Characterization of transcription factors important for fatty acid and lipid metabolism in the phytopathogen *Fusarium graminearum*

Dissertation

A thesis submitted to the
Fachbereich Biologie, Universität Hamburg
for the degree of
doctor rerum naturalium

by

Le, Thi Thu Giang

Ha noi, Viet nam

Hamburg, 2011

December 22nd, 2010

To Whom it May Concern

This letter is to certify that the English in the thesis titled "Characterization of transcription factors important for fatty acids and lipid metabolism in the phytopathogen Fusarium graminearum" submitted to the Fachbereich Biologie, Universität Hamburg for the degree of doctor rerum naturalium by Le, Thi Thu Giang fulfills the language requirements of the University of Hamburg.

Sincerely

Prof Dr. Michael St. John
University of Hamburg
Department of Biology
Institute for Hydrobiology and Fisheries Science

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der Fakultät für Mathematik, Informatik und Naturwissenschaften
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auf Antrag von Prof. Dr. W. SCHÄFER
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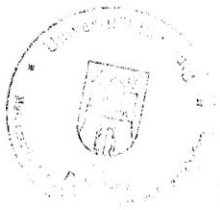
Prof. Dr. C. VOIGT
Tag der Disputation: 25. Februar 2011

Hamburg, den 10. Februar 2011



Professor Dr. Axel Temming
Leiter des Fachbereichs Biologie

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ABBREVIATIONS	FULL NAME
%	Percentage
Δ	Delta/mutant
aa	amino acid
BLAST	Basic Local Alignment Search Tool
bp	base pairs
°C	Degree Celsius
C	Carbon chain
cDNA	complementary Deoxyribonucleic Acid
CDS	Coding sequence
CM	Complete medium
CMC	Carboxymethylcellulose
CT	Control
CTF	Cutinase Transcription Factor
CTAB	Cetyl trimethyl ammonium bromide
CUT	Cutinase
cv	Cultivated variety; cultivar
CWDE (s)	Cell Wall Degrading Enzyme (s)
DEPC	Diethylpyrocarbonate
DIG	Digoxygenin
DNA	Deoxyribonucleic Acid
dNTPs	Desoxynucleotide triphosphate (s)
DON	Deoxynivalenol
dpi	days post inoculation
EC	Enzyme Commission
ECT	Ectopic strains
EDTA	Ethylenediaminetetraacetic acid
et al.	et alii = and others
FAR	Fatty acid regulator
F_{do}	downstream fragment

FGL	*Fusarium graminearum* lipase
FHB	Fusarium Head Blight
Fig.	Figure
FOX	Fatty acid OXidation
F_{up}	Upstream fragment
gDNA	genomic DNA
Gpmk1	Gibberella pathogenicity MAP kinase 1
HYG	Hygromycin B phosphotransferase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	kilo base pair (= 1000 bp)
KO	Knock-out
L	Liter
LB	Luria-Bertani medium
LR	Lipase regulator
M	Molar (mol/L)
MAP	Mitogen Activated Protein
MAPK	Mitogen Activated Protein Kinase
min	minute
MIPS	Munich Information center for Protein Sequences
ml	millilitre
mM	millimolar
MM	Minimal medium
mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
NIV	Nivalenol
nptII	neomycin phosphotransferase
OD	Optical Density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PEX	PEroXin

pH	Potentia Hydrogenii
pNP	para-Nitrophenol
qPCR	quantitative PCR
RNA	Ribonucleic acid
rpm	round per minute
RT-PCR	Reverse transcriptase PCR
SCFA	Short chain fatty acid
SDS	Sodium Dodecylsulfate
SNA	Synthetic Nutrient Agar
Tab.	Table
TBE	TRIS-Borate-EDTA
TF	Transcription factor
Tm	Annealing Temperature
Tri	Trichothecene synthase gene
Tris	Tris-(hydroxymethyl) aminomethane
UV	Ultra violet
v	Volume
v/v	Volume per volume
w/v	Weight per volume
WGO	Wheat Germ Oil
WT	Wild type
X-gal	Bromo-chloro-indolyl-galactopyranoside
YPG	Yeast-extract Peptone Glucose
ZEA	Zearalenone

1. Introduction

1.1. Fusarium head blight

Fusarium head blight (FHB) is one of most important diseases of small grain cereals. It was first described in 1884 in England, called wheat scab, and later, tombstone disease, because of the chalky, lifeless appearance of the infected kernels (Clear and Patrick, 2009; Goswami and Kistler, 2004; Leonard and Bushnell, 2003). FHB has become of increasing international importance in recent years. FHB can affect wheat, barley, oats, rye, corn, triticale, canary seed and some forage grasses.

FHB is caused by several *Fusarium* species such as *Fusarium graminearum, F. culmorum, F. poae, F. avenacearum, F. sporotrichioides, and F. nivale* (Osborne and Stein, 2007). Epidemic levels of FHB have occurred in America, Asia, Canada, Europe and Australia, causing world-wide losses in food supplies (Goswami and Kistler, 2004; Kikot et al., 2009; O'Donnell et al., 2000; O'Donnell et al., 2004; Osborne and Stein, 2007; Starkey et al., 2007; Uhlig et al., 2007; Yang et al., 2008; Yli-Mattila et al., 2009). Yield losses caused by FHB may reach 50 – 60% on infected fields (Osborne and Stein, 2007; Windels, 2000). In the 1990s, yield losses caused by epidemic FHB led to losses estimated at $ 3 billion dollars in the USA and $ 220 million dollars in Quebec and Ontario, Canada (Windels, 2000). In China, it is estimated that FHB affected up to 7 million hectares and 2.5 million tons of grain in epidemic years (Duveiller et al., 2007; Hongxiang et al., 2008).

The FHB infection does not only result in reduced yield because of shrunken grains but also in reduced milling and malting quality and the contamination of grains with several trichothecene mycotoxins including deoxynivalenol (DON), nivalenol (NIV), and zearalenone (Zea), (Bennett and Klich, 2003; Edwards, 2004; Sherwood and Peberdy, 1974; Tanaka et al., 1986) that are toxic to plants, humans and animals.

1.2. The phytopathogenic fungus *Fusarium graminearum*

Fusarium graminearum (anamorph *Gibberella zeae*) is a homothallic (self-fertile) ascomycete with ubiquitous geographic distribution. It is the most important causal

agent of Fusarium head blight of wheat, barley, corn, and rice (Jurgenson et al., 2002; Leonard and Bushnell, 2003).

F. graminearum is capable of producing several trichothecene mycotoxins, including deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) (Jurgenson et al., 2002; Pineiro et al., 1995; Sydenham et al., 1991; Trail, 2009). *F. graminearum* causes low grain weight, the primary economic and health consequences of the disease are due to mycotoxin contamination, primarily DON (Trail, 2009). DON is a potent inhibitor of protein synthesis in eukaryotic organisms and causes a variety of acute and chronic toxicoses when ingested in sufficient quantities by human or animals (Bluhm et al., 2007). Because of the highly toxic nature of DON, the world regulatory agencies have imposed strict guidelines and tolerances regarding its presence in grain and foods. For example, Chinese Ministry of Health advises a limit of DON level in finished flour product intended for human consumption is less than 1 mg kg^{-1}. Similar guidelines have also been set for the United States (1 mg kg^{-1}), European Union (0.5 mg kg^{-1}), Austria (0,5 mg kg^{-1}), and Canada (2 mg kg^{-1}) (Bluhm et al., 2007; Ilgen et al., 2009; Trail, 2009; Zhang et al., 2009).

In barley, the presence of *F. graminearum* can also lead to uncontrolled foaming (gushing) of beer caused by an unknown fungal component. The presence of *F. graminearum* in maize has resulted in high levels of zearalenone and other mycotoxins in the “distillers’ dried grains with solids”, remnants from maize-based ethanol production fed to cattle and pigs. Zearalenone causes estrogenic effects in animals, including humans, and is regulated in some countries. Although zearalenone is of concern to the U.S. Food and Drug administration, there are currently no regulatory standards limiting its levels in grain (Marcia et al., 2008; Trail, 2009).

F. graminearum overwinters as mycelia and spores on seed, and as mycelia and perithecia in infested residue. Ascospores produced in perithecia and conidia produced from mycelia during warm, moist weather are disseminated by wind to the plant host. Diseased seeds cause seedling blight, and soilborne inoculum causes seedling blight and root rot. *F. graminearum* develops in warm, humid weather

during formation and ripening of the kernels. Prolonged wet weather during and after anthesis favors infection. The macroconidia serve as secondary inoculum. Grain that becomes wet in swath favors *F. graminearum* development. The epidemic is more severe when rye follows maize (Lee et al., 2006; Nyvall, 1999). The life cycle of *F. graminearum* can be summarized as follows (Fig.1)

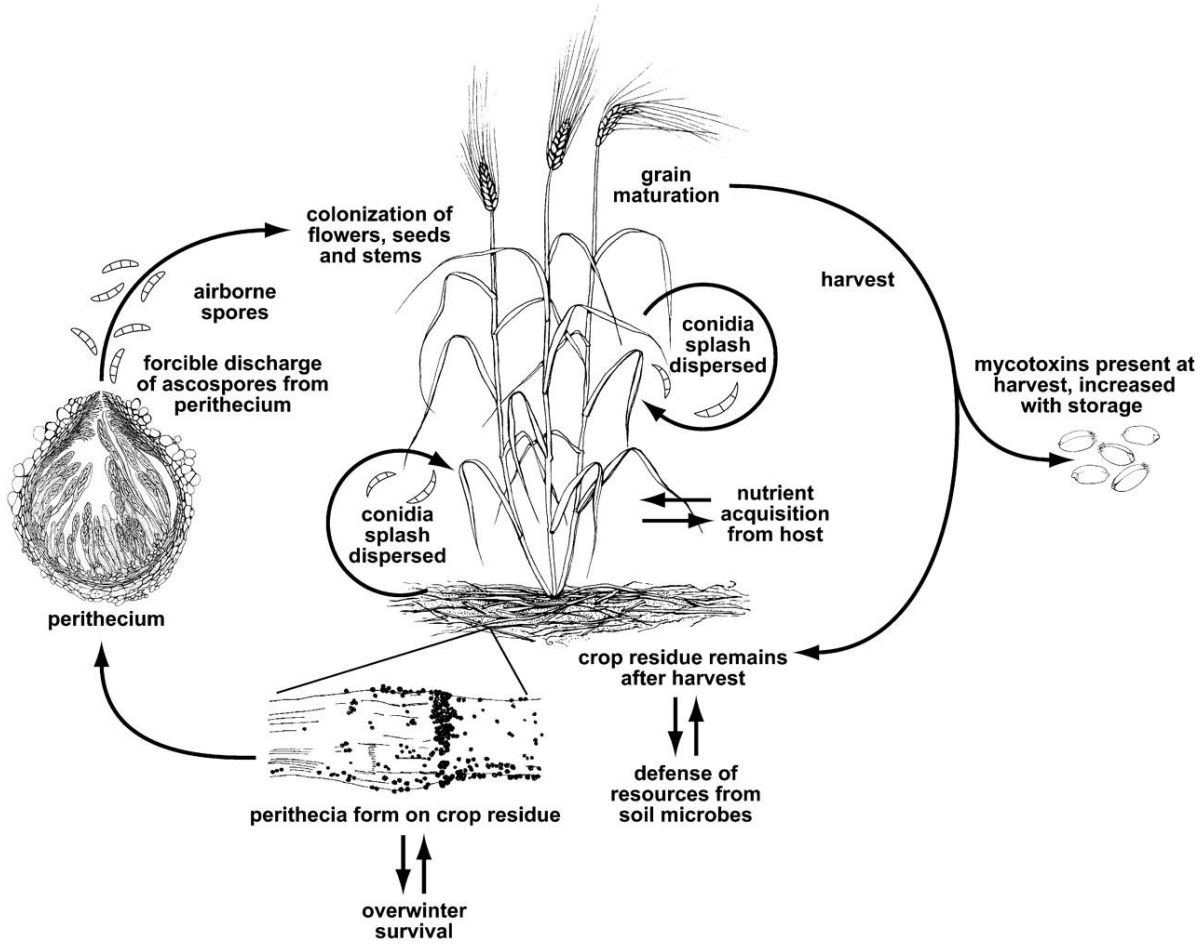


Figure 1: The life cycle of *F. graminearum* (sexual phase, *Gibberella zeae*), causal agent of Fusarium head blight on wheat (Trail, 2009)

1.3. Lipase

Lipase [EC 3.1.1.3] is defined as a carboxylesterase, which catalyzes both the hydrolysis and the synthesis of esters formed from glycerol and long chain fatty acids (Fig. 2) (Jaeger and Reetz, 1998). Unlike esterase, lipase prefers long chain fatty acids and is activated in the interface of lipid substrate and water. Therefore,

lipase is also called an interfacial enzyme (Jaeger et al., 1999; Jaeger and Eggert, 2002; Verger, 1976). Lipases are ubiquitous enzymes found in bacteria, fungi, plants, and animals. Microbial lipases are currently receiving much attention with the rapid development of enzyme technology. Lipases constitute the most important group of biocatalysts for biotechnological applications. There are various industrial applications of microbial lipases in detergents, food, flavours, biocatalytic resolution of pharmaceuticals, ester and amino acid derivatives, making of fine chemicals, agrochemicals, use as biosensor, bioremediation and cosmetics and perfumery (Gilham and Lehner, 2005; Hasan et al., 2006).

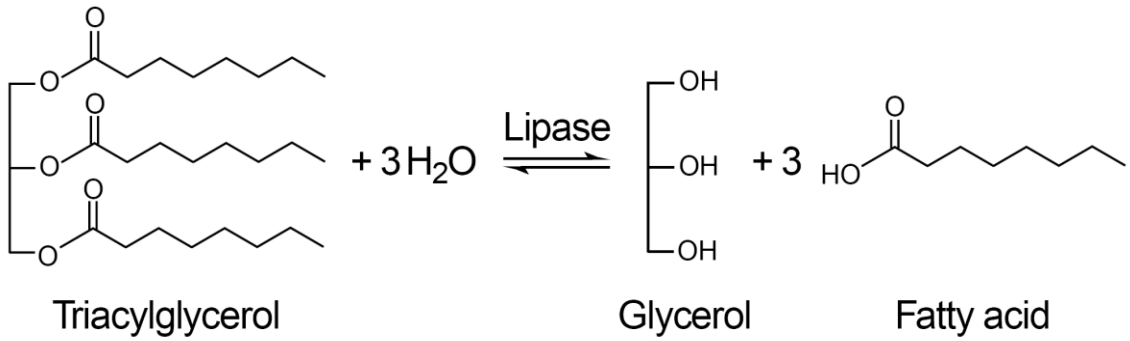


Figure 2: The catalytic action of lipase. A triglyceride can be hydrolysed to from glycerol and fatty acids, or the reverse (synthesis) reaction can combine glycerol and fatty acids to form the triglyceride (Jaeger and Reetz, 1998).

Works on fungal lipases started as early as the 1950s by Lawrence, Brockerhoff and Jensen. Lipases were found in *Aspergillus, Candida, Rhizopus, Mucor, Geotrichum, Penicillium* and *Humicola* species. Many workers have exploited fungi as valuable sources of lipase due to the following properties: thermal stability, pH stability, substrate specificity and activity on organic solvents (Ghosh et al., 1996).

Beside potential applications, recent studies have proven that lipases are involved in pathogenicity in several microbial plant pathogens (Paper et al., 2007). Fungal lipases and esterases may be involved in providing carbon sources during plant adhesion to and penetration of the plant surface (Pietro et al., 2009). The first evidence of the involvement of a secreted lipase in plant virulence was shown in the fungal pathogen *Botrytis cinerea*. A secreted lipase was induced during early stages

of infection. When specific anti-lipase antibodies were added to a conidial suspension of *B. cinerea*, lesion formation was completely suppressed on tomato leaves (Comménil et al., 1999; Comménil et al., 1998; Comménil et al., 1995; Pietro et al., 2009; Subramoni et al., 2010). However, a recent study has shown that *LIP1* disruption mutants of *B. cinera* did not prevent the fungus from infecting the plant. Even double mutants which lacked both *LIP1* and the cutinase gene *CUTA* and were largely devoid of extracellular esterase activity, still retained full virulence in various host plant systems (Pietro et al., 2009; Reis et al., 2005). In *Alternaria brassicicola*, a lipase in spores is shown to play crucial role in the infection of cauliflower leaves. When anti-lipase antibodies were added to a conidial suspension of *A. brassicicola* prior to inoculation, black spot lesions were reduced by 90% on intact cauliflower leaves, but not on leaves from which the surface wax had been removed (Berto et al., 1999). An extracellular lipase, *NhL1*, of the fungal pea pathogen *Fusarium solani* f. sp. *pisi* has been cloned, characterized and its transcriptional regulation has been studied. Importantly, the expression of the *NhL1* gene was induced during infection indicating a possible function in virulence (Nasser Eddine et al., 2001). The fungus *Ustilago maydis* converts from yeast like non-pathogenic cell-type to a pathogenic filamentous form capable of colonizing and causing diseases to maize. Recently, they found that growth in the presence of lipids promotes a filamentous phenotype meaning that the response to lipids as carbon source is an important component of the infection process (Klose et al., 2004). In *Burkholderia glumae*, they found that AHL quorum sensing in *B. glumae* AU6208 regulates secreted *LIPA* lipase and toxoflavin, the phytotoxin produced by *B. glumae*. *B. glumae* AU6208 *LIPA* mutants were no longer pathogenic to rice, indicating that the lipase is an important virulence factor (Devescovi et al., 2007). Intracellular lipases were shown to be involved in appressoria formation by *Magnaporthe grisea* (Thines et al., 2000). It has been shown that *Blumeria graminis* secreted lipase reveals the importance of host epicuticular wax components for fungal adhesion and development. Expression of *LIP1* is dramatically up regulated during the early stage of fungal development and pre treatment of wheat leaves with *LIP1*, thereby removing leaf surface wax, severely compromises components of fungal pathogenicity, including conidial

adhesion, appressorium formation, and secondary hyphal growth. These results suggested that *LIP1* activity releases cues from the host surface to promote pathogen development and infection (Feng et al., 2009).

In *F. graminearum*, gene disruption of *FGL1*, a secreted lipase, led to strongly decreased lipolytic activity in early time points of wheat germ oil induction and the lipase *FGL1* was found to be required for successful colonization during infection of wheat and maize (Voigt et al., 2005). MAP kinase Gpmk1 is known to regulate the pathogenicity process of the fungus. The disruption of the Gpmk1 MAP kinase leads to an apathogenic phenotype on wheat (Jenczmionka et al., 2003). Determination of secreted lipolytic activity of the Δ*gpmk1* mutants showed that Gpmk1 was responsible for the overall induction of secreted lipolytic activities in culture (Jenczmionka and Schaefer, 2005), and most likely regulates the onset of lipase gene *FGL1*. Nguyen has shown that disruption of two secreted lipase, *FGL2* and *FGL5,* in *F. graminearum* lead to an avirulence phenotype on wheat and maize (Nguyen, 2008). However, disruption of *LIP1*, another secreted lipase of *F. graminearum* involved in utilization of saturated fatty acids, showed no effect on virulence during colonization of wheat heads. It was therefore concluded that this lipase was most probably involved in fungal nutrient acquisition but not in pathogenesis (Feng et al., 2005).

1.4. Cutinase

Cutinases [EC 3.1.1.74] are enzymes produced by several phytopathogenic fungi and pollen which is able to hydrolyse ester bonds in the cutin polymer. Cutinases are also able to hydrolyse a large variety of synthetic esters and show activity on short and long chains of emulsified triacylglycerols as efficiently as pancreatic lipases. Contrary to lipases, the activity of which is greatly enhanced in the presence of a lipid-water interface, cutinases do not display, or display little, interfacial activation, being active on both soluble and emulsified triglycerides (Longhi and Cambillau, 1999).

The plant cuticle forms a hydrophobic coating that covers nearly all above-ground parts of terrestrial plants and constitutes the interface between plant and

environment. The main structural component of the plant cuticle is cutin, a polyester consisting of hydroxyl and epoxy fatty acids of n-C16 and n-C18 types (Kolattukudy et al., 1995; Pietro et al., 2009; Rocha et al., 2008). The cuticle structure has been thought to protect plants from invasive microbial pathogens (Chassot et al., 2008). To penetrate the cutin barrier, fungal pathogens use a combination of physical pressure and enzymatic degradation by extracellular cutinases (Pietro et al., 2009). Cutinase was shown to be present at the site of fungal penetration of the host plant cuticle and specific inhibition of cutinase was shown to protect plants against fungal penetration and consequently infection (Soliday and Kolattukudy, 1983). But the role of cutinase in fungal pathogenicity is subject to debate (Pietro et al., 2009; Schaefer, 1993). It has been observed that application of cutinase antibodies to germinating spores inhibited infection of pea by *F. solani* (Maiti and Kolattukudy, 1979). *Mycosphaerella* spp., a parasitic fungus that affects papaya fruits, only damages the fruit if the fruit skin is mechanically breached before inoculation, suggesting that the cutin layer is important for infection protection. Dickman has shown that expression of a cutinase (Cut1) from *F. solani* in the fungus *Mycosphaerella* enhances the infection the intact host plant (Dickman et al., 1989). This study demonstrated that cutinase is important for the fungus to breach the cutin layer of the host plant. Cutinase was indicated to be required for spore adhesion on the rust fungus *Uromyces viciae-fabae* and suggested to be involved in adhesion in *Colletotrichum graminicola* and *Blumeria graminis* f. sp *hordei* (Deising et al., 1992; Pascholati et al., 1993; Pascholati et al., 1992; Skamnioti and Gurr, 2007). Cutinase has been shown to be involved in carbon acquisition during subcuticular growth of *Venturia inaequalis* (Koller et al., 1991). Furthermore, disruption of the cutinase *CUT1* gene in *F. solani* f. sp. *pisi* led to a mutant which did not produce cutinase and was reduced in pathogenicity on sectioned pea sterms (Rogers et al., 1994). However, the function of cutinase in fungal pathogens is controversial. For example, Stahl and Schäfer demonstrated that the cutinase-deficient mutants by transformation-mediated gene disruption of single cutinase gene of *F. solani* f. sp. *pisi* were lacking a functional cutinase gene but had no difference in pathogenicity and virulence on pea compared to the wild type (Stahl and Schäfer, 1992; Stahl et al., 1994).

Inmunofluorescence studies of cutinase secretion by phytopathogenic fungi *F. solani* f. sp. *pisi* and *Collectotrichum gloeosporioides* indicated that the secretion of cutinase was directed towards the region that penetrates the host. In *F. solani*, which penetrates plant tissue without appressorium formation, cutinase was targeted to the growing tip of germinating spores. In *C. gloeosporioides*, cutinase secretion was directed to the infection peg that arises from the appressorium (Podila et al., 1995). Treatment of the serine esterase inhibitors ebelactone A and B resulted in 50% reduction of an esterase with cutinase activity and decrease in pathogenicity of *P. brassicae*. These results suggest a functional role of cutinase in pathogenicity of *P. brassicae* on brassicaceae (Davies et al., 2000). *CUT2*, a cutinase in *Magnaporthe grisea* was dramatically up regulated during appressorium maturation and penetration. It was found that *CUT2* mediates the formation of the penetration peg. Furthermore, the *CUT2* mutant strain was severely less pathogenic than the wild type (Skamnioti and Gurr, 2007). Lee et al. indicated that overexpression of a redox-regulated cutinase gene, *MfCUT1*, increases virulence of the brown rot pathogen *Monolinia fructicola* on Prunus spp. (Lee et al., 2010). Therefore, the cuticle appears to be involved in signal exchange between plant and pathogen and important for plant defence and development (Skamnioti and Gurr, 2007).

1.5. The transcription factor Zn2Cys6 family in fungi

Transcription factors are proteins that bind to specific DNA sequences to regulate the expression of genes. Transcription factors can be activators or repressors of gene expression and they perform the functions alone or in association with other proteins in a complex (Latchman, 2008). They contain DNA binding domains which belong to several super-families, each with a specific mechanism of DNA binding (Itzkovitz et al., 2006; Karin, 1990; Latchman, 1997). According to the sequence and structure of their DNA-binding domains, transcription factors are often grouped into different families, such as helix-turn-helix, basic helix-loop-helix, leucine zipper or bZIP proteins, β-ribbon and several zinc finger motifs (Carey and Smale, 1999; Latchman, 1997; Moxley et al., 2003). Zinc-binding proteins form one of the largest families of transcriptional regulators in eukaryotes, displaying variable secondary

structures and enormous functional diversity (Miller et al., 1985; Shelest, 2008). Zinc cluster proteins contain several functional domains apart from the cysteine-rich DBD, including the regulatory and activation domains. The typical structure of a zinc finger protein is depicted in figure 3.

Figure 3: Functional domains of zinc cluster proteins. Zinc cluster proteins can be separated into three functional domains: the DBD, the regulatory domain, and the acidic region. In addition, the DBD is compartmentalized into subregions: the zinc finger, the linker, and the dimerization domain. These regions contribute to DNA-binding specificity and to protein-DNA and protein-protein interaction. MHR, middle homology region (MacPherson et al., 2006).

The zinc finger transcription group, which requires zinc ions in order to bind to the target DNA, is categorized into three classes based on the DNA binding motifs, such as class I (Cys2His2); class II (Cys4); class III (Cys6) (MacPherson et al., 2006). Class III (Cys6) zinc finger proteins contain a DNA-binding domain (DBD) that consists of six cysteine residues bound to two zinc atoms as CysX2CysX6CysX5-12CysX2CysX6-8Cys (MacPherson et al., 2006), and hence these have the names zinc cluster, zinc binuclear cluster, or Zn2Cys6 proteins.

The Zn2Cys6 cluster protein has been found in many ascomycetes: *Fusarium, Magnoporthe, Trichoderma, Saccharomyces, Aspergillus, Kluyveromyces, Neurospora, Schizosaccharomyces, Candida, Pichia, Colletotrichum, Cercospora, Penicillium, Sordaria* and *Hansenula* species (MacPherson et al., 2006). The Zn2Cys6 cluster proteins have been indentified exclusively in fungi (Drobná et al., 2008) and the first- and best-studied in this cluster is Gal4p, a transcriptional activator of genes involved in the catabolism of galactose in *S. cerevisiae* (MacPherson et al., 2006).

Most transcription factors that contain a Zn2Cys6 binuclear zinc cluster DNA binding domain in the Gal4p family recognize a CGG motif (Schjerling and Holmberg, 1996; Todd and Andrianopoulos, 1997). This CGG motif recognized by the proteins has

been classified into three different patterns; palindromic repeats (CGG_CCG; also called inverted repeats), direct repeats (CGG_CGG), or everted repeats (CCG_CGG). They can also interact with DNA as monomers, homodimers, or heterodimers (Fig. 4) (Lohr et al., 1995; Schjerling and Holmberg, 1996; Shelest, 2008; Todd and Andrianopoulos, 1997). However, each individual protein in this family displays variation in the number of nucleotides between the two CGG repeats. For example, GAL4p and LAC9p bind to $CGGN_{11}CCG$, PPR1p and UAY bind to $CGGN_6CCG$, and PUT3p binds to $CGGN_{10}CCG$ (Todd and Andrianopoulos, 1997).

