization of 2,7-dichlorofluorescin fluorescence in dissociated mammalian brain neurons: estimation on intracellular content of hydrogen peroxide. *Brain Res.* 635(1-2):113-117.

Potters G, Pasternak TP, Guisez Y, Plame KJ, Jansen MA. 2007. Stress-induced morphogenic responses: growing out of trouble? *Trends Plant Sci.* 12:98-105.

Routaboul J-M, Fischer SF, Browse J. 2000. Trienoic fatty acids are required to maintain chloroplast function at low temperatures. *Plant Physiol*. 124(4):1697-1705.

Schmid-Siegert E, Loscos J. 2012. Inducible malondialdehyde pools in zones of cell proliferation and developing tissues in *Arabidopsis. J. Biol. Chem.* 287: 8954-8962.

Schmid-Siegert E, Stepushchenko O, Glauser G, Farmer E.E. 2016. Membranes as structural antioxidants: Recycling of malondialdehyde to its source in oxidation-sensitive chloroplast fatty acids. *J. Biol. Chem.* 291: 13005-13013.

Shanon MC. 1986. New insights in plant breeding efforts for improved salt tolerance. *Hort. Technol*. 6:96–99.

Shi H, Ishitani M, Kim C, Zhu JK. 2000. The *Arabidopsis thaliana* salt tolerance gene SOS1 encodes a putative Na^+/H^+ antiporter. *Proc Natl Acad Sci USA*. 97(12):6896-6901.

Spiteller G. 1996. Enzymic lipid peroxidation a consequence of cell injury. *Free Radic. Biol. Med.* 21:1003-1009.

Sun F, Zhang W, Hu H, Li B, Wang Y, Zhao Y, Li K, Liu M, Li X. 2008. Salt modulates gravity signaling pathway to regulate growth direction of primary roots in *Arabidopsis*. *Plant Physiol.* 146(1):178-188.

Swanson S and Gilroy S. 2010. ROS in plant development. *Physiol Plant.*

138:384–392.

Swanson S and Gilroy, S. 2010. ROS in plant development. *Physiol Plant.* 138:384–392.

Triantaphylides C, Havaux M. 2009. Singlet oxygen in plants: production, detoxification and signaling. *Trends Plant Sci*. 14:219–228.

Triantaphylides C, Krischke M, Hoeberichts FA, Ksas B, Gresser G, Havaux M, Van Breusegem F, Mueller MJ. 2008. Singlet oxygen is the major reactive oxygen species involved in photooxidative damage to plants. *Plant Physiol*. 148:960–968.

Tsukagoshi H, Busch W, Benfey, PN. 2010. Transcriptional regulation of ROS controls transition from proliferation to differentiation in the root. *Cell*. 143:606–616.

Tsukagoshi H. 2012. Defective root growth triggered by oxidative stress is controlled through the expression of cell cycle-related genes. *Plant Sci*. 197:30-39.

Vanstraelen M, Benkova E. 2012. Hormonal interactions in the regulation of plant development. *Annu Rev Cell Dev Biol*. 28:463-487.

Vick BA and Zimmerman DC. 10984. Biosynthesis of jasmonic acid by several plant species. *Plant Physiol.* 75(2):458-461.

Wang ZY. 2012. Brassinosteroids modulate plant immunity at multiple levels. *Proc. Natl. Acad. Sci. USA.* 109:7–8.

Weber H, Chételat A, Reymond P, Farmer EE. 2004. Selective and powerful stress gene expression in *Arabidopsis* in response to malondialdehyde. *Plant J.* 37:877–888.

West G, Inze D, Beemster GTS. 2004. Cell cycle modulation in the response of the primary root of *Arabidopsis* to salt stress. *Plant Physiol*. 135:1050-1058.

Wojciechowsk WV. 2014. Respiratory care sciences: an integrated approach. *Cengage learning*; 5th edition. 752.

Yoshimitsu Y, Tanaka K, Fukuda W, Asami T, Yoshida S, Hayashi K, Kamiya Y, Jikumaru Y, Shigeta T, Nakamura Y, Matsuo T, Okamoto S. Transcription of *DWARF4* plays a crucial role in auxin-regulated root elongation in addition to brassinosteroid homeostasis in *Arabidopsis thaliana*. *PLoS ONE*. 6:e23851.

Zhang J, Liu H, Sun J, Li B, Zhu Q, Chen S, Zhang H. 2012. *Arabidopsis* fatty acid desaturase *FAD2* is require for salt tolerance during seed germination and early seedling growth. *PLoS One.* 7:e30355.

Zhang JT, Zhu JQ, Zhu Q, Liu H, Gao XS, Zhang HX. 2009. Fatty acid desaturase-6 (*FAD6*) is required for salt tolerance in *Arabidopsis thailana. Biochem. Biophys. Res. Commun.* 390: 469-474.

Chapter IV: PUFAs and their role in susceptibility to *Botrytis cinerea.*

IV.1. Screening for *fad* mutant resistant to *B. cinerea* in 7 and 14 day-old plants.

Plant-fungal interactions are considered to be as old as the terrestrial vascular plants. According to evolutionary studies, the colonization of the land by plants happened with the help of fungi 400 - 460 million years ago (Redecker *et al.,* 2000; Remy *et al.,* 1994). Most plant-fungal interactions are considered to be symbiotic, promoting growth and stress tolerance in plants and providing carbohydrates to the fungus (Buscot *et al.,* 2000). However, some fungal species became plant pathogens and these have led to fast evolution of resistance mechanisms in the plant kingdom (Jones and Dangl, 2006).

B. cinerea is one of the most studied and abundant necrotrophic pathogens that affects more than 200 plant species and causes serious agricultural loss each year (Jarvis, 1977). The efficiency of this fungus is caused by its rapid attachment on the host surface and fast germ tube development that promotes penetration (Epstein *et al.,* 1997). The asexual spores of *B. cinerea* (conidia) are produced on conidiophores and are easily dispersed in nature. Conidial attachment occurs in two phases: immediate adhesion – caused by hydration with the help of weak adhesive forces (Doss et al., 1993) and delayed adhesion – induced by secretion of extracellular matrix-degrading enzymes a couple of hours after (Doss *et al.,* 1995; Doss *et al*., 1999). Development of the germ tubes depends on multiple factors, such as: surface hardness, hydrophobicity,

topography and structural components. If some of these factors are missing, germ tube growth is arrested, if not, the fungus forms an appressorium as a next step of its development (De Zwaan *et al*., 1999; Staples *et al.,* 1987; Talbot *et al.,* 1996; Wessels *et al.,* 1991; Tucker *et al.,* 2001).

Plants, on the other hand, have developed several defense mechanisms against necrotrophic fungi such as *B. cinerea*: a) prevention of pathogen penetration (Fleishman *et al,* 1995); b) increased levels of reactive oxygen species (ROS; Malolepsza and Urbanek, 2002); c) induction of defense hormones, such as jasmonate (Thomma *et al.,* 1998), ethylene (ET; Feys *et al.,* 2000), salicylic (Audenaert *et al.,* 2002) and abscisic acids (ABA; Pandey *et al.,* 2005).

This study aimed to uncover other possible sources of defense against *B. cinerea*. Diunsaturated fatty acids were chosen as possible candidates due to their strong involvement in cuticle synthesis, that forms the first layer of defense against many pathogens. We chose 7 and 14 day-old *Arabidopsis* seedlings to study the role of DUFAs in *B. cinerea* resistance.

To address these issues, three *Arabidopsis* oleate desaturase mutants were used: two alleles of FAD2 (*fad2-1*, *fad2-3)* unable to synthetize 18:2 FAs in the endoplasmic reticulum, and one allele of FAD6 (*fad6*) that prevents the generation of 16:2 and 18:2 fatty acids in plastids. In addition, we used as

positive controls mutants deficient in production of triunsaturated fatty acids (*fad3-2 fad7-2 fad8*), jasmonate (the allene oxide synthase mutant: *aos*) and for cutin biosynthesis (a glycerol-3-phosphate acyltransferase double insertion mutant*: gpat4 gpat8*). *B. cinerea* application was conducted on 7-day-old cotyledons and 14-day-old first true leaves; plants were analysed 48 hours post-inoculation by counting the number of organs with lesions (Fig.4.1).

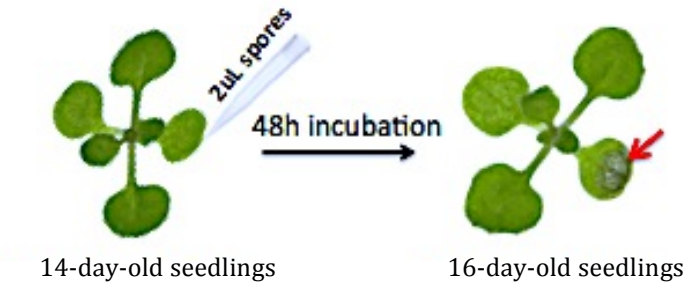


Figure 4.1. *B. cinerea* pathogenesis on *Arabidopsis* seedlings. Lesions were detected visually 48 hours post treatment.

Wild type (Columbia) seedlings including mutants described earlier were tested for *B. cinerea* susceptibility when they were 9- and 16-days-old. Results are presented in Figure 4.2.

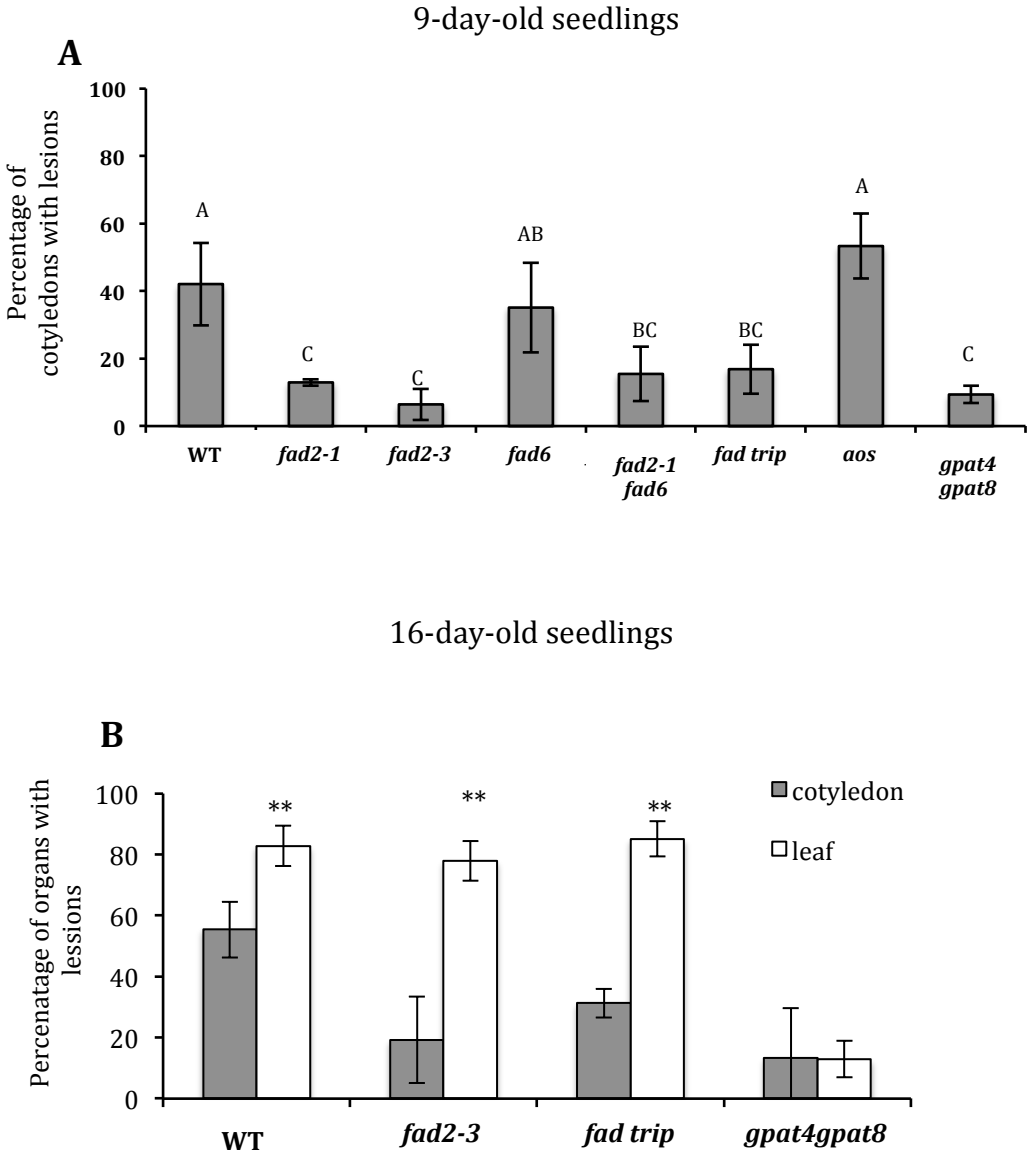


Figure 4.2. *B. cinerea* resistance bioassay on *Arabidopsis* seedlings. (**A**) 9-day- and (**B**) 16-day-old seedlings were used in this assay. 2 µL of *B. cinerea* spore suspension (5×10^5 s/ml) was applied on one cotyledon or first true leaf of each seedling and incubated for 48 h. The number of organs with lesions was scored. (**A**) Error bars represent the means of four biological replicates (± SD), each from a pool of ≈ 120 seedlings. Letters indicate statistically significant differences between pairs as determined by Tukey's HSD test ($p < 0.05$). (**B**) Error bars represent the means of four biological replicates (± SD), each from a pool of 70 seedlings. Statistical significance in pair-wise comparison control *vs* treatment was evaluated by Student's test, where ** $p \leq 0.01$.

The results obtained revealed strong increase in resistance of *fad2* and *gpat4 gpat8* mutants to *B. cinerea* in relation to the WT at 7-day-old stage, whereas the *fad6* mutant showed no significant difference to WT. Surprisingly, *fad trip* and *fad2-1 fad6* mutants also showed resistance to this pathogen. This was unexpected due to the absence of JA in these mutants. Jasmonates are very important hormones for defences against necrotrophic pathogens (Thomma *et al.,* 1998). The *aos* mutant showed enhanced susceptibility upon *B. cinerea* treatment. These results indicate that resistance of *fad trip* and *fad2-1 fad6* mutants was caused by changes in fatty acid composition and was not related to jasmonate responses.

B. cinerea application on the WT revealed a 20% increase in susceptibility of leaves compared to cotyledons. Similarly to WT, *fad2-3- and fad trip* mutants showed an average of 60% increase in susceptibility in leaves, showing the same levels as WT. Finally, the *gpat4 gpat8* double mutant's levaes remained resistant to *B. cinerea*. To estimate roles of fatty acids in increased susceptibility on the first true leaves, we conducted fatty acid profiling of WT and *fad2-3* mutant at both the 7-day- and 14-day-old stages (Fig.4.3).

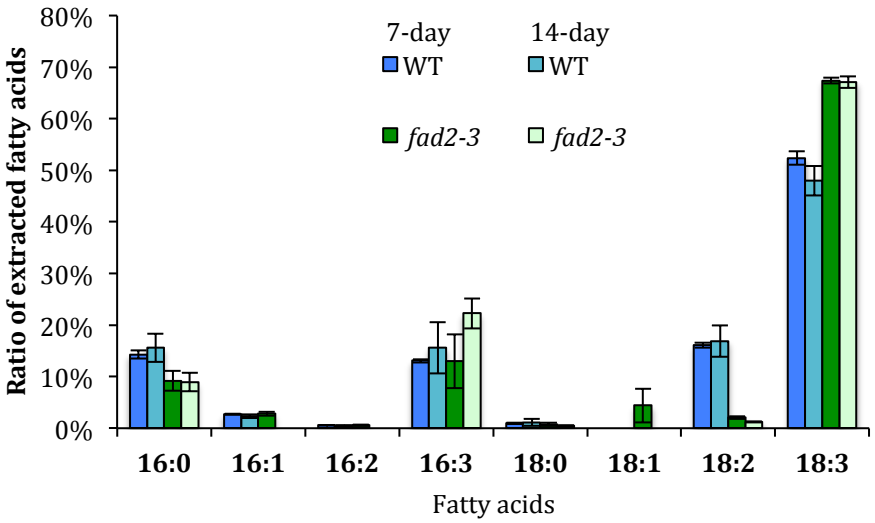


Figure 4.3. Fatty acid composition of WT and *fad2-3* mutant performed at 7- and 14-day-old stage. Error bars represent the means of of 4 - 6 biological replicates (± SD); each pool contained approximately 40 seedlings.

The major difference was detected in the levels of 18:1 and 18:3 fatty acids. At the stage of 7-days *fad2-3* mutants had 4% of oleic acid, whereas at the stage of 14 days, these levels were below limit of quantification. Increased levels of 18:3 FAs at the stage of 14-days-old seedlings could not explain *B. cinerea* resistance. No correlation was found between increase in the levels of 18:3 FAs and higher levels of jasmonic acid – a major defence hormones involved in resistance to necrotrophic pathogens. This leads to the conclusion that 18:1 fatty acid could play an important role in resistance to *B. cinerea*.

IV.2. Does cuticle permeability lead to *B. cinerea* resistance in *fad* mutants?

B. cinerea enters within the plant by using cutinase, an enzyme that degrades the cuticle. To avoid activation of defence response genes in host, *B. cinerea* produces oxalate that prevents generation of reactive oxygen species (Commenil *et al.,* 1998; Liu *et al.,* 1998). In Table 4.1, we summarize current knowledge concerning cuticle mutants and their resistance or susceptibility to this necrotrophoic pathogen using earlier published data from Serrano *et al.* (2014).

Table 4.1. Characterized cuticle mutants and their responses to *B. cinerea*.

Mutant	*B. cinerea* susceptibility	Resistance factor	References
lcr (lacerata)	-	Fungitoxic diffusate, ROS	Voisin *et al.,* 2009
lacs2 -long-chain acyl-CoA synthetase	-	Fungitoxic diffusate;SAR ; 6-week old	Bessire, *et al.,* 2007; Schnurr *et al.,* 2004
ace/hth – adhesion of calyx edges /hothead	+		Celine Chassot thesis, 2006, University of Freiburg
bdg (bodyguard)	-	Fungitoxic diffusate;ROS	Chassot *et al.*, 2008
fdh (fiddlehead)	-		Voisin *et al.,* 2009

acbbp1 (acyl-coa-binding protein)	+		Xue *et al.,* 2014
pec1 (permeable cuticle1)	-	Fungitoxic diffusate; ROS	Chassot *et al.*, 2008
acp4 (acil carrier protein)	-	ROS, SAR	Bessire, *et al.,* 2007; Schnurr *et al*., 2004
gly1 (glycerol-3-phosphate dehydrogenase)	+	SAR	Xia *et al.,* 2010
cdk8 (cycle -dependent kinase8)	-	PDF1.2	Zhu *et al.,* 2014
aba2 (ABA biosynthesis)	-	ROS, ABA down regulation	L'Haridon *et al.,* 2011
aba3 (ABA biosynthesis)	-	ROS, ABA down regulation	L'Haridon *et al.,* 2011

Symbols indicate: susceptibility (+) and resistance (-) to *B. cinerea;* SAR: systematic acquire resistance.