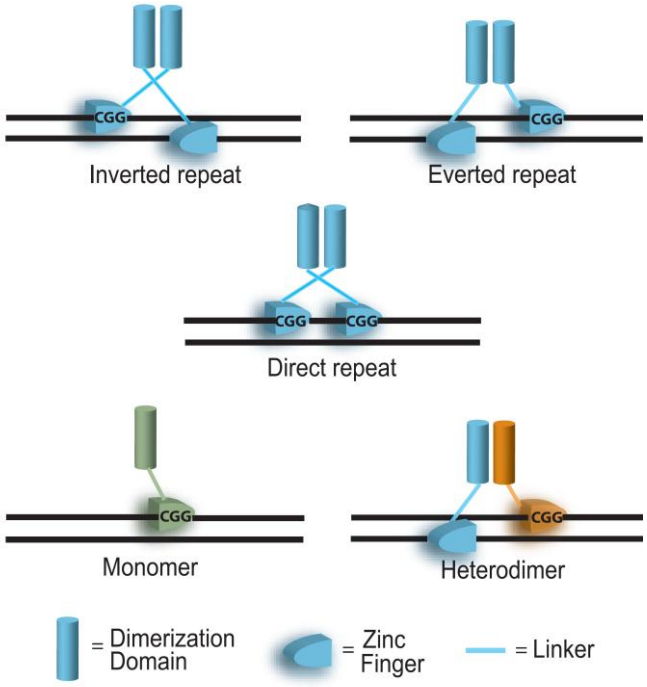


Figure 4: A model for zinc cluster protein DNA recognition. Zinc cluster proteins preferentially bind to CGG triplets that can be oriented in three different configurations: the inverted, everted, and direct repeats. The orientation of CGG triplets and the nucleotide spacing between the triplets are the two major determinants of DNA-binding specificity. Zinc cluster proteins can also bind as monomers (in green) as well as homodimers (two molecules in blue) and heterodimers (one molecule in blue and one in orange) (MacPherson et al., 2006).

1.6. Fatty acid metabolism in fungi

The metabolism of fatty acids is important for growth and virulence of many fungi, including plant and animal pathogens such as *F. oxysporum* (Rocha et al., 2008) or *Candida albicans* (Ramirez and Lorenz, 2009). In mammals, beta-oxidation of long

chain and medium/short chain fatty acids occurs in peroxisomes and mitochondria, respectively (Eaton et al., 1996; Wanders et al., 2001). The nuclear transcription factors PPAR α, γ, and β have been known to regulate the lipid metabolism in mammals and humans. In yeasts such as *Saccharomyces cerevisiae* and *C. albicans*, fatty acid metabolism occurs only in the peroxisomes (Hiltunen et al., 2003; Ramirez and Lorenz, 2009). Metabolism of fatty acids in *S. cerevisiae* is regulated by Oaf1 and Pip2. As claimed by Raminez (Ramirez and Lorenz, 2009), *C. albicans* does not contain a homolog of Oaf1 but has a single homolog of cutinase transcription factor (Ctf) family. In other fungi such as *A. nidulans,* long chain and short chain fatty acids metabolism exists in both peroxisomes and mitochondria. It has been demonstrated by gene deletion approach that null mutations of the gene controlling the utilization of carbon sources are attenuated in virulence in fungal pathogens such as *C. albicans*, *M. grisea*, *F. oxysporum.* Regulation of carbon metabolism is more complex in filamentous fungi such as *A. nidulans*. In *A. nidulans*, two proteins with homology to *C. albicans Ctf1* or *F. solani* Ctf1α, FarA and FarB from the zinc finger transcription factor family have been shown to regulate the expression of genes involved in metabolism of short chain and long chain fatty acids (Hynes et al., 2006). The zinc finger transcription factor Ctf1 from the vascular wilt fungus *F. oxysporum* is a functional orthologue of Ctf1α that controls expression of cutinase genes and virulence in the pea stem pathogen *F. solani* f. sp. *pisi* (Rocha et al., 2008). Deletion of *CTF1* led to reduced expression of the *CUT1* and *LIP1* genes, encoding a putative cutinase and lipase, respectively. These results suggest that *F. oxysporum CTF1* mediates expression of genes involved in fatty acid hydrolysis. However, expression of *LIP1* during root infection was not dependent on *CTF1*, and virulence of the *CTF1* mutants on tomato plants and fruits was not different from that of the wild type. Thus, in contrast to *CTF1* in the stem pathogen *F. solani*, *CTF1* is not essential for virulence in the root pathogen *F. oxysporum* (Rocha et al., 2008).

Genes involved in regulation of fatty acid degradation and peroxisomal biogenesis seem to play similar roles. In *S. cerevisiae,* Oaf1 and Pip2 and in *A. nidulans,* FarA and FarB have been shown to regulate genes which are required for growth on lipids

and for expression of genes necessary for β-oxidation. *C. albicans CTF1* is necessary for growth on fatty acids, regulates expression of several genes encoding enzymes of β-oxidation, including *FOX2*, and when mutated, confers a mild attenuation of virulence. Thus, phenotypic and genotypic observations highlight important differences in the regulatory network for alternative carbon metabolism in *C. albicans* compared to the paradigms developed in other model fungi (Ramirez and Lorenz, 2009). Additionally, Poopanitpan et al. showed that *POR1*, an ortholog of FarA of *A. nidulans*, in *Yarrowwia lipolytica* regulates the β-oxidation enzymes such as a peroxisomal 3-oxoacyl-CoA thiolase, a peroxisomal acetoacetyl-CoA thiolase, and an acyl-CoA oxidase, (Poopanitpan et al., 2010). These studies provided evidence that transcription factors regulating fatty acid metabolism also regulate fungal pathogenesis.

1.7. Aim of the study

Previous studies showed that secreted lipases are important virulence factors of the *F. graminearum* (Nguyen, 2008; Voigt et al., 2005). However, little is known about the mechanism how the lipases function as virulence factors. Interestingly, lipases have been long identified and characterized in many fungi. However, the lipase regulators are not identified yet to date. To better understand the lipase function, this study was focused on the identification of transcription factors which are potentially regulators of lipases expression. In this study, the proteins, which are the lipase transcriptional regulators in the fungus *F. graminearum,* were identified and characterized. Eight putative transcription factors were identified from the whole genome of the fungus and characterized their functions by gene deletion. Among the putative transcription factors, *FAR1, FAR2*, and *LR1* are involved in regulation of several lipases including a virulence factor *FGL1*. *FAR1* and *FAR2* also regulate the carbon source catabolism in the fungus, implicating their global regulatory functions. More importantly, these transcription factors are involved in virulence of the fungus, perhaps through regulation of known virulence and other unknown virulence factors. Thus, the study also paves the way for future studies on other virulence factors of the fungus.

2. Material and Methods

2.1. Enzymes and chemicals

Restriction enzymes, DNA-modifying enzymes, Taq polymerase, SYBRGreen master mix, and the RNA extraction kit were obtained from Fermentas (St. Leon Roth, Germany), NEB (New England Biolabs, USA), promega (Mannheim, Germany), 5 prime (Hamburg, Germany), MACHEREY-NAGEL (Düren, Germany), EVEGENE (Hamburg, Germany), and invitrogen (Darmstadt, Germany).

Chemicals used in culture media and buffers were obtained from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany), Fluka (Buchs, Switzerlandand), and Sigma-Aldrich (Steinheim, Germany). Chemicals used in Southern and northern blot were obtained from Roche (Mannheim, Germany).

2.2. Microbial strains and culture conditions

Escherichia coli competent cells XL1-blue used for plasmid preparation and cloning procedures were purchased from DNA Cloning Services (Biocentrum Klein Flottbek, Hamburg, Germany). *F. graminearum* strain 8/1 and PH1 were obtained from T. Miedaner (Miedaner et al., 2000) and H. Giese, Arhus (Denmark), and maintained on SNA plates (Nirenberg, 1981). Induction of conidiation was performed by placing a mycelium plug (0.5 cm^2) or pipetting a drop of conidia suspension either on SNA plates, incubating for 2 weeks at 18 °C under near-UV light (TLD 36 W-08; Philips, Eindhoven, The Netherlands) and white light (TL 40 W-33 RS; Philips) with a 12-h photoperiod or in CMC liquid culture at room temperature with shaking at 150 rpm for 7 days. For long time storage, conidia were kept in water and stored at –70 °C. Bacteria were cultivated in sterile Luria Bertani (LB) medium (Sambrook et al., 1989), either as liquid culture or on agar plates. 25 g of the LB broth or 40 g of the LB agar mixture (Difco) were dissolved in 1 l deionized water. For selection of transformed bacterial cultures, the antibiotic Ampicilin (100 μg/ml) was added after sterilization. For blue/white selection of transformed bacteria, 50 μg/ml X-Gal (solubilized in dimethylformamid) and 200 μM IPTG were supplemented after sterilization of the media.

Sterile CM or YPG was used for pre-cultivation of fungal strains and prepared as follows:

CM medium (Leach et al., 1982):

- Solution A (100x): 100 g/l $Ca(NO_3)_2$ x 4 H_2O.
- Solution B (100x): 20 g/l KH_2PO_4; 25 g/l $MgSO_4$ x $7H_2O$; 10 g/l NaCl (sterilized by filtration).
- Solution C: 20% (w/v) Glucose (sterilized by filtration through 0.2 μm filter).
- Suspension D (100x): 60 g/l H_3BO_3; 390 mg/l $CuSO_4$ x $5H_2O$;13 mg/l KI; 60 mg/l $MnSO_4$ x H_2O; 51 mg/l $(NH_4)_6Mo_7O_{24}$ x $4H_2O$; 5.48 g/l $ZnSO_4$ x $7H_2O$; 932 mg/l $FeCl_3$ x 6 H2O; 2ml Chloroform (added for sterilization of the solution).
- Solution E: 1 g Yeast extract; 0.5 g Casein, hydrolyzed by enzymatic cleavage; 0.5g Casein, hydrolyzed by acid degradation.

To prepare 1 l CM, 10 ml of solution A was added to 929 ml H_2O and was sterilized in the autoclave. For solid CM media, 16 g/l granulated agar was supplemented before autoclaving. Then 10 ml of the solution B, 50 ml of the solution C, 1 ml of the suspension D and the complete solution E were added. For selection of the transformants, 100-250 μg/ml Hygromycin B or Geneticin were added to the solid medium, respectively.

MM medium: Modification of CM medium for DON assays *in vitro*

- Solution B (100x): 20 g/l KH_2PO_4; 25 g/l $MgSO_4$ x $7H_2O$; 10 g/l NaCl (sterilized by filtration).
- Solution C: 20% (w/v) sucrose (sterilized by filtration through 0.2 μm filter).
- Suspension D (100x): 60 g/l H_3BO_3; 390 mg/l $CuSO_4$ x $5H_2O$;13 mg/l KI; 60 mg/l $MnSO_4$ x H_2O; 51 mg/l $(NH_4)_6Mo_7O_{24}$ x $4H_2O$; 5.48 g/l $ZnSO_4$ x $7H_2O$; 932 mg/l $FeCl_3$ x 6 H2O; 2ml chloroform (added for sterilization of the solution).

To prepare 1 l MM medium, 10 ml of solution B, 50 ml of the solution C, and 1 ml of the suspension D were added to 939 ml sterilized H_2O, 50 ml of the solution C, 1 ml of the suspension D.

CMC medium (Cappellini and Peterson, 1965): Component of 1 l CMC are as follows: 1g NH_4NO_3; 1g KH_2PO_4; 0,5 $MgS0_4x7H_2O$; 1g yeast extract; 15 g carboxymethylcellulose.

YPG medium (Sambrook et al., 1989): 1% Yeast extract; 2% Pepton; 2% Glucose. To prepare solid agar plate, 16% granulated agar was added before autoclaving.

SNA medium (Nirenberg, 1981): Components of 1 l SNA are as follows: 1 g KH_2PO_4; 1 g KNO_3; 0.5 g $MgSO_4$ x 7 H_2O; 0.5 g KCl; 0.2 g Glucose; 0.2 g Saccharose; 1 l H_2O; 16 g granulated agar (used for solid agar plate).

Modified SNA medium: Components of 1 l modified SNA are as follows: 1g KH_2PO_4; 1 g KNO_3; 0.5 g $MgS0_4$ x $7H_20$; 0.5 g KCl; 1 L H_2O; 16 g granulated agar (used for solid agar plate).

Media prepared for lipotytic plate assays: Modification SNA medium was used by adding either 1% wheat germ oil (WGO), 1% Triolein, 1% Olive oil, or 1% Tributyrin as the sole carbon source.

All oligonucleotide primers used in this study were designed by using PrimerSelect program (DNASTAR software, USA). PCRs were performed using non-proofreading Taq DNA Polymerase (purchased from Fermentas or 5 prime) whose terminal transferase activity adds extra A nucleotides to the 3'-ends of PCR products. Therefore fusion primers were designed just after T to avoid mismatch mutations at the 3’ ends of PCR products (Clark, 1988).

Primers used in this study are listed in the following table:

Note: upF = primer 1; upR = primer 2; upnF = primer 5; doF = primer 3; doR = primer 4; donR = primer 6 in figure 5.

Table 1. Primers for generation of disruption construct

Primer name	Sequence (5'→ 3')	Description
FAR1upF	CACACGCTCAAGTCTCACAGT	Forward primer to amplify upstream region of *FAR1* (FGSG_01936)
FAR1upR	agatgccgaccgaacaagagctgtccccA GTATTAGGTAGGTGCCCAGG	Reverse primer to amplify upstream region of *FAR1* gene; Lower case letters are overlapping oligonucleotides of HYG
FAR1doF	caatgctacatcacccacctcgctccccGAA CATAAGACGCAACGAGTG	Forward primer to amplify downstream region of *FAR1* gene; Lower case letters are overlapping oligonucleotides of HYG
FAR1doR	TTGGCACGCTAATAAATGATG	Reverse primer to amplify downstream region of *FAR1* gene
FAR1upnF	c**tctaga**GATGATACGACGGGATT ACAG	Forward primer to generate plasmid pGEM-*FAR1* of *FAR1* gene; bold letters: *Xba*I site
FAR1donR	c**tctaga**GGGTCATTCAATCATCCT GTA	Reverse primer to generate plasmid pGEM-*FAR1* of *FAR1* gene; bold letters: *Xba*I site
FAR2upF	TCCTTGAATAGAATGCCCAG	Forward primer to amplify upstream region of *FAR2* (FGSG_07192)
FAR2upR	agatgccgaccgaacaagagctgtccccG GTTGTGGTGATATGACTGTGA	Reverse primer to amplify upstream region of *FAR2* gene; Lower case letters are overlapping oligonucleotides of HYG
FAR2doF	caatgctacatcacccacctcgctccccGAT GGATTACTTTTTGGATTGG	Forward primer to amplify downstream region of *FAR2* gene; Lower case letters are overlapping oligonucleotides of HYG
FAR2doR	CTTGCATATCCCATTCCTCTC	Reverse primer to amplify downstream region of *FAR2* gene
FAR2upnF	ATAGAAATCCCCACACGCA	Forward primer to generate plasmid pGEM-*FAR2* of *FAR2* gene
FAR2donR	AATTTCGCATGTTTCTGTGG	Reverse primer to generate plasmid pGEM-*FAR2* of *FAR2* gene

Table 1. Primers for generation of disruption construct (continued)

Primer name	Sequence (5'→ 3')	Description
LR1upF	GAGCGCCTACATATTACGGG	Forward primer to amplify upstream region of *LR1* (FGSG_09318 or FGSG_17192)
LR1upR	agatgccgaccgaacaagagctgtcccccC AACATGAAATCGTGCGTAGA	Reverse primer to amplify upstream region of *LR1* gene; Lower case letters are overlapping oligonucleotides of HYG
LR1doF	caatgctacatcacccacctcgctccccAAT GCTCTTGAGGCTGGTTC	Forward primer to amplify downstream region of *LR1* gene; Lower case letters are overlapping oligonucleotides of HYG
LR1doR	GTACATAACCTCCCCCTTCGT	Reverse primer to amplify downstream region of *LR1* gene
LR1upnF	c**tctaga**AAAGAAGATTCGCCCCA GTC	Forward primer to generate plasmid pGEM-*LR1* of *LR1* gene; bold letters: *Xba*I site
LR1donR	c**tctaga**ACCAAGGAGGAGAACGC TAAG	Reverse primer to generate plasmid pGEM-*LR1* of *LR1* gene; bold letters: *Xba*I site
LR2upF	CAAACATATCCGCAATCACAG	Forward primer to amplify upstream region of *LR2* (FGSG_03207)
LR2upR	agatgccgaccgaacaagagctgtccccG ACCGGAAATAATGACACCC	Reverse primer to amplify upstream region of *LR2* gene; Lower case letters are overlapping oligonucleotides of HYG
LR2doF	caatgctacatcacccacctcgctccccTCT CCTGCCATCTCAACTCC	Forward primer to amplify downstream region of *LR2* gene; Lower case letters are overlapping oligonucleotides of HYG
LR2doR	GTGTTGGTTGATAGCGACTCC	Reverse primer to amplify downstream region of *LR2* gene
LR2upnF	c**aagctt**AGCGATATACCGAGTGT GGC	Forward primer to generate plasmid pGEM-*LR2* of *LR2* gene; bold letters: *Hin*dIII site
LR2donR	c**aagctt**CCAACCCCAAATGATACT GAG	Reverse primer to generate plasmid pGEM-*LR2* of *LR2* gene; bold letters: *Hin*dIII site

Table 1. Primers for generation of disruption construct (continued)

Primer name	Sequence (5'→ 3')	Description
LR3upF	CTCGACACCACTATTGCCAC	Forward primer to amplify upstream region of *LR3* (FGSG_00227)
LR3upR	agatgccgaccgaacaagagctgtccccCCACTCACCTTATCGTTGCC	Reverse primer to amplify upstream region of *LR3* gene; Lower case letters are overlapping oligonucleotides of HYG
LR3doF	caatgctacatcacccacctcgctccccGCAACATTCTTTCTATCCAGCC	Forward primer to amplify downstream region of *LR3* gene; Lower case letters are overlapping oligonucleotides of HYG
LR3doR	ACCATCTCTTGGACCGTTGC	Reverse primer to amplify downstream region of *LR3* gene
LR3upnF	c**tctaga**TTACTACAACGGCAGCGGAG	Forward primer to generate plasmid pGEM-*LR3* of *LR3* gene; bold letters: *Xba*I site
LR3donR	c**tctaga**GTCATGCTTATCTGGGCGG	Reverse primer to generate plasmid pGEM-*LR3* of *LR3* gene; bold letters: *Xba*I site
LR4upF	TGGCACCAACTAGATTTCGTC	Forward primer to amplify upstream region of *LR4* (FGSG_08034)
LR4upR	agatgccgaccgaacaagagctgtccccTAGGGTGGTTGATATTGCTGG	Reverse primer to amplify upstream region of *LR4* gene; Lower case letters are overlapping oligonucleotides of HYG
LR4doF	caatgctacatcacccacctcgctccccAATGAGCAATCAGCGGAATAC	Forward primer to amplify downstream region of *LR4* gene; Lower case letters are overlapping oligonucleotides of HYG
LR4doR	TCCAGGGACATGAACAAGAGA	Reverse primer to amplify downstream region of *LR4* gene
LR4upnF	c**aagctt**TCGACAAATCTTTCTTCCGC	Forward primer to generate plasmid pGEM-*LR4* of *LR4* gene; bold letters: *Hin*dIII site
LR4donR	c**aagctt**TAAGATCAGACACCACCGCC	Reverse primer to generate plasmid pGEM-*LR4* of *LR4* gene; bold letters: *Hin*dIII site

Table 1. Primers for generation of disruption construct (continued)

Primer name	Sequence (5'→ 3')	Description
LR5upF	TTACTGTAGGTGTTGGCGGTG	Forward primer to amplify upstream region of *LR5* (FGSG_08064)
LR5upR	agatgccgaccgaacaagagctgtccccA TGATGTTAGGGTTAGGTTGGAC	Reverse primer to amplify upstream region of *LR5* gene; Lower case letters are overlapping oligonucleotides of HYG
LR5doF	caatgctacatcacccacctcgctccccGC ATGACATTTGGGGTTGG	Forward primer to amplify downstream region of *LR5* gene; Lower case letters are overlapping oligonucleotides of HYG
LR5doR	GAGGATTCTTGGCTGTGGTG	Reverse primer to amplify downstream region of *LR5* gene
LR5upnF	c**tctaga**ATGCGTAGTTAGCAGAG GTGTC	Forward primer to generate plasmid pGEM-*LR5* of *LR5* gene; bold letters: *Xba*I site
LR5donR	c**tctaga**TGTAATGTTGGAGAACT GGAGC	Reverse primer to generate plasmid pGEM-*LR5* of *LR5* gene; bold letters: *Xba*I site
LR6upF	TGGTTCCTGGGACTGAGATTTA C	Forward primer to amplify upstream region of *LR6* (FGSG_15882)
LR6upR	agatgccgaccgaacaagagctgtccccC AACGTTGCTACTCTACTACCCG	Reverse primer to amplify upstream region of *LR6* gene; Lower case letters are overlapping oligonucleotides of HYG
LR6doF	caatgctacatcacccacctcgctccccTG TGGGTGAATCAGGGCTATC	Forward primer to amplify downstream region of *LR6* gene; Lower case letters are overlapping oligonucleotides of HYG
LR6doR	AAAAGGCCTCTGGTGACATCTA C	Reverse primer to amplify downstream region of *LR6* gene
LR6upnF	c**tctaga**TTTATTCAGCACCACAA CGGAC	Forward primer to generate plasmid pGEM-*LR6* of *LR6* gene; bold letters: *Xba*I site
LR6donR	c**tctaga**AGCGATGTAACTGGGGT AACG	Reverse primer to generate plasmid pGEM-*LR6* of *LR6* gene; bold letters: *Xba*I site

Table 2. Primers for RT-PCR and qPCR

Primer name	Sequence (5'→ 3')	Description
YgF	GTTGGCGACCTCGTATTGG	Forward primer for RT-PCR of Hygromycin gene (HYG)
HyR	CTTACCACCTGCTCATCACCT	Reverse primer for RT-PCR of HYG
β-TubF	TGCTGTTCTGGTCGATCTTG	Forward primer for RT-PCR of Betatubulin (FGSG_06611)
β-TubR	ATGAAGAAGTGAAGTCGGGG	Reverse primer for RT-PCR of Betatubulin
FAR1rtF	AAACAAAACACTGGAAGAGCG	Forward primer for RT-PCR of *FAR1*
FAR1rtR	GGTTATGTATCCCGCTCTGTC	Reverse primer for RT-PCR of *FAR1*
FAR2rtF	ACCACCATTTCAGCCCTTCA	Forward primer for RT-PCR of *FAR2*
FAR2rtR	ATTCAATCGGCACACGCTTC	Reverse primer for RT-PCR of *FAR2*
LR1rtF	CTCTATGCTGGCCTCTCCTG	Forward primer for RT-PCR of *LR1*
LR1rtR	AGTCACTTTTGCTGTCCATCG	Reverse primer for RT-PCR of *LR1*
LR2rtF	AAGATTTGCGATTATTGTCCG	Forward primer for RT-PCR of *LR2*
LR2rtR	TATCTGGTTGAGAGCGAGCAT	Reverse primer for RT-PCR of *LR2*
LR3rtF	GGCACGCAAATATCACTACCT	Forward primer for RT-PCR of *LR3*
LR3rtR	AGATTTCAGCAGCCTTACGC	Reverse primer for RT-PCR of *LR3*
LR4rtF	AACGCTTGGTATTGGATTGG	Forward primer for RT-PCR of *LR4*
LR4rtR	ATAGCGTCCTCTGGTGTCTCA	Reverse primer for RT-PCR of *LR4*
LR5rtF	TACAGATGTTTTGGGCGTTG	Forward primer for RT-PCR of *LR5*
LR5rtR	AGCTCTTCATTTGTTTCACCAG	Reverse primer for RT-PCR of *LR5*
LR6F	ATGAATAACTCCCAGCAACATTC	Forward primer for RT-PCR of *LR6*
LR6rtR	ATTCGCTTTCTTCGAGGTTTAG	Reverse primer for RT-PCR of *LR6*
CUT1rtF	CAAGTATGGCCGAAATGGAG	Forward primer for RT-PCR of *CUT1* (FGSG_03457)
CUT1rtR	GAAGCATCGGTCTGGTAAAGC	Reverse primer for RT-PCR of *CUT1*

Table 2. Primers for RT-PCR and qPCR (continued)

Primer name	Sequence (5'→ 3')	Description
CUT2rtF	CTTCTGCCTGTCCTCCTGTG	Forward primer for RT-PCR of *CUT2* (FGSG_02890)
CUT2rtR	AACCAGTGCAGACCAAATCG	Reverse primer for RT-PCR of *CUT2*
PEX11rtF	ATCTTGGGCTGTTGGTATCG	Forward primer for RT-PCR of *PEX11* (FGSG_09281)
PEX11rtR	CGTCGTCGAGGTATTTGTTGT	Reverse primer for RT-PCR of *PEX11*
FOX2rtF	ATCATTAGCGGAGTTTTGCG	Forward primer for RT-PCR of *FOX2* (FGSG_09643)
FOX2rtR	ACTGGTGTCGAATTGGCATAC	Reverse primer for RT-PCR of *FOX2*
ß-tubulin q1F	TGTCGACGACCAGTTCTCAGC	Forward primer for qPCR of Beta tubulin
ß-tubulin q1R	CGATGTCGGCGTCTTGGTAT	Reverse primer for qPCR of Beta tubulin
FGL1q2F	ATGCCCATCTCCACTACTTCCA	Forward primer for qPCR of *FGL1* (FGSG_05906)
FGL1q2R	ATCGGTCATGGTAGCCCTCTC	Reverse primer for qPCR of *FGL1*
FGL1rtF	CACCCCTTGACATCTACACCTAC	Forward primer for RT-PCR of *FGL1*
FGL1rtR	GCGGCCTGGCATGAGTCTTGATA	Reverse primer for RT-PCR of *FGL1*
FGL2rtF	GAAAATGTTATCGTCACCACG	Forward primer for RT-PCR of *FGL2* (FGSG_01240)
FGL2rtR	CTTGATATCGTCCTCTACCCC	Reverse primer for RT-PCR of *FGL2*
FGL6rtF	AAGCCAGTATCCCGAAGTG	Forward primer for RT-PCR of *FGL6* (FGSG_03846)
FGL6rtR	AATTGCAGTGCTGTTTCTCG	Reverse primer for RT-PCR of *FGL6*
FGL9rtF	AGGAATCAACACCACCGAG	Forward primer for RT-PCR of *FGL9* (FGSG_03687)
FGL9rtR	CAGCAGTGTAGCCAAGTCCA	Reverse primer for RT-PCR of *FGL9*
FGL16rtF	CACATTCCCGACGCTGAC	Forward primer for RT-PCR of *FGL16* (FGSG_11386)
FGL16rtR	TGCACAACAGGATACTTGGG	Reverse primer for RT-PCR of *FGL16*
LIP1rtF	CCAACGTGACCACTACATCC	Forward primer for RT-PCR of *LIP1* (FGSG_01603)
LIP1rtR	CTGTTCTTATTCGCCGTCTC	Reverse primer for RT-PCR of *LIP1*

Table 3. Primers for cloning and sequencing of *FAR1*, *FAR2*, *LR1* and nptII

Primer name	Sequence (5'→ 3')	Description
M13F	GTAAAACGACGGCCAG	Forward primer for sequencing from pGEMT vector
M13R	CAGGAAACAGCTATGAC	Reverse primer for sequencing from pGEMT vector
nptIIF	g**GCTAGC**ATCATCGATGAATTCATGCC	Forward primer for cloning Geneticin gene; bold letters: *Nhe*I site
nptIIR	g**GCTAGC**GAACATCAGTTTGAGTCCGTG	Reverse primer for cloning Geneticin gene; bold letters: *Nhe*I site
FAR1F	c**tgatca**GACGTGGACATGTCGTCGG	Forward primer for cloning *FAR1* gene; bold letters: *Bcl*I site
FAR1R	c**tgatca**AGCCATACTACTCAGCCCAGG	Reverse primer for cloning *FAR1* gene; bold letters: *Bcl*I site
FAR1F2	GACGCCAACTACGAAGATGACC	Forward primer for sequencing *FAR1* gene
FAR1R1	CGATCGCGAGTGAAAAGAGAC	Reverse primer for sequencing *FAR1* gene
FAR2F3	ATGGCTATGACAACGGAATCAT	Forward primer for cloning *FAR2* gene
FAR2R3	CTATGCCGATGCGGTAATATC	Reverse primer for cloning *FAR2* gene
FAR2F2	GGTTACACAACGCGGAAAGC	Forward primer for sequencing *FAR2* gene
Far2R1	CTTTCTGGTCATCTGGCGTTTC	Reverse primer for sequencing *FAR2* gene
LR1F3	ATGTCCAACGAAGATCCTTCTGTT	Forward primer for cloning *LR1* gene
LR1R3	TCAGTGTCTCTTCATCACTGCCA	Reverse primer for cloning *LR1* gene
LR1F2	AACACCACGAGGATCACCAAG	Forward primer for sequencing *LR1* gene
LR1R1	TCGCACAACCTGGGAGAAGTA	Reverse primer for sequencing *LR1* gene

2.4. Cloning and sequence of *F. graminearum* genes from genomic DNA and complement DNA (cDNA)

To prepare mycelia for DNA and RNA isolation, *F. graminearum* conidia were cultured in CM or YPG medium at 28 °C with shaking at 150 rpm for 3 days. Mycelium was filtered using a 200 µm diameter sieve, washed with water and dried with paper towels. DNA was isolated by using CTAB solution (2% CTAB, 1 M Tris HCl, 5 M NaCl, 0.5 M EDTA) as described (Kidwell and Osborn, 1992). Total RNA was isolated by using Nucleospin RNA II Kit (MACHEREY-NAGEL) and single stranded cDNA was synthesized by using SuperScript II Rnase H-Reverse Transcriptase (Invitrogen) and oligo dT18 as the primer (Fermentas). To prepare cDNA from wheat germ oil induced conditions, the washed mycelium was inoculated with 2% (v/v) wheat germ oil in 50 ml pure water at 28 °C with shaking at 150 rpm (Voigt et al., 2005), and samples were taken at 4 hours after inducing as indicated in the result part. RNA and cDNA were similarly isolated and synthesized as described above.