As shown in Table 4.1, most cuticle mutants are resistant to *B. cinerea*. This could be explained by fast induction of antifungal compounds (Bessire *et al.,* 2007) or constitutive overexpression of genes involved in lipid transfer (LPT), peroxidase (PER) and proteinase inhibitors (PI) (Chassot *et al.,* 2007).

Unfortunately, these results do not explain why some cutin biosynthesis mutants do not show resistance to this pathogen.

Plant waxes were also shown to play a role in fungal development on the host; for example, terpenoids found in the wax layer of avocado fruits induced appressorium formation in *Colletrichum gloeosporioides* (Podila *et al.*, 1993; Kolattukudy *et al.,* 1995). C22 FAs, fatty alcohols or alkanes, present in the wax layer of rice leaves promoted development of *Magnaporthe grisea* (Hegde and Kolattukudy, 1997). In grapes, wax composition was shown to change during maturation and this correlated with increased *B. cinerea* susceptibility in mature grapes. This effect was linked to high levels of primary alcohols and oleanic acid in young berries and their diminution in mature grapes (Commenil *et al.,* 1996). *B. cinerea* resistance is a complicated process that requires overlap of different defence pathways that are strongly variable depending on the plant genotypes.

Hence, the purpose of this part was to disengage the role of the cuticle in resistance to *B. cinerea* in *fad* mutants. We performed cuticle permeability assays to describe the effect of deficiency of di- or triunsaturated fatty acids on cuticle formation (Fig. 4.4).

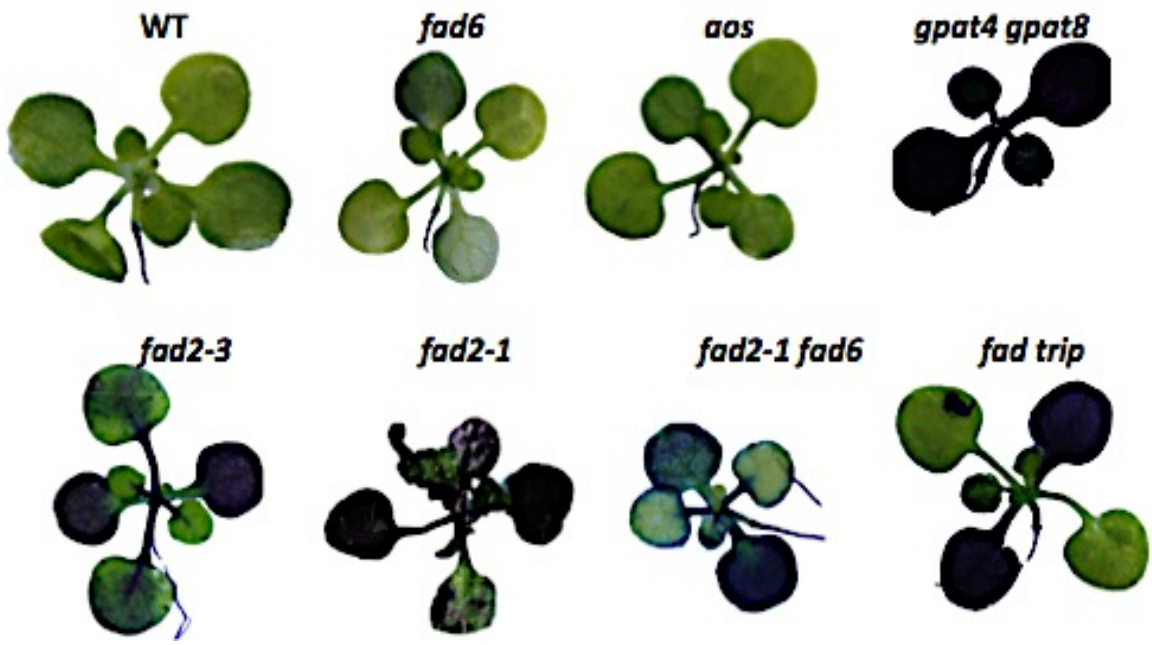


Figure 4.4. Cuticle permeability assay in 14-day-old seedlings. Plants were harvested and immediately placed in toluidine blue solution for 15 minutes.

Toluidine blue staining of 14-day-old seedlings permits the visualization of cuticle permeability in cotyledons and fully expanded true leaves. WT, *fad6* and *aos* did not stain, meaning that cuticle function is not affected in these plants. *fad2-3, fad2-1, fad2-1 fad6* and *gpat4 gpat8* double mutants have cuticle permeability defects in both cotyledons and first true leaves, whereas *fad trip* permeability effected only in the cotyledons.

Transmission electron microscopy (TEM) was performed to describe how the cuticle ultrastructure is affected in *fad2-3*, *fad trip* and *gpat4 gpat8* mutants. (Fig.4.5)

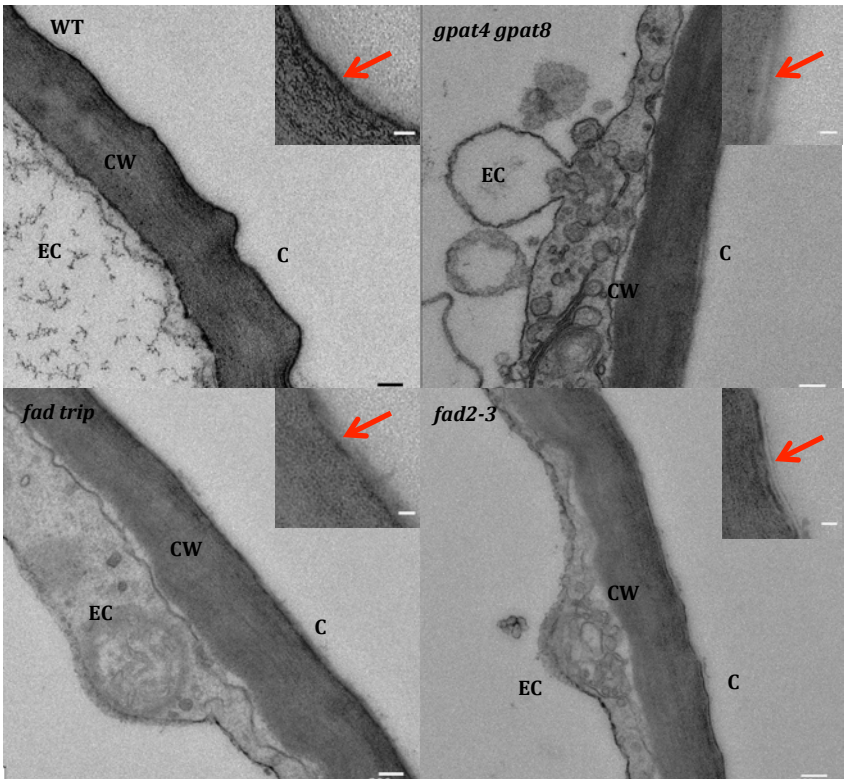


Figure 4.5. Cuticle ultrastructure of 7-day-old *Arabidopsis* cotyledons. C – cuticle, CW- cell wall; EC- epidermal cell. Small boxes represent zoomed in part with the cuticle; scale bars 50 μm. Arrows indicate the cuticle layer. This experiment was repeated three times with similar results in all cases, this graph is representative of one of these experiments; scale bars 200 μm.

Cuticle of the WT plants is seen as dark electron dense layers, whereas in the *gpat4 gpat8* double mutant these are absent and instead there is a light grey layer that is not tightly attached to the cell wall. Concerning *fad trip* and *fad2-3* mutants, they both have thin electron dense layers of the cuticle with less electron dense patches.

To identify which cuticle monomers were affected in *fad* mutants, we performed a quantification of aliphatic polyester monomers from the shoots of 7-day-old seedlings (Fig. 4.6).

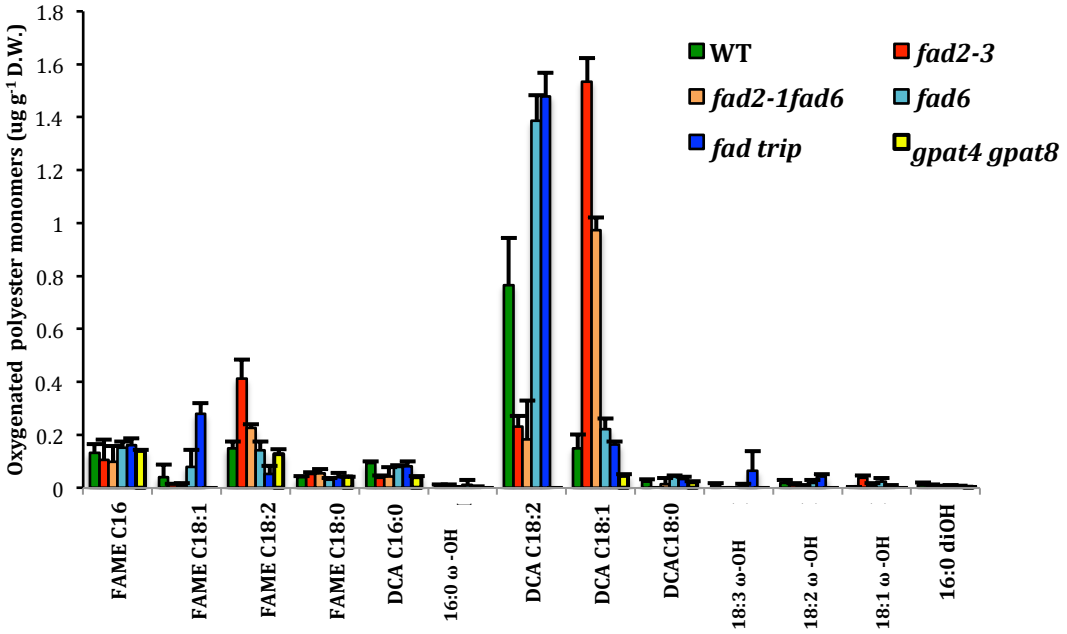


Figure 4.6. Quantification of aliphatic polyester monomers in 7-day-old *Arabidopsis* shoots. Error bars represent the means of six biological replicates (± SD), each containing a pool of ≈ 1920 seedlings. FAME - fatty acid methyl ester; DCA - dicarboxylic acid; ω-OH – omega hydroxyl fatty acid. This experiment was repeated three times with similar results in all cases, this graph is representation of one of these experiments.

Polyester monomer profiles revealed a strong overall reduction of cutin monomers in the *gpat4 gpat8* double mutant, highlighting a very strong permeability in cotyledon and leaves of these plants. *fad2-3 and* the *fad2-1 fad6* double mutant displayed an 2.5 times lower levels of DCA 18:2 and 8 times higher levels of DCA 18:1 compared to the WT. Surprisingly, *fad2-3*

mutants showed an over accumulation of C18:2 FAME, that was not observed in *fad2-1 fad6* double mutant. Both mutants had almost no detectable levels of 18:1 FAME.

The *fad trip* mutant that showed a permeable cuticle phenotype at the cotyledon stage displayed an over-accumulation of 18:2 DCA, similar to *fad6* mutant, i.e. three times higher levels of 18:1 FAME and two times reduction in 18:0 FAME. The *fad6* mutant showed almost no difference in its polyester profile compared to the WT, except as mentioned earlier, a 1.5 times higher level of 18:2 DCA. This change did not appear to affect cuticle permeability in this plant.

Due to the involvement of waxes in *B. cinerea* resistance, we performed scanning electron microscopy to visualize wax layers in WT and *fad* mutants (Fig. 4.7).

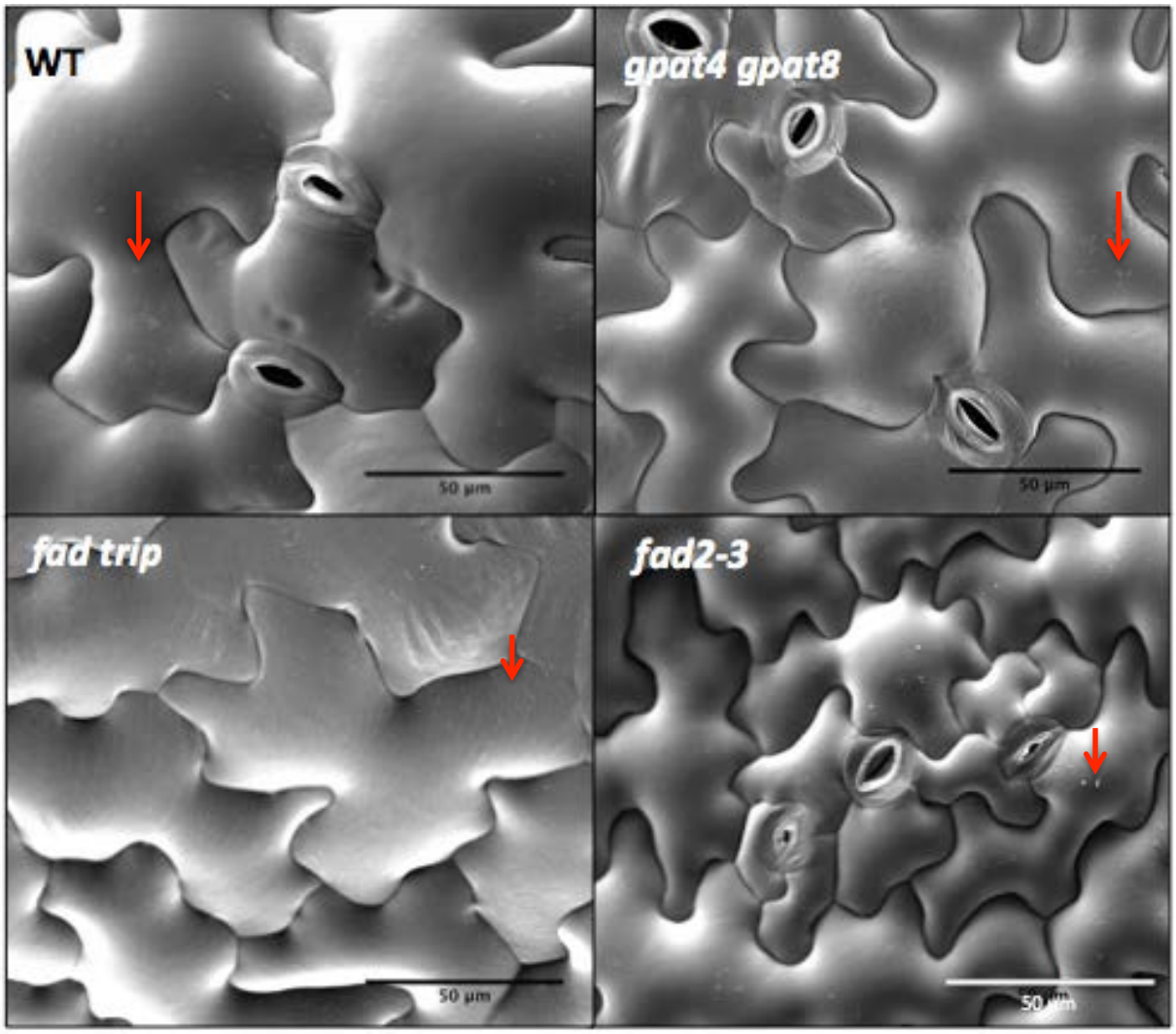


Figure 4.7. Scanning electron microscopy images of 7-day-old *Arabidopsis* cotyledons. Arrows indicate wax crystals. Five cotyledons were analyzed for each genotype; the images shown are representative.

These images clearly show that there is almost no wax layer on *Arabidopsis* cotyledons and small patches of crystals chaotically organized on the surface, similar to the situation for *Arabidopsis* leaves (Jenks *et al*., 1995; Lee and Suh *et al*., 2014).

In summary, aliphatic polyester monomer profiling revealed strong changes in the cuticle composition of *fad* mutants. Monomer compositions differed in *fad2-3, fad2-1 fad6*, *fad trip* and *gpat4 gpat8* mutants. No changes in wax composition were observed. As a conclusion, no polyester monomers could explain resistance to *B. cinerea*. Visualization of waxes on the surface of cotyledons also did not explain resistance to this pathogen. One more time, our results confirm that host-pathogen interaction is a very complicated mechanism that still has to be uncovered.

IV.3. Induction of ROS and defense hormones upon *B. cinerea* infection in *fad* mutants.

ROS are known to play an important role in the control of plant development (Gapper and Dolan, 2006) and in responses to abiotic and biotic stresses (Apel and Hirt, 2004; Mittler *et al*., 2004), by inducing defense response genes (Levine *et al.,* 1994).

The role of ROS in *B. cinerea* resistance remains unknown. According to one hypothesis *B. cinerea*, like other necrotrophic pathogens, can suppress ROS production, preventing induction of antifungal compounds and defence response genes at the early steps of fungal penetration (Germeier *et al.,* 1994;

Pezet *et al.,* 2004). However, some cuticle-deficient mutants have high basal ROS levels that lead to the constant upregulation of major defense genes (Bessire *et al.,* 2007; Chassot *et al.,* 2007; L'Haridon *et al.,* 2011; Liu *et al*, 2015) and hence induce *B. cinerea* resistance. The aim of this part was to analyse ROS levels in *fad* mutants upon *B. cinerea* infection and to compare it with the expression of common hormonal marker genes induced upon *B. cinerea* application.

We used 7-day-old *Arabidopsis* cotyledons either with mock treatment (24h treatment with potato dextrose broth (PDB)), or infected with *B. cinerea* in PDB for 24h. To detect changes in the ROS levels, we used ROS-ID^{TM} Total ROS/Superoxide Detection Kit (Enzo Life Sciences AG, Switzerland) as described by Marschal and Tudzynski (2014). It is important to note that this technique has not, to our knowledge, been used before on plants, however it provided promising results in the present study (Fig.4.8). Due to the previously published results showing stronger ROS fluorescence with later infection time points (L'Haridon *et al.,* 2011), we performed our assay 24 h after the treatment.

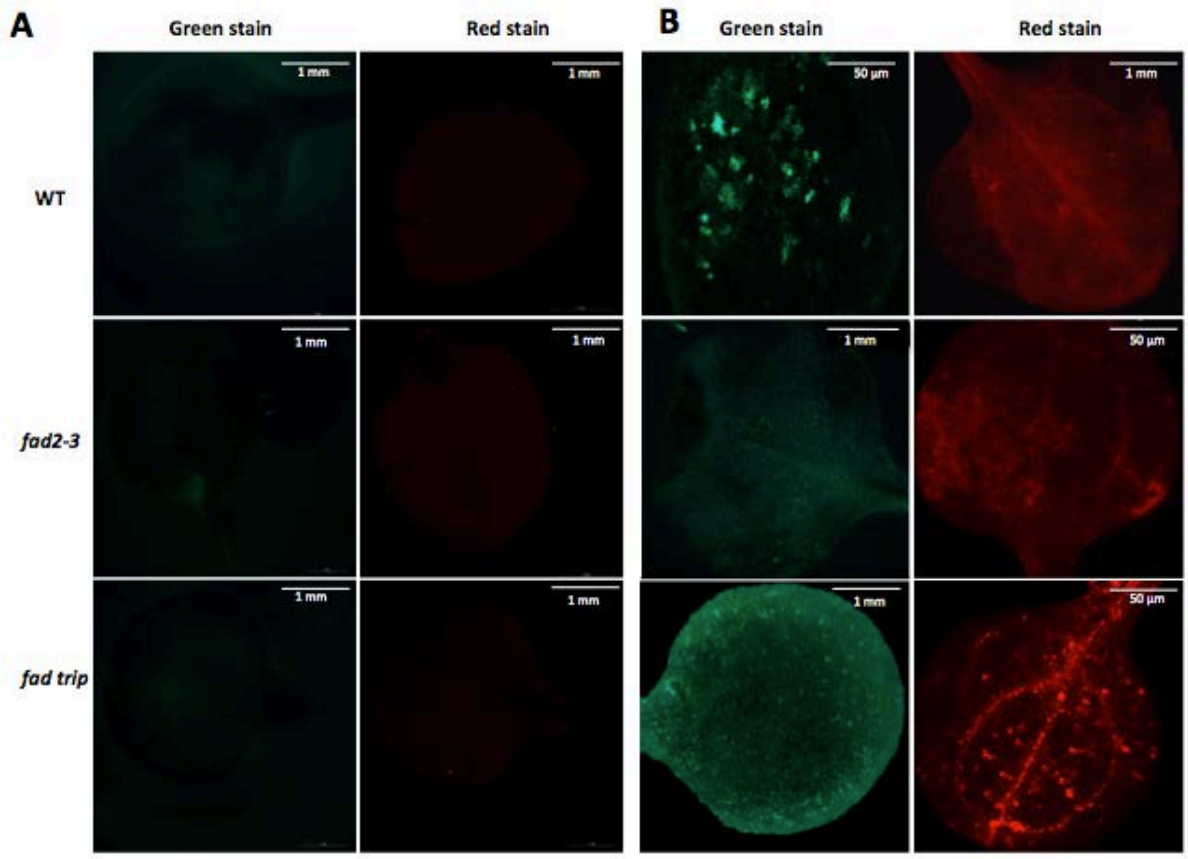


Figure 4.8. ROS staining of 8-day-old cotyledons. (**A**), 24 h post PDB application and (**B**) 24 h post *B. cinerea* application in PDB. Treated seedlings were vacuum infiltrated in ROS staining solution for 30 min. Hydrogen peroxide, peroxynitrite and hydroxyl radicals were visualized *via* wavelengths 490 nm/ 525 nm (Ex/Em) and called as 'green stain', superoxide via 550 nm/ 620 nm (Ex/Em), 'red stain'. Each photo is representative of ten analysed cotyledons.

No visual difference was found between the WT, *fad trip* and *fad2-3* mutant upon PDB treatment. Cotyledons infected with *B. cinerea* showed stronger induction of analyzed ROS species. Patchy fluorescent spots on the WT cotyledons revealed areas of fungal penetration. In this case the detected fluorescence is likely to come from both plant and pathogen, since both of them induce ROS during the pathogenesis process. The *fad2-3* mutants showed

less fluorescence with both dyes, a result that could be explained by a lower ratio of fungal penetration (not tested in the present study). The *fad trip* mutant displayed the strongest induction of ROS compared to WT and *fad2-3* mutant. This could be explained by the absence of TUFAs in this plant and its inability to conduct ROS buffering due to absence of these fatty acids (Mene-Saffrane *et al.,* 2007; Schmid-Siegert *et al.,* 2016).

In summary, WT, *fad trip* and *fad2-3* mutants showed no difference in ROS fluorescence after the mock treatment. 24 h post *B. cinerea* application induces stronger fluorescence of ROS in *Arabidopsis* cotyledon. We noticed a gradient of increased green and red staining in WT, *fad2-3- and fad trip* mutants.

The next step was to correlate increased ROS levels with hormonal changes (Fig.4.9). In order to detect changes in the levels of JA and ET – the (ethylene- and jasmonate-responsive plant defensin) *PDF1.2* marker gene was used (Penninckx *et al.,* 1996,1998). *PDF1.2* expression was seen at the site of infection and at the distal regions leading to the similar kinetics at both parts (Penninckx *et al.,* 1996, Ferrari *et al.,* 2003). To analyse the potential role of the SA pathway in *B. cinerea* resistance, we used the *PR1* (pathogenesis-related protein 1) marker gene. This gene is activated upon pathogen infection downstream of systematic acquired resistance (SAR; Von Loon *et al.,* 1994). In contrast to *PDF1.2*, *PR1* is expressed only in the region of pathogen

penetration (Ferrari *et al.,* 2003). Finally, to test the involvement of ABA, we used the *ABA1* (encoding an zeaxanthin epoxidase involved in ABA biosynthesis), *aba1* mutants in *Arabidopsis* show *B. cinerea* susceptibility that might be caused by JA and ET changes this mutant (Korolev *et al,* 2004).

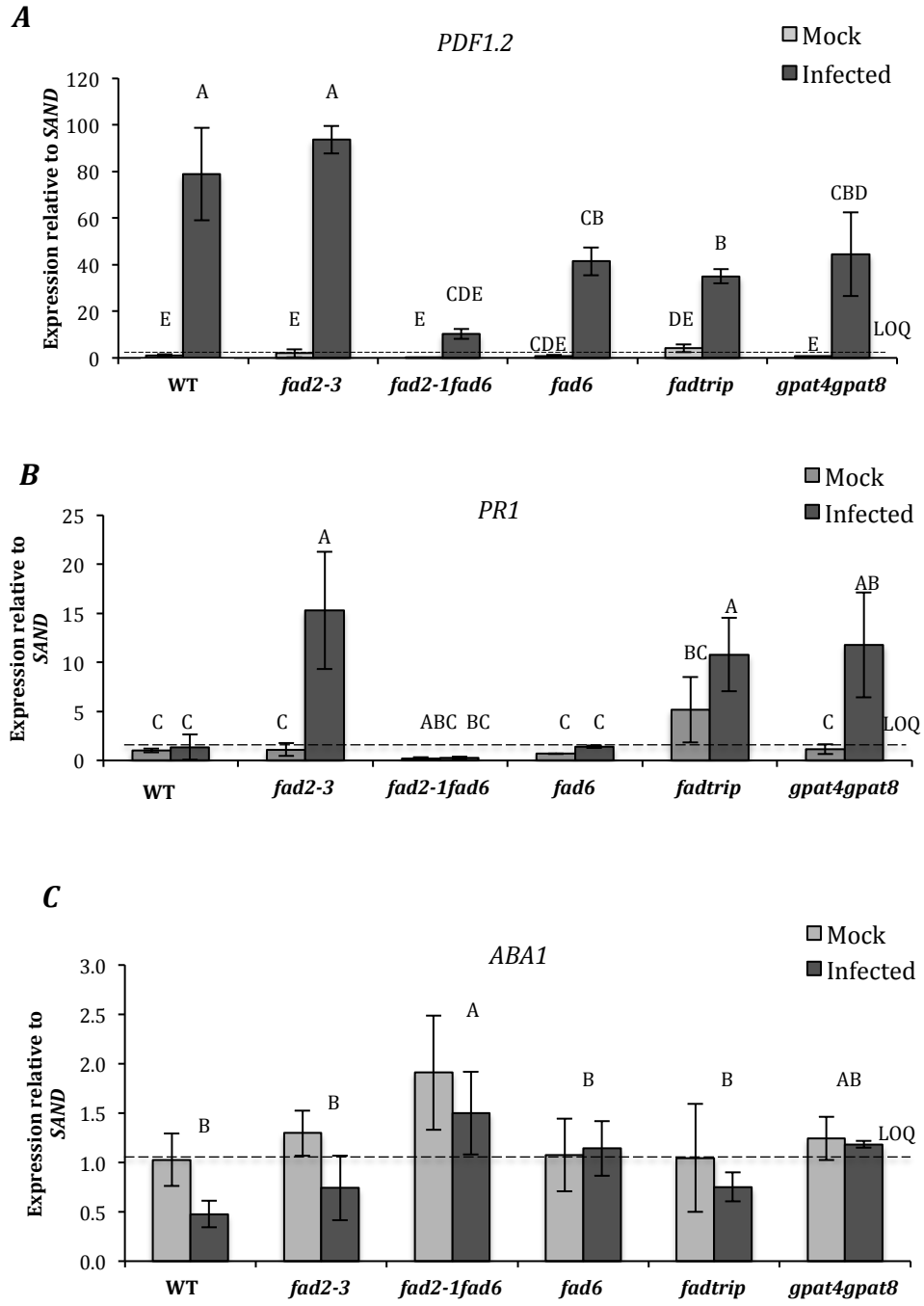


Figure 4.9. Induction of defence marker genes upon *B. cinerea* infection in 8-day-old seedlings. qRT-PCR of *PDF1.2* (**A**), *PR1* (**B**) and *ABA1* (**C**) expression in 7-day-old *Arabidopsis* seedlings 24 h after *B. cinerea* application. Transcripts were normalized to *SAND* and displayed relative to the expression in WT mock. Error bars represent the means of six biological replicates (±SD), each containing a pool of ≈ 30 seedlings. The dashed line indicates the limit of quantification (LOQ) is indicated. (**A**-**B**) Letters indicate statistically significant differences between pairs, as determined by Tukey's HSD test ($p < 0.05$). (**C**) No significant interaction between treatment and genotypes was revealed. Tukey's HSD test was performed on genotypes, revealed significant effect of infection ($p < 0.0008$).

According to the qPCR results, no similar pattern of hormonal response was revealed between mutants showing resistance to *B. cinerea*. Expression of the *PDF1.2* marker gene was similar between WT and *fad2-3* mutants, whereas *fad2-1 fad6, fad6* and *fad trip* showed much lower *PDF1.2* gene expression compare to the WT. This result could be explained by potentially lower levels of JA in these mutants, due to the complete absence or strong reduction of triunsaturated fatty acids. Significantly lower levels of *PDF1.2* in the *gpat4 gpat8* double mutant require further investigation. Indeed, according to fatty acid profiling, no difference was found between WT and *gpat4 gpat8* double mutant (Fig.4.10), leading to the idea that ET levels might be affected in this mutant.

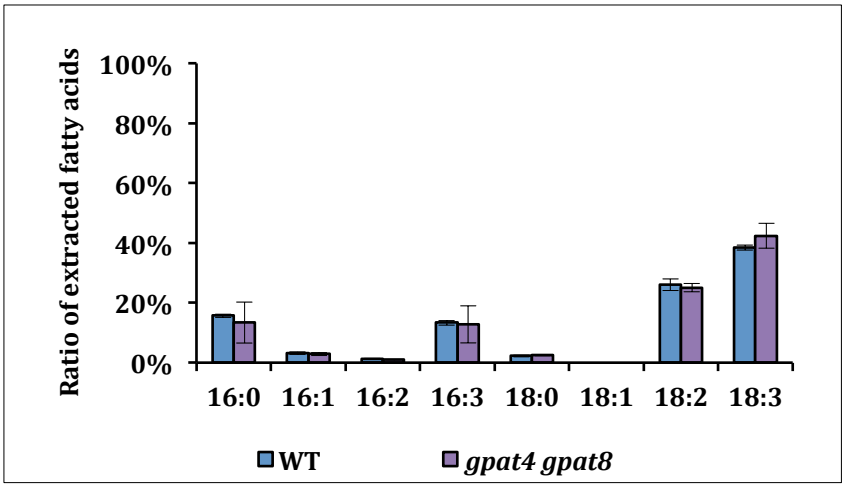


Figure 4.10. Fatty acid composition in 7-day-old seedlings of WT and the *gpat4 gpat8* double mutant. Error bars represent the means of six biological replicates (± SD); each contained a pool of ≈ 50 seedlings.

According to previously published results, SA was not strongly induced upon *B. cinerea* infection in WT plants (Birkenbihl *et al.,* 2012). We confirmed this result with 7-day-old WT seedlings by conducting qPCR with the SA-responsive the *PR1* marker gene (a similar pattern upon *B. cinerea* infection was observed in *fad6* and *fad2-1 fad6* double mutants). Surprisingly, *fad2-3* and *gpat4 gpat8* double mutants showed more than 10-fold *PR1* induction upon *B. cinerea* infection. Concerning *fad trip*, it revealed higher basal levels of *PR1* expression and 10-fold induction upon treatment.

During *B. cinerea* infection, ABA signalling is strongly down regulated (Windram *et al.,* 2012), due to its antagonistic relations with ET, which is induced upon infection (Yang *et al.,* 2005). A similar pattern of down regulation of the *ABA1* gene upon *B. cinerea* infection was observed on WT,

fad2-3, *gapt4 gpat8* and *fad2-1 fad6* double mutants, with the exception of a 2-fold higher level of *ABA1* in *fad2-1 fad6* plants. Surprisingly, the *fad6* mutant displayed no down regulation of the *ABA1* gene upon *B. cinerea* infection, leading to assumption that ethylene levels in this mutant are lower than in WT plants during infection process. As shown above (Fig.4.8), mild changes in ROS levels in *fad2-3* mutants and strong basal levels of green stain in *fad trip* did not lead to upregulation of genes such as a *PDF1.2* or *ABA1*, but did lead to strong induction of *PR1*. Interestingly, SA is involved in the local defence to *B. cinerea*, by decreasing lesion size, but not the overall survival rate of infected seedlings (Ferrari *et al.,* 2003).

IV.4. Changes in topography of the cotyledon surface can lead to *B. cinerea* resistance.

Early pathogenesis is a crucial step that requires spore attachment to the host surface, spore germination, formation of appressoria and further penetration (Kuo and Hoch, 1996). Development of the germ tube depends on multiple factors, such as surface hardness, hydrophobicity, topography and structural components. It is important to note that if one or more of these factors are perturbed, germ tube growth is reduced or arrested. If all the required conditions are present, the fungus forms an appressorium as a next step of its development (DeZwaan *et al*., 1999; Staples *et al.,* 1987; Talbot *et al.,* 1996;

Wessels *et al.,* 1991; Tucker *et al.,* 2001). For these various reasons, we examined cotyledon topography in relation to *B. cinerea* penetration in WT plants and fatty acid desaturase mutants.

To examine immediate adhesion and hyphal growth on *fad* mutants, we performed adhesion assays (Fig.4.11). For this purpose, cotyledons were incubated with *B. cinerea* spore suspension for 4 h after which seedlings were washed in 100% ethanol for 1 h to remove unattached spores from the cotyledon surface.

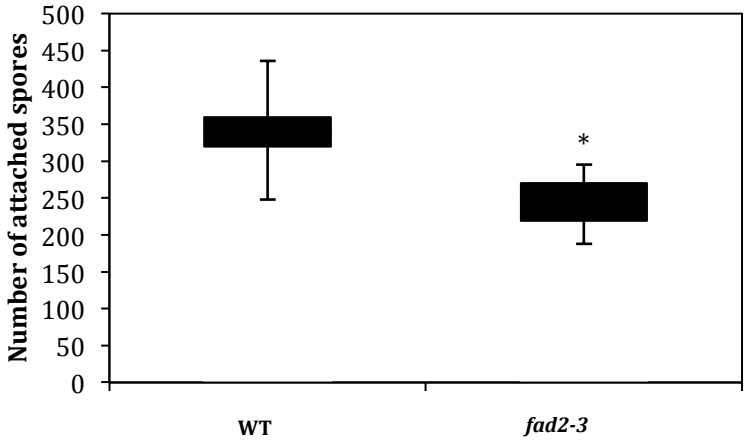


Figure 4.11. *B. cinerea* adhesion assay. WT and *fad2-3* cotyledons were infected with spores of *B. cinerea*, the amount of attached spores per cotyledon was quantified 4 h after spore application. Error bars represent the means of ten biological replicates (± SD), each initially containing a pool of ≈ 500 spors of *B. cinerea*; Statistical significance in pair-wise comparison was evaluated by Student's test, where * $p < 0.05$.

The initial concentration of applied *B. cinerea* spores was 500 spores/µl. The number of attached spores on WT cotyledons equalled approximately 350

spores, whereas on *fad2-3* seedlings these were approximately 250 spores ($p < 0.05$; Fig. 4.11). This result could explain a partial resistance to *B. cinerea* in the *fad2-3* mutant. It led us to the idea that a modified topography in *fad2-3* cotyledons might be responsible for this difference.

The second step was to test the effect of delayed adhesion by analysing development of germ tubes several hours post infection. First, we conducted a time course assay to estimate the speed of *B. cinerea* development on WT and *fad* mutants (Fig. 4.12).

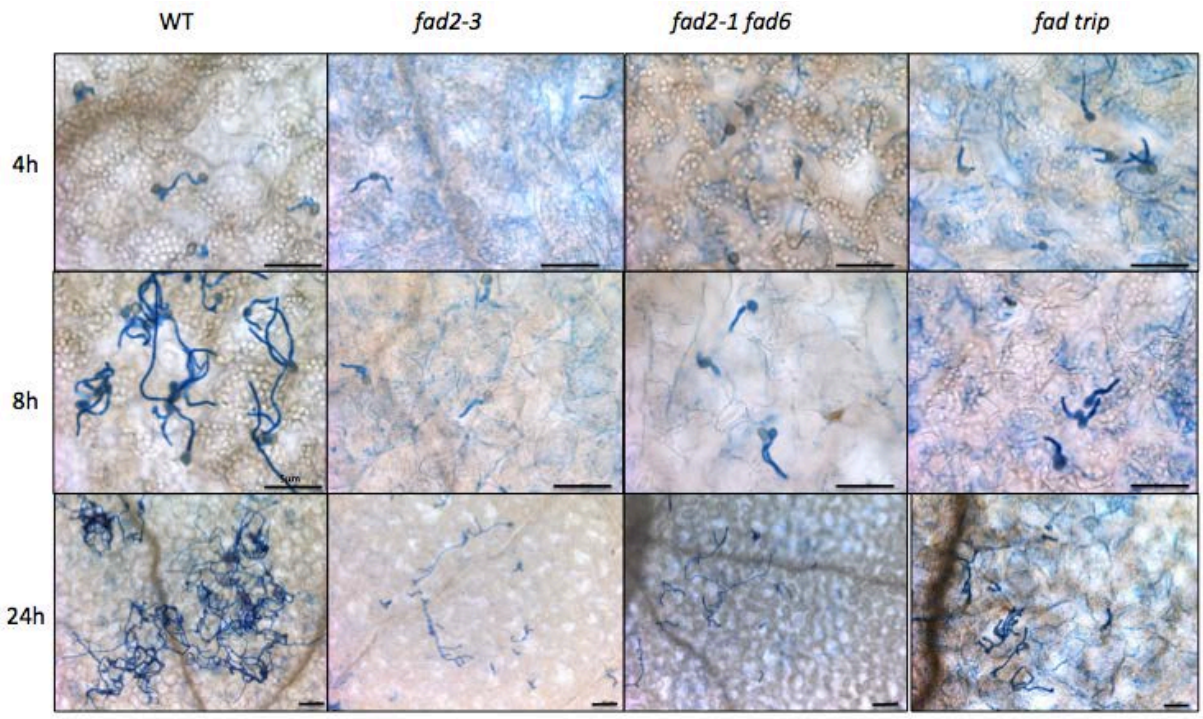


Figure 4.12. *B. cinerea* development: a time course assay on 7-day-old cotyledons. Pictures were taken at three different time points: 4, 8 and 24 hours post inoculation. For 4 and 8 hours a 40X standard objective was used and for 24 hours a 20XDIN standard objective. Hyphae were stained with Trypan blue. The experiment was repeated 3 times. Scale bar 50 μm.