The open reading frames (ORF) of the *FAR1*, *FAR2* or *LR1* were amplified by PCR from gDNA as well as from cDNA as templates using its specific primer pairs (see list of primers). The PCR program was an initial denaturation step for 4 min at 94°C, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at a gradient (Eppendorf Mastercycler) Tm = 54 – 58 °C for 45 s, and an appropriate elongation time, 3 min 30 s, at 68°C; the final elongation step was performed at 68 °C for 10 min. The DNA sequences from gDNA as well as from cDNA were subcloned into pGEM-T (Promega), and then sequenced using M13F/R primers (Invitrogen) and internal primers shown in section 2.3.

2.5. Detection of extracellular lipolytic activity of gene disruption strains

To measure the lipolytic activity of the gene disruption strains and the reference strains, approximately 1.2-1.5 g of air-dried washed mycelia were inoculated in flasks containing 50 ml water supplemented with 2% (v/v) of wheat germ oil for lipase induction. Induction was conducted at 28°C and 150 rpm for 24 hours. Samples were taken as indicated in the result section. Lipolytic activity of the

samples' supernatants was measured by using para-nitrophenyl palmitate (pNPP, Sigma, St. Louis, MO, USA) as substrate (Kok et al., 1993). To prepare the enzyme reaction solution, an appropriate amount of p-nitrophenyl esters was first dissolved in 2-propanol; the substrate was then mixed with Bis-Tris Propane buffer (50 mM Bis-Tris Propane, 0.1% (w/v) Gum arabic and 0.1% (v/v) Triton X-100) to reach final concentration of 2 mM. To assay lipolytic activity, 100 µl of the concentrated supernatant were incubated with 900 µl reaction solution. The assay was carried out in 1 ml cuvettes at 37 °C for a period of 1 h as indicated. Relative lipolytic activity was determined as the differences of OD_{410} values between samples and control.

Measurements of lipolytic activity and relative lipolytic activity in the presence of sodium butyrate were similarly performed. Pre-cultures of wild type mycelia were prepared as described above. The washed mycelia were incubated for one additional day in water with the supplementation of either 2% (v/v) wheat germ oil; 50 mM sodium butyrate; 50 mM sodium butyrate plus 2% (v/v) wheat germ oil. Lipolytic activity of samples taken after every 2 hours of induction was assayed.

2.6. Gene disruption methods in *F. graminearum* by double homologous recombination

Plasmid construction: To disrupt the genes in *F. graminearum*, the double homologous recombination constructs were generated as described in the figures below. The pGEM-TF plasmids were used to disrupt the entire open reading frames of the TF genes. Generation the pGEM-TF plasmids were performed using a fusion polymerase chain reaction (PCR) approach. From 0.4 - 1kb bp upstream and downstream fragments of the TF genes were cloned by PCR from genomic DNA (gDNA) using the primers listed above. The Hygromycin (HYG) cassette was generated by digestion of pGEM-HYG plasmid (Dr. A. Lösch, University of Hamburg, Germany) and the PCR fragments were either gel-purified or directly used. Fusion of the upstream and downstream fragment with HYG cassette was performed with 200 ng of each PCR fragment mixed with 400 ng HYG cassette in a 25 µl PCR reaction without addition of primers, as the fragments acted as primers and templates for the elongation. The detailed program was performed following Fig

5. The PCR products were ligated into vector pGEM-T (Promega) to generate pGEM-*FAR1*-HYG, pGEM-*FAR2*, pGEM-*LR1*, pGEM-*LR2*, pGEM-*LR3*, pGEM-*LR4*, pGEM-*LR5*, and pGEM-*LR6* plasmids. The pGEM-*FAR1*-HYG construct was treated with *Nhe*I and replaced the whole HYG cassette by the Geneticin (nptII) cassette to create pGEM-*FAR1* with Geneticin selection marker.

Before fungal transformation, the recombinant pGEM-TF plasmids were digested with appropriate enzymes to release the disruption construct. A solution of 30 – 50 µl of 10-30 µg DNA was used for *F. graminearum* transformation.

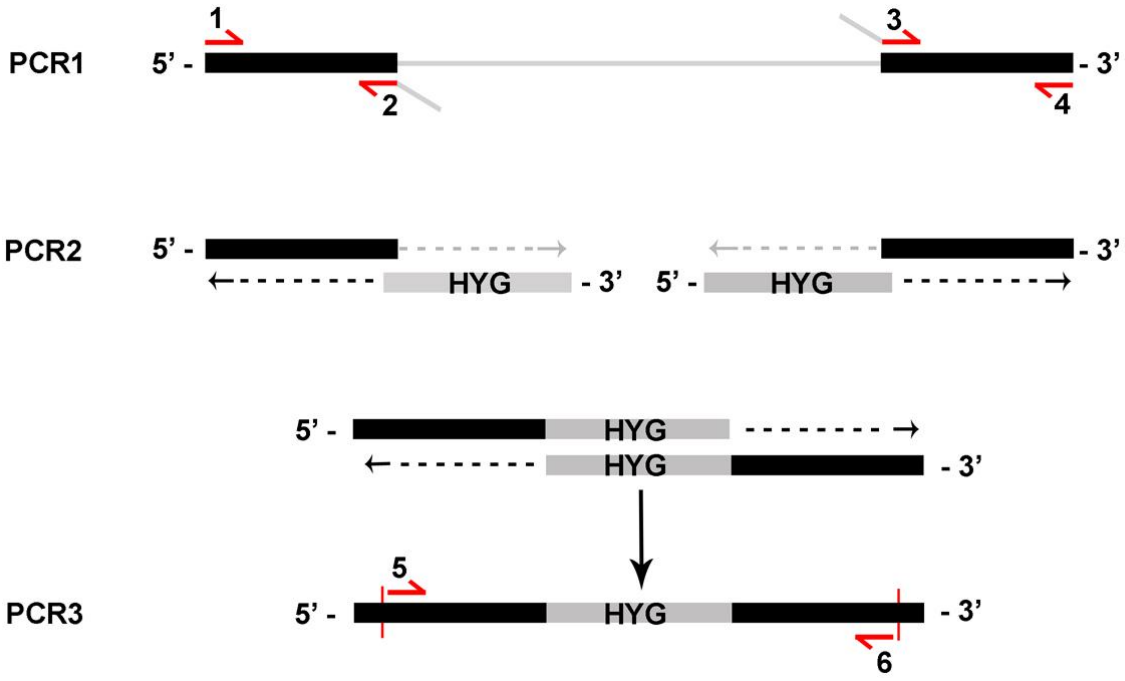


Figure 5: Strategy to generate a double homologous recombination construct (disruption construct). PCR1: two flanking fragments of a target gene are amplified with the use of primer pair 1 and 2 for the 5' homologous fragment, and primer pair 3 and 4 for the 3' homologous fragment. PCR2: Two flanking fragments amplified from PCR1 are fused with the HYG fragment in one PCR reaction. In the first PCR cycles, each flanking fragment is fused with the HYG fragment, which results in up-HYG and HYG-do fragments. In the following PCR cycles, the HYG fragments anneal and the entire fragment is elongated. PCR3: propagation of disruption constructs by the use of nested primers 5 and 6 for up-HYG-do fragment.

Program of PCR1:

Cycles	Thermal settings
1x	94 ^{o}C, 4 min
35x	94 ^{o}C, 0.5 min; 52 ^{o}C to 58 ^{o}C, 0.75 min; 68 ^{o}C, 1 – 1.5 min
1x	68 ^{o}C,10 min

Program of PCR2:

Cycles	Thermal settings
1x	94 ^{o}C, 4 min
20x	94 ^{o}C, 0.5 min; 60 ^{o}C, 2 min; 68 ^{o}C, 3.5 – 4 min
1x	68 ^{o}C, 10 min

Program of PCR3:

Cycles	Thermal settings
1x	94 ^{o}C, 4 min
35x	94 ^{o}C, 0.5 min; 52 ^{o}C to 58 ^{o}C, 0.75 min; 68 ^{o}C, 3.5 – 4 min
1x	68 ^{o}C, 10 min

2.7. Transformation-mediated gene disruption

In this study, various gene disruption constructs were generated by double homologous recombination constructs. These constructs, which all contain the Hygromycin resistance cassette HYG except *FAR1*, which contains the Geneticin resistance cassette nptII as selective marker were used to transform with wild type *F. graminearum* strain PH1 via the protoplast transformation method. *FAR2* construct was transformed into both wild type *F. graminearum* strain PH1 and 8/1.

The protoplast transformation method was performed as described previously (Maier et al., 2005; Proctor et al., 1995) 100 ml of YEPD medium (0.3% yeast extract, 1% bacto peptone, 2% D-glucose) were inoculated with 1x10^{6} conidia and incubated overnight at 28°C, 150 rpm. The mycelium was collected by filtering with a 200 µm diameter sieve or miracloth and was washed by rinsing three times with double-distilled water. Then, 0.5-2 g of mycelia were resuspended in 20 ml Driselase/Lysing enzymes (Invitrogen; 2.5%: 0.5% in 1.2 M KCl), and digested 2-3 h at 30°C, 80 rpm.

Undigested hyphal material was removed from the protoplast suspension by filtration first through a 100 µm Wilson-sieve then through a 40 µm Wilson-sieve or three layers of cheesecloth. The protoplasts were pelleted by centrifugation for 10 min at 2000 rpm at room temperature, washed one time with 10ml STC (20% sucrose, 10 mM Tris-HCl, pH 8.0, 50 mM $CaCl_2$), centrifuged again and then resuspended in STC at $1x10^8$ protoplasts per ml. For transformation, 200 µl STC of protoplast suspension were supplemented with 10 µg plasmid DNA. Samples were mixed and incubated at room temperature for 20 min. 1 ml PEG (40% polyethylene glycol 4000, 60 % STC) was mixed with the suspension and incubated at room temperature for 20 min. The protoplast suspension was mixed gently into 5 ml TB3 medium (200g sucrose, 0.3% yeast extract, 0.3% casamino acids) and shaken overnight at room temperature at 100 rpm for cell wall regeneration. After 12– 24 h, regenerated protoplasts were collected by centrifugation at 5000 rpm for 10 min at room temperature and 5 ml of TB3 were removed. The regenerated protoplasts were mixed in 1.5% TB3 agar at 50°C with Hygromycin B or Geneticin, (100-250 µg/ml) and plated out in 596 mm petridishes (10 ml/plate). After 12-24 h, the plates were overlaid with the same amount of 1.5% TB3 agar with double amount of Hygromycin B or Geneticin as the day before. Transformants were obtained after 2 days post-transformation. They were transferred to fresh plates of CM medium with 100-250 µg/ml Hygromycin B or Geneticin, respectively, and incubated at 28°C. The transformants were purified by single-spore isolation.

2.8. Southern blot analysis

Genomic DNA of wild-type and knock-out (KO) strains was isolated and restricted overnight with appropriate enzymes. The digested DNA was then separated on an 0.8% agarose gel by electrophoresis with a voltage of 70 V for 6 to 7 hours. DNA was transferred by capillary blotting onto Hybond NX membrane (Amersham Biosciences, Little Chalfont, UK) and then hybridized with a DIG (digoxygenin)-labeled (Roche, Mannheim, Germany) DNA probe, which was specifically amplified by PCR from the coding sequence of the transcription factor genes. Detection and

visualization procedures were carried out following the manufacturer's manual (Roche).

To confirm single copy integration of the HYG-cassette, re-hybridization with a HYG probe was performed for some mutants

2.9. Pathogenicity tests of lipase-KO strains

2.9.1. Virulence assays on wheat heads

Susceptible spring wheat *c.v*. Nandu (Lochow-Petkus, Bergen-Wohlde, Germany), Florida and Melissos (Strube, Soellingen, Germany) were chosen for wheat head infection assays. Wheat plants were cultivated in a green house and then transferred to an infection chamber with optimized conditions. A suspension of 200 conidia was inoculated in each of two central spikelets at the early stages of anthesis (Jenczmionka and Schaefer, 2005). The inoculated spikes were enclosed in small plastic bags misted with water during the first 3 days and monitored up to three weeks in the infection chambers. For each disruption mutant strain, three independent knockout strains were used in parallel with the wild type strain. 20 µl pure water were used as negative control.

2.9.2. Virulence assays on maize cobs

Maize inbred line A188 (Green and Philips, 1975) was grown in the green house (temperature: 26°C – 30°C, humidity: 70% - 85%, natural daily photoperiod with additional artificial light when required). Before inoculation, silks were manually pollinated to ensure optimal pollination. Each maize cob was inoculated by injecting conidia suspensions into the silk channel of primary ears using a syringe and cannula (Reid et al., 1995). The infection was performed with 4 ml of conidial suspension at a concentration of 4×10^4 conidia per ml of *F. graminearum* wild-type strain and disruption mutant strains, respectively. Maize cobs inoculated with 4 ml of pure water were used for negative control. The inoculated cobs were enclosed in plastic bags during the first 3 days and the inoculation lasted for five weeks. Disease severity assessments were determined as described by Reid and Hamilton (Reid et al., 1995).

2.10. Phenotypic assays

2.10.1. Growth assays

The growth rate of disrupted mutant strains was determined by either placing a mycelial plug (diameter 0.6 cm^2) on agar plates or dropping 5000 conidia in 50 ml of CM and YPG medium as well as in modified SNA medium supplemented with either 1-2% (v/v) wheat germ oil (WGO); 1-2% Triolein; 1-2% Olive oil; 1-2% Tributyrin as the sole carbon sources. The cultures were incubated at 28^oC for 3-10 days. Diameter of mycelial colonies or dry weight of mycelia was measured by using a technical ruler or scale.

2.10.2. Histone deacetylase activity assays

To measure the histone deacetylase activity of the gene disrupted strains and the *F. gramineaum* wild-type PH1, 5x10^3 conidia were cultured in flasks containing 50 ml YPG for 3 days. The mycelia were collected by filtering with a 200 μm diameter sieve or miracloth, washed by rinsing three times with double-distilled water and dried by putting them into a 200 μm diameter sieve containing the mycelia on sterile whatman paper. Then 1g of dry mycelia were induced in SNA supplemented 50 mM sodium butyrate for 4 hours. The same amount of dry mycelium was only cultured in SNA for control. Otherwise, 5x10^3 conidia were cultured in flasks containing 50 ml CM supplemented with 50 mM sodium butyrate for 3 days. Culturing in CM was used for control. All the mycelia were collected, washed by rinsing three times with double-distilled water and then thoroughly dried with a water-jet vacuum pump and immediately frozen in liquid nitrogen. The frozen mycelia were ground to powder by hand in liquid nitrogen and the powder was suspended in 0.2 g/ml of buffer B (15mM Tris HCl, pH 8.0, 0.25mM EDTA, 1mM 2-Mecapthoethanol, 10% (v/v) glycerol) as described (Brosch et al., 2001).

The procedure of measuring HDAC activity was done following the introduction of the Histone deacetylase assay kit (Sigma-Aldrich, Steinheim, Germany).

2.10.3. DON assay *in vitro*

To measure the concentration of deoxynivalenol *in vitro*, 5 x 10^3 conidia of the *F. gramineaum* wild-type PH1 or gene disruption strains were cultured in YPG for 3 days. The mycelia were collected by filtering with a 200 μm diameter sieve or miracloth, washed by rinsing three times with double-distilled water and dried by putting into a 200 μm diameter sieve containing the mycelia on sterile whatman paper. Then 1.2g of dry mycelia were induced in MM supplemented with 5mM $(NH_4)_2S0_4$ or 2 mg/ml extracted fraction of wheat protein enriched with gliadin for 24 hours. 50 μl of supernatants were taken and measured for concentration of DON, following the instruction from RIDAscreen DON kits (R-Biopharm AG, Darmstadt, Germany).

2.10.4. DON assay *in planta*

To obtain samples for measuring DON concentration on wheat spikes, the high yielding, FHB-susceptible German spring wheat cultivar Nandu was chosen for wheat head infection assays. The inoculation procedure was following virulence assays on wheat heads 2.9.1. Four spikes each were inoculated with the *F. gramineaum* wild-type PH1 or gene disruption strain. As a negative control, spikes were inoculated with pure water. The inoculated samples were collected for 5 days post-inoculation (dpi). 200 mg of the infected spikelets were ground to a powder under liquid nitrogen, dried by speedvac for overnight at -70°C, and then 50 mg dried homogenized tissue was dissolved in 500 μl of distilled water. The extract was manually mixed by hand for 30 min and centrifuged. 50 μl of the supernatant was used in the test for Deoxynivalenol quantification with RIDASCREEN DON Kit (R-Biopharm AG, Darmstadt, Germany).

2.10.5. DON assay in wheat kernels

The production of the mycotoxins DON was induced on wheat kernels as follows: Fifteen grams of kernels and 10 ml water were autoclaved in a 300 ml Erlenmeyer flask. Autoclaved kernels were inoculated with 2 x 10^4 conidia from each of the following strains *F. graminearum* wild-type PH1 and *Δfar2* mutant. Uninoculated

samples served as the control. Incubation was carried out at 28 ^{0}C in the dark for 5, 7, 14 days. Later on, inoculated wheat kernels were treated with the same procedure as described in 2.10.4., DON assay *in planta.*

2.10.6. Determination of the amount of mycelium in mycotoxin samples

To determine the amount of mycelium in inoculated wheat spikes and infected wheat kernels, cereal samples were ground in liquid nitrogen as described above. 10-20 mg of ground samples were used for DNA isolation, which was performed according to the manufacture's instruction of the AVEGENE plant kit (Hamburg, Germany). Pure, ground *F. graminearum* wild-type PH1 mycelium was used as standard. Resultant DNA served as template in qPCR using ß-tubulin primers (ß-Tub q1F and R), calculating the number of ß-tubulin gene copies per milligram mycelium of the standard.

2.11. Expression analysis

2.11.1. Expression analysis by RT-PCR

In order to study the expression of the genes of interest (goi) *in vitro* and *in planta*, RNA was isolated with Nucleospin RNA II Kit (MACHEREY-NAGEL). For RT-PCR, SuperScript II Rnase H^{-} Reverse Transcriptase (Invitrogen) were used according to the manufacturer's instructions. The resulting single stranded cDNA was then used as template in PCR reactions to prove gene transcription in different samples. The PCR reactions were carried out with specific primers R-internal and F-internal. The cycler program consisted of an initial denaturation step for 3 min at 94°C, followed by 25-30 cycles of denaturation at 94°C for 30 s, annealing at 55-58°C for 30 s, and extension at 72°C for 30 s; the final elongation step was performed at 72°C for 10 min. The level of constitutively expressed β-tubulin mRNA served as reference (β-TubF, β-TubR).

To study the expression of the cutinase transcription factor genes in wheat cultivar Nandu during infection, samples were collected from spikes inoculated with conidia from *F. graminearum* wild type PH1 strain 3 d, 7 d, 10 d and 14 d post-inoculation (dpi). Uninoculated spikes served as reference. For RNA isolation, only directly

inoculated spikelets and intermediary rachis parts were used and frozen in liquid nitrogen for subsequent RNA isolation. All subsequent procedures for RNA isolation and RT-PCR were performed as described above.

2.11.2. Expression analysis by quantitative real-time PCR

To study the expression of the transcription factors, lipases, cutinase and beta oxidation genes *in vitro* and *in planta*, RNA was isolated with the Nucleospin RNA II Kit (MACHEREY-NAGEL). Samples were collected from *in vitro* cultures, 4 hours wheat germ oil induction and from spikes 5 days post inoculation and frozen in liquid nitrogen for subsequent RNA isolation. For RT-PCR, SuperScript II Rnase H^- Reverse Transcriptase (Invitrogen) was used according to manufacturer's instructions. The resulting single stranded cDNA was later used as template in quantitative real-time PCR (qPCR) reactions. The single stranded cDNA was normalized in dependence of the level of the constitutively expressed ubiquitin gene (Trevaskis et al., 2003). qPCR reactions were carried out with gene specific primers (Table 1) using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) in a volume of 20 µl. Quantification for gene-expression was performed with gene specific cDNA fragments of known concentration. QPCR reactions were carried out in a light Cycler 480 (Roche, Karlsruhe, Germany). The PCR program was as follows: incubation for 2 min at 50°C, then 2 min at 95°C, followed by 40-50 cycles of denaturation at 94°C for 30 s, annealing at 55-58°C for 30 s and extension at 72°C for 15 s, followed by melting curve analysis to check specificity of fragment amplification. All measurements were repeated twice with two replicates each. Relative changes in the gene expression were calculated with the comparative Cp method (using the LC 480 software for light Cycler 480, Roche, Karlsruhe, Germany)

To study the expression of the transcription factors, lipases, cutinase and beta oxidation genes in 50 mM sodium acetate, 50 mM sodium propionate, 50 mM sodium butyrate, 1% wheat germ oil, 1% triolein or 1% oleic acid, mycelia of *F. graminearum* wild type PH1 strain were collected at 6 h inducing. All subsequent procedures for RNA isolation and qPCR were performed as described above, including the primers used in qPCR reactions.

2.11.3. Northern Blot

To compare the *FGL1* expression in the *F. gramineaum* wild-type PH1 and *⊿lr1* knockout mutant strain, total RNA was isolated from 4h wheat germ oil culture by using Nucleospin RNA II Kit (MACHEREY-NAGEL) and separated on a 1% TBE-agarose gel by electrophoresis with a voltage of 70 V (approximately 20 μg/lane were loaded). RNA was transferred by capillary blotting onto Hybond NX membrane (Amersham Biosciences, Little Chalfont, UK) and then hybridized with a DIG (digoxygenin)-labeled (Roche, Mannheim, Germany) DNA probe. All procedures were performed according to the Southern blot procedure, except that all solutions were treated by diethylpyrocarbonate (DEPC) to remove RNAses. TBE buffer was prepared in DEPC treated water. Detection of RNA transcripts of *FGL1* was carried out by using the *FGL1* DNA probe, which was generated by PCR.

2.13. Accession numbers

Sequence data of *LR1* mRNA-complete Cds and gDNA from *F. graminearum* strain PH1 have been deposited in Genbank under the following accession numbers: HQ218320 and HQ201954, respectively.

3. Results

3.1. Computer-based identification of transcription factor genes of *F. graminearum*

The first cutinase transcription factor (CTF) was characterized in *F. solani* (Li and Kolattukudy, 1997). Later on, they found that this family of fungal transcription factors also regulated lipase, cutinase and the fatty acid metabolism (Hynes et al., 2006; Poopanitpan et al., 2010; Ramirez and Lorenz, 2009; Rocha et al., 2008). Based on the annotated sequences of *F. graminearum* from the Broad Institute in 2003 (www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html) (Trail, 2009), and the MIPS *F. graminearum* Genome Database (http://mips.helmholtz-muenchen.de/genre/proj/FGDB/), the transcription factor was sought with the key word “cutinase transcription factor”. A list of 10 entries was retrieved, 8 of them containing the Zn2Cys6 cluster motif with a conserved sequence of six cysteine residues spaced in the form CysX2CysX6CysX5-12CysX2CysX6-8Cys (MacPherson et al., 2006) were chosen to study. Table 4 shows a list of 8 transcription factors from FGDB. The 2 transcription factors were named *FAR1* and *FAR2* (**F**atty **a**cid **r**egulator) based on their homology to the FarA and FarB in *A. nidulans*. The 6 remaining transcription factors were named from Lr1 to Lr6 (**L**ipase **r**egulator).

Table 4. List of 8 transcription factors from the genomic sequence of *F. graminearum* strain PH-1. Some common features of the 8 transcription factors were found in FGDB such as access entries, genome coordinates, and number of base pairs (bp).

Name	FGDB entry	Genome Coordinate	Length (bp)	Comment
FAR1	FGSG_01936	6341045 - 6344229 (W)	3185	**This study**
FAR2	FGSG_07192	2682307 - 2685579 (W)	3273	**This study**
LR1	FGSG_09318	1138233 - 1135172 (C)	3062	**This study**
LR2	FGSG_03207	4268385 - 4270726 (W)	2342	**This study**
LR3	FGSG_00227	677902 - 675386 (C)	2517	**This study**
LR4	FGSG_08034	3046533 - 3048606 (W)	2074	**This study**
LR5	FGSG_08064	2982476 - 2985167 (W)	2692	**This study**
LR6	FGSG_15882	5753115 - 5755930 (W)	2816	**This study**

(W): + strand; (C): - strand; source: MIPS *F. graminearum* Genome Database (Guldener et al., 2006).

3.2. Transcription factors sequence homology

Alignment of the 8 transcription factors revealed that all contain a “Zin2Cys6 zinc cluster” DNA-binding motif and are located in the N-terminus from amino acid (aa) residues 56-85, 54-82, 55-85, 13-44, 54-82, 29-59, 15-44, and 25-54 for Far1, Far2, Lr1, Lr2, Lr3, Lr4, Lr5, and Lr6, respectively. They possess the well conserved motif of six cysteine residues in the form of CysX2CysX6CysX7-9CysX2CysX6-8Cys (Figure 6). Mutagenesis of these cysteines in GAL4, HAP1, LEU3, UME3, NIT4, AmdR, and FacB has shown that they are required for DNA binding and/or function (Defranoux et al., 1994; Johnston and Dover, 1987; Todd and Andrianopoulos, 1997). Although they have a high similarity in the Zn2Cys6 DNA-binding motif region, the sequences outside of the DNA-binding motif region are variable in amino

acid. Percentage identity between 8 transcription factors is only from 25.5% to 10.2%. The amino acid preceding the first cysteine is alanine for all transcription factors. This residue of HAP1, a transcription factor of yeast, was necessary for both the specificity of recognition of the target genes and the function of the protein (Defranoux et al., 1994). The amino acid preceding the fourth cysteine is proline in all transcription factors. Mutagenesis of the conserved proline to a leucine in GAL4 abolished DNA binding and function. However, substitution of the conserved proline to leucine, glutamine, or arginine in NIT4, and to arginine in HAP1, only reduced the function. The conserved proline is absent in AlcR of *A. nidulans* and PRIB of *L. edodes*, but both of these proteins are able to bind DNA. So the conserved proline is not absolutely required (Todd and Andrianopoulos, 1997).