No difference was found between WT and *fad* infected cotyledons 4 h post infection, whereas infection at later time points (8 and 24 h) revealed increased hyphal growth on WT cotyledons compared to the *fad* mutants. To be able to estimate the difference between at these time points we performed hyphae length measurements at 4 and 8 h post infection (Fig. 4.13). The 24 h time point was excluded from the experiment, due to the difficulty of measuring hyphae lengths on and in WT plants caused strong overlay of hyphae.

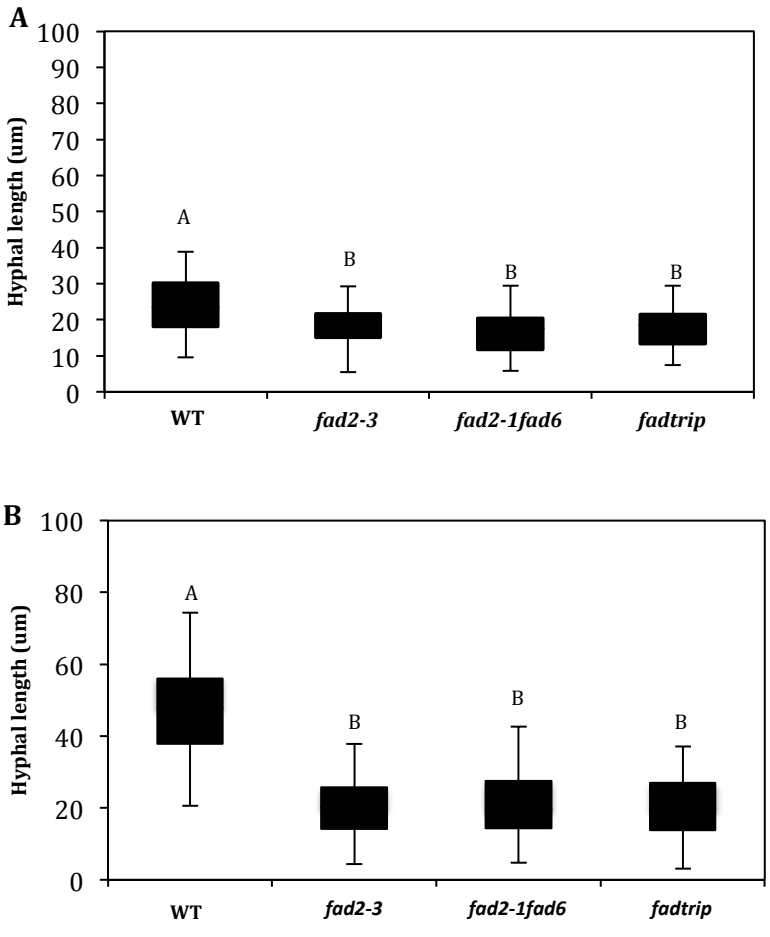


Figure 4.13. *B. cinerea* hyphal length assay at two different time points. 2µL suspension of 5×10^5 spores was applied to single cotyledons of 7-day-old seedlings (**A**) 4 h and (**B**) 8 h post application. Error bars represent the means of 100 *B. cinerea* hyphae measured on 10 cotyledons (± SD). Letters indicate statistically significant differences between pairs as determined by Tukey's HSD test ($p < 0.05$).

According to our results, the average length of hyphal 4 h post infection was 25 µm on WT cotyledons and 20 µm on the *fad* mutants. After 8 h, hyphal length increased two times on WT, whereas on *fad* mutants, hyphal growth was arrested at the stage of 4 h post infection. We concluded that delayed adhesion reduces *B. cinerea* development on *fad* mutants.

Earlier papers considered leaf topography as an important factor promoting pathogen development (DeZwaan *et al*., 1999; Staples *et al.,* 1987; Talbot *et al.,* 1996). To examine if cell morphology in *fad* mutants was affected, we used confocal microscopy to visualize cotyledon epidermal cells of WT and cuticle mutants at the stage of 7-day-old seedlings (Fig.4. 14).

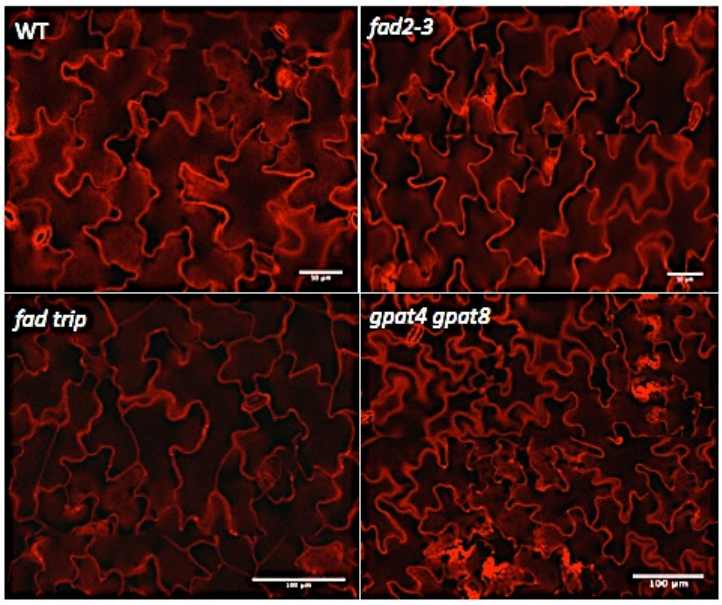


Figure 4.14. Pavement cell morphology of 7-day-old cotyledons, stained with propidium iodide. Pavement cells have different morphology depending on their location, for this reason, only the region near to cotyledon tip was chosen for the analysis (n = 10 cotyledons). Scale bar 50 μm.

Visually, pavement cells of cuticle mutants have a different morphology to those of the WT: smaller cells with fewer lobes. To obtain quantitative results, we used Lobefinder_V1-4.0 software (Wu *et al.,* 2016). Single pavement cells were selected in ImageJ and converted to roi files, further analyses were carried out in Lobefinder (Fig.4.15).

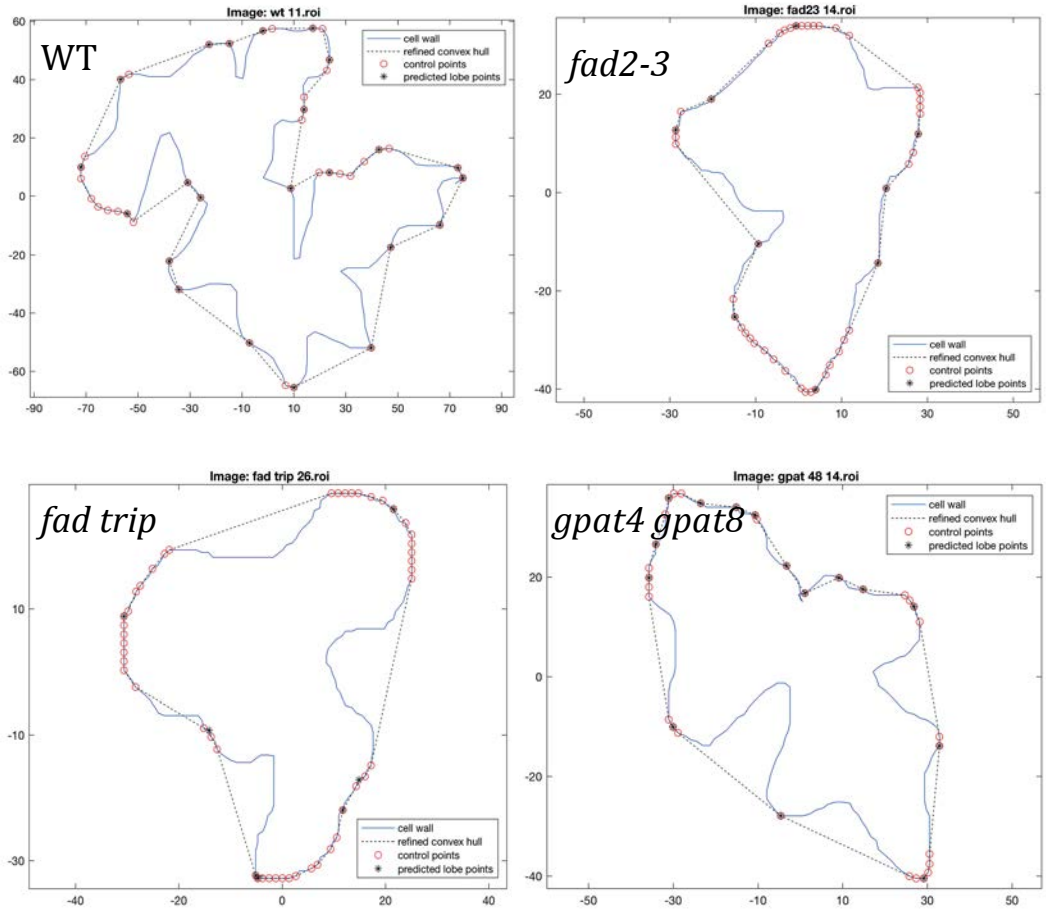


Figure 4.15. Example of a single pavement cell analyzed with Lobefinder_V1-4.0 software.

The numeric data were saved in xlsx format and analyzed as described in Materials and Methods. Quantified parameters of pavement cells are shown below (Fig.4.16).

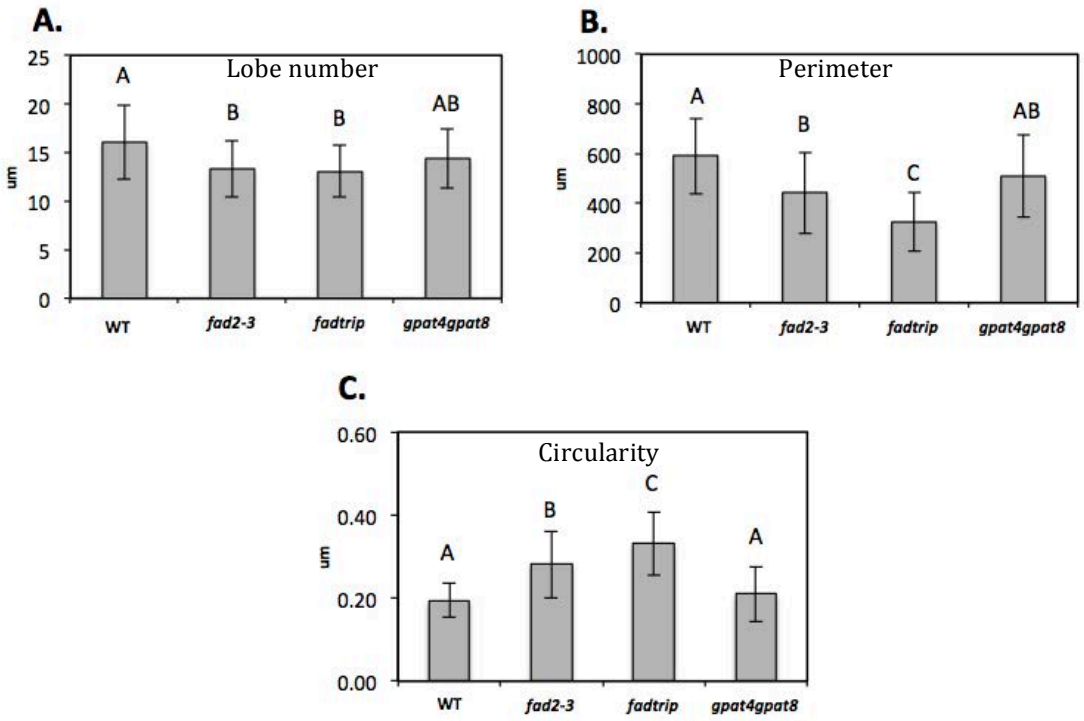


Figure 4.16. Quantitative analysis of pavement cell parameters in 7-day-old cotyledons. (**A**) Lobe number, (**B**) perimeter and (**C**) circularity were measured. Error bars represent the means of 45-50 pavement cells measured on 10 cotyledons (±SD). Letters indicate statistically significant differences between pairs as determined by Tukey's HSD test ($p < 0.05$).

fad mutants had reduced lobe numbers, cellular perimeters and increased circularity compared to the WT cells, no significant difference was found between WT and *gpat4 gpat8* cotyledons. This could be caused by morphological and turgor changes of the cell (Hamant *et al*., 2008, Routier-Kierzkowska *et al*., 2012, Sampathkumar *et al*., 2014). For this reason we decided to use atomic force microscopy to examine the spatial distribution of mechanical properties such as height, elasticity and adhesion of *Arabidopsis thaliana* cotyledons (Fig.4.17).

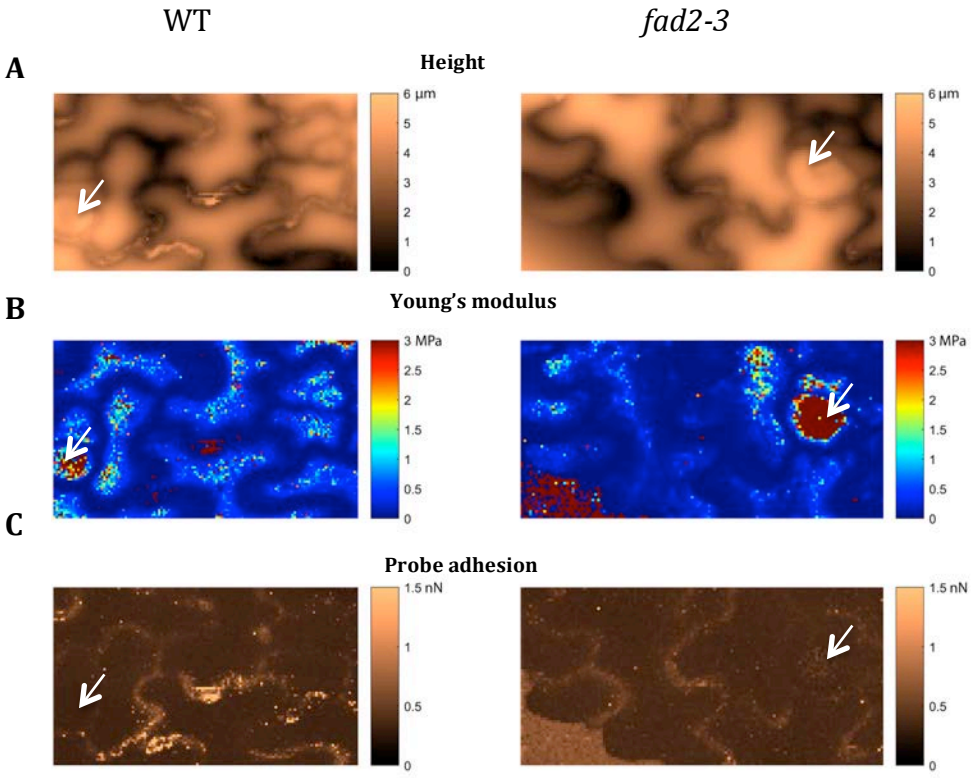


Figure 4.17. Atomic force microscopy images of 7-day-old *Arabidopsis* cotyledons. (A) Height, (B) Young's modulus (stiffness) and (C) adhesion maps were obtained. Five cotyledons were analysed in this work, representative images were chosen for each map. Arrows indicate stomata. Experiment performed by Petar Stupar, Laboratory of the Physics of Living Matter, EPFL, Lausanne.

These images show the difference in elastic modulus (Young's modulus), a measure of stiffness in *fad2-3* mutants compared to WT cotyledons. However, only slight changes were noticed in the adhesion and no difference in the height maps. Due to the observed difference in adhesion and stiffness maps we performed quantitative analysis of these features (Fig.4.18).

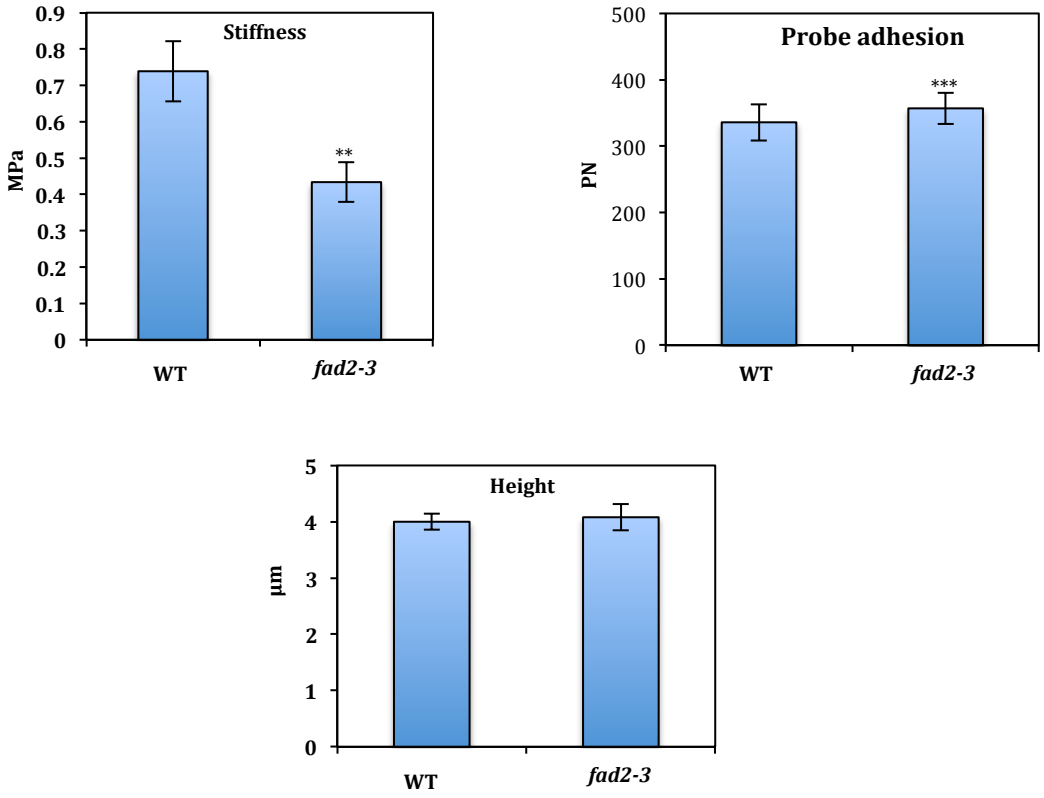


Figure 4.18. Quantitative analysis of atomic force microscopy results. (**A**) Young's modulus, (**B**) adhesion and (**C**) height were measured. Error bars represent the means of 15 to 20 pavement cells parameters obtained from five cotyledons (±SD). Statistical significance in pair-wise comparison control *vs* treatment was evaluated by Student's test, where ** $p \leq 0.01$; *** $p \leq 0.001$. Measurenments performed by Petar Stupar, Laboratory of the Physics of Living Matter, EPFL, Lausanne.