Figure 6: Comparison of 8 transcription factors that contain a Zn2Cys6 zinc cluster DNA-binding motif in *F. graminearum*. The aa sequences of 8 transcription factors were aligned by ClustalX in MegAlign of DNASTAR and viewed by GeneDOC (version 2.7) program. Conserved cysteine residues are shaded and marked with a star, other conserved residues are shaded in grey. The deduced amino acid sequences of the transcription factors were retrieved from FGDB as follows: *FAR1* (FGSG_01936), *FAR2* (FGSG_07192), *LR1* (FGSG_09318), *LR2* (FGSG_03207), *LR3* (FGSG_00227), *LR4* (FGSG_08034), *LR5* (FGSG_08064), *LR6* (FGSG_15882).

3.3. Characterization of *FAR1* and *FAR2* genes

3.3.1. Conserved protein sequence of *FAR1* and *FAR2* in fungi

FAR1 and *FAR2* transcription factors are highly conserved proteins in fungi. They were investigated in *F. solani, F. oxysorum, A. nidulans, C. albicans* and *Y. lipolytica* (Hynes et al., 2006; Poopanitpan et al., 2010; Ramirez and Lorenz, 2009; Rocha et al., 2008). An alignment of entire amino acid sequences of this group was performed

with ClustalV in MegAlign of DNASTAR to generate the phylogenetic tree file. Then the phylogenetic tree was opened by Figtree_v1.3.1. The phylogenetic tree was divided into three branches as *FAR1*, *FAR2* homologs, and Oaf1/Pip2 of *S. cerevisiae*. *F. graminearum FAR1* (FgFar1) is closely related to other filamentous fungi such as *F. oxysporum FAR1* (93.9%), *F. solani FAR1* (88.4%) or *F. verticillioides FAR1* (91%) but is less closely related to yeast such as *C. albicans* Ctf1 (32%) and *S. cerevisiae* Oaf1/Pip2 (10.8 and 10.6%, respectively). *F. verticillioides FAR1* is highly similar to FgFar1 but has no Zn2Cys6 nuclear motif, the same is true for *C. globosum FAR1* and *A. fumigatus FAR1*. *F. graminearum FAR2* (FgFar2) showed the highest similarity to *F. oxysporum FAR2* (92.7%), *F. verticillioides FAR2* (91.5%) or *F. solani FAR2* (85%). *A. terreus FAR2* has no Zn2Cys6 nuclear motif.

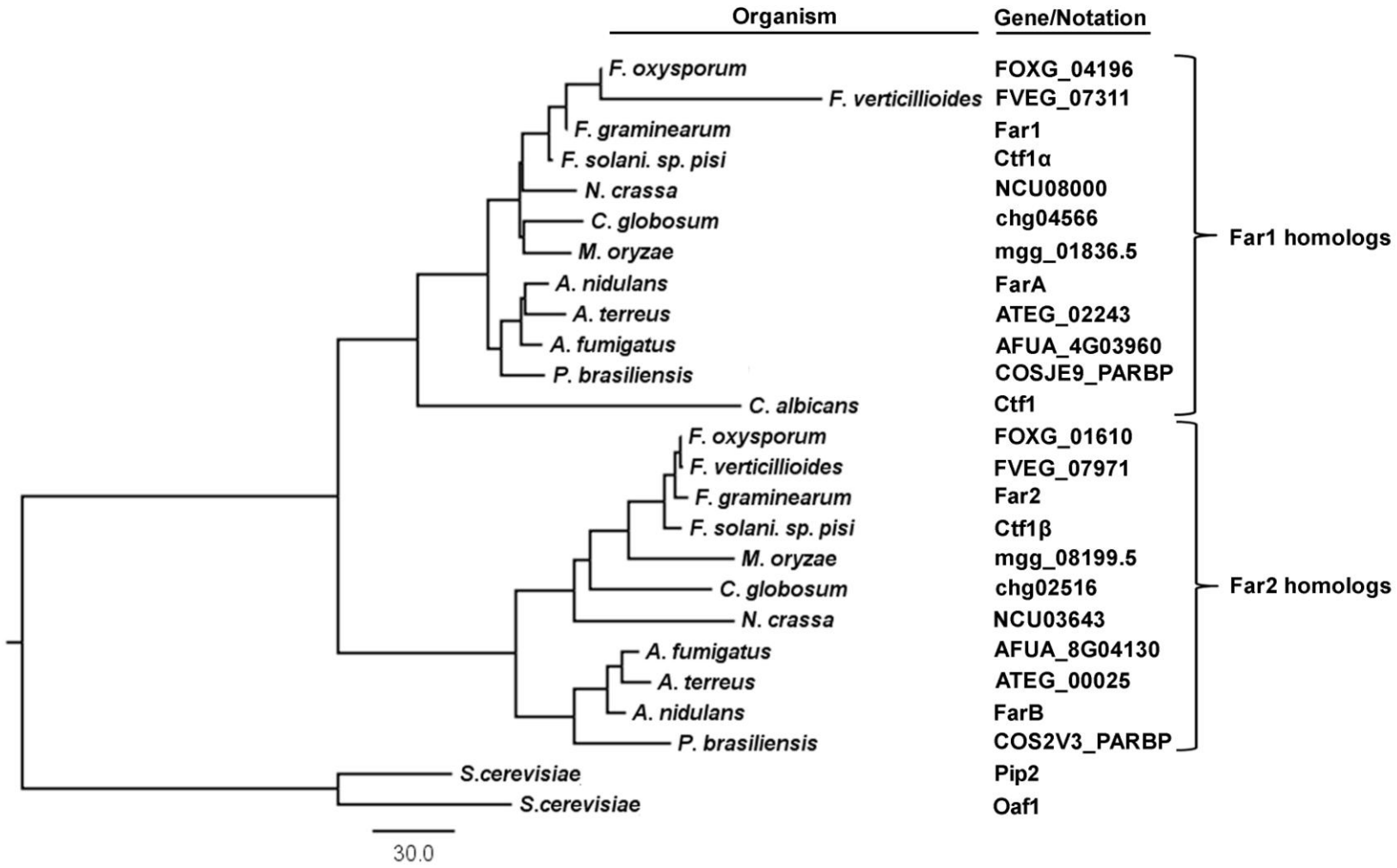


Figure 7: Phylogenetic relationship of fungal Far1/Far2 transcription factors harboring the Zn2Cys6 DNA-binding motif. The protein sequences obtained were aligned with the multiple sequence alignment program ClustalV in MegAlign of DNASTAR. Then the phylogenetic tree was generated by Figtree_v1.3.1. Organisms and gene names or sequence notations are indicated.

3.3.2. Generation of *FAR1, FAR2*, and *FAR2/FAR1* disruption strains

To characterize the role of *FAR1* and *FAR2*, the wild type *F. graminearum* PH1 strain was transformed with the plasmids pGEM-*FAR1* and pGEM-*FAR2*, respectively, by protoplast-mediated transformation. Two independent transformations were performed and more than ten primary colonies were generated in each transformation. Eight colonies of either transformation were picked up and grown in CM with the appropriate selection marker. The total DNA of transformants was isolated and analyzed by PCR for the absence of the *FAR1* or *FAR2*. Among these transformants, five of them have lost the *FAR1* or *FAR2* gene (data not shown). Southern blot was used to confirm the gene deletion. In the first hybridization of Southern blot, the *FAR1* and *FAR2* probe were used to confirm the deletion of the endogenous genes in the transformants. As shown in the figure 8B (deletion of *FAR1*, strains 1-3) and 8C (deletion of *FAR2*, strain 1-3), the *FAR1* and *FAR2* gene have been disrupted. The Southern blot also showed that *FAR1* or *FAR2* was not deleted in the ectopic transformants (ECT). To finally proof that the one copy of the selective marker cassette was integrated in the gene locus, Southern blots were re-hybridized with respective selective maker probe, Geneticin (nptII) probe for *FAR1* and Hygromycin (HYG) probe for *FAR2*. The selective marker cassette was located in the *FAR1* or *FAR2* loci in the gene disrupted strains, whereas it was randomly integrated in an unknown locus of the genome in the ectopic transformants (Figure 8B and C). The results show that the *FAR1* and *FAR2* gene have been successfully replaced by the selective marker cassette and the endogenous genes were present in the ectopic strains which were also used for transformation control.

FAR1 and *FAR2* have high amino acid sequence similarity (61% and 43% identity with FarA and FarB in *A. nidulans*, respectively) and it has been shown that their orthologs play overlapping functions in *A. nidulans* (Hynes et al., 2006). To better understand the functions of the genes, the *FAR1* and *FAR2* double mutant was generated by using the *FAR1* deletion strain (*Δfar1*, strain 1) as the background for disruption of *FAR2*. Transformation of the *Δfar1* strain with the plasmid pGEM-*FAR2*

was similarly performed by transformation of protoplasts which resulted in 35 transformants. Twenty four transformants were analyzed by PCR for the absence of *FAR2*. It was shown that 6 transformants, in which *FAR2* was absent, were found (data not shown). As shown in figure 8D, the hybridization with the *FAR2* probe was negative in three transformants, while it was positive in the WT, the original *Δfar1*, and two ectopic transformants. Re-hybridization of the Southern blot with the *FAR1* probe was also performed to confirm the absence of *FAR1* in the disrupted strains (Δ*far2*/Δ*far1*) as well as in the ectopic ones (Figure 8D, middle panel). It was also shown that the selective marker was located in the *FAR2* locus in the double gene disrupted strains and it was integrated in an unknown locus in the genome of ectopic strains (Figure 8D, lower panel).

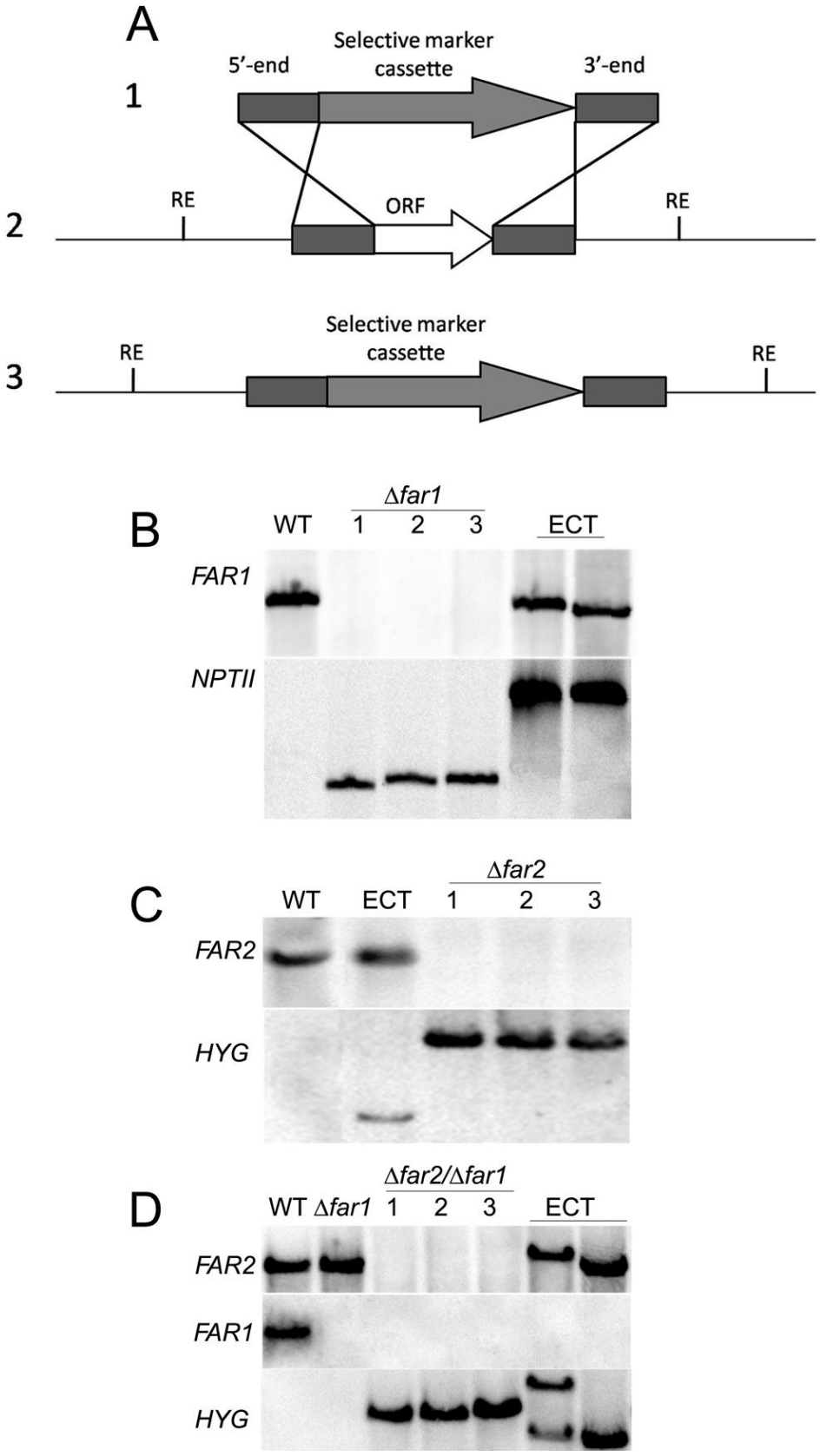
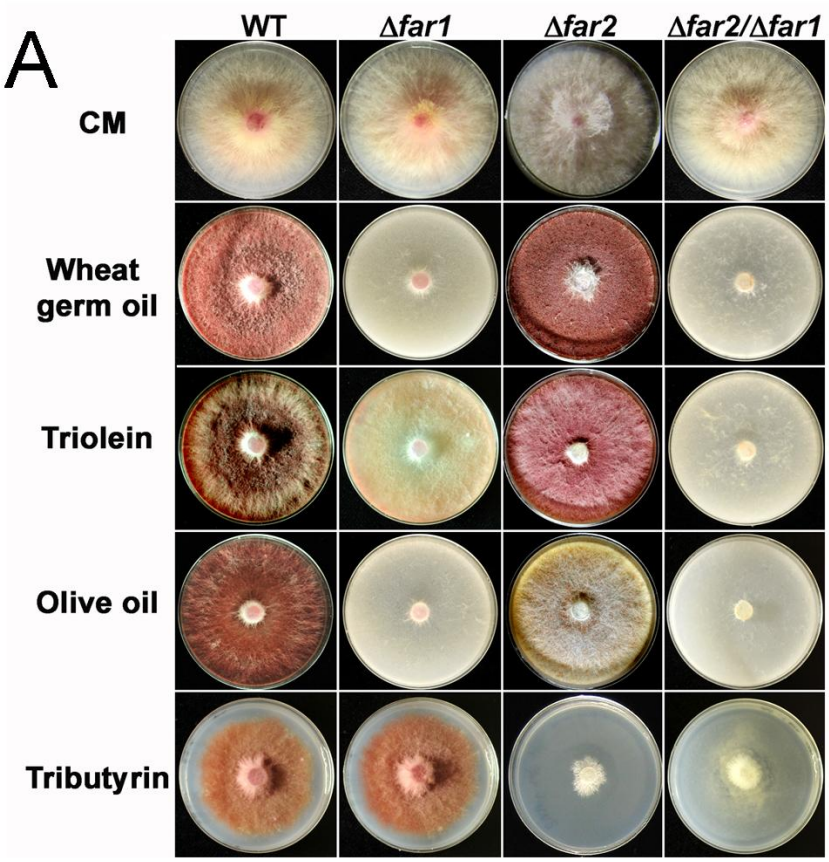

A
1
5'-end
Selective marker cassette
3'-end
2
RE
ORF
RE
3
Selective marker cassette
RE
RE
B
Δfar1
WT
1
2
3
ECT
FAR1
NPTII
C
Δfar2
WT
ECT
1
2
3
FAR2
HYG
D
Δfar2/Δfar1
WT
Δfar1
1
2
3
ECT
FAR2
FAR1
HYG

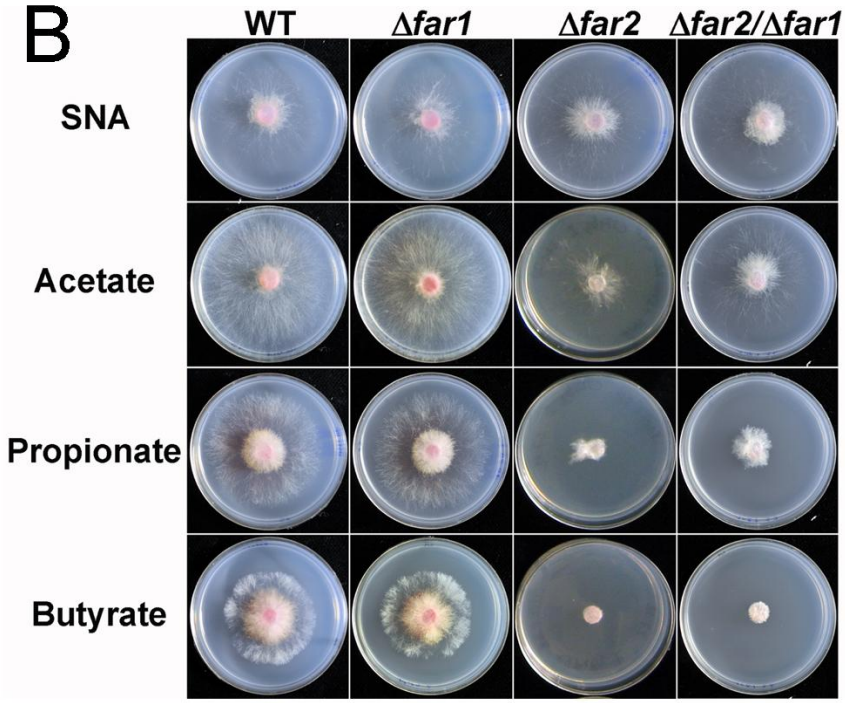
Figure 8: Disruption of *FAR1, FAR2*, and both *FAR1* and *FAR2* genes in *F. graminearum*. A) Illustration of gene disruption strategy. A1, representation of disruption construct; A2, genotype of wild type; A3, genotype of disrupted strains. Selective marker cassette used for *FAR1* disruption was Geneticin (nptII); selective marker cassette used for *FAR2* disruption was Hygromycin (HYG). RE, restriction enzyme; ORF, open reading frame. B) Southern blot analysis of *FAR1* disrupted *(*Δ*far1*) strains, wild type (WT), and ectopic (ECT) strains. Upper panel illustrates the hybridization with *FAR1* probe, showing the absence of the native gene in the disrupted strains (strain 1, 2, 3), its presence in the WT and ECT. Lower panel illustrates the re-hybridization with Geneticin probe to show the replacement of the selection marker (Geneticin, nptII) in the *FAR1* locus. C) Southern blot analysis of *FAR2* disrupted (Δ*far2*) strains, WT, and ECT strains. Upper panel: hybridization with *FAR2* probe, showing the absence of the gene in the disrupted strains (strain 1, 2, 3), and its presence in the WT and ECT strains. Lower panel: re-hybridization with Hygromycin probe to show the replacement of the selection marker (Hygromycin, HYG) in the *FAR2* locus. D) Southern blot analysis of *FAR1* and *FAR2* double disrupted strains (Δ*far2*/Δ*far1*) using Δ*far1* strain as the background. Upper panel: hybridization with *FAR2* probe, showing the absence of *FAR2* in the disrupted strains (strain 1, 2, 3), and its presence in the WT, Δ*far1*, and ECT strains. Middle panel: re-hybridization with *FAR1* probe. Lower panel: re-hybridization with HYG probe.

3.3.3. *FAR1* and *FAR2* regulate fatty acid metabolism

To examine the involvement of *FAR1* and *FAR2* in lipid metabolism processes, the growth of wild-type, Δ*far1,* Δ*far2*, and *Δfar2/Δfar1* strains was tested on minimal medium containing various lipids or fatty acids as the sole carbon source. The growth of all these strains on CM was not affected by the gene deletions (Figure 9A). The WT and disrupted strains grew also equally on minimal medium SNA without fatty acids (Figure 9B). Thus, deletion of *FAR1* or *FAR2* did not affect the fungal growth in rich media. However, the WT and the corresponding ectopic strains (data not shown) grew better when SNA medium was supplemented with lipids or with fatty acids as the sole carbon sources (Figure 9A, B). In contrast, the growth of disrupted strains Δ*far1* and Δ*far2* strains were remarkably affected in SNA supplemented with lipids or fatty acids as the sole carbon sources. The growth of the Δ*far1* strain was reduced in minimal media supplemented with complex long fatty acid chains such as wheat germ oil, olive oil or triolein (triglyceride formed from oleic acid). Its growth was not impaired in media supplemented with short chain fatty acids (Figure 9A, B). The fungal biomass of Δ*far1* strain grown in lipids and fatty acid-containing media was also measured. As shown in figure 9C and D, mycelial weight of Δ*far1* was significantly reduced in liquid SNA supplemented with long chain

fatty acid sources such as wheat germ oil (WGO), triolein or oleic acid, but not with short chain fatty acids such as butyrate. The results suggest that *FAR1* is required for long chain fatty acid utilization and it is not necessary for short chain fatty acid metabolism. In contrast to Δ*far1* strain, the growth of *Δfar2* strain was not affected in media supplemented with long chain fatty acids; its growth was severely affected in media supplemented with short chain fatty acids (Figure 9A, B). The data suggest that *FAR2* is required for short chain fatty acid utilization. It has been reported that *A. nidulans* FarA and FarB have overlapping functions in long chain and short chain fatty acid metabolism. However, it is found that the *FAR1* and *FAR2* in *F. graminearum* play separate roles in regulating long chain and short chain fatty acid metabolism. Thus, the *FAR1* and *FAR2* double gene disrupted strain (*Δfar2/Δfar1*) was generated and tested its growth in minimal medium supplemented with long chain fatty acids (e.g. oleic acid) and short chain fatty acids (e.g. butyrate). The growth of *Δfar2/Δfar1* strain was impaired in utilization of both long chain and short chain fatty acids. This result was confirmed by measuring the fungal biomass grown in the SNA supplemented with long chain fatty acids (Figure 9C). The phenotypes of this strain were the overlapping phenotypes of the *Δfar1* and *Δfar2* strains. Taken together, the data suggest that *FAR1* and *FAR2* regulate long chain and short chain fatty acid metabolism, respectively.


A
WT
Δfar1
Δfar2
Δfar2/Δfar1
CM
Wheat germ oil
Triolein
Olive oil
Tributyrin


B
WT
Δfar1
Δfar2
Δfar2/Δfar1
SNA
Acetate
Propionate
Butyrate

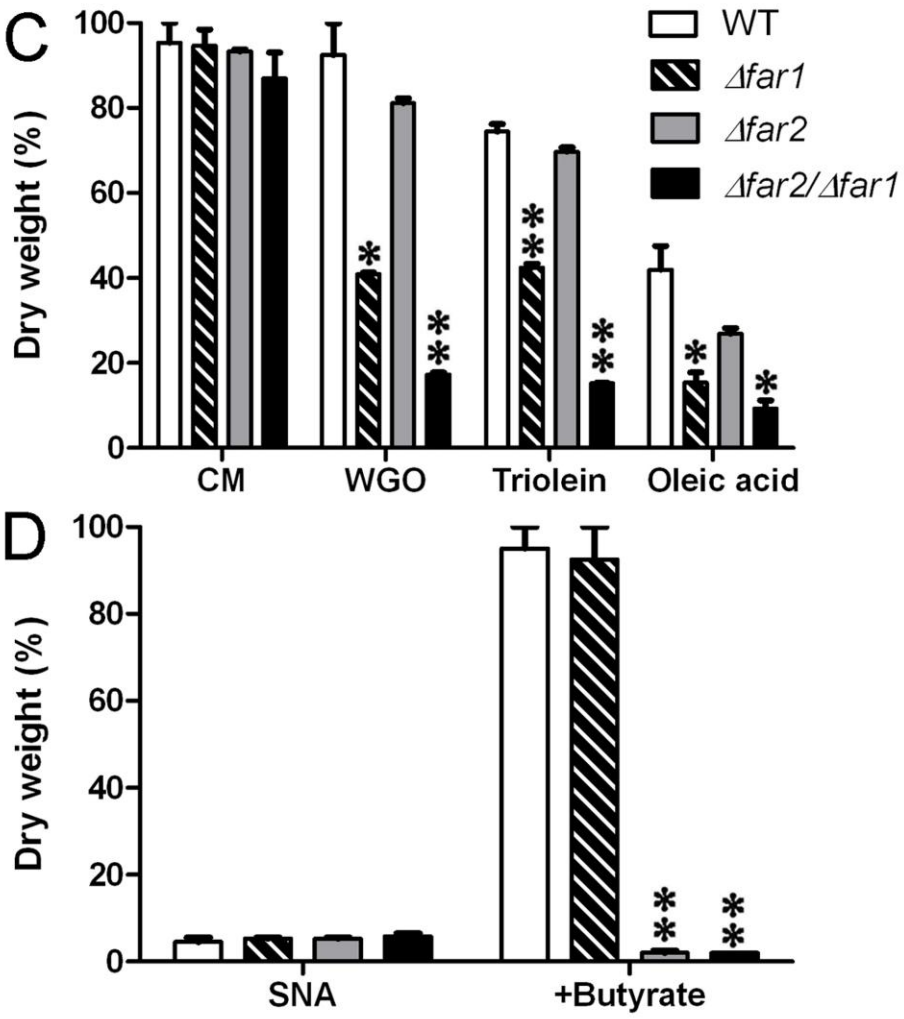
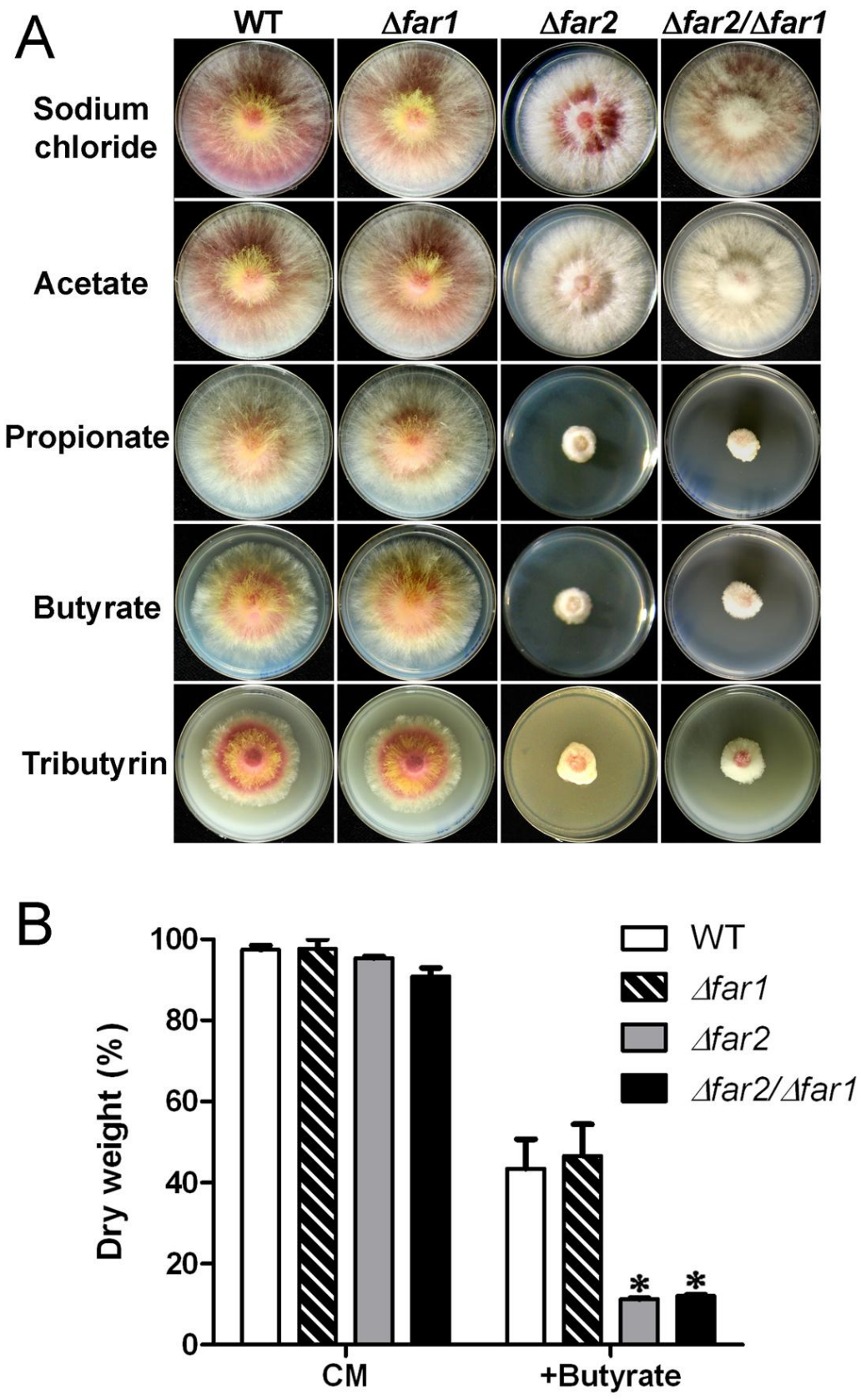


Figure 9: Growth of disrupted strains on complete medium (CM) and minimal medium SNA, SNA supplemented with 1% (v/v) indicated lipids and 50 mM short chain fatty acids. Growth of disrupted strains on minimal medium (SNA) supplemented with long chain fatty acids (A) and short chain fatty acids (B) as the sole carbon source. A section of agar with mycelia of the WT and disrupted strains was inoculated on the media for 5 days at 28^{o}C. The *Δfar1* strain was reduced in growth in lipid containing media with long chain fatty acids such as triolein (oleate) and exhibited a normal growth the same as WT in media with short chain fatty acid. The *Δfar2* strain was reduced in growth in media with short chain fatty acids. The double disrupted strain (*Δfar2/Δfar1*) showed reduced growth in both long chain and short chain fatty acid containing media. Biomass of fungal strains in liquid CM and SNA without or with the indicated lipids (C) and short chain fatty acids (D) as the sole carbon source. Mycelia of the strains were harvested after 3 days, 5 days and 10 days of growth at 28^{o}C, respectively. Mycelia were air-dried and weighed. In comparison with WT, the biomass of *Δfar1* and *Δfar2/Δfar1* strains was decreased in media supplemented with the lipids, whereas biomass of *Δfar2* strain was not significantly reduced. The *Δfar1* strain grew normal as WT in the media whereas *Δfar2* and *Δfar2/Δfar1* strains were markedly reduced in growth in media with short chain fatty acids. ∗P< 0.05; ∗∗P< 0.01.

3.3.4. *FAR2* is important for fungal growth and conidia germination in response to SCFAs

As indicated above, the growth of WT in SNA media supplemented with short chain fatty acids (SCFAs) such as propionate or butyrate was reduced compared to the growth in acetate containing media (Figure 9B). Thus, the growth of the WT and the disrupted strains was tested on complete media CM supplemented with the SCFAs. Indeed, the results showed that butyrate or its lipid form tributyrin inhibits the growth of the WT and the disrupted strains (Figure 10A). Tributyrin showed a greater inhibitory effect than butyrate to the growth of the strains. Fungal biomass of the WT or the *Δfar1, Δfar2*, and *Δfar2/Δfar1* strains was significantly reduced in the presence of butyrate (Figure 10B). Notably, the growth of *Δfar2* and *Δfar2/Δfar1*, but not *Δfar1* strains was strongly suppressed by propionate or butyrate, suggesting that *FAR2* is important for SCFAs degradation (Figure 10A). The growth of WT in complete medium CM was also reduced in the presence of tributyrin or butyrate (Figure 10A). This result indicates that butyrate exerts the inhibitory activity to fungal growth and deletion of *FAR2* leads to profound susceptibility to the compound. We also found that conidia germination of the WT and *Δfar1* strains was slightly inhibited by SCFAs (Figure 10A and data not shown). Interestingly, deletion of *FAR2* led to conidia germination arrest in the *Δfar2*, and *Δfar2/Δfar1* strains in the presence of SCFAs (Figure 10C). Among the SCFAs tested, butyrate exerted the greatest effect on conidia germination of the *FAR2* deleted strains after 8 days of incubation. About 1% of conidia of *FAR2* deleted strain were able to germinate after 8 days of incubation with butyrate, but with a delayed growth (data not shown). Butyrate has been found to inhibit histone deacetylases. Thus, it was postulated that *FAR2* might regulate the expression of histone deacetylases of the fungus. The histone deacetylase activity of the WT, ECT and *Δfar2* strains was measured. However, the histone deacetylase activity among these strains was not different (Figure 11). Thus, *FAR2* might regulate other genes which are important for the detoxification or metabolism of butyrate.


A
WT
Δfar1
Δfar2
Δfar2/Δfar1
Sodium chloride
Acetate
Propionate
Butyrate
Tributyrin
B
Dry weight (%)
100
80
60
40
20
0
WT
Δfar1
Δfar2
Δfar2/Δfar1
*
*
CM
+Butyrate

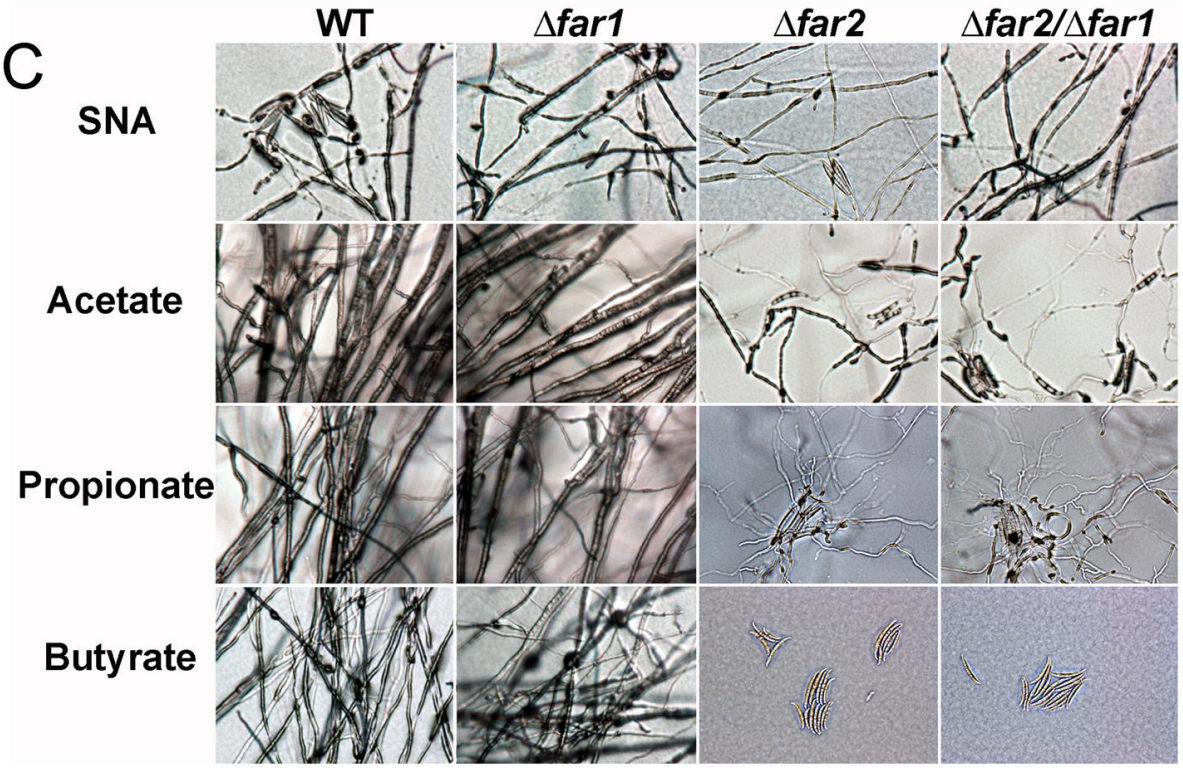


Figure 10: Butyrate inhibits fungal growth and conidia germination. A) Growth of disrupted strains on complete medium (CM) supplemented with the indicated short chain fatty acids as the sole carbon source. A section of agar with mycelia of the WT and disrupted strains were inoculated in the media for 3 days at 28°C. The *Δfar1* strain grew normal as WT in the media whereas *Δfar2* and *Δfar2/Δfar1* strains were markedly impaired in growth in the presence of propionate, butyrate or butyrin, but not in the presence of acetate. B) Biomass of the WT and disrupted strains in liquid CM media supplemented with butyrate. Fungal strains were cultured in CM with or without 50mM sodium butyrate for 3 days at 28°C. Mycelia were harvested, air-dried and weighed. The WT and *Δfar1* strains were able to use butyrate as the carbon source for growth, while *Δfar2* and *Δfar2/Δfar1* strains were unable to use butyrate as their biomass was significantly reduced. ∗P< 0.05. C) Germination of WT and disrupted strains in SNA with presence of short chain fatty acids. Conidia of WT and the disrupted strains were plated on SNA and incubated at 28°C for 8 days. In comparison with WT, Δ*far1* strains were only impaired in germination, while Δ*far2* and Δ*far2*/Δ*far1* strains were markedly reduced in germination in the presence of the fatty acids. Conidial germination of Δ*far2* and Δ*far2*/Δ*far1* strains was greater impaired with butyrate. Pictures were taken by microscope (Leica) with 10X magnification.

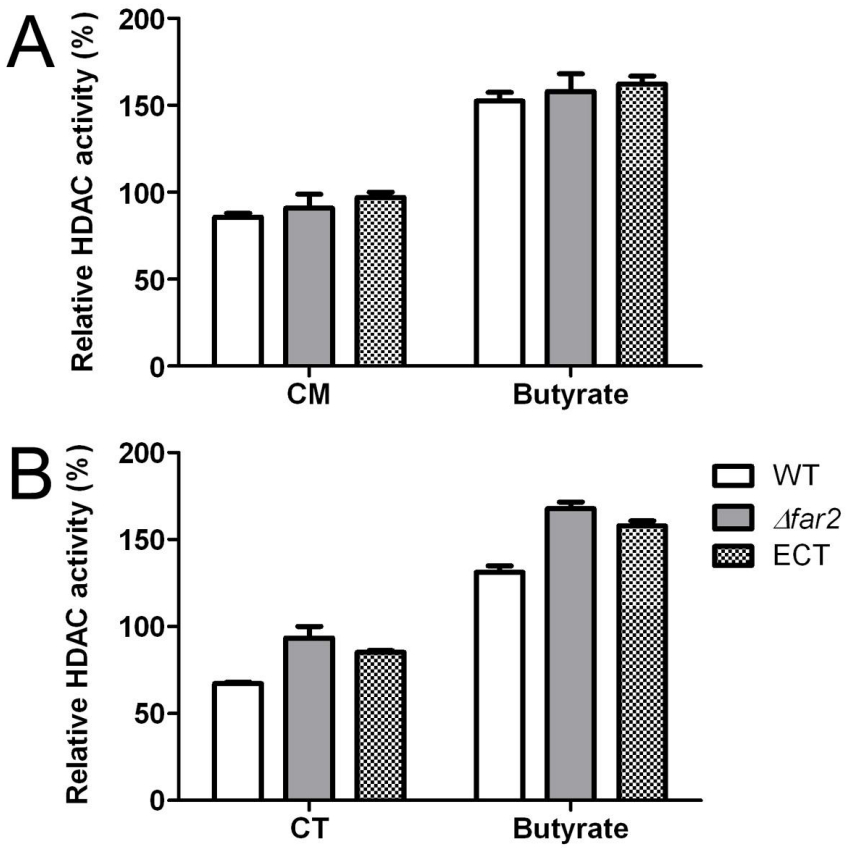


Figure 11: Histone deacetylase (HDAC) activity in wild-type and Δ*far2* strains. A) Culturing in CM plus sodium butyrate for 3 days. B) Culturing 3 days in YPD, then inducing by sodium butyrate for 4 hours. The HDAC activity of all samples was calculated per 1mg total protein.

3.3.5. Enhanced susceptibility of *FAR2* deleted strains to fungicides

Butyrate might affect the chitin synthesis in the *FAR2* deleted strain (Hoberg et al., 1983) and *FAR2* might regulate genes important for cell wall or membrane synthesis. Thus, the susceptibility of the strains in the presence of several antifungal drugs was tested. The growth of the Δ*far1,* Δ*far2*, and Δ*far2*/Δ*far1* strains was tested in complete media with the presence of the commonly used fungicides tebuconazole and azoxystrobin. The results showed that with the concentration of 0.4 µg/ml tebuconazole and 0.125 mg/ml azoxystrobin the wild type growth was reduced compared to control without the drugs (Figure 12A). Wild type biomass was reduced by 24 % and 78.5 % by treatment of tebuconazole and azoxystrobin, respectively.

Enhanced susceptibility of the Δ*far1* strain to the drugs was not observed (Figure 12A). However, deletion of *FAR2* from the WT or Δ*far1* strains caused the fungus to be more susceptible to the drugs. Biomass of *FAR2* deleted strains (*Δfar2 or Δfar2/Δfar1* strains) was significantly reduced in the presence of the drugs compared with WT or Δ*far1* strains (Figure 12B). The susceptibility of these strains to cell wall stressors Congo red and Calcofluor white was also tested (Figure 13). However, enhanced susceptibility of gene deleted strains to these compounds was not seen. These results suggest that unlike *FAR1*, *FAR2* could be important for drug resistance of the fungus and *FAR2* might regulate other genes which are important for antifungal drug resistance.

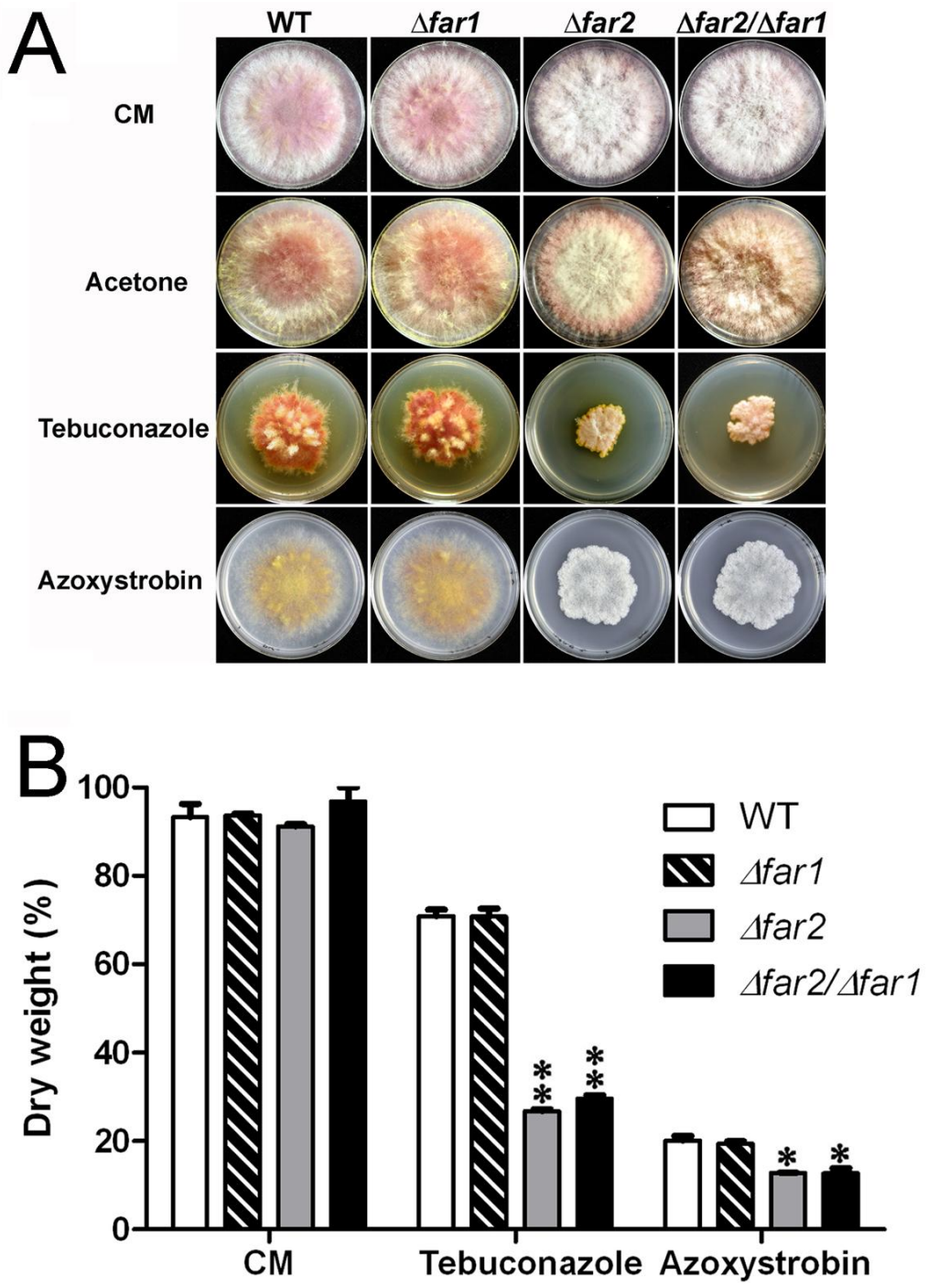


Figure 12: Susceptibility of *FAR2* deletion mutant to antifungal drugs. A) The sensitivity of the fungal strains to 0.4 µg/ml tebuconazole and 0.125 mg/ml azoxystrobin was tested in CM media. It showed that deletion of *FAR2* led to more susceptibility to the fungicide compared to the wild type (WT). Deletion of *FAR1* did not make the fungus more susceptible to the drugs. B) The biomass of *FAR2* deletion strain was reduced compared to WT and the *FAR1* deletion strains. ∗P< 0.05; ∗∗P< 0.01.

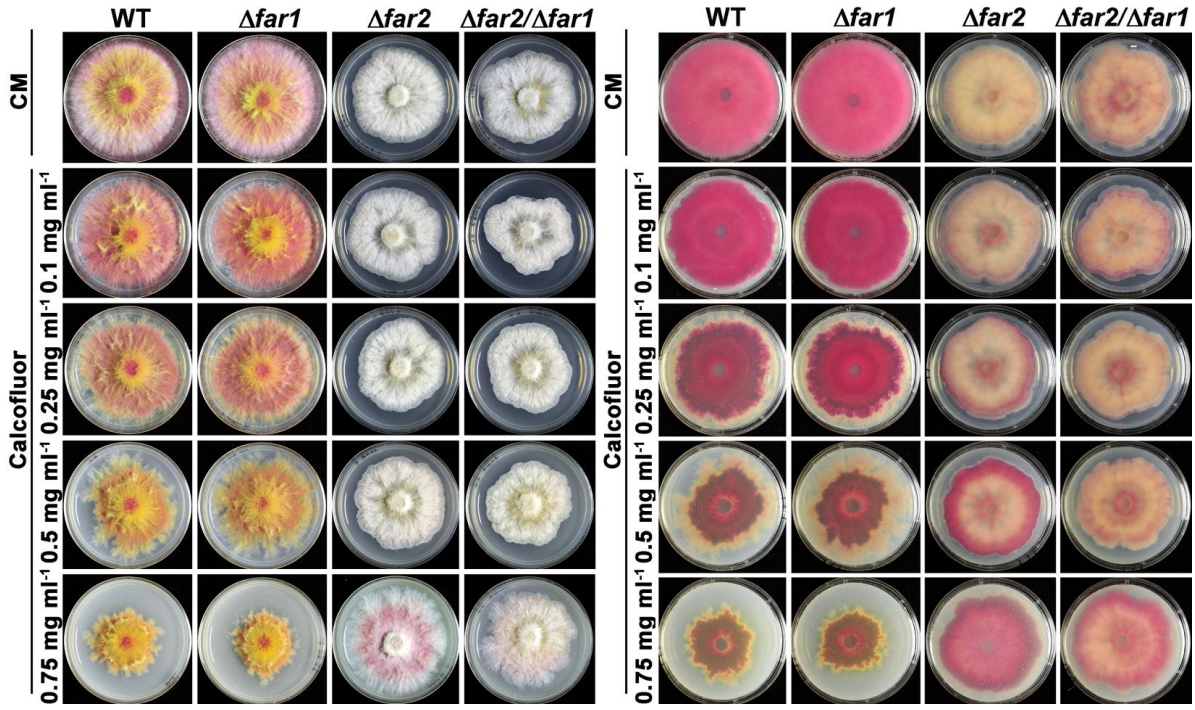


Figure 13: Colony morphology during growth on CM with Calcofluor of wild-type, Δ*far1,* Δ*far2* and Δ*far2*/Δ*far1* strains. A) Top of plate. B) Bottom of plate. The pictures were taken at 4 days post-inoculation.

As indicated above, Δ*far2* and Δ*far2/*Δ*far1* strains were inhibited by butyrate and tebuconazole. In the next experiment, wild-type and mutant strains were cultured in CM supplemented with a combination of butyrate and tebuconazole at lower concentrations. Δ*far2* and Δ*far2/*Δ*far1* strains were strongly reduced in growth in CM media containing 0.2 μg/ml tebuconazole supplemented with 5 and 10 mM butyrate compared to slightly reduced growth in the wild-type and Δ*far1* strains (Figure 14A). Especially the Δ*far2* and Δ*far2/*Δ*far1* strains showed almost no growth in CM media containing 0.4 μg/ml tebuconazole supplemented with 5 and 10 mM butyrate compared to slightly reduced growth in 5 mM butyrate and strongly reduced growth in 10 mM in both the wild-type and Δ*far1* strains (Figure 14B). This result indicated once more that Δ*far2* and Δ*far2/*Δ*far1* strains are more sensitive to tebuconazole and butyrate. The result suggested that butyrate is a promising compound to inhibit the fungal pathogen.

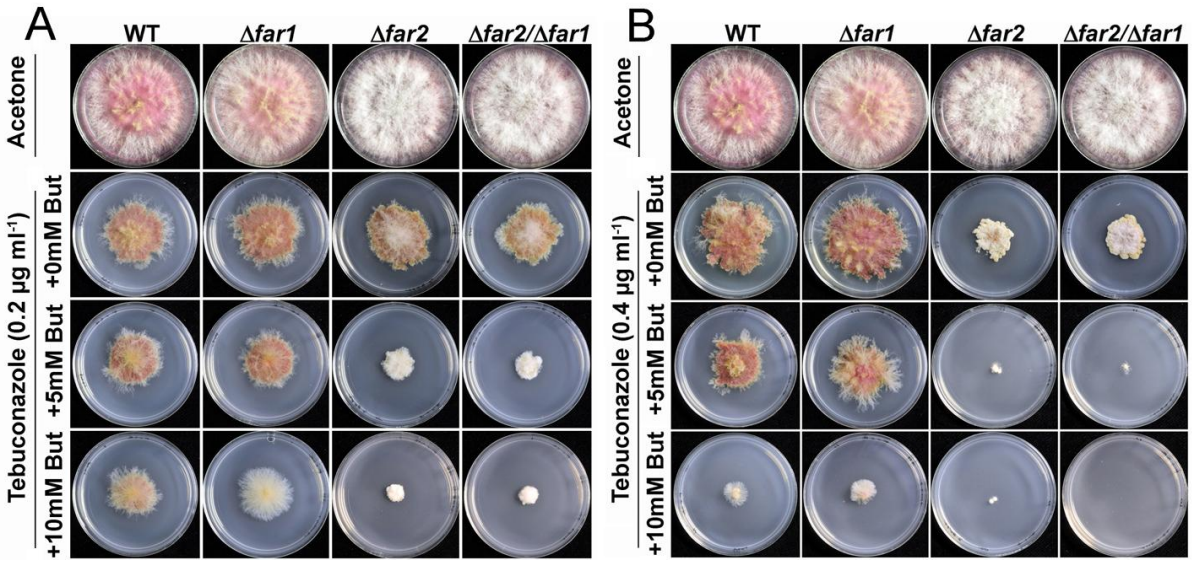


Figure 14: Susceptibility of *FAR2* deletion mutant to antifungal drugs and butyrate. A) The sensitivity of the fungal strain to 0.2 µg/ml tebuconazole supplemented with 5 and 10 mM butyrate (But) was tested in CM media for 5 days. B) The sensitivity of the fungal strain to 0.4 µg/ml tebuconazole supplemented with 5 and 10 mM butyrate was tested in CM media for 10 days. It showed that the fungus was more sensitive to the combination of tebuconazol and butyrate, especially Δ*far2* and Δ*far2*/Δ*far1* mutants.

3.3.6. *FAR1* and *FAR2* are not necessary for perithicia formation

The fertility of Δ*far1,* Δ*far2* and Δ*far2*/Δ*far1* strains was determined by comparing the amount of perithecia produced by the mutants on carrot agar medium with the amount produced by the *F. graminearum* wild-type PH1 strain. Conidia of the wild-type and mutant strains were inoculated on carrot agar for 21 days. The result was no difference in perithecia formation between wild-type and mutant strains. All strains produced an average of 25 clumps/plate (Figure 15). The experiment was performed on 4 plates for each strain. Conidia production of wild-type and mutants was also examined. All wild-type and Δ*far1,* Δ*far2* and Δ*far2*/Δ*far1* strains showed no difference in conidia production (data not shown).

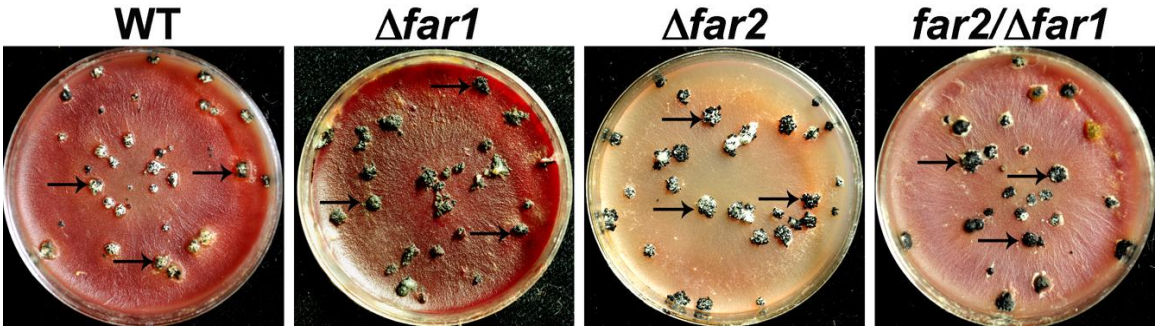


Figure 15: Perethicia formation of wild-type, Δ*far1,* Δ*far2* and Δ*far2*/Δ*far1* strains on carrot agar. Perithecia can be seen as black structures on the surface of the plate inoculated with wild-type and mutant strains. The black arrows mark some of the perithecia clusters. The pictures were taken at 21 days post-inoculation.

3.3.7. *FAR1* and *FAR2* transcript detection during wheat germ oil induction and plant infection

FAR1 and *FAR2* were detected in the presence of wheat germ oil. During host infection, *FAR2* was strongly expressed at all time points examined. *FAR1* was slightly expressed at 3 dpi, 7 dpi, 10 dpi and strongly expressed at 14 dpi (Figure 16). No *FAR1* and *FAR2* transcripts were detected in uninoculated control plants.