Figure 4.18 show that mean Young's modulus is different between WT and *fad2-3* mutants and was 0.7 and 0.4 MPa respectively. Adhesion parameters were also affected and were 0.3- and 0.4 nN in WT and mutants respectively. Cell height was the only feature that did not change in WT and mutants cotyledons and was 4 µm in both cases. These results revealed multiple non-uniformities that could be explained by heterogeneous composition of the cell surface in the WT and the *fad2-3* mutant. Unfortunately, such a slight change

in morphology of the pavement cells cannot explain resistance to *B. cinerea* in *fad2-3* mutant, but might explain slight changes in adhesion of *B. cinerea* spores on cotyledons of *fad2-3* mutants relative to the WT (Fig.4.11).

IV. 5. Surface metabolites and their role in *B. cinerea* resistance.

Early stages of *B. cinerea* development are crucial for the successful process of pathogenesis (Perryman *et al.,* 2002). Plants have developed multiple defense mechanisms to prevent fungal spreading: release of volatiles, induction of ROS and defence genes. For example, (E)-2-hexanal and β-phellandrene volatiles identified from *Solanum lycopeisicum* were shown to have an inhibitory effect on the germination and hyphal growth of *B. cinerea* (He *et al*., 2005). On another hand, *B. cinerea* has developed multiple layers of adaptation to parasitize on the host that makes it very efficicent necrotrophic pathogen. However, when plant undergoes developmental changes such as, impaired secondary cell wall function in *Arabidopsis* mutants, *B. cinerea* development is strongly supressed in these plants (Hernandez-Blanco *et al.,* 2007). The aims of this subchapter were to identify compounds found on the surface of *Arabidopsis* cotyledons and relate it to *B. cinerea* resistance in *fad* seedlings.

The first step of this work was to extract metabolites localized on the surface of WT and *fad2-3* plants. We applied a drop of isopropanol (2 μl) on each cotyledon, collecting it 30 sec after application. 784 cotyledons were used for the surface analyses. Further samples (196) were used to analyse the metabolite composition of the entire seedling. The extracts were analysed using ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC–qTOF-MS/MS) in collaboration with Gaétan Glauser at Platform of Analytical Chemistry, University of Neuchâtel. For the positive control, we used fresh weight powder obtained from whole WT, *fad2-3- and fad trip* seedlings shoots and compared it to surface metabolites extracted with gently placed isopropanol drops. To visualize the similarities between WT and *fad2-3* samples we used a principal component analysis (PCA; Fig 4.19).

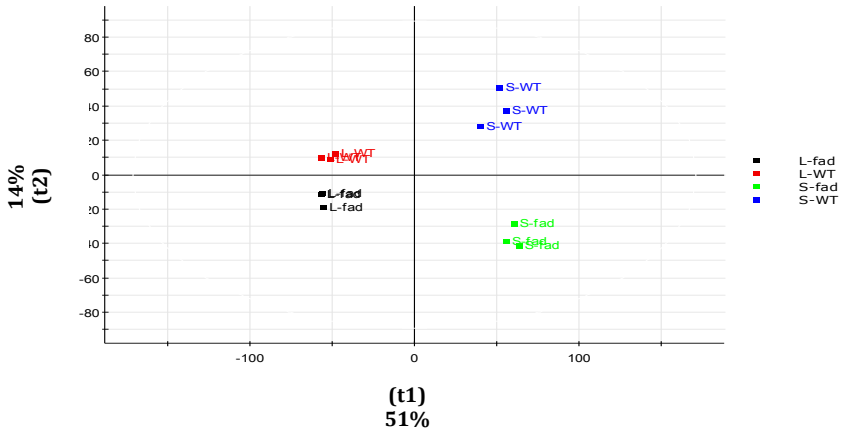


Figure 4.19. PCA score plot from surface (S) and whole shoot (L) metabolites of 7-day-old WT and *fad2-3* seedlings. t[1] and t[2] are first and second principle components, with percentage of explained variance.

The score plot analysis revealed 51% difference between metabolites located on the cotyledon surface and within the whole shoot. The difference between genotypes base on t2 score was 14%. Based on the results we were able to generate a list of metabolites that were more abundant on the surface of WT (Tab.4.2, **A**) or *fad2-3* cotyledons (Tab.4.2, **B**).

Table 4.2. List of metabolites differently abundant on the surface of cotyledons.

Primary ID	Retention Time	Mass	p[1]	p[2]
0.42_96.9604	0.42	96.9604	0.049395	-0.0482446
7.69_115.9202	7.69	115.9202	0.0561692	0.0313873
0.37_175.0241	0.37	175.0241	0.127511	0.0488703
0.51_175.0241	0.51	17`5.0241	0.0819731	0.00919991
3.20_173.1172	3.2	173.1172	0.0631811	-0.0020076
7.61_200.8580	7.61	200.858	0.057357	-0.0307473
1.37_271.1653	1.37	271.1653	0.0540017	-0.0315947
5.11_297.1523	5.11	297.1523	0.0602876	-0.0123178
0.54_306.0757	0.54	306.0757	0.0767897	-0.0225199
5.43_311.1675	5.43	311.1675	0.0950371	0.182941
5.65_311.1674	5.65	311.1674	0.0593553	0.0288497
6.35_325.1830	6.35	325.183	0.0761216	-0.0110057
7.23_339.1990	7.23	339.199	0.0469393	0.0635867
7.73_339.1988	7.73	339.1988	0.0624628	-0.0241187
7.22_355.1573	7.22	355.1573	0.0691702	0.0706257
1.28_420.0452	1.28	420.0452	0.301344	0.0444207
1.58_434.0605	1.58	434.0605	0.110374	0.00198554
1.91_448.0766	1.91	448.0766	0.10657	0.00438866
1.43_447.0524	1.43	447.0524	0.182129	0.0981691
2.25_462.0920	2.25	462.092	0.251875	0.0088241
2.58_476.1076	2.58	476.1076	0.300901	0.0337789
1.92_480.0629	1.92	480.0629	0.075229	-0.000402789

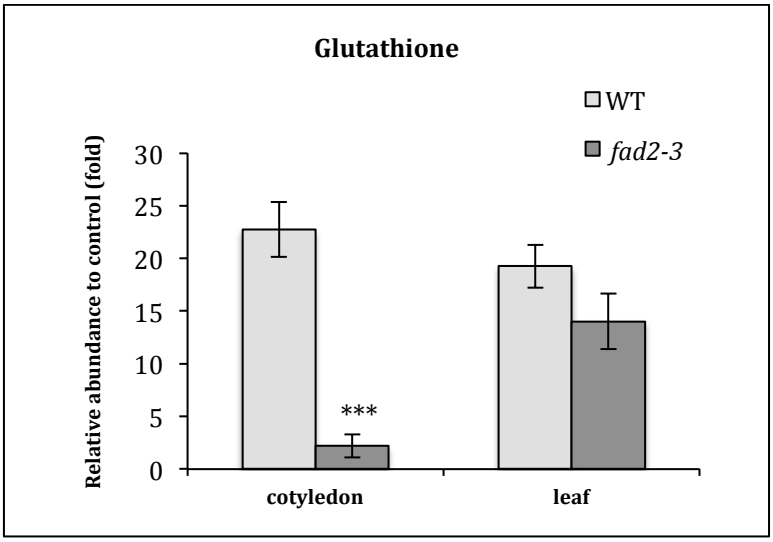
1.66_477.0631	1.66	477.0631	0.050346	-0.0665822
2.14_494.0783	2.14	494.0783	0.153499	-0.0139228
1.28_488.0328	1.28	488.0328	0.0592286	-0.00150966
2.25_530.0797	2.25	530.0797	0.0598475	-0.00510187
2.58_544.0952	2.58	544.0952	0.0675826	0.00213135
2.07_701.1319	2.07	701.1319	0.0671114	-0.0436028
1.28_863.0796	1.28	863.0796	0.0580936	0.0078035

Primary ID	Retention Time	Mass	p[1]	p[2]
2.10_133.0139	2.1	133.0139	-0.0719767	-0.00649105
0.38_135.0291	0.38	135.0291	-0.0568916	-0.0628453
0.59_173.0086	0.59	173.0086	-0.0494958	0.0169776
0.38_194.0482	0.38	194.0482	-0.0860043	0.0505904
8.29_200.8580	8.29	200.858	-0.0526127	-0.109828
7.74_200.8579	7.74	200.8579	-0.0506031	-0.234611
2.08_223.0604	2.08	223.0604	-0.160571	-0.149679
5.47_311.1671	5.47	311.1671	-0.0957646	0.0353388
2.09_339.0711	2.09	339.0711	-0.221309	-0.0802656
1.65_385.1130	1.65	385.113	-0.125053	0.0658945
4.33_431.1831	4.33	431.1831	-0.105223	0.130447
0.57_436.0400	0.57	436.04	-0.0675544	0.0524221
0.64_452.0358	0.64	452.0358	-0.0886851	-0.0511424
3.80_449.1489	3.8	449.1489	-0.128415	0.231524
3.61_449.1488	3.61	449.1488	-0.140641	0.267151
1.27_494.0819	1.27	494.0819	-0.110853	-0.00673135
1.39_492.1025	1.39	492.1025	-0.12882	0.160839
1.90_497.3337	1.9	497.3337	-0.0582332	0.0289323
1.55_508.0981	1.55	508.0981	-0.137131	0.00795124
0.42_565.0476	0.42	565.0476	-0.0524982	-0.00720483
2.46_591.1709	2.46	591.1709	-0.0786776	0.00910763
2.58_591.1709	2.58	591.1709	-0.112459	0.0631813
2.58_613.1524	2.58	613.1524	-0.0486447	0.00346483
2.09_677.1349	2.09	677.1349	-0.126528	-0.0191261
6.63_681.2953	6.63	681.2953	-0.11395	-0.00458775
2.07_679.1502	2.07	679.1502	-0.139561	-0.0896932

2.10_701.1320	2.1	701.132	-0.0597376	-0.0444339
1.59_771.2341	1.59	771.2341	-0.051502	0.0898185
7.18_822.5271	7.18	822.5271	-0.075844	-0.0710086

(**A**) WT and (**B**) *fad2-3* seedlings were analysed in this experiment. List was created automatically based on principal component plots: p1 and p2 vectors. These vectors indicate relation of different variables to each another.

To select compounds potentially involved in *B. cinerea* resistance we performed metabolomics analysis from the surface of true leaves of WT and *fad2-3* two-week old seedlings. This approach was chosen due to the loss of resistance in the first true leaves of *fad2-3* mutants. Metabolites obtained from the surface of first true leaves were compared to the ones obtained from the cotyledons. Only compounds that show more than two fold increase were chosen for further analysis (Fig 4.20).



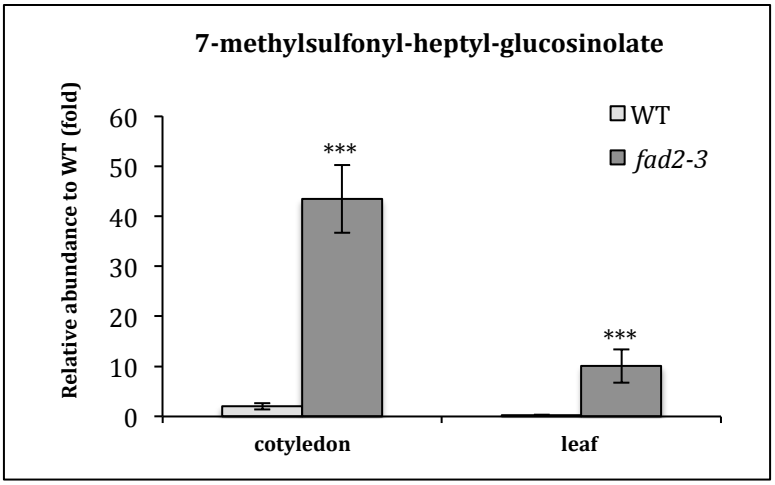


Figure 4.20. Relative quantification of two metabolites differently present on the surface of WT and *fad2-3* cotyledons and leaves. Error bars represent the means of 3 - 4 biological replicates; one biological replicate consisted of isopropanol drops collected from 784 cotyledons and 196 leaves (± SD). Statistical significance in pair-wise comparison was evaluated by Student's test, where *** $p \leq 0.001$.

With the help of a surface metabolomics approach we were able to identify two compounds that could be involved in *B. cinerea* resistance in *fad2-3* mutant. Glutathione is one of the most important sulfur-containing antioxidants and redox buffers in plants (Bergmann and Rennenberg, 1993). It is a tripeptide thiol (ϒ-glutamyl-cysteiyl-glycine) that was shown to play multiple roles in the plant such as: uptake, transport and storage of reduced sulfur, detoxification of ROS, xenobiotics, heavy metals and other substances that can damage cell functioning (Noctor *et al*., 1998; Tausz 2001, Blokhina *et al.,* 2003; Kopriva and Rennenberg, 2004; Tausz *et al.,* 2004).

The other possible candidate may be 7-methylsulfinylheptyl glucosinolate (7MSOH). The mass of this compound is 494.083 in negative mode and its fragmentation products are consistent with such a structure. However, this is a tentative identification that needs conformation by, for example, nuclear magnetic resonance. This compound belongs to the group of secondary metabolites containing sulfur and nitrogen, common in order of *Capparales* and involved in herbivore and pathogen resistance (Rask *et al.,* 2000; Redovnikovic *et al.,* 2008). Levels of glucosinolates are differently distributed in *Arabidopsis* leaves. The highest concentration of glucosinolates was found in the midvein and the outer lamina, whereas one of the lowest on the surface of *Arabidopsis* leaves (Shroff *et al.,* 2008; Soenderby *et al.,* 2010; Shroff *et al.,* 2015). 7MSOH is an aliphatic glucosinolate that is synthetized from pentahomomethionine, that is not strongly abundant in *Arabidopsis* shoot and roots making in average 15 to 20 pmol mg^{-1} of FW (Hogge *et al.,* 1988; Maruyama-Nakashita *et al.,* 2008). 7MSOH is not strongly abundant in *Arabidopsis* leaves and no biological role for this glucosinolate in nature was found so far (Maruyama-Nakashita *et al.,* 2006). Once the cell is damaged glucosinolates are hydrolyzed by myrosinases (thioglucosidase) to form isothiocyanates, thiocyanates or nitriles (Rask *et al.,* 2000). 7MSOH might play a role in preventing *B. cinerea* development on the surface of *Arabidopsis* seedlings.

IV. 6. What is the *B. cinerea* entry point in *Arabidopsis* cotyledons?

B. cinerea is an opportunistic fungus that can infect host organisms through wounded regions, sites previously infected by other pathogens, intact epidermal cells or, by direct penetration through cuticle into the cell wall (McKeen, 1974; van Kan, 2006). The purpose of this subchapter was to identify the entrance points of *B. cinerea* on the cotyledons of the WT and cuticle mutants at the 7-day-old seedling stage.

We used scanning electron microscopy to follow development and appressorium formation of *B. cinerea.* A 6 h post - inoculation time point was chosen to follow germ tube growth (considering previous results, it is an optimal time point). Fig. 4.21 shows a 7-day-old WT cotyledon with developing *B. cinerea* hyphae on it.

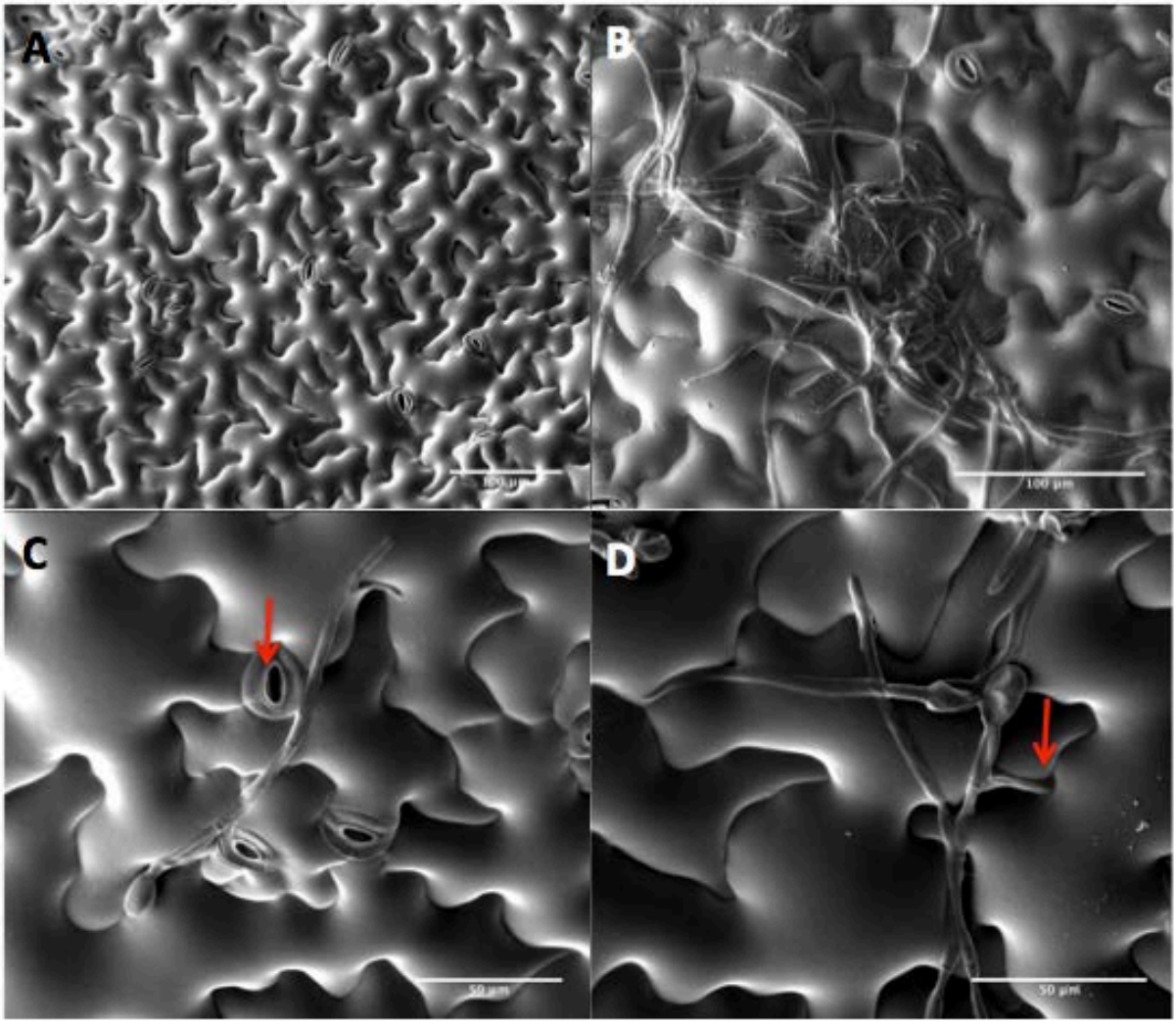


Figure 4.21. Scanning electron micrographs of 7-day-old *Arabidopsis* cotyledons. (**A**) Control, central part of cotyledon, (**B**) 6 h post *B. cinerea* inoculation, (**C**) hyphal growth on the surface of cotyledon avoiding stomata as an entrance point, (**D**) Appressorium formation at the boundary of two pavement cells.