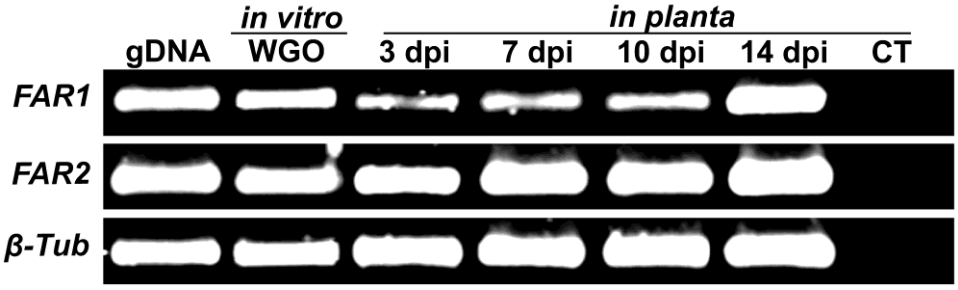


Figure 16: Expression analysis of *FAR1* and *FAR2*. PCR amplification with *FAR1* and *FAR2* specific primers. Template cDNAs were generated from isolated RNA of *F. graminearum* wild-type culture that was incubated for 4h at 28 ^{0}C and 150 rpm in media containing H_20+2% wheat germ oil or from RNA isolated of wheat spikes infected with *F. graminearum* 3 days post inoculation (3dpi), 7 dpi, 10 dpi, 14 dpi and cDNA from uninfected wheat spikes (negative control, CT). Genomic *F.graminearum* DNA was used as control. β-tubulin-specific primers (β-tub) were used as expression control. Semi-quantitative RT-PCR was performed with 30 cycles for all samples.

3.3.8. Induction of gene expression by different fatty acids

As demonstrated above that *FAR1* and *FAR2* are the regulators of fatty acid/lipid metabolism. Thus, *FAR1* and *FAR2* might regulate genes involved in lipid/fatty acid utilization. Thus, the expression response of *FAR1, FAR2* genes together with several genes such as secreted lipases *FGL1* and *LIP1*, which are important for lipid hydrolysis, and *FOX2* and *PEX11* which are important for fatty acid oxidation in the WT under induction of different fatty acids was analyzed. Expression of *FAR1, FGL1, LIP1, PEX11, FOX2* was inhibited by short chain carbon fatty acids (Figure 17). Unexpectedly, expression of *FAR2* which is important for short chain fatty acid utilization was also strongly suppressed by propionate and butyrate, but not by acetate. Expression of *FAR1, FGL1, LIP1, PEX11, FOX2* was induced by long chain fatty acids (Figure 17). Expression of *FAR2* was not upregulated by long chain fatty acids. These data suggest that *FAR1* regulates the expression of lipases and genes important for beta-oxidation.

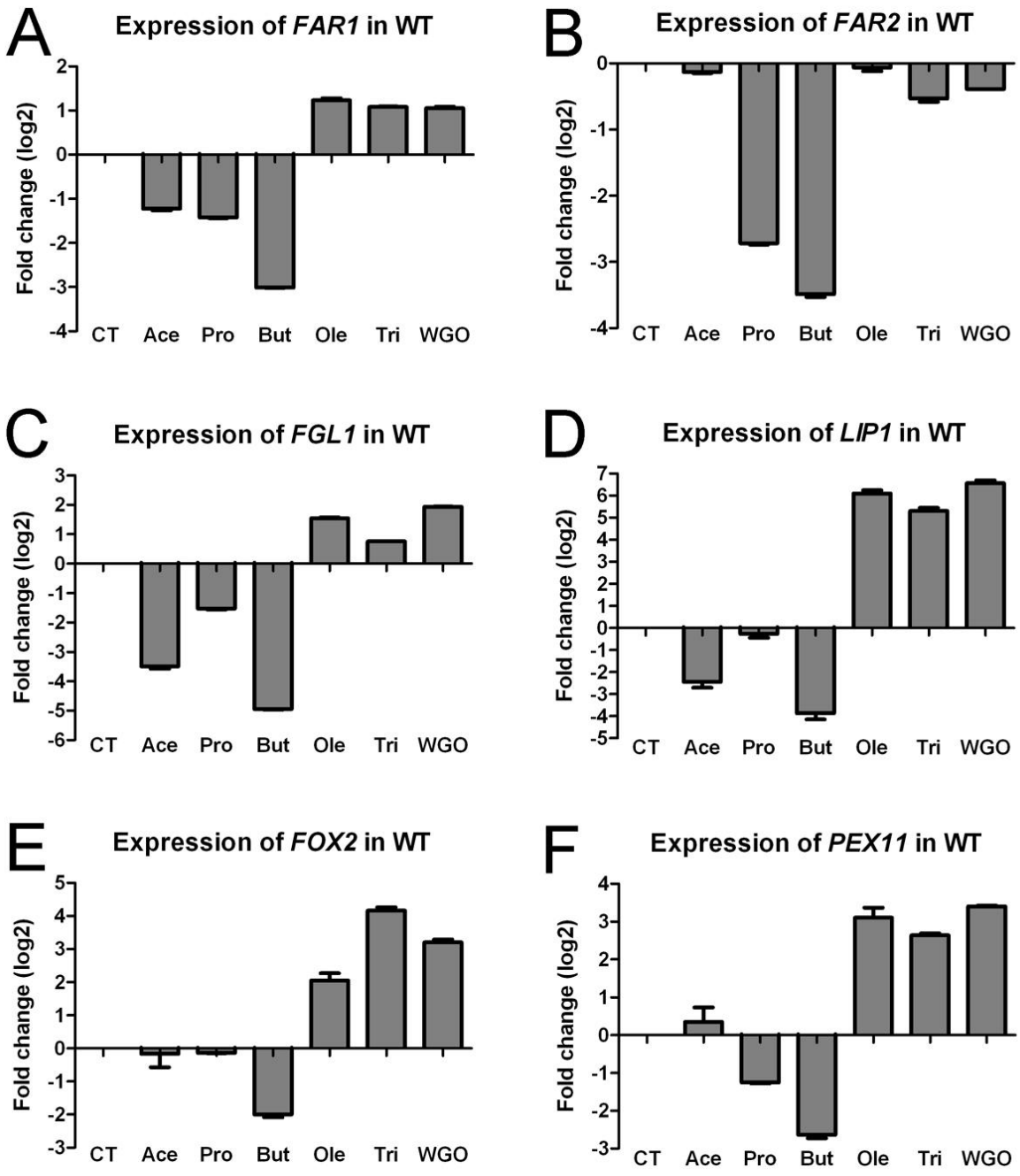


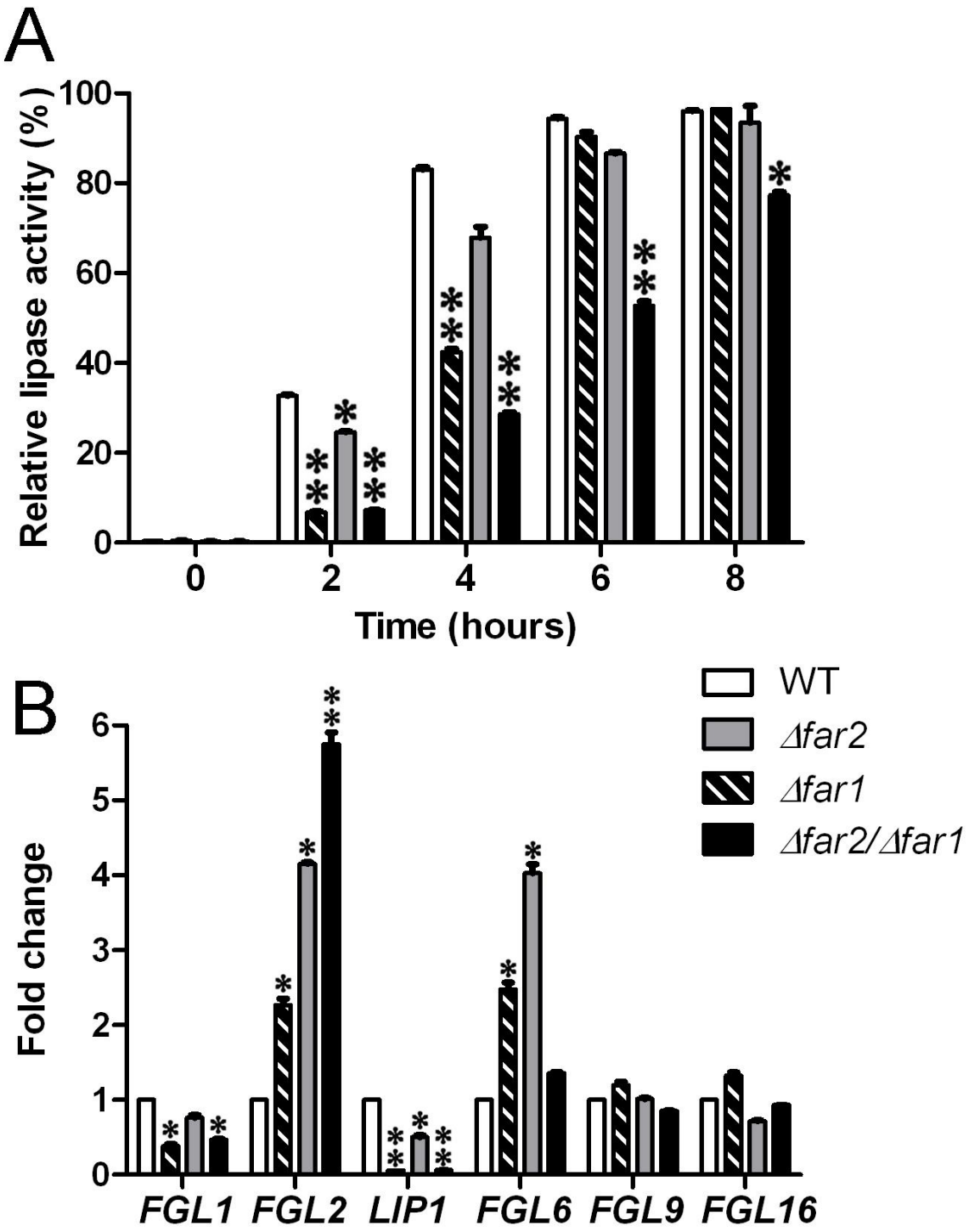
Figure 17: Transcription of the *FAR1* (A), *FAR2* (B), *FGL1* (C), *LIP1* (D), *PEX11* (D), and *FOX2* (E) in the wild type in response to carbon sources. Transcript expression level of *FAR1, FAR2, FGL1, LIP1, PEX11*, and *FOX2* was assessed using real-time quantitative PCR. Transcript expression of *FGL1, LIP1, PEX11*, and *FOX2* was significantly down-regulated by short chain carbon fatty acids, but up-regulated by long chain fatty acids. Expression of these genes was normalized to their expression under conditions without fatty acids (H2O). CT (H2O), Ace (Acetate), Pro (Propionate), But (Butyrate), (Oleate), Tri (Triolein), WGO (Wheat germ oil). Experiments were repeated twice with triplicates.

3.3.9. *FAR1* and *FAR2* regulate genes important for fatty acid/lipid metabolism

Previous studies showed that *F. graminearum* expresses lipolytic enzymes when grown in lipid containing media (Jenczmionka and Schaefer, 2005; Nguyen et al., 2010). Since *FAR1* and *FAR2* are important for fatty acid metabolism and expression of several lipases correlated with *FAR1* expression, the lipase activity of the supernatant of WT, *Δfar1, Δfar2,* and *Δfar2/Δfar1* strains induced by wheat germ oil was tested. The results indicated that secreted lipase activity at early times of induction of *Δfar1* and *Δfar2/Δfar1* strains was significantly reduced (Figure 18A). Lipase activity of *Δfar1* strain was reduced from 25 to 40% after 2 and 4 hours of induction, and from 8 to 15% in the *Δfar2* strain. Lipase activity of *Δfar2/Δfar1* strain was decreased from 25 to 55% after 2 to 4 hours of induction. Notably, lipase activity of the *Δfar2/Δfar1* strain was still significantly reduced after 6 and 8h of induction. These data indicate that *FAR1* plays a major role in regulation of lipase activity while *FAR2* plays a minor role in this process.

To better understand the genes targeted by the regulation of *FAR1* and *FAR2*, the transcription expression of several lipases, cutinases, and genes involved in peroxisomal oxidation of fatty acids such as *PEX11* and *FOX2* in the WT and the *Δfar1, Δfar2*, and *Δfar2/Δfar1* strains was analyzed by quantitative PCR. Among five tested lipases, transcript expression of *FGL1, LIP1* was significantly decreased in the *Δfar1, Δfar2*, and *Δfar2/Δfar1* strains (Figure 18B). Expression levels of these lipases were more profoundly reduced in the *Δfar1* strain compared to the *Δfar2* strain, indicating that *FAR1* is the major regulator of *FGL1* and *LIP1* expression. *FGL1* and *LIP1* have been shown to constitute the major extracellular lipase activity of the fungus. Taken together with the reduced lipase activity in the *Δfar1, Δfar2*, and *Δfar2/Δfar1* strains, it is shown that *FAR1* and *FAR2* are involved in regulation of transcriptional expression of the major secreted lipases *FGL1 and LIP1*. In contrast, expression of *FGL2 and FGL6* was significantly increased in the *Δfar1, Δfar2*, and *Δfar2/Δfar1* strains. Thus, this increase of expression of *FGL2, FGL6* could compensate the reduced lipase activity in the gene deleted strains at 8h and 24h (Figure 18A and data not shown). The expression of cutinases *CUT1* and *CUT2* was

also affected by deletion of *FAR1* and *FAR2* (Figure 18C). Notably, transcript expression of *CUT2* was severely reduced in the *FAR1* deleted strains; its expression was also reduced in the *FAR2* deleted strains. These data indicate that *FAR1* and *FAR2* also are the regulators of cutinases. Lipids could be used as the sole carbon source for *F. graminearum*. To utilize lipids, the fungus must secrete lipases for hydrolysis to release free fatty acids. Fatty acids could be then taken up and oxidized by certain proteins in peroxisomes or mitochondria. Expression of *PEX11* and *FOX2* was also significantly reduced by deletion of *FAR1* or *FAR2* (Figure 18D). Expression of these genes was more profoundly decreased in the *FAR1* deleted strains (*Δfar1 and Δfar2/Δfar1* strains) compared with the *FAR2* deleted strain (*Δfar2* strain). These results indicate that *FAR1* is the major regulator responsible for lipid hydrolysis and fatty acid oxidation. *FAR2* also plays a minor role in the pathway. Taken together, it is concluded that *FAR1* and *FAR2* globally regulate the expression of genes involved in lipid metabolism from utilization of exogenous lipid sources (lipases, cutinases) to beta-oxidation (*PEX11, FOX2*) of the fatty acids to produce energy. *FAR1* seems to be the major regulator of this pathway.


A
Relative lipase activity (%)
100
80
60
40
20
0
0
2
4
6
8
Time (hours)
B
Fold change
6
5
4
3
2
1
0
WT
Δfar2
Δfar1
Δfar2/Δfar1
FGL1
FGL2
LIP1
FGL6
FGL9
FGL16

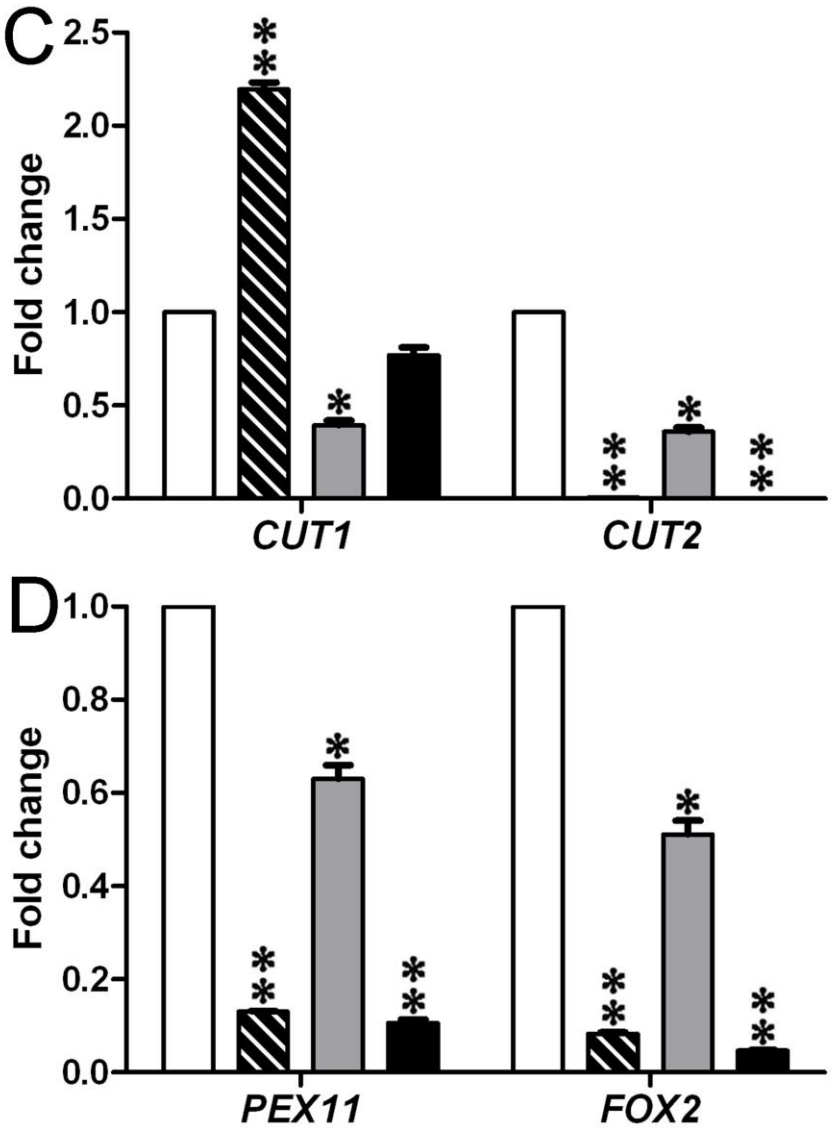


Figure 18: Reduction of extracellular lipolytic activity and transcription expression of different genes involved in lipid hydrolysis and fatty acid oxidation in the *FAR1* and *FAR2* and *FAR2/FAR1* disrupted strains. A) Lipase activity of the lipid-induced culture supernatants of the WT, *Δfar1, Δfar2*, and *Δfar2/Δfar1* strains was evaluated using pNPP as the substrate. Lipase activity of *Δfar1* and *Δfar2/Δfar1* strains was significantly reduced at early induction time. Slight reduction of lipase activity was observed in the *Δfar2* strain. Graphs show the mean of three independent assays with triplicates. B) Transcription level of lipases *FGL1, FGL2, LIP1, FGL6, FGL9, FGL16*. C) Transcription level of cutinases *CUT1, CUT2*. D) Transcription level of peroxisomal genes *PEX11, FOX2*. Expression of these genes was assessed by real-time quantitative PCR. Transcript expression of *FGL1, LIP1, CUT1, PEX11, FOX2* in the *Δfar1, Δfar2*, and *Δfar2/Δfar1* strains was normalized to its expression in the wild type. Experiments were repeated twice with triplicates. ∗P< 0.01; ∗∗P< 0.001.

3.3.10. Reduced virulence of *Δfar1, Δfar2*, and *Δfar2/Δfar1* strains on wheat

F. graminearum secretes lipases as a virulence factor to infect wheat. *FAR1* and *FAR2* were revealed to regulate the expression of these virulence genes *in vitro*. Thus, the infection of the *Δfar1, Δfar2*, and *Δfar2/Δfar1* strains on wheat heads was tested. Three wheat cultivars were used for the infection. As shown in figure 19, infections of *FAR1* and *FAR2* deleted strains or the double deletion strain were reduced in the wheat heads. The infection of these mutant strains significantly varied between the cultivars. In Melissos, the infection of the mutant strains was strongly reduced and the spreading of the inoculum was more often found in the upper spikelets of the infected spikes (Figure 19A). Infection of the *Δfar2/Δfar1* strain was restricted to the inoculated sites of the spikes. However, the infection of these mutants sometimes spreads to other parts of inoculated wheat heads. To give a better assessment of the infection of these strains, the number of infected spikelets and non-infected ones was counted. As shown in the figure 19B, the number of spikelets infected by the deletion strains was significantly reduced compared to the number infected by WT. In Nandu and Florida, infection of the *FAR1* or *FAR2* deleted strains was not significantly reduced (Figure 19C, E). These data were confirmed by counting the number of infected spikelets (Figure 19 D, F). However, the results showed that the infection of *FAR1* and *FAR2* double deletion strains (*Δfar2/Δfar1* strain) was significantly decreased in these two wheat cultivars. The infected wheat spikelets were reduced by about 50% after three weeks of infection. The infection of the *Δfar1, Δfar2*, and *Δfar2/Δfar1* strains on maize cobs was also tested. However, no reduced infection was seen by the mutant strains (Figure 20). Taken together, it is concluded that *FAR1* and *FAR2* differentially regulate the infection of *F. graminearum* on wheat and the reduction of virulence of the mutants varied between different cultivars.

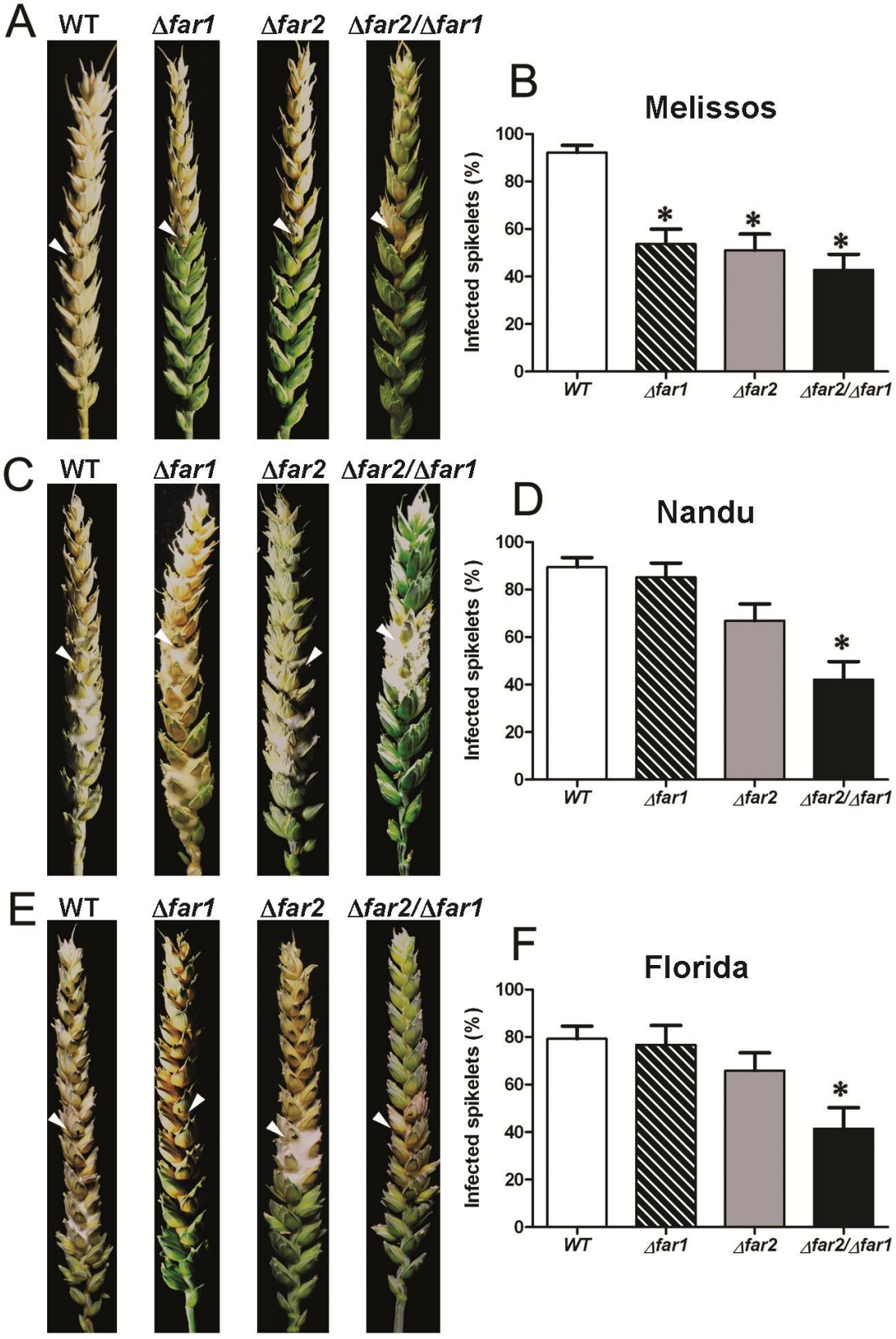

A
WT
Δfar1
Δfar2
Δfar2/Δfar1
B
Melissos
Infected spikelets (%)
100
80
60
40
20
0
*
*
*
WT
Δfar1
Δfar2
Δfar2/Δfar1
C
WT
Δfar1
Δfar2
Δfar2/Δfar1
D
Nandu
Infected spikelets (%)
100
80
60
40
20
0
*
WT
Δfar1
Δfar2
Δfar2/Δfar1
E
WT
Δfar1
Δfar2
Δfar2/Δfar1
F
Florida
Infected spikelets (%)
100
80
60
40
20
0
*
WT
Δfar1
Δfar2
Δfar2/Δfar1

Figure 19: Reduced infection of *FAR1* and *FAR2* deletion strains on wheat heads. A) Representation of wheat heads (Melissos) infected with the wild type (WT), *Δfar1, Δfar2*, or *Δfar2/Δfar1* strains. B) Number of infected spikelets (expressed by %) harvested from infection of wheat cv Melissos with these strains. C) Representation of wheat heads (nandu) infected with the WT, *Δfar1, Δfar2*, or *Δfar2/Δfar1* strains. D) Number of infected spikelets (expressed by %) harvested from infection of wheat heads cv Nandu with these strains. E) Representation of wheat heads (Florida) infected with the WT, *Δfar1, Δfar2*, or *Δfar2/Δfar1* strains. F) Number of infected spikelets (expressed by %) harvested from infection of wheat heads cv Florida with these strains. All wheat heads were point-inoculated in the two central spikelets with 400 conidia of the wild type (WT), *Δfar1, Δfar2*, or *Δfar2/Δfar1* strains. Infection assay was terminated after 21 days. Experiments were repeated 10 times with three spikes per strain. Pictures were captured by a digital camera (Nicon). Arrow heads point to the inoculated sites. ∗P< 0.001.