B. cinerea enters the plant through intercellular spaces. The same process has been observed on broad bean leaves (Mansfield and Richardson, 1981), onion (Gourgues *et al.,* 2004) and Chinese wild *Vitis* (Wan *et al.,* 2015). To further examine this we made transversal sections of cotyledons inoculated with *B. cinerea* (Fig.4.22).

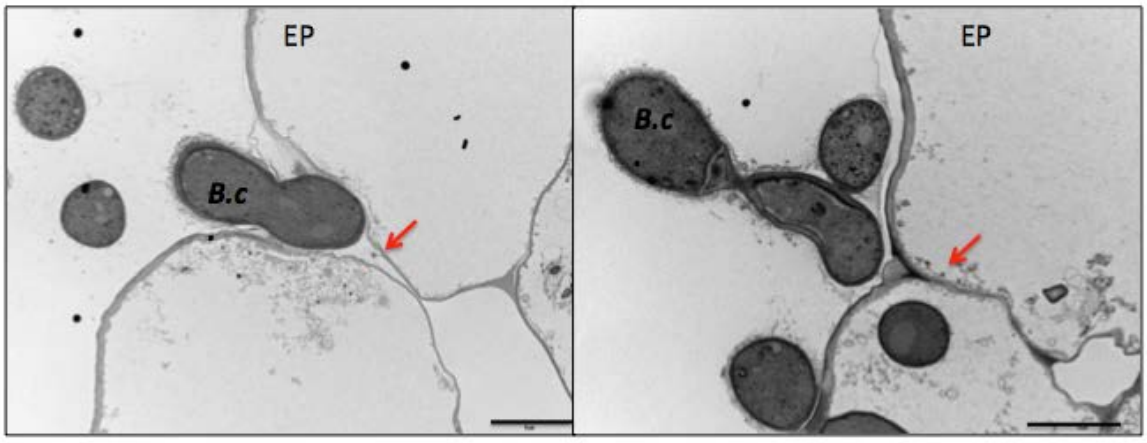


Figure 4.22. Transversal sections of WT cotyledons inoculated with *B. cinerea* for 24h. (**A**), appressorium formation in the intercellular region of two epidermal cells. (**B**), *B. cinerea* entrance in *Arabidopsis* cotyledon. B.c.- *Botrytis cinerea*, EP – epidermal cell, red rows indicate intercellular space. Scale bar 5µm.

This clearly showed that *B. cinerea* penetration started in intercellular spaces. To identify the entrance point of *B. cinerea* in cuticle mutants, cotyledons of *fad2-3- and gpat4 gpat8* mutants were inoculated with *B. cinerea* for 24 h with further preparation of transversal sections and visualization under a transmission electron microscope (Fig.4.23).

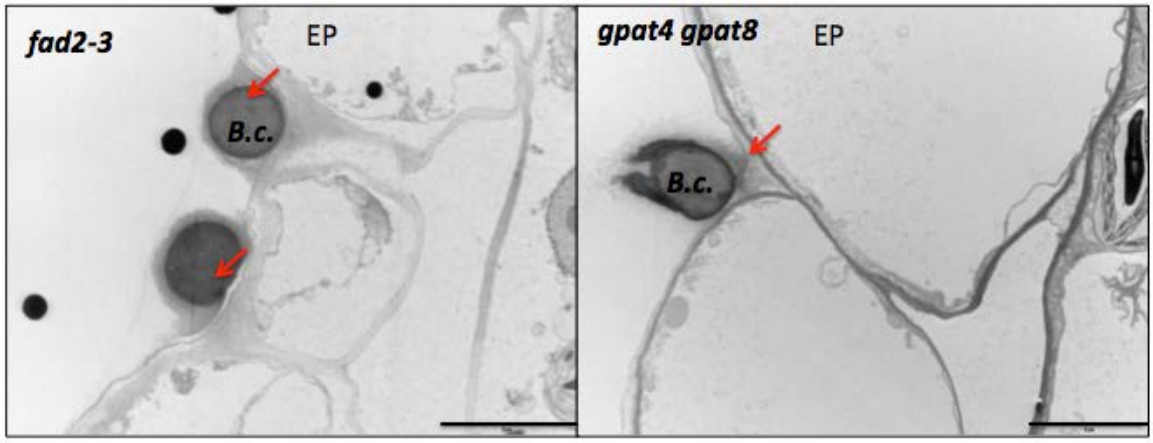


Figure 4.23. Transversal section of cuticle deficient mutants. (**A**) *fad2-3 and* (**B**) *gpat4 gpat8* cotyledons were inoculated with *B. cinerea* (B.c). EP – epidermal cell, red rows indicate *Botrytis cinerea*. Scale bar 5µm.

On both images *B. cinerea* formed mucilage (light grey layer of undefined shape, surrounding spores) - an early step of germ tube development (Hawker and Hendy, 1963). These results support the hypothesis that *B. cinerea* enters *Arabidopsis* cotyledons through the intercellular spaces of epidermal cells.

The next question addressed in this study was to identify preferable cell type for *B. cinerea* entrance. For this purpose we used *Botrytis cinerea* enhanced GFP fluorescent B05 strain (*Bcgfp1*), kindly provided by Prof. Matthias Hahn (Technical University of Kaiserslautern). The fluorescent *B. cinerea* strain was applied to *fad2-3* and WT cotyledons and incubated for 6 h followed by visualization on a Zeiss LSM700 confocal microscope (Zeiss, Feldbach, Switzerland; Fig. 4.24).

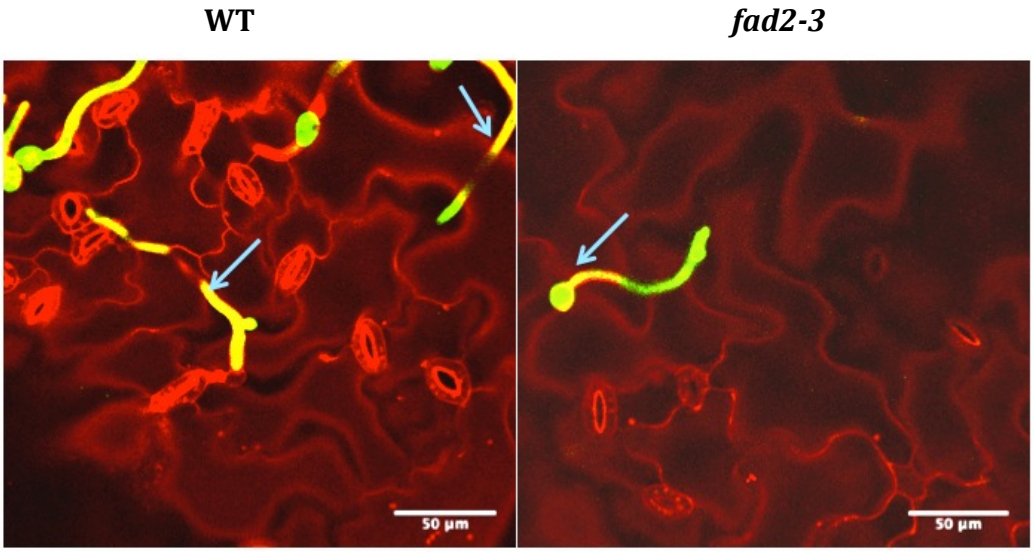


Figure 4.24. *B. cinerea* entrance through the intracellular regions of *Arabidopsis* pavement cells. Cellular borders were stained with propidium iodide. *Bcgfp1* strain was visualized 6h post application. Arrows indicate entrance points of *B. cinerea*.

This image clearly shows that *B. cinerea* enters through the intercellular spaces of *Arabidopsis* pavement cells. We analysed 10 cotyledons of WT and *fad2-3* mutant. No difference in *B. cinerea* entrance on these genotypes was observed.

IV.7. Discussion.

Triunsaturated fatty acids were shown to have an important role in resistance to necrotrophic fungi, as precursors of jasmonic acid (Thomma *et al.,* 1998), whereas the function of diunsaturated fatty acids in this process remained unknown. However, there is only some evidence concerning their involvement in the conidiation process in *Aspergillus parasiticus* (Wilson *et al.,* 2014). DUFAs are major components of the surface defense layer, i.e. in the cuticle

(Bonaventure *et al.,* 2004). Our study further investigated the roles of DUFAs resistance to the necrotrophic fungus *Botrytis cinerea*.

Cuticle deficiency and its role in *B. cinerea* resistance. We showed that cotyledons of mutants deficient in 18:2 (*fad2-3*); 16:3 and 18:3 (*fad trip*) or both DUFAs and TUFAs (*fad2-1 fad6*) have permeable cuticle. Increased permeability of *fad* cotyledons could be explained by changes in composition of major structural polyester components. However, no common cutin monomer defect, compared to WT, was detected in the mutants analysed. Moreover, the *gpat4 gpat8* double mutant showed no differences in FA composition compared to WT, but this mutant displayed the highest cuticle permeability in cotyledons and the first true leaves, together with the most reduced levels of cutin monomers. Toluidine blue staining of the first true leaves showed permeability defect only in *fad2-3* and *fad2-1 fad6* mutants, showing a specific role of TUFAs in the polyester composition of *Arabidopsis* cotyledons, but not true leaves. If only cuticle permeability would be involved in *B. cinerea* resistance, *fad trip* plants would be the only ones to show susceptibility to this pathogen, however leaves of *fad2-3* mutants also show this phenotype. These results lead to conclusion that cuticle permeability might be one of the factors to promote *B. cinerea* resistance, but additional mechanisms should be activated to fight this pathogen.

The role of ROS in *B. cinerea* resistance at the stage of 7-days old seedlings. Another question that we addressed in this research was to understand what leads to *B. cinerea* resistance in *fad* mutants at the stage of 7-day-old seedlings. To address this problem, we analysed internal ROS levels in our mutants. Their upregulation was shown to play a crucial role in the resistance to *B. cinerea* by the activation of defence response genes (Malolepsza and Urbanek, 2002). We used malondialdehyde as a marker for nonenzymatic lipid oxidation. Surprisingly, no difference was found in MDA levels in the WT and the *fad2-3* mutant in non-stressed conditions, but the *fad2-1 fad6* double mutant had MDA levels close to the limit of quantification and *fad trip* levels were lower than in the WT, consistent with previously published results (Schmid-Siegert *et. al.,* 2012). These finding indicate that *fad2-1 fad6* and *fad trip* mutants are present under constant oxidative stress, that might lead to the higher internal levels of the defence hormones, such as SA or JA. However, no difference in the hormonal levels should be found between WT and *fad2-3* seedlings. We assume that *fad2-3* can have different mechanism of *B. cinerea* resistance to *fad2-1 fad6* and *fad trip* seedlings.

Defense hormones and their induction upon *B. cinerea* treatment. Most of the defence marker genes examined did not show a difference upon *B. cinerea* infection at cotyledon stage between WT and mutants. The exception was the *PR1* gene that showed strong basal and induced resistance in *fad trip* mutants,

as well as 5-fold induction in *fad2-3* seedlings. These differences might be explained by possibly higher levels of ROS such as, for example, H_2O_2 in *fad trip* seedlings (Schmid-Siegert *et. al.,* 2012), leading to the synthesis of salicylic acid (Nawrath and Metraux, 1999; Yalpani *et al.,* 1991). Salicylic acid is an important component of plant defence against *B. cinerea* that inhibits fungal entrance at the site of infection (Ferrari *et al.,* 2003), but does not promote resistance to this fungus at the later stages of infection. Consequently, we concluded that *B. cinerea* resistance in *fad* mutants is not simply due to increase in the levels of defence hormones upon *B. cinerea* infection.

Changes in morphology of epidermal cells correlated with *B. cinerea* resistance Previously it was shown that some necrotrophic fungi cannot penetrate into the plant due to surface changes or cell topography (DeZwaan *et al*., 1999; Staples *et al*., 1987; Talbot *et al*., 1996). In this work we showed that *fad* mutants have a different pavement cell morphology compared to the WT. Using atomic force microscopy to analyse pavement cells revealed differences in adhesion and stiffness parameters of *fad2-3* mutants compared to the WT. These results did not explain the arrested growth of *B. cinerea* hyphae in the *fad2-3* mutant, but may relate to the partial spore adhesion defect in this mutant.

Different to the WT metabolites on the surface of *fad2-3* cotyledons. To understand *B. cinerea* resistance in the *fad2-3* mutant surface metabolomic analysis was performed. This technique allowed us to identify metabolites that were abundant on the surface of *fad2-3* cotyledons compared to the WT. Of these 7-methylsulfinylheptyl glucosinolate is of strong interest. Surprisingly, previous works on aliphatic glucosinolates did not show involvment of these compounds in *B. cinerea* resistance (Tierens *et al.,* 2001). The inability to identify the potential involvement of compounds such as 7-methylsulfinylheptyl glucosinolate in fungal resistance previously, could be explained by a low abundance of this glucosinolate in WT plants. Over-accumulation of this compound on the surface of *fad2-3* cotyledons may be promoted by the permeable cuticle that permits chemical transport onto the cotyledon surface. The might also be an indirect effect of DUFA deficiency that causes over-accumulation of methyl-derived glucosinolates.

***B. cinerea* entrance in *Arabidopsis* cotyledons.** The last question of this work was to identify the entrance point of *B. cinerea* in WT and *fad* mutants. According to results of scanning electron microscopy, *B. cinerea* enters into the plant through the intercellular spaces of pavement cells. This is consistent with images from previous publications (Mansfield *et al.,* 1981, Gourgues *et al.,* 2004, Wan *et al.,* 2015). To estimate if there was cell preference for penetration, we applied Bc*gfp* on cotyledons of WT and *fad2-3* mutants. No

cell preference was identified. However, we confirmed that *B. cinerea* indeed enters through the intercellular spaces. Surprisingly, developing hyphae did not growth directly into the mesophyll layer. Instead they sometimes re-emerged from the cotyledon after the first entrance through not only the intercellular spaces but also through the epidermal cells directly.

IV.8. Experimental procedures.

Plant growth conditions, genotypes and chemicals - Wild-type *Arabidopsis* (WT *Columbia* background) was used. The following alleles in the *Columbia* background were received from the ABRC: *fad2-1* (CS8041), *fad2-3* (SK18137), *fad6* (CS207), *fad2-1 fad6* and *fad2-3 fad6* double mutants were obtained by crosses in this work, *gpat4 gpat8* double mutant seeds were kindly provided by Frederic Beisson (Li *et al.,* 2007, Li-Beisson *et al,* 2009), *fad3-2 fad7-2 fad8* mutant seeds from McConn and Browse (1996) and *aos* was from Park *et al.* (2002). Seeds were stratified for 2 days at 4 ^{0}C and then grown at 21 ^{0}C under 100 μE m^{-2} s^{-1} of light with photoperiods depending on the application (seedlings: 14 h light, 10 h dark (long days); on soil for seed propagation: 24 h light (continuous days). Seedlings were grown on half-strength Murashige and Skoog solid media (1/2 X MS, 2.15 g/L, pH 5.7; Duchefa Biochemie, Haarlem, The Nederlands) supplemented with 0.5 g/L of MES hydrate (Sigma, Buchs,

Switzerland) and 0.7 %. No sucrose was added to the media.

Infection with Botrytis cinerea – two strains of *B. cinerea* were used in this work: BMM, kindly provided by C. Nawrath and B 05.10 fused to the GFP (Bc*gfp1*) provided by M. Hahn. The fungus was grown on potato dextrose for 10 days. Conidia were filtered and diluted to $5x10^5$ spores per µl in half PDB. 2 µl of this suspension was applied on cotyledons of 7day old *Arabidopsis* grown in petri dishes with ≈ 100% humidity. After infection Petri dishes were incubated for 48 hours in long day conditions. Infection rate was scored visually by counting the number of seedlings (cotyledons or first true leaves) with lesions. All the chemicals were obtained from Sigma-Aldrich (unless mentioned below).

Fatty acid composition analysis – 7- and 14-day-old seedlings were collected (≈30 mg) and placed in 15 ml Pyrex SVL capped test tubes (Milian, Switzerland). 1 ml of 2.5% H_2SO_4 solution, in methanol was added to the samples. Then, samples were incubated for 90 min at 80 ^{0}C, 1.5 ml 0.9% NaCl and 1 ml of hexane (≥ 97.0% (GC), Sigma-Aldrich, Buchs, Switzerland) were added. The solution was vortexed briefly (2 sec) then centrifuged for 5 min at 1000 rpm. 250 µl of the upper phase containing fatty acid methyl esters were collected. 1 µl of a 1:5 dilution was analysed by GCMS (initial T = 150 ^{0}C for 3 min, with a following increase from 150 ^{0}C to 200 ^{0}C).

Cuticle permeability assay – 14-day old seedlings were placed in 6-well plates with 1 ml of Toluidine blue solution (500 mg TB dissolved in 1 L of water and 0.001% of Tween 20), incubated for 15 min and gently rinsed with water (Tanaka *et al.,* 2004).

Transmission electron microscopy – 7-day-old *Arabidopsis* cotyledon discs (1.5 mm in diameter) were fixed and sectioned as indicated earlier (Fabre *et al.,* 2015). Micrographs were taken with a Philips CM100 TEM (FEI, Eindhoven, the Netherlands) at an acceleration voltage of 80 kV with a TVIPS TemCam-F416 digital camera (TVIPS GmbH, Gauting, Germany).

Polyester analysis – aliphatic polyester composition was performed on 7-day-old seedlings (only the areal part of seedling was used for this analysis). 2000 shoots were collected and pooled in liquid nitrogen to obtain one biological replicate. Six replicates were made for each genotype. Then, the polyester composition was analysed after trans esterification by base-catalysis and following acetylation as described previously (Li-Beisson *et al.,* 2013).

ROS detection assay – 7-day-old seedlings were divided into two groups. For the first group, *B. cinerea* suspension (2 µl) in PDB media was applied to cotyledons. For the second group (used as a control) only PDB (2 µl) was applied to cotyledons. Both groups were then incubated for 24 prior to staining

by ROS-ID™ Total ROS/Superoxide Detection Kit (Enzo Life Sciences AG, Switzerland) as described in the manual. Seedlings were vacuum infiltrated for 30 min under dark conditions. ROS species were visualized *via* wavelengths 490 / 525 (Ex / Em), superoxide via 550 / 620 (Ex / Em).

Gene expression analysis – 7-day-old seedlings were either infected with *B. cinerea* or only PDB solution was applied (as a control) with 24 h incubation. Finally, seedlings were collected and stored in liquid nitrogen. 30 to 50 seedlings were used per one biological replicate. RNA isolation and quantitative RT-PCR was performed as described earlier (Gfeller *et al.*, 2011). *PDF1.2, PR1* and *ABA1* transcripts were quantified as described earlier (Solano *et al.,* 1998, Hilfiker *et al.,* 2014, Chen *et al.,* 2013).

B. cinerea adhesion assay – 1 µL drops of *B. cinerea* spore suspension ($5x10^5$ spores/ml) were applied onto 10 cotyledons of WT and *fad* mutants, incubated for 4 h, then seedlings were washed with 100% ethanol with gentle shaking for 1 h. Cotyledons were stained by dipping 7-days-old cotyledons in preheated solution of Trypan blue (500 mg/L Trypan blue with 1 ml/L of Tween 20 dissolved in water) for 15 sec and directly rinsed in H_2O and visualized under Leica 5000 microscope. Modified and adapted from Doss (1995).