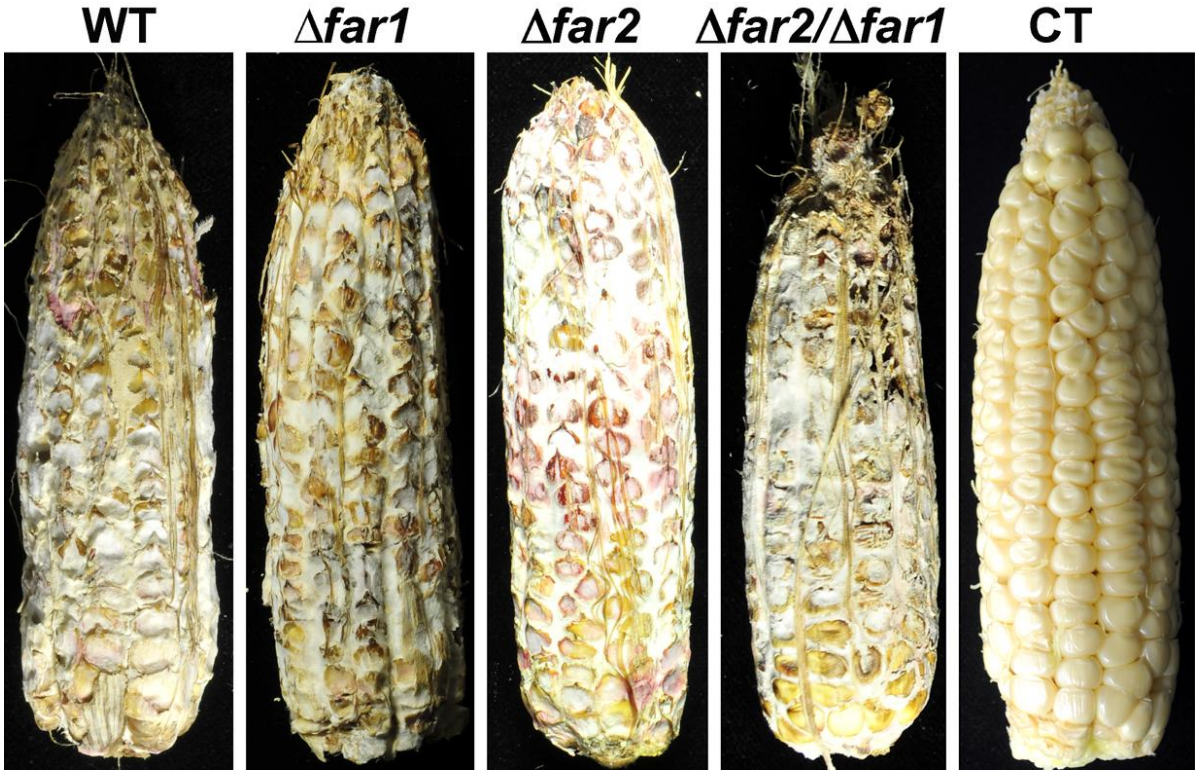
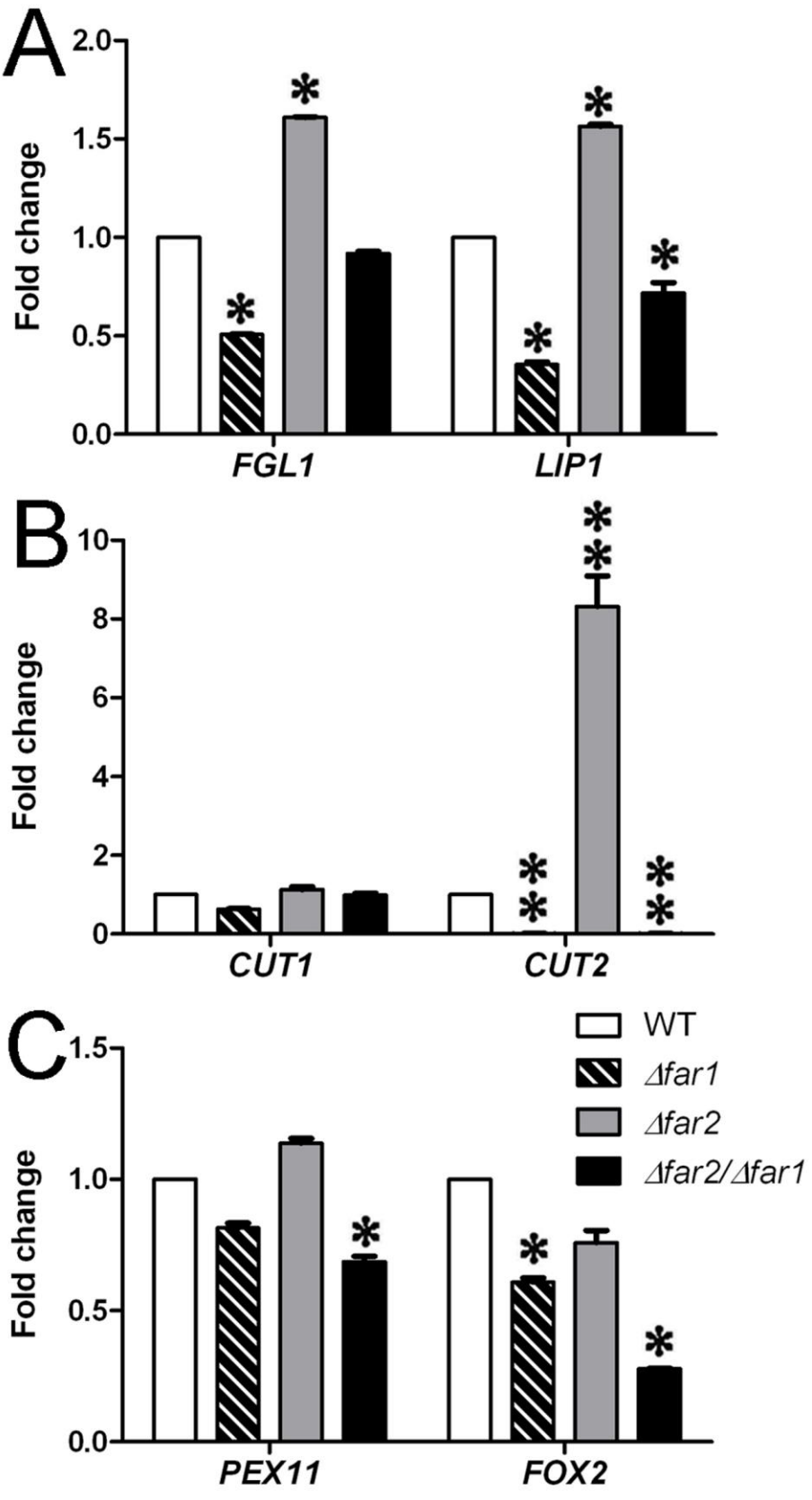


Figure 20: Infection of maize ears with *F. graminearum* wild-type, Δ*far1,* Δ*far2* and Δ*far2*/Δ*far1* strains. Inoculation of ears was performed by injecting 4 ml of conidial suspension (4 x 10^4 conidia x ml^{-1}) of wild-type (WT), Δ*far1,* Δ*far2* and Δ*far2*/Δ*far1* strains into the silk channel. As a control (CT), 4 ml of water were injected. Figure shows cobs 5 weeks post infection. Pathogenicity tests were repeated five-times for each strain. All mutant strains exhibited a similar infection as wild type strain.

3.3.11. Reduced expression of virulence factors in *FAR1* and *FAR2* deleted strains *in planta*

To better understand the mechanism of reduced virulence in the *FAR1* and *FAR2* deleted strains, the expression of the lipases, cutinases, and genes responsible for beta oxidation as well as the mycotoxin production *in planta* and *in vitro* was assessed. It was found that the transcription expression of *FGL1* and *LIP1* was decreased by 50% in the *FAR1* deletion strain compared with that of WT strain (Figure 21A). The data was found consistent with *in vitro* assays. Unexpectedly, *FGL1* and *LIP1* expression levels were increased by 50% in *FAR2* deletion strain and unchanged in the *FAR2/FAR1* deletion strain compared with those of WT. It was also found that the expression of *CUT2* gene in the *FAR1* and the *FAR2/FAR1* deletion strains was reduced (Figure 21B). Yet, the expression of *CUT2* was also up-regulated in the *FAR2* deletion strain. Expression of the peroxisomal genes *PEX11* and *FOX2* was also down regulated in the *FAR1* and *FAR2/FAR1* deletion strains (Figure 21C). Thus, the down-regulation of lipases, cutinases, and peroxisomal genes could be the major factors responsible for the reduced virulence of the *FAR1* deletion strain (*Δfar1* and *Δfar2/Δfar1* strains), but not of the *Δfar2* strain. The levels of DON produced from the strains during induction with ammonium sulphate (Figure 21D) (Ilgen et al., 2009), extracted fraction of wheat protein enriched with gliadin (Figure 21E), or with wheat spikes (Figure 21F) (Voigt et al., 2007) were measured. Interestingly, it was found that DON levels were significantly reduced in *FAR2* and *FAR2/FAR1* deletion strains in ammonium sulphate and extracted fraction of wheat protein enriched with gliadin induction conditions (Figure 21D, E). DON production is an important factor contributing to the virulence of the fungus in wheat. The DON level of *FAR2* strain was not reduced when inoculated on harvested wheat spikes or wheat kernels. The DON level of the *FAR1* deletion strain was not reduced under all examined conditions (Figure 21). The data indicated that the reduced virulence of the *FAR1* deletion strain might be due to the reduced expression of several secreted enzymes and enzymes in the beta-oxidation process. The reduced infection of the *FAR2* deletion strain might instead be related to mycotoxin production.


A
Fold change
2.0
1.5
1.0
0.5
0.0
FGL1
LIP1
B
Fold change
10
8
6
4
2
0
CUT1
CUT2
C
Fold change
1.5
1.0
0.5
0.0
PEX11
FOX2
WT
Δfar1
Δfar2
Δfar2/Δfar1

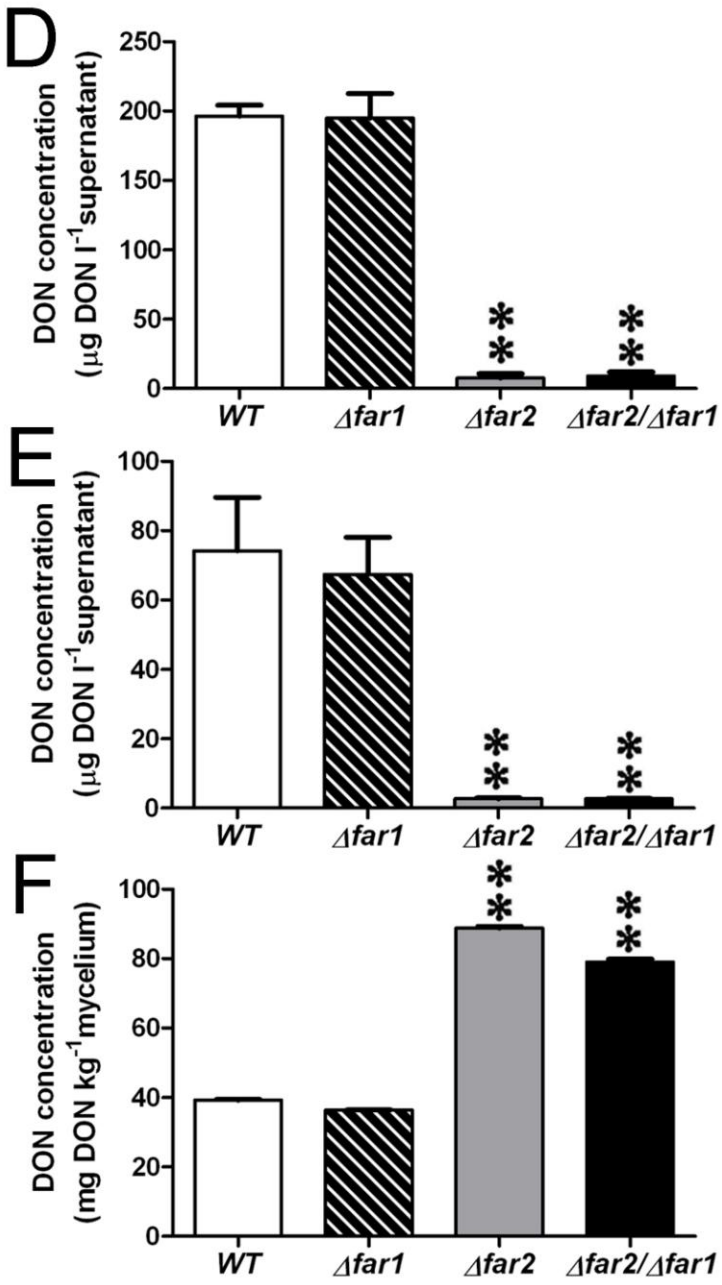


Figure 21: Transcript expression of lipases, cutinases, *FOX2*, and *PEX11* and DON levels in the *FAR1* and *FAR2* deletion strains. Transcription expression level of *FGL1, LIP1* (A), *CUT1, CUT2* (B), *PEX11*, and *FOX2* (C) was assessed using real-time quantitative PCR. DON levels of Δ*far1,* Δ*far2*, and Δ*far2/*Δ*far1* strains induced by $(NH_4)_2SO_4$ (D), extracted fraction of wheat protein enriched with gliadin (E), and wheat spikes (F). DON levels induced with 5mM $(NH_4)_2SO_4$ or 2 mg/ml extracted fraction of wheat protein enriched with gliadin were measured after 24 hours inoculation. DON levels induced with intact wheat spikes were measured after for 5 days of inoculation. DON levels induced with wheat kernels were measured after 7 and 14 days of inoculation. Levels of DON were calculated per liter culture supernatant (in D and E) or kg of mycelia (in F). ∗P< 0.01; ∗∗P< 0.001.

3.4. Characterization of *LR* genes 1, 2, 3, 4, 5, and 6

3.4.1. Disruption of *LR* genes 1, 2, 3, 4, 5, and 6

To create disruption mutants of *LR1,* the wild type *F. graminearum* PH1 was transformed with the plasmid pGEM-*LR1*, linearized with *Xba*I, using protoplast-mediated transformation, which resulted in more than 50 primary transformants. Twenty four transformants were screened by PCR with primers LR1rtF/R (see 2.3) for the absence of *LR1* gene which showed that 3 transformants had no *LR1* gene (data not shown). The wild type, three knockouts, and 5 ectopic transformants were analyzed by Southern blot, which showed that the *LR1* had been replaced by the HYG cassette in the knockout transformants, while *LR1* was present in the ectopic and the WT strain (Figure 22B). The replacement of the *LR1* gene by the HYG cassette was confirmed by PCR with LR1upF-HyR and yGF-LR1doR primers, which showed positive results in the knockout transformants, but negative results in the wild type strain and one ectopic transformant. One ectopic transformant was positive with yGF-LR1doR primer pairs; that means that the construct was integrated in the downstream fragment but did not delete the *LR1* gene (Figure 22C). These results demonstrate that *LR1* was deleted from the wild type strain. To learn about the function of the gene, the double mutant *FAR1* and *LR1* was generated using *Δlr1* (strain 3) as the background for disruption of *FAR1*. The transformation of the *Δlr1* strain with the plasmid pGEM-*FAR1* was similarly performed by transformation of protoplasts which resulted in 60 transformants. The 6 transformants were analyzed by PCR for the absence of *FAR1*, while it was present in the WT, *Δlr1* and 3 ectopic transformants. A second PCR with LR1rt primers was also performed to confirm the absence of *LR1* in the disrupted strains (*Δfar1*/*Δlr1*) as well as in the ectopic ones (Figure 22D). To confirm that the selective marker was integrated in the *FAR1* locus in the double disrupted strains, PCR with FAR1upF-nptIIR and nptIIF-FAR1doR primer were performed, which both showed positive results in the knockout transformants, while negative results in the wild type strain and one ectopic transformant. One ectopic transformant was positive with FAR1upR-nptIIR primer pairs, that means the construct was integrated in the upstream fragment but did not

delete the *FAR1* gene. The deletion vector (VT) was used as a negative PCR control (Figure 22E).

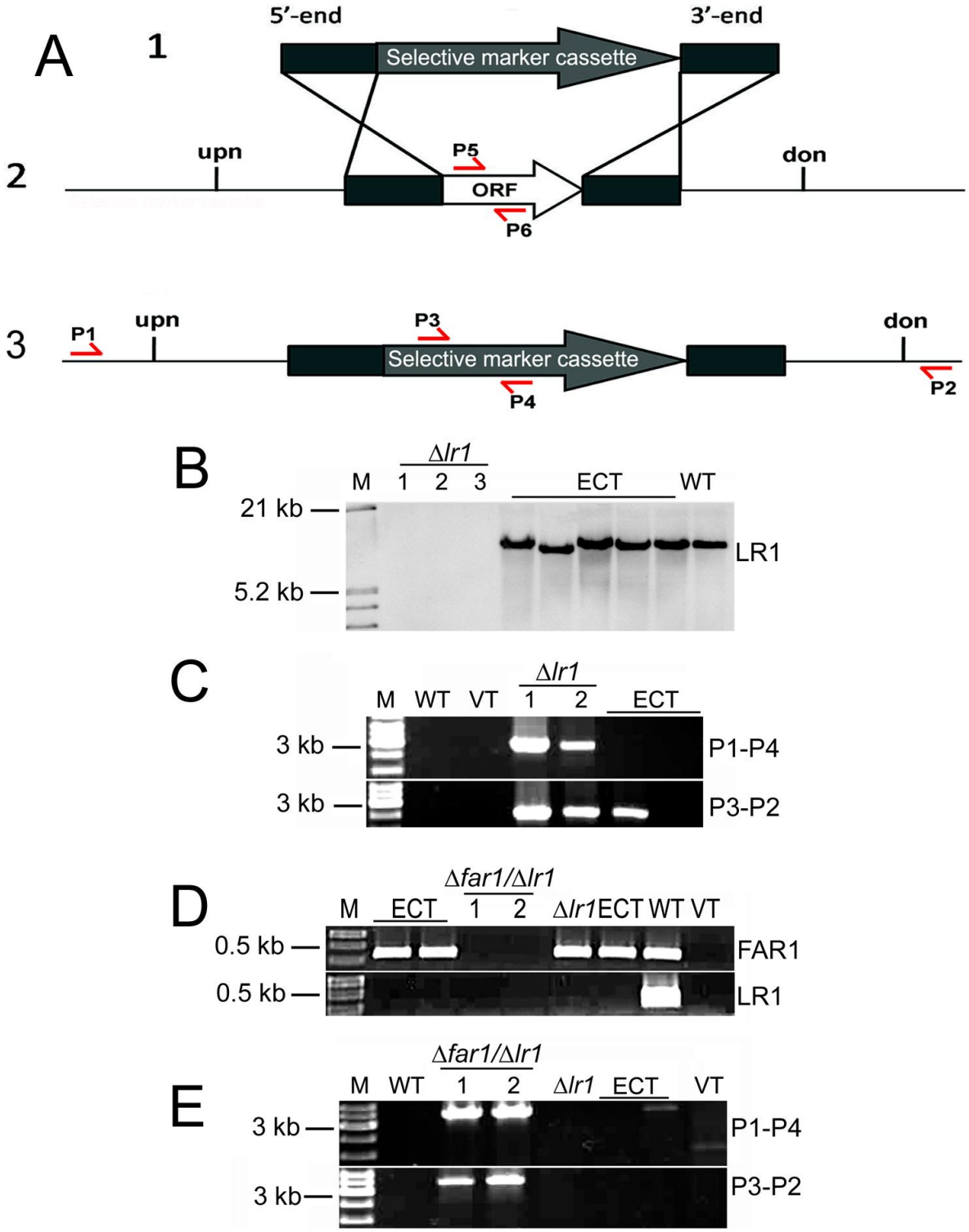


Figure 22: Disruption of *LR1* and *FAR1/LR1* genes in *F. graminearum*. A) Illustration of the gene disruption strategy. A1, representation of disruption constructs; A2, genotype of wild type; A3, genotype of disrupted strains. Selective marker cassette used for *LR1* disruption was Hygromycin; selective marker cassette used for *FAR1* was Geneticin. Upn, up-nested region for integration; don, do-nested region for integration; ORF, open reading frame. B) Southern blot analysis of *LR1* disrupted strains (*Δlr1*), wild type (WT), and ectopic integration (ECT). The hybridization with *LR1* gene specific probe was shown the absence of the gene in the disrupted strains (strain 1, 2, 3), whereas in the WT and ECT the gene was present. C) For PCR analysis of *Δlr1* primers LR1upF-HyR and YgF-LR1doR were used to locate the HYG cassette in the mutant strains, which showed positive results in the knockout transformants, negative ones in the wild type strain and ectopic transformants. D) PCR analysis of *Δfar1*/*Δlr1* double mutants using FAR1rtF/R primers was shown the absence of the gene in the disrupted strains (strain 1, 2), whereas in the WT and ECT the gene was present. A second PCR with LR1rt primers was performed to confirm the absence of *LR1* in the disrupted strains (*Δfar1*/*Δlr1*) as well as in the ectopic strains. E) PCR analysis of *Δfar1*/*Δlr1* was used FAR1upF-nptIIR and nptIIF-FAR1doR primers to locate NPTII (Geneticin) cassette in the mutant strains, which showed positive results in the knockout transformants, while it was negative in the wild type strain and ectopic transformants. The disruption vector (VT) was used for negative PCR for control. M: dig-labeled marker VII (Roche) and 1kb DNA ladder (Fermantas).

A similar strategy was used for the disruption of the other *LR* genes. After rapid PCR screening, nine *LR2* transformants were chosen and their genomic DNA was digested by *Kpn*I. A 3641 bp DNA fragment was expected for the wild type and a 2135 bp fragment for disruption mutants. The first hybridization with an *LR2* probe confirmed the gene deletion (strain 3, 8). The Southern blot also showed that *LR2* was not deleted in the ectopic transformants (ECT) (strain 1, 2, 4, 5, 6, 7 and 9) (Figure 23B upper panel). To show that the one copy of the HYG cassette was integrated in the gene locus, the Southern blot was re-hybridized with the HYG probe (Figure 23B lower panel). The results show that the *LR2* gene has been successfully replaced by the HYG cassette.

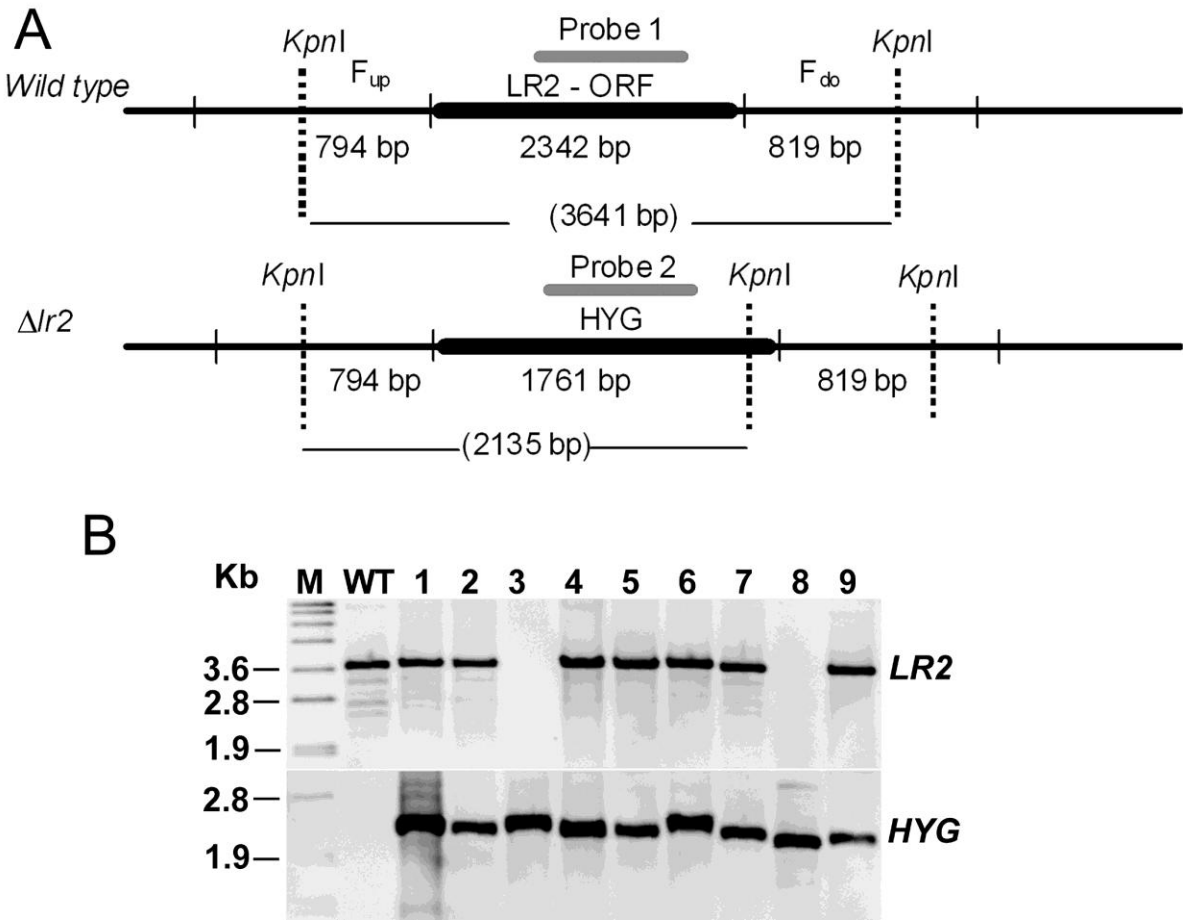


Figure 23: Disruption of *LR2* in *F. graminearum*. A) Illustration of the gene disruption strategy A. The selective marker cassette used for *LR2* disruption was Hygromycin (HYG); open reading frame (ORF); upstream flanking fragment (F_{up}); downstream flanking fragment (F_{do}). B) Southern blot analysis of *LR2* disruption strains (*Δlr2). Kpn*I was used to digest genomic DNA samples isolated from the wild type and putative *LR2* gene disruption (*Δlr2*) mutants as well as ectopic strains (ECT). The first hybridization with *LR2* gene specific probe (probe 1) was used to detect the native *LR2* gene. The second hybridization used a HYG-specific probe (probe 2) to locate the HYG cassette in the mutant strains. Probe 1 was synthesized by PCR with the use of primers LR2rtF/R and wild type DNA as templates. Probe 2 was synthesized by PCR with the use of primers HYGrtF/R and pGEM-HYG as template. Kb and bp: kilo base pair and base pair. Line 3 and 5: *LR2* disrupted mutants *(Δlr2);* 1, 2, 4-7 and 9: ectopic strains (ECT). WT*:* wild type, M: dig-labeled marker VII (Roche).

Four genomic DNA samples of *LR3* transformants and wild type were digested by *Hin*dIII. A 5042 bp DNA fragment was expected for the wild type and a 4129 bp fragment for disruption mutants. The hybridization with the *LR3* probe confirmed the gene deletion (strain 2). The Southern blot also showed that *LR3* was not deleted in

the ectopic transformants (ECT) (strain 1, 3, and 4) (Figure 24B upper panel). To show that the HYG cassette was present in the genome, PCR with HYGrtF/R primer pairs was performed (Figure 24B lower panel). The results show that *LR3* has been successfully replaced by HYG cassette.