B. cinerea development assay – Spores were applied on 7-day-old seedlings and visualized with Trypan blue at: 4, 8 and 24 h post inoculation. To obtain stain solution, 20 mg of Trypan blue was dissolved in 10 ml of lactic acid, 10 ml glycerol, followed by addition of 10 ml warm water-saturated phenol. To perform staining, seedlings were dipped for 15 to 25 seconds in the warm solution, followed by further 30 min wash in 70 % ethanol to remove excesive stain and to remove chlorophyll. Cotyledons were visualized under a bright field microscope. Hyphal length was measured using the ImageJ freehand tool option.

Cryo-scanning electron microscopy – 7-day-old shoots were fixed with a mixture of 50 % colloidal graphite (Scientific, Essex, United Kingdom) and 50 % Tissue Tek OCTTM (Sakura Finetek, Torrance, USA) at room temperature and then cryofixed. The observations were performed as described previously (Mazurek *et al.,* 2013).

Atomic force microscopy – 7-day-old cotyledons of *Arabidopsis thaliana* (WT and *fad2-3*) were dissected in a moist environment. Each cotyledon was fixed with double-sided tape in a small Petri-dish and quickly covered with ultra-pure water. Measurements were done on NanoWizard III (JPK Instruments, Berlin, Germany) mounted on an Axiovert inverted optical microscope (Zeiss, Jena, Germany). Quantitative Imaging (QI) mode and DNP-10 (Bruker Probes,

Camarillo, USA) cantilevers with spring constants of 0.06 N/m were used. Properly calibrated (glass indentation, thermal tuning) cantilevers indented the samples with 2.5 nN force at 40 μm/s with Z length of 2 μm. Resolution of 0.7 μm per pixel was kept constant throughout imaging. All measurements were conducted within 1h of cotyledon attachment. JPK SPM Data Processing software (JPK Instruments, Berlin, Germany) was used for force-curve fitting, while Matlab was used for data presentation. The Hertz model was used for force-curve fitting, assuming the Poisson ratio of 0.5, and pyramidal geometry of the tip (front angle of 15^0). For histogram representations of pavement cells only a masking parameter was applied on the height, stiffness and adhesion maps.

Metabolomic analysis - was carried out by UHPLC-QTOF-MS on an Acquity UPLC coupled to a Synapt G2 QTOF mass spectrometer (Waters). An Acquity UPLC BEH C18 column (50 x 2.1 mm, 1.7 μm; Waters) was employed at a flow rate of 400 μL/min and maintained at a temperature of 25°C. The following gradient was used with 0.05 % formic acid in water as mobile phase A and 0.05% formic acid in acetonitrile as mobile phase B: 0.0 - 7.0 min 2-100 % B, 7.0 - 9.0 min 100% B, 9.0 - 11.0 min 2% B. The injection volume was 2.5 μL. The QTOF was operated in electrospray negative mode using the MS^E mode. Mass spectrometric parameters were as follows: mass range 85 - 1200 Da, scan time 0.2 s, source temperature 120°C, capillary voltage -2.0 kV, cone

voltage -25V, extraction cone -4.5 V, desolvation gas flow and temperature 800 L/h and 400 ^{0}C, respectively, cone gas flow 20 L/h, collision energy 4 eV (low energy acquisition function) and 10 - 35 eV (high energy acquisition function), collision gas (argon) 7 x 10^{-3} mbars. A 400 ng/mL solution of the synthetic peptide leucine-enkephaline in water: acetonitrile: formic acid (50 : 50 : 0.1) was infused constantly into the mass spectrometer as internal reference to ensure accurate mass measurements. Data were recorded by Masslynx v.4.1. Marker detection was performed using Markerlynx XS (Waters) with the following parameters: initial and final retention time 0.0 and 9.0 min, mass range 85 - 1200 Da, mass window 0.02 Da, retention time window 0.06 min, intensity threshold 500 counts, automatic peak width and peak-to-peak baseline noise calculation, deisotoping function applied. Data were mean - centred and Pareto - scaled before applying principal component analysis. Markers of interest were tentatively identified based on their molecular formula determination and fragments obtained by collision-induced dissociation.

Statistical analysis - Statistical significance in pair-wise comparison was evaluated by Student's test, where * $p \leq 0.05$; ** $p \leq 0.01$; ***$p \leq 0.001$, multiple comparison analysis of variances (ANOVA) followed by Turkey's HSD test was performed using JMP – statistical analysis software.

IV.9. Literature.

Apel, K, and Hirt H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol*. 55:373–399.

Audenaert K, de Meyer GB, Hofte MM. 2002. Abscisic acid determines basal susceptibility of tomato to *Botrytis cinerea* and suppresses salicylic acid-dependent signaling mechanisms. *Plant Physiol.* 128: 491–501.

Bergmann L, Rennenberg H. 1993. Glutathione metabolism in plants. In : *Sulfur Nutrition and Assimilation in Higher Plants*. The Hague : SPB Academic Publishing. pp.102–123.

Bessire M, Chassot C, Jacquat AC, Humphry M, Borel S, Petetot JM, Métraux JP, Nawrath C. 2007. A permeable cuticle in *Arabidopsis* leads to a strong resistance to *Botrytis cinerea*. *EMBO J*. 26:2158-2168.

Birkenbihl RP, Diezel C, Somssich IE. 2012. Arabidopsis WRKY33 is key transcriptional regulator of hormonal and metabolic responses toward *Botrytis cinerea* infection. *Plant Physiol.* 159:266–285.

Blokhina O, Virolainen E, Fagerstedt KV. 2003. Antioxidants, oxidative damage and oxygen deprivation stress. *Annals of Botany*. 91:179 -194.

Bonaventure G, Beisson F, Ohlrogge J, Pollard M. 2004. Analysis of the aliphatic monomer composition of polyesters associated with Arabidopsis epidermis: occurrence of octadeca-cis-6, cis-9-diene-1,18-diene as the major component. *Plant J.* 40(6):920-930.

Buscot F, Munch JC, Charcosset JY, Gardes M, Nehls U, Hampp R. 2000. Recent advances in exploring physiology and biodiversity of ectomycorrhizas highlight the functioning of these symbioses in ecosystems. *FEMS Microbiol Rev*. 24(5):601-614.

Chassot C, Buchala A, Schoonbeek HJ, Métraux, JP, Lamotte O. 2008. Wounding of *Arabidopsis* leaves causes a powerful but transient protection against *Botrytis* infection. *Plant J.* 55:555–567.

Chena L, Zhanga L, Lia D, Wanga F, Yu D. 2013. WRKY8 transcription factor functions in the TMV-cg defence response by mediating both abscisic acid and ethylene signalling in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*. 110:1963-1971.

Comménil P, Belingheri L, Audran JC, Collas A, Dehorter B. 1996. Mise en evidence d'une activité anti-*Botrytis* dans les cires épicuticulaires de jeunes baies de *Vitis vinifera*, variété pinot noir. *J Int Sci Vigne Vin*. 30(1):7-13.

Comménil P, Belingheri L, Dehorter B. 1998. Antilipase antibodies prevent infection of tomato leaves by *Botrytis cinerea*. *Physiol. Mol. Plant Pathol*. 52:1-14.

DeZwaan TM, Carroll AM, Valent B, Sweigard JA. 1999. *Magnoporthe grisea* pth11p is a novel plasma membrane protein that mediates appressorium differentiation in response to inductive surface cues. *Plant Cell*. 11:2013-2030.

Doss RP, Potter SW, Chastagner GA, Chrisian JK. 1993. Adhesion of non-germinated *Botrytis cinerea* conidia to several substrata. *Appl. Environ. Biol.* 59:1786-1791.

Doss RP, Potter SW, Soeldner AH, Christian JK, Fukunaga LE. 1995. Adhesion of germlings of *Botrytis cinerea. Appl. Environm. Microbiol.* 61:260-265.

Doss RP. 1999. Composition and enzymatic activity of the extracellular matrix secreted by germlings of *Botrytis cinerea. Appl. Environm. Microbil.* 65:404-408.

Epstein L, Nicholson RN. 1997. Adhesion of spores and hyphae to plant surfaces. *The Mycota. V. Plant relationships.* 11-25.

Fabre G, Garroum I, Mazurek S, Daraspe J, Mucciolo A, Sankarl A, Humbel BM, Nawrath C. 2016. The ABCG transporter PEC1/ABCG32 is required for the formation of the developing leaf cuticle in *Arabidopsi*s. *New Phytol*. 209(1):192-201.

Ferrari S, Plotnikova JM, De Lorenzo G, Ausubel FM. 2003. *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires *EDS4* and *PAD2,* but not *SID2*, *EDS5* or *PAD4. Plant J*. 35:193–205.

Feys BJ, Parker JE. 2000. Interplay of signaling pathways in plant disease resistance. *Trends Genet*. 16(10):449-455.

Flaishman MA, Kolattukudy PE. 1995. Timing of fungal invasion using host's ripening hormone as a signal. *Proc. Natl. Acad. Sci USA*. 91:6579 –6583.

Gapper C, Dolan L. 2006. Control of plant development by reactive oxygen species. *Plant Physiol.* 141:341–345.

Germeier C, Hedke K, Von Tiedemann A. 1994. The use of pH indicators in diagnostic media for acid-producing plant-pathogens. *Z. Pflanzenkrankh. Pflanzensch*.101:498–507.

Gfeller A, Baerenfaller K, Loscos J, Chetelat A, Baginsky S, Farmer EE. 2011. Jasmonate controls polypeptide patterning in undamaged tissue in wounded *Arabidopsis* leaves. *Plant Physiol.* 156(4):1797-1807.

Gourgues M, Brunet-Simon A, Lebrun MH, Levis C. 2004. The tetraspanin *BcPls1* is required for appressorium-mediated penetration of *Botrytis cinerea* into host plant leaves. *Mol Microbiol*. 51:619–629.

Govrin EM and Levine A. 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Curr. Biol*. 10:751–757.

Halkier BA, Gershenzon J. 2006. Biology and biochemistry of glucosinolates. *Ann Rev Plant Biol.* 57:303–333.

Hamant O, Heisler MG, Jönsson H, Krupinski P, Uyttewaal M, Bokov P, Corson F, Sahlin P, Boudaoud A, Meyerowitz EM, Couder Y, Traas J. 2008. Developmental patterning by mechanical signals in *Arabidopsis*. *Science.* 322:1650-1655.

Hara K, Yokoo T, Kajita R, Onishi T, Yahata S, Peterson KM, Torii KU, Kakimoto T. 2009. Epidermal cell density is auto-regulated via a secretory

peptide, EPIDERMAL PATTERNING FACTOR2 in *Arabidopsis* leaves. *Plant Cell Physiology*. 50:1019–1031.

Hawker LE and Hendy RJ. 1963. An electron microscope study of germination of conidia of *Botrytis cinerea*. *Journal of General Microbiology.* 33:43-46.

He PQ, Zhang PY, Chen KS, Li GY. 2005. Inhibitory effects of several volatiles of *Lycopersicon esculentum* on *Botrytis cinerea. Acta Bot. Yunnan.* 27:315–320.

Hegde Y and Kolattukudy PE. 1997. Cuticular waxes relieve self-inhibition of germination and appressorium formation by the candida of *Magnaporthe grisea*. *Physiol. Mol. Plant Pathol*. 51:75-84.

Hernández-Blanco C, Feng DX, Hu J, Sánchez-Vallet A, Deslandes L, Llorente F, Berrocal-Lobo M, Keller H, Barlet X, Sánchez-Rodríguez C, Anderson LK, Somerville S, Marco Y, Molina A. 2007. Impairment of cellulose synthases required for *Arabidopsis* secondary cell wall formation enhances disease resistance. *Plant Cell*. 19(3):890-903.

Hilfiker O, Groux R, Bruessow F, Kiefer K, Zeier J, Reymond P. 2014. Insect eggs induce a systemic acquired resistance in *Arabidopsis*. *Plant J*. 80:1085–1094.

Hogge LR, Reed DW, Underhill EW, Haughn GW. 1988. HPLC separation of glucosinolates from leaves and seeds of *Arabidopsis thaliana* and their identification using thermospray liquid chromatography-mass spectometry. *J Chromatgr Sci*. 26:551–556.

Hopkins RJ, van Dam NM, van Loon JJA. 2009. Role of glucosinolates in insect-plant relationships and multitrophic interactions. *Ann Rev Entomol*. 54:57–83.

Jarvis WR. 1977. *Botryotinia* and *Botrytis* species: Taxonomy, Physiology and Pathogenicity, A guide to the Literature. Monograph No. 15, Canada Department of Agriculture, Ottawa, Canada. pp.206.

Jenks MA, Tuttle HA, Eigenbrode SD, Feldmann KA. 1995. Leaf epicuticular waxes of the *Eceriferum* mutants in *Arabidopsis*. *Plant Physiol*. 108:369-377.

Jones JD, Dangl JL. 2006. The plant immune system. *Nature.* 444(7117):323-329.

Jones MJ, Epstein L. 1989. Adhesion of *Nectria Haematocca* macroconidia. *Phys. Mol. Plant Pathol*. 35: 453- 461.

Kolattukudy PE, Rogers LM, Li DX, Hwang CS, Flaishman MA. 1995. Surface signaling in pathogenesis. *Proc. Natl. Acad. Sci. U.S.A*. 92:4080-4087.

Kopriva S, Rennenberg H. 2004. Control of sulfate assimilation and glutathione synthesis: interaction with N and C metabolism. *J. Exp. Bot.* 55:1831-1842.

Korolev N, Rav David D, Elad Y. 2004. Involvement of plant hormones in the biocontrol achieved by *Trichoderma harzianum*. *IOBC WPRS Bulletin*. 27(8):363-366.

Kuo KC, Hoch HC. 1996a. Germination of *Phyllosticta ampelicida pycnidiospores*: prerequisite of adhesion to the substratum and the relationship of substratum wettability. *Fung Genet Biol*. 20:18–29.

L'Haridon F, Besson-Bard A, Binda M, Serrano M, Abou-Mansour E, Balet F, Schoonbeek HJ, Hess S, Mir R, Leon J, Lamotte O, Metraux JP. 2011. A permeable cuticle is associated with the release of reactive oxygen species and induction of innate immunity. *PLoS Pathog*. 7(7):e1002148.

Lee SB and Suh MC. 2014. Cuticular wax biosynthesis is up-regulated by the *MYB94* transcriptional factor in *Arabidopsis*. *Plant Cell Physiol*. 56(1):48-60.

Levine A, Tenhaken R, Dixon R, Lamb C. 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell.* 79:583–*593.*

Li YH, Beisson F, Koo AJK, Molina I, Pollard M, Ohlrogge J. 2007. Identification of acyltransferases required for cutin biosynthesis and production of cutin with suberin-like monomers. *Proceedings of the National Academy of Sciences, USA*. 104:18339–18344.

Li-Beisson Y, Pollard M, Sauveplane V, Pinot F, Ohlrogge J, Beisson F. 2009. Nanoridges that characterize the surface morphology of flowers require the synthesis of cutin polyester. *Proceedings of the National Academy of Sciences, USA*. 106:22008–22013.

Li-Beisson Y, Shorrosh B, Beisson F, Andersson MX, Arondel V, Bates PD, Baud S, Bird D, Debono A, Durrett TP, FrankeRB, Graham IA, Katayama K, Kelly AA, Larson T, Markham JE, Miquel M, Molina I, Nishida I, Rowland O,

Samuels L, Schmid KM, Wada HJ, Welti R, Xu C, Zallot R, Ohlrogge J. 2013. Acyl-lipid metabolism. *The Arabidopsis Book/American Society of Plant Biologists*. 11:e0161.

Liu S, Oeljeklaus S, Gerhardt B, Tudzynski B. 1998. Purification and characterisation of glucose oxidase of *Botrytis cinerea*, *Physiol. Mol. Plant Pathol*. 53:123-132.

Liu T, Longhurst AD, Talavera-Rauh F, Hokin SA, Barton MK. 2016. The Arabidopsis transcription factor ABIG1 relays ABA signaled growth inhibition and drought induced senescence. *eLife. 5:e13768.*

Malolepsza U, Urbanek H, 2000. The oxidants and antioxidant enzymes in tomato leaves treated with o-hydroxyethylorutin and infected with *Botrytis cinerea*. *European Journal of Plant Pathology*. 106:657–65.

Malolepsza U, Urbanek H. 2002. *o*-Hydroxyethylorutin-mediated enhancement of tomato resistance to *Botrytis cinerea* depends on a burst of reactive oxygen species. *J Phytopathol.* 150:616-624.

Mansfield JW and Richardson A. 1981. The ultrastructure of interactions between *Botrytis* species and broad bean leaves. *Physiol. Plant Pathol.* 19:41-48.

Maruyama-Nakashita A, Nakamura Y, Tohge T, Saito K, Takashi H. 2008. *Arabidopsis SLIM1* is a central transcriptional regulator of plant sulphur response and metabolism. *Plant Cell*. 18:3235-3251.

Marschall R and Tudzynski P. 2014. A new and reliable method for live imaging and quantification of reactive oxygen species in *Botrytis cinerea:* Technological advancement. *Fungal Genet Biol*. 71:68-75

Mazurek S, Mucciolo A, Humbel BM, Nawrath C. 2013. Transmission Fourier transform infrared microspectroscopy allows simultaneous assessment of cutin and cell-wall polysaccharides of *Arabidopsis* petals. *Plant J*. 74:880–891.

McKeen WE. 1974. Mode of penetration of epidermal cell walls of *Vicia faba* by *Botrytis cinerea*. *Phytopathology*. 64:461-467.

Mène-Saffrané L, Dubugnon L, Chételat A, Stolz S, Gouhier-Darimont C, Farmer EE. 2007. Nonenzymatic oxidation of trienoic fatty acids contributes to reactive oxygen species management in *Arabidopsis*. *J Biol Chem.* 284: 1702–1708.

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F**.** 2004. Reactive oxygen gene network of plants. *Trends Plant Sci.* 9:490–*498.*

Muller R, de Vos M, Sun JY, Sonderby IE, Halkier BA, Wittstock U, Jander G.2010. Differential effects of indole and aliphatic glucosinolates on lepidopteran herbivores. *J Chem Ecol.* 36(8):905–913.

Nadeau JA and Sack F. 2002. Control of stomatal distribution on the *Arabidopsis* leaf surface. *Science*. 296:1697–1700.

Nawrath C, Metraux JP. 1999. Salicylic acid induction-deficient mutants of *Arabidopsis* express *PR-2* and *PR-5* and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell*. 11:1393–1404.

Noctor G, Arisi A-C M, Jouanin L, Foyer CH. 1998. Manipulation of glutathione and amino acid biosynthesis in the chloroplast. *Plant Physiol*. 118:471-482.

Pandey GK, Grant JJ, Cheong YH, Kim BG, Li L, Luan S. 2005. ABR1, an *APETALA2*-domain transcription factor that functions as a repressor of ABA response in Arabidopsis. *Plant Physiol.* 139(3):1185-1193.

Park JH, Halitschke R, Kim HB, Baldwin IT, Feldmann KA, Feyereisen R. 2002. A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in *Arabidopsis* due to a block in jasmonic acid biosynthesis. *Plant J*. 31(1):1-12.

Penninckx IAMA, Eggermont K, Terras FRG, Thomma BPHJ, De Samblanx GW, Buchala A, Métraux J-P, Manners JM, Broekaert WF. 1996. Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. *Plant Cell.* 8:2309–2323.

Penninckx IAMA, Thomma BPHJ, Buchala A, Métraux J-P, Broekaert WF. 1998. Cooperative activation of jasmonate and ethylene response pathways in parallel is required for induction of a plant defensin gene in *Arabidopsis*. *Plant Cell*. 10:2103–2114.

Perryman S, Fitt BDL, Harold JFS. 2002. Factors affecting the development of *Botrytis cinerea* (grey mold) on linseed (*Linum usitatissimum*) buds, flowers and capsules. *Ann. appl. Biol.* 140:1-12.

Pezet R, Viret O, Gindro K. 2004. Plant-microbe interaction: the *Botrytis* grey mould of grapes. Biology, biochemistry, epidemiology and control management. *Advances in Plant Physiology*. 7:179–193.

Podil GK, Rogers LM, Kolattukudy PE. 1993. Chemical signals from avocado surface wax trigger germination and appressorium formation in Colletotrichum gloeosporioides. *Plant Physiol.* 103:267-272.

Rask L, Andre´asson E, Ekbom B, Eriksson S, Pontoppidan B, Meijer J. 2000. Myrosinase: gene family evolution and herbivore defense in *Brassicaceae*. *Plant Mol Biol.* 42:93–113.

Redecker D, Kodner R, Graham LE. 2000. Glomalen fungi from the Ordovician. *Science.* 289(5486):1920-1921.

Redovnikovic I. R., Glivetic T., Delonga K., Vorkapic-Furac J. 2008. Glucosinolates and their portential role in plant. *Period. Biol.* 110:297–309.

Remy W, Taylor TN, Hass H, Kerp H. 1994. Four hundred-million-year old vesicular arbuscular mycorrhizae. *Proc Natl Acad Sci USA.* 91(25):11841-11843.

Routier-Kierzkowska AL, Weber A, Kochova P, Felekis D, Nelson BJ, Kuhlemeier C, Smith RS. 2012. Cellular force microscopy for *in vivo* measurements of plant tissue mechanics. *Plant Physiol*. 158(4):1514-1522.

Sampathkumar A, Krupinski P, Wightman R, Milani P, Berquand A, Boudaoud A, Hamant O, Jönsson H, Meyerowitz EM. 2014. Subcellular and supracellular mechanical stress prescribes cytoskeleton behavior in Arabidopsis cotyledon pavement cells. *Elife*. 16(3):e01967.

Schmid-Siegert E, Loscos J. 2012. Inducible malondialdehyde pools in zones of cell proliferation and developing tissues in *Arabidopsis*. *J. Biol. Chem.* 287: 8954-8962.

Schmid-Siegert E, Stepushchenko O, Glauser G, Farmer E.E. 2016. Membranes as structural antioxidants: Recycling of malondialdehyde to its source in oxidation-sensitive chloroplast fatty acids. *J. Biol. Chem.* 291:13005-13013.

Schnurr, J., Shockey, J., and Browse, J. 2004. The acyl-CoA synthetase encoded by *LACS2* is essential for normal cuticle development in Arabidopsis. *Plant Cell.* 16:629–642.

Sela-Buurlage MB, Epstein L, Rodriguez RJ. 1991. Adhesion of ungerminated *Colletotricum musae* conidia. *Physiol. Mol. Plant Pathol.* 39:345-352.

Serrano M, Coluccia F, Torres ML, Haridon F, Metraux JP. 2014. The cuticle and plant defense to pathogens. *Front.PlantSci*. 5:274.

Shroff R, Vergara F, Muck A, Svatos A, Gershenzon J. 2008. Nonuniform distribution of glucosinolates in *Arabidopsis thaliana* leaves has important consequences for plant defense. *Proc Natl Acad Sci U S A*. 2008. 105(16):6196-201.

Shroff R, Schramm K, Jeschke V, Nemes P, Vertes A, Gershenzon J, Svatos A. 2015. Quantification of plant surface metabolites by matrix-assisted laser desorption-ionization mass spectrometry imaging: glucosinolates on *Arabidopsis thaliana* leaves. Plant J. 81(6):961-972.

Solano R, Stepanova A, Chao Q, Ecker JR. 1998. Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. *Genes Dev.* 12:3703–3714.

Sonderby IE, Burow M, Rowe HC, Kliebenstein DJ, Halkier BA. 2010. A complex interplay of three *R2R3 MYB* transcription factors determines the profile of aliphatic glucosinolates in *Arabidopsis*. *Plant Physiol*. 153(1):348-63.

Staples RC, Hoch HC. 1987. A possible role for microtubules and microfilaments in the induction of nuclear division in bean rust uredospore germlines. *Expl Mycol.* 6:293-302.

Tanaka T, Tanaka H, Machida C, Watanabe M, Machida Y. 2004. A new method for rapid visualization of defects in leaf cuticle reveals five intrinsic patterns of surface defects in *Arabidopsis*. *Plant Journal*. 37:139–146.

Talbot NJ, Kershaw MJ, Wakley GE, de Vries OMH, wessels JGH, Hamer JE. 1996. *MPGI* encodes a fungal hydrophobin involved in surface interactions during infection-related development of *Magnoporthe grisea*. *Plant Cell.* 8:985-999.

Tausz M. 2001. The role of glutathione in plant response and adaptation to natural stress. Significance of Glutathione to Plant Adaptation to the Environment. 101–122.

Tausz M, Šircelj H, Grill D. 2004. The glutathione system as a stress marker in plant ecophysiology: is a stress-response concept valid? *J Exp Bot*. 55:1955–1962.

Thomma BPHJ, Eggermont K, Penninckx IAMA, Mauch-Mani B, Vogelsang R, Cammue BPA, Broekaert WF. 1998. Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proc Natl Acad Sci USA*. 95:15107–1511.

Tierens K.F.M, Thomma B.P.H.J, Brouwer M, Schmidt J, Kistner K, Porzel A, Mauch-Mani B, Cammue B.P.A, Broekaert W. F. 2001. Study of the role of antimicrobial glucosinolate - derived isothiocyanates in resistance of *Arabidopsis* to microbial pathogens. *Plant Physiol.* 125(4):1688-1699.

Tucker SL, Talbot NJ. 2001. Surface attachment and pre-penetration stage development by plant pathogenic fungi. *Annu Rev Phytopathol*. 39: 375-417.

Van Kan JAL. 2005. Infection stages of *Botrytis cinerea*. Proc. VIIIth IS Postharvest Phys. Ornamentals. Acta Hort. 669, ISHS.

Van Loon LC, Pierpoint WS, Boller T and Conejero V. 1994. Recommendations for naming plant pathogenesis-related proteins. *Plant Mol. Biol. Rep.* 12:245–264.

Voisin D, Nawrath C, Kurdyukov S, Franke RB, Reina-Pinto JJ, Efremova N, Will I, Schreiber L, Yephremov A. 2009. Dissection of the complex phenotype in cuticular mutants of Arabidopsis reveals a role of SERRATE as a mediator. *PLoS Genet*. 5(10): e1000703.

Wan R, Hou X, Wang X, Qu J, Singer SD, Wang Y, Wang X. 2015. Resistance evaluation of Chinese wild Vitis genotypes against Botrytis cinerea and different responses of resistant and susceptible hosts to the infection. *Front Plant Sci*. 26(6):854.

Wessels JGH, de Vries OMH, Asgeirdotir SA, Schuren FHJ. 1991. Hydrophobin genes involved in formation of aerial hyphae and fruit bodies in *Schizophylum*. *Plant Cell*. 3:793:799.

Willmer CM and Fricker M. 1996. Stomata. Second edition. Chapman and Hall, London. pp. 375.

Wilson RA, Calvo AM, Chang PK, Keller NP. 2004. Characterization of the *Aspergillus parasiticus* delta12-desaturase gene: a role for lipid metabolism in the *Aspergillus*-seed interaction. *Microbiology*. 150(9):2881-1889.

Windram O, Madhou P, McHattie S, Hill C, Hickman R, Cooke E, Jenkins DJ, Penfold CA, Baxter L, Breeze E, Kiddle SJ, Rhodes J, Atwell S, Kliebenstein DJ, Kim YS, Stegle O, Borgwardt K, Zhang C, Tabrett A, Legaie R, Moore J, Finkenstadt B, Wild DL, Mead A, Rand D, Beynon J, Ott S, Buchanan-Wollaston V, Denby KJ. 2012. *Arabidopsis* defense against *Botrytis cinerea*: chronology and regulation deciphered by high-resolution temporal transcriptomic analysis. *Plant Cell*. 24:3530–3557.

Wu TC, Belteton SA, Pack J, Szymanski DB, Umulis DM. 2016. LobeFinder: A Convex Hull-Based Method for Quantitative Boundary Analyses of Lobed Plant Cells. *Plant Physiol*. 171(4):2331-2342.

Xia Y, Yu K, Navarre D, Seebold K, Kachroo A, Kachroo P. 2010. The *glabra1* mutation affects cuticle formation and plant responses to microbes. *Plant Physiol*. 154:833–846.

Xue Y, Xiao S, Kim J, Lung SC, Chen L, Tanner JA, Suh MC, Chye ML. 2014. *Arabidopsis* membrane-associated acyl-CoA-binding protein *ACBP1* is involved in cuticle formation. *J Exp Bot*. 65(18):5473-5483.

Yalpani N, Silverman P, Wilson TMA, Kleier DA, Raskin I. 1991. Salicylic acid as a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacoo. *Plant Cell*. 3:809-818.

Yang Z, Tian L, Latoszek-Green M, Brown D, Wu K. 2005. *Arabidopsis ERF4* is a transcriptional repressor capable of modulating ethylene and abscisic acid responses. *Plant Mol Biol.* 58:585–596.

Zhu Y, Schluttenhoffer CM, Wang P, Fu F, Thimmapuram J, Zhu JK, Lee SY, Yun DJ, Mengiste T. 2014. Cyclin-dependent kinase8 differentially regulates plant immunity to fungal pathogens through kinase-dependent and –independent functions in *Arabidopsis*. *Plant Cell.* 26(10):4149-4170.

Chapter V: Concluding remarks

The focus of this work was to understand the roles of diunsaturated fatty acids (DUFAs) in seedling establishment and defence. Previous work revealed that plants lacking DUFAs could not survive on soil (McConn *et al.,* 1998). In this thesis we generated plants with extremely low levels of DUFAs (< 1% total FAs) that had dwarfish and yellowish phenotypes but were viable on soil and able to produce seeds. So far no specific morphological phenotypes have been reported for *Arabidopsis fad2* mutants during their first weeks of development. In this work we found that *fad2* mutants show reduced root growth and that this correlated with high internal ROS levels. The most surprising finding of this research concerned aerial tissues. Both *fad2-3* and *fad trip* seedlings were strongly resistant to the necrotrophic pathogen *Botrytis cinerea*. We focused our research on cotyledons and found high levels of a glucosinolate (potentially 7-methylsulfinylheptyl-glucosinolate) on the surface of *fad2-3* cotyledons. This could potentially have a fungitoxic affect on *B. cinerea*. The thesis covered a broad range of questions related to polyunsaturated fatty acids and their involvement in plant development and adaptation to stresses. However, even more questions need to be addressed to better understand roles of DUFAs in plant. This chapter contains perspectives for further work on DUFAs.

V.1. Characterization of *Arabidopsis* PUFA-deficient *fad* mutants.

In the chapter II of this thesis I compared phenotypic and biochemical characteristics of *fad2* and *fad6* mutants to the WT. Fatty acid analysis of the WT identified changes in fatty acid composition during the 3- to 7-day-old stage. These changes probably reflect semi-autotrophic growth during the first days, followed by switch to fully autonomous growth on the 5^{th} to 6^{th} day of seedling development. One of the open questions from this part was what are the mechanisms used by the plant to control fatty acid changes? Are these processes controlled at the translational or transcriptional level? To separate these processes qPCR can be performed to analyse gene expression and western blot could be performed to visualize changes in protein levels. However, this was not addressed and the thesis instead concentrated on the questions more related to the roles of DUFAs in plants.

An interesting finding was the bushy phenotype of *fad2-3* mutants observed during the flowering period. Notably, this was not observed in *fad2-1* mutant. The absence of this phenotype in *fad2-1* mutants could be caused by a weaker (hypomorphic) allele in this plant that leads to slightly higher levels of PUFAs in *fad2-1* plant compared to *fad2-3,* which appears to be a null mutant. Quantitative FA analysis using C18 and C16 standards will be required to test this hypothesis and this may be challenging since DUFA levels are low in

fad2-1 mutant. The bushy phenotype could be related to changes in the levels of auxin, strigolactone or cytokinin in this mutant. These hormones control rosette morphology (Fellner, 1996; Prochazka and Truksa, 1999; Nordström *et al.,* 2004; Brewer *et al.,* 2009 Mordhorst *et al.,* 1998; Nogue *et al.,* 2000). All of these hypotheses require further analysis, for example, by performing hormonal mesurments at the flowering stage to identify which hormone causes to the bushy morphology of *fad2-3* mutants. This could be followed by detailed analysis of the biosynthesis of this hormone to identify the stage that could potentially involve FAs. Performance of these experiments could possibly lead to a better understanding of plant development and role of DUFAs in this process.

V.2. The effect of *Arabidopsis* DUFA-deficient *fad2* mutants on seedling growth.

In the Chapter III of this thesis I identified a role of PUFAs in root development using *fad2* mutants. The reduced root growth in these seedlings was explained by almost complete absence of PUFAs that are known to be ROS scavengers (Mène-Saffrané *et al.,* 2007; Schmid-Siegert *et al.,* 2016). High internal ROS levels in *fad2* mutants were suggested by ROS staining. These high ROS levels would lead to constant oxidative stress. The most obvious hypothesis is that once another external stress is added, for example, salt stress, it leads to stronger suppression of the root growth in *fad2* mutants

compared to WT seedlings (Zhang *et al.,* 2012; this work). Analysis of root growth characteristics in this thesis showed that this effect is mainly caused by a reduction in the number of meristem cells and reduced cortex cell lengths in the mutants, due to over accumulation of ROS that leads to oxidative stress followed by decrease in the levels of growth hormones. This work can be continued in multiple ways. First of all, the *FAD2* gene could be expressed in different cell types of *fad2-3* roots to attempt to rescue root length. Then *fad2-1* or/ and *fad2-3* could be crossed with respiratory burst oxidase homologs (*rboh*) mutants to obtain plants with reduced ROS levels in the meristem and elongation zones (Lee *et al.,* 2013).

The almost complete absence of PUFAs and their replacement by 18:1 FAs could lead to the changes in root polyester composition in *fad2-1* and *fad2-3* mutants. This might affect root suberinization and casparian strip maturation that can be investigated by fluorol yellow and propidium iodide staining. If changes in suberin levels are detected, it would be interesting to apply pathogens to WT and *fad2* roots to examine the role of monounsaturated fatty acids in root defence.

V.3. PUFAs and their role in resistance to *Botrytis cinerea.*

In the last experimental chapter (IV) of this thesis I identified resistance of *fad2-3*, *fad2-1 fad6* and *fad trip* cotyledons to the necrotrophic pathogen *Botrytis cinerea*. These mutants were found to have permeable cotyledon cuticles, but no detectable changes in the internal ROS levels or upregulation of common hormonal marker genes in aerial tissues were found. The increased resistance of *fad2* mutants activated at the early stages of *B. cinerea* infection. Changes in pavement cell topography of *fad2-3* mutants can explain a partial adhesion defect of *B. cinerea* on *Arabidopsis* cotyledons. However, the major resistance of *fad2-3* to *B. cinerea* may be associated with surface metabolites. In this work one such metabolite (tentatively identified as the glucosinolate 7MSOH) was found. The structure of this compound needs to be established. To test whether this compound has a fungitoxic activity additional experiments need to be done. For example, incorporation of the glucosinolate into *B. cinerea* growth media followed by application on WT cotyledons and quantifying number of seedlings showing resistance. Another possible experiment to extend this research could be to perform surface metabolite analysis on *fad trip* and *fad2-1 fad6* cotyledons and first true leaves. This should identify similar or different compounds involved in *B. cinerea* resistance in these mutants. Beyond this, it would be interesting to identify *B. cinerea* mutants that show resistance to a 7MSOH, followed by full

transcriptomic analysis compared to control *B. cinerea* strain. These results will help us to better understand internal mechanisms that are involved in fungal susceptibility to this glucosinolate.

V.4. Literature.

Brewer PB, Dun EA, Ferguson BJ, Rameau C, Beveridge CA. 2009. Strigolactone acts downstream of auxin to regulate bud outgrowth in pea and *Arabidopsis*. *Plant Physiol.* 150:482–493.

Fellner M. 1999. Research on mechanisms of auxin action: Isolation and characterization of auxin-related mutants. In: Strnad M, Pec P, Beck E (Ed). *Adv. Reg. Plant Growth Devel.* Peres Publ., Prague. pp.139–155.

Lee Y, Rubio MC, Alassimone J, Geldner N**.** 2013. A mechanism for localized lignin deposition in the endodermis. *Cell*. 153:402-412.

Mène-Saffrané L, Dubugnon L, Chételat A, Stolz S, Gouhier-Darimont C, Farmer EE. 2007. Nonenzymatic oxidation of trienoic fatty acids contributes to reactive oxygen species management in *Arabidopsis*. *J Biol Chem.* 284:1702–1708.

McConn M, Browse J. 1996. The critical requirement of linolenic acid is pollen development, not photosynthesis, in an *Arabidopsis* mutant. *Plant Cell*. 8:403-416.

Mordhorst AP, Voerman KJ, Hartog MV, Meijer EA, van Went J, Koornneef M, de Vries SC. 1998. Somatic embryogenesis in *Arabidopsis thaliana* is facilited by mutations in genes repressing meristematic cell divisions. *Genetics*. 149:549-563.

NogueÂ F, Grandjean O, Craig S, Dennis E, Chaudhury A. 2000. Higher levels of cell proliferation rate and cyclin *CycD3* expression in the *Arabidopsis amp1* mutant. *Plant Growth Regul.* 32:275-283.

Nordstrom A, Tarkowski P, Tarkowska D, Norbaek R, Astot C, Dolezal K, Sandberg G. 2004. Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: a factor of potential importance for auxin-cytokinin-regulated development. *Proc. Natl. Acad. Sci. U.S.A.* 101:8039–8044.

Procházka S, Truksa M. 1999. Phytohormones and shoot apical dominance. *Adv. Reg. Plant Growth Devel.* Peres Publ., Prague. pp.221–231.

Schmid-Siegert E, Stepushchenko O, Glauser G, Farmer E.E. 2016. Membranes as structural antioxidants: Recycling of malondialdehyde to its source in oxidation-sensitive chloroplast fatty acids. *J. Biol. Chem.* 291:13005-13013.

Zhang J, Liu H, Sun J, Li B, Zhu Q, Chen S, Zhang H. 2012. *Arabidopsis* fatty acid desaturase *FAD2* is require for salt tolerance during seed germination and early seedling growth. *PLoS One* 7:e30355

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