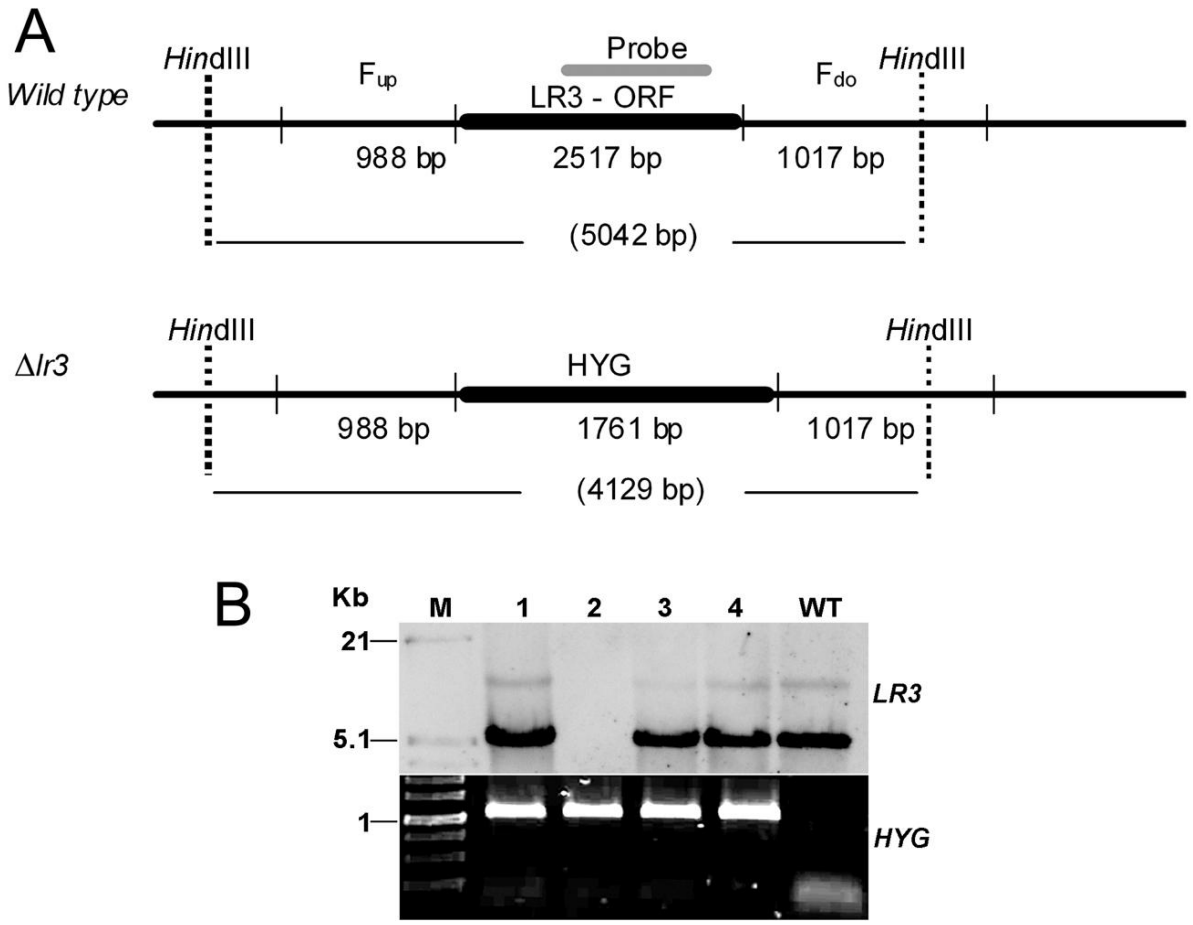


Figure 24: Disruption of *LR3* in *F. graminearum*. A) Illustration of the gene disruption strategy. The selective marker cassette used for *LR3* disruption was Hygromycin (HYG); open reading frame (ORF); upstream flanking fragment (F_{up}); downstream flanking fragment (F_{do}). B) Southern blot analysis of *LR3* disruption strains (*Δlr3). Hin*dIII was used to digest genomic DNA samples isolated from the wild type and putative *LR3* gene disruption (*Δlr3*) mutants as well as ectopic strains (ECT). The first hybridization with a *LR3* gene specific probe was used to detect the native *LR3* gene. The probe was synthesized by PCR with the use of primers LR3rtF/R and wild type DNA as template. The second PCRs used HYG-specific internal primer as described in the list of primers 2.3 to confirm the presence of the HYG cassette in the mutant strains. Kb and bp: kilo base pair and base pair. Line 2: *LR3* disruption mutants *(Δlr3);* 1, 3 and 4: ectopic strains (ECT). WT*:* wild type, M: dig-labeled marker III (Roche) and 1kb DNA ladder (Fermentas).

Seven genomic DNA samples of *LR4* transformants and the wild type were digested by *Xba*I. A 11585 bp DNA fragment was expected for the wild type and a 11199 bp fragment for the disruption mutants. The first hybridization with a *LR4* specific probe was confirmed the gene deletion (strain 2, 3, 4, and 6). The Southern blot also showed that *LR4* was not deleted in the ectopic transformants (ECT) (strain 1, 5, and 7) (Figure 25B upper panel). To show that one copy of the HYG cassette was integrated in the gene locus, the Southern blot was re-hybridized with the HYG probe (Figure 25B lower panel). The results show that the *LR4* gene has been successfully replaced by the HYG cassette. Since the band of HYG cassette was bigger than expected, it was probably integrated several times.

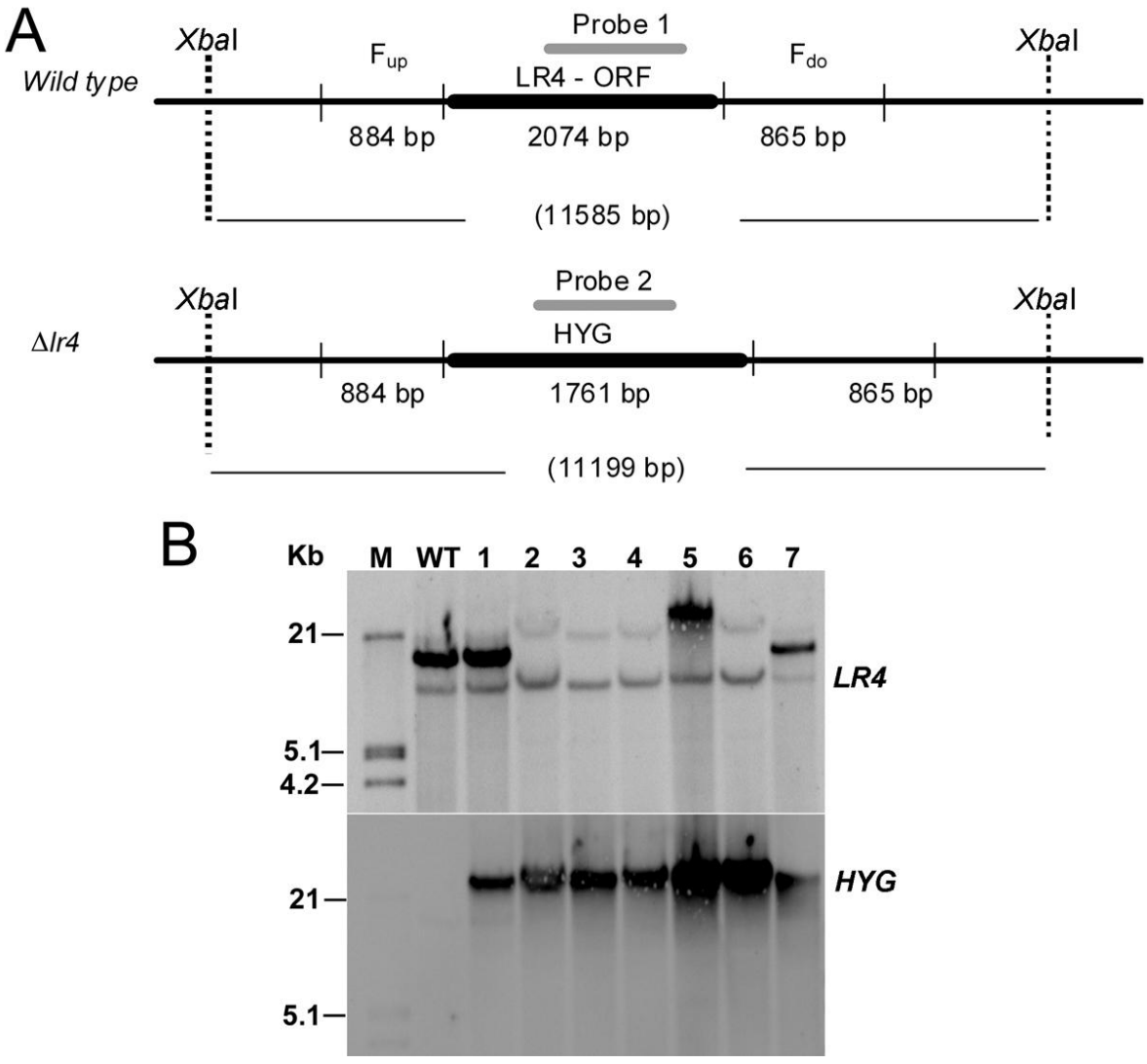


Figure 25: Disruption of *LR4* in *F. graminearum*. A) Illustration of the gene disruption strategy A. The selective marker cassette used for *LR4* disruption was Hygromycin (HYG); open reading frame (ORF); upstream flanking fragment (F_{up}); downstream flanking fragment (F_{do}). B) Southern blot analysis of *LR4* disruption strains (*Δlr4). Xba*I was used to digest genomic DNA samples isolated from the wild type and putative *LR4* gene disruption (*Δlr4*) mutants as well as ectopic strains (ECT). The first hybridization with a *LR4* gene specific probe (probe 1) was used to detect the native *LR4* gene. The second hybridization used a HYG-specific probe (probe 2) to locate the HYG cassette in the mutant strains. Probe 1 was synthesized by PCR with the use of primers LR4rtF/R and wild type DNA as template. Probe 2 was synthesized by PCR with the use of primers HYGF/R and pGEM-HYG as template. Kb and bp: kilo base pair and base pair. Line 2-4 and 6: *LR4* disrupted mutants *(Δlr4);* 1, 5 and 7: ectopic strains (ECT). WT*:* wild type, M: dig-labeled marker III (Roche).

Seven genomic DNA samples of *LR5* transformants and the wild type were digested by *Hin*dIII. A 4124 bp DNA fragment was expected for the wild type and a 3146 bp fragment for disruption mutants. The first hybridization with the *LR5* probe confirmed the gene deletion (strain 2, 5, and 7). The Southern blot also showed that *LR5* was not deleted from the ectopic transformants (ECT) (strain 1, 3, 4, and 6) (Figure 26B upper panel). To show that one copy of the HYG cassette was replaced in the gene locus, the Southern blot was re-hybridized with the HYG probe (Figure 26B lower panel). The results show that *LR5* has been successfully replaced by the HYG cassette.

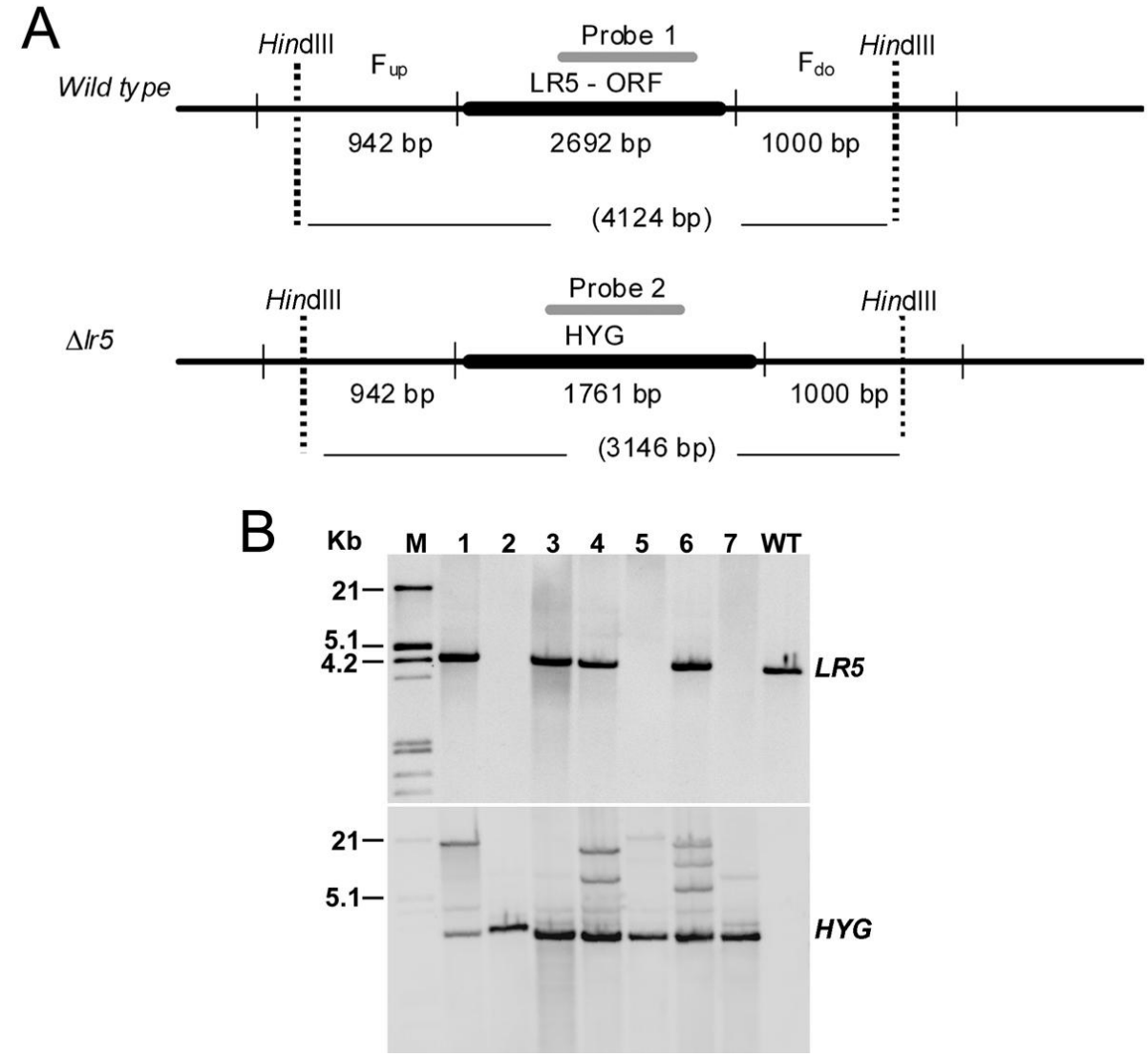


Figure 26: Disruption of *LR5* in *F. graminearum*. A) Illustration of the gene disruption strategy A. The selective marker cassette used for *LR5* disruption was Hygromycin (HYG); open reading frame (ORF); upstream flanking fragment (F_{up}); downstream flanking fragment (F_{do}). B) Southern blot analysis of *LR5* disruption strains (*Δlr5).* *Hin*dIII was used to digest genomic DNA samples isolated from the wild type and putative *LR5* gene disruption (*Δlr5*) mutants as well as ectopic strains (ECT). The first hybridization with a *LR5* gene specific probe (probe 1) was used to detect the native *LR5* gene. The second hybridization used a HYG-specific probe (probe 2) to locate the HYG cassette in the mutant strains. Probe 1 was synthesized by PCR with the use of primers LR5rtF/R and wild type DNA as template. Probe 2 was synthesized by PCR with the use of primers HYGF/R and pGEM-HYG as template. Kb and bp: kilo base pair and base pair. Line 2, 5 and 7: *LR5* disrupted mutants *(Δlr5);* 1, 3, 4 and 6: ectopic strains (ECT). WT*:* wild type, M: dig-labeled marker III (Roche).

PCR was used to confirm of the deletion of *LR6* gene. In strain 1 and 2 the absence of *LR6* was shown using primers LR6rtF/R, while it was still detectable in the WT and 2 ectopic transformants. To confirm that the selective marker was integrated in the *LR6* locus in the disrupted strains, PCR with LR6upF-HyR and yGF-LR6doR primers was performed, which showed positive results in the knockout transformants (strain 1 and 2), while it was negative in the wild type strain and ectopic transformants. The disruption vector (VT) was used as negative PCR control (Figure 27B).

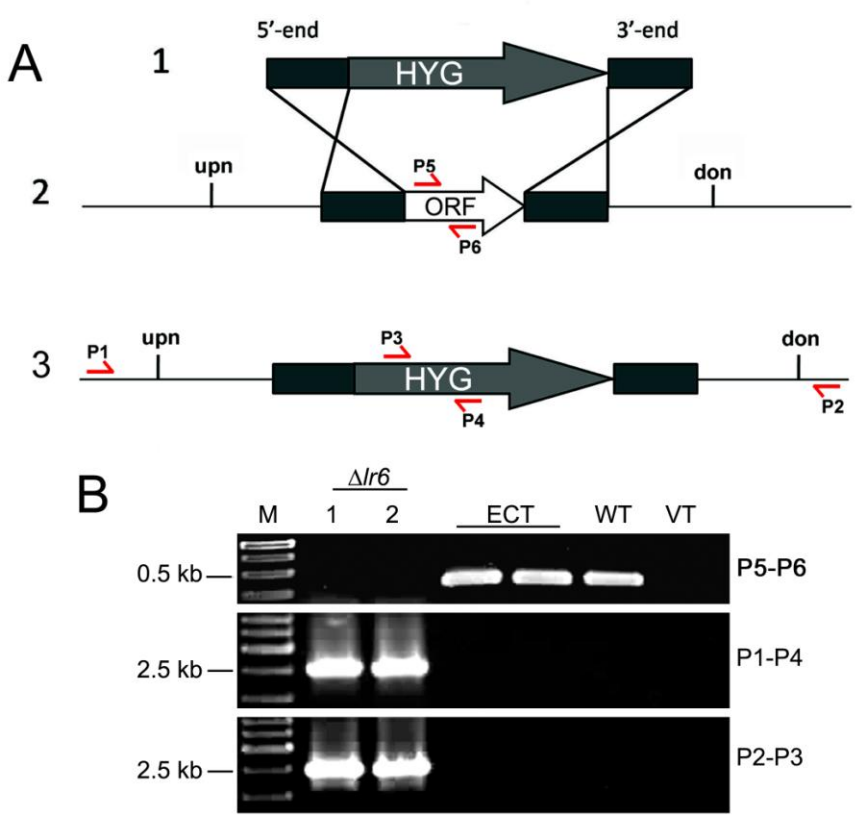


Figure 27: Disruption of *LR6* in *F. graminearum*. A) Illustration of the gene disruption strategy. A1, representation of disruption constructs; A2, genotype of wild type; A3, genotype of disrupted strains. The selective marker cassette used for *LR6* disruption was Hygromycin (HYG); up-nested stream region for integration of construct (upn); do-nested stream region for integration of construct (don); open reading frame (ORF). B) Upper band, PCR analysis of *Δlr6* disruption mutants using LR6rtF/R to show the absence of the gene in the disruption strains (strain 1, 2), whereas in the WT and ECT the gene was present. Middle and lower band, PCR analysis of *Δlr6* was using LR6upF-HyR and yGF-LR6doR primers to locate the HYG cassette in the mutant strains, which showed positive results in the knockout transformants, while negative ones in the wild type strain and ectopic transformants. The disruption vector (VT) was used for negative PCR control. M: 1kb DNA ladder (Fermantas).

3.4.2. Differential expression of *LR* genes *in vitro* and *in planta*

To test expression of the *LR* genes, transcriptional expression of these genes was examined in the wild type strain. Initially, the expression of these genes induced with wheat germ oil (Voigt et al., 2005) was analyzed. Figure 28 shows the semi-quantitative PCR results from expression of the genes. Among the *LR* genes, *LR1* and *LR6* were strongly expressed compared to the other *LR* genes. Nonetheless, the results showed that these *LR* genes were transcribed and differentially expressed in response to wheat germ oil. Then, the expression of these genes on wheat cv Nandu spikes infected with *F. graminearum* at different time points was analyzed. The relative amount of gene expression was also measured using semi-quantitative PCR. In consistence with the *in vitro* analysis, the expression of *LR1* and, *LR6* was detected 3 days post infection (dpi). Expression of these two genes was constant and culminated at 14 dpi. Expression of other *LR* genes was also detected as early as after 3 dpi. However, expression of these genes was retained at very low levels at 10 dpi and significantly increased at 14 dpi with exception of *LR2*. Expression of this gene was not highly elevated at 14 dpi compared to other *LR* genes (Figure 28). These results indicate that *LR* genes were differentially expressed *in vitro* and *in planta*. Expression of *LR1* and *LR6* was significantly higher under both conditions. Genomic DNA was also used as template to prove the presence of *LR* genes in the genome of *F. graminearum* and assure similar primer efficiency.

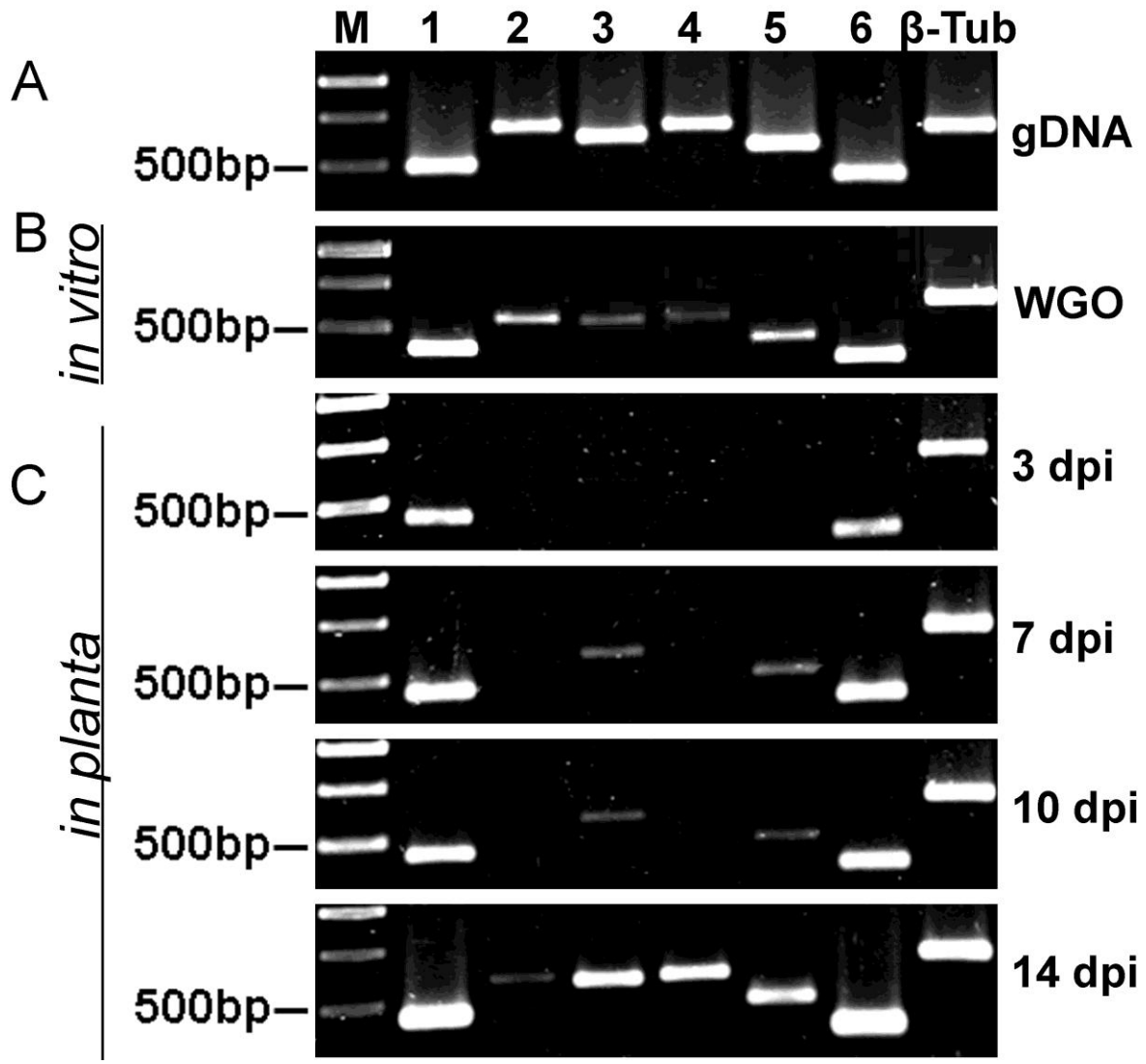


Figure 28: Expression analysis of *LR* genes. A) PCR products of the *LR* genes were specifically amplified from gDNA of *F. graminearum* strain PH1. B) Expression of *LR* genes *in vitro*. Total RNA from wheat germ oil-induced mycelia of wild type *F. graminearum* was extracted after 4 hours. C) Expression of *LR* genes *in planta*. Total RNA was isolated from inoculated wheat spikes. Single stranded cDNA was generated and used as templates in semi-quantitative PCR. Equal amounts of cDNA were used as template and the same elongation cycles were used in the PCR reactions (30 cylces). 10 µl of PCR solution was loaded on 1.2 % (w/v) agarose gel. Dpi: day post infection. β-Tub: housekeeping gene beta-tubulin used as control. M1: 1kb ladder (Fermentas). Line 1-6: *LR1, LR2, LR4, LR5, LR3* and *LR6*.

3.4.3. *LR* family is dispensable for fungal growth

To dissect the role of the *LR* genes, these were deleted using Hygromycin as the selectable marker in the *F. graminearum* PH1 strain. Additionally, the double

deletion of *FAR1* and *LR1* genes was also generated using *FAR1* strain as the background. The deletion of the *LR* genes was confirmed by PCR and Southern blot analysis (Figure 22). These mutant strains, the ectopic strains generated from each transformation (data not shown), and the wild type were used in the phenotype tests.

Previous studies suggested that the Zn2Cys6 family plays a significant role in regulation of genes involved in lipid metabolism. Thus, the growth of the gene disruption strains was examined in complete media (CM) and in minimal media (SNA) supplemented with different oils as the sole carbon sources. As shown in figure 29, the growth of the mutant strain was not reduced in CM media, suggesting that these genes are dispensable for fungal growth in rich media. It was also observed that the growth of these mutant strains in SNA media was similar to WT growth. Additionally, the mutant strains grew normal as the WT in SNA supplemented with oils. These results suggest that these genes are not important for lipid catabolism in the fungus.

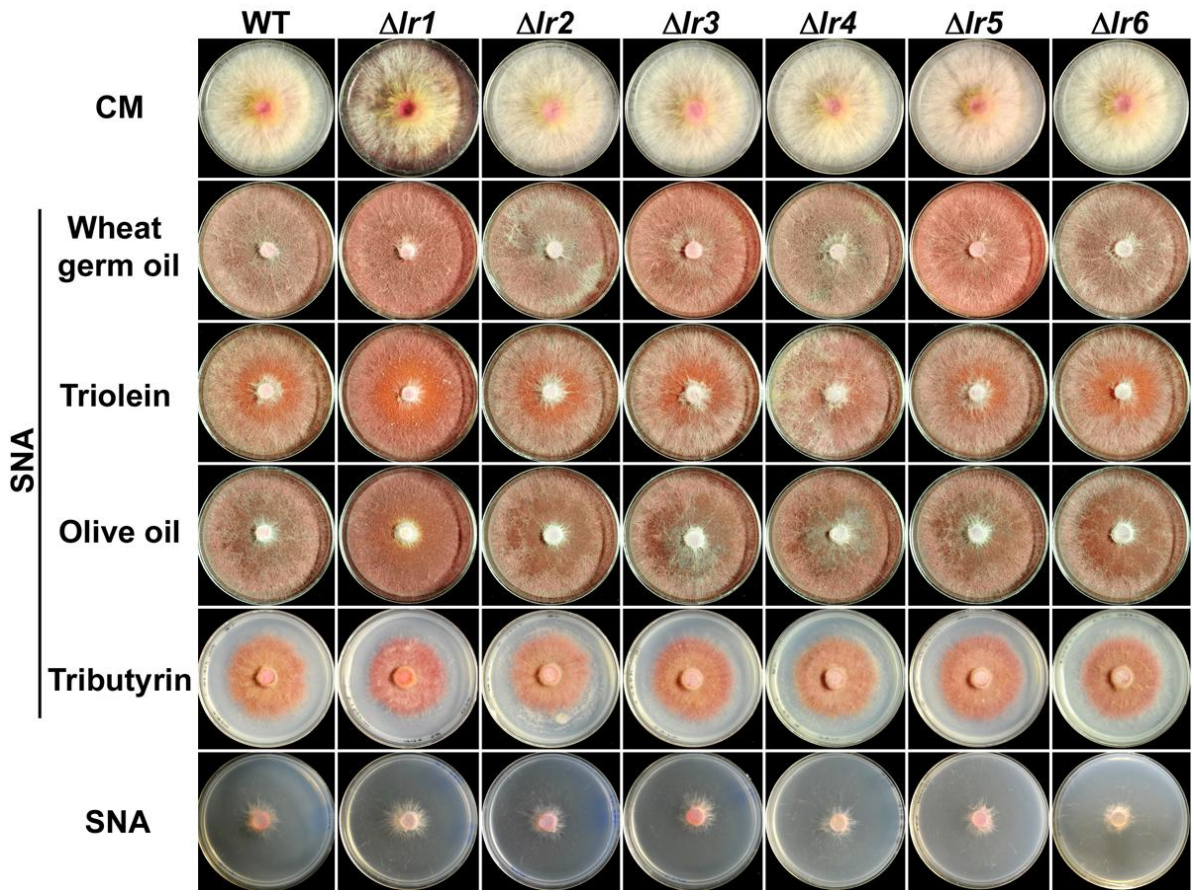


Figure 29: Growth of disruption strains on complete medium (CM) and minimal medium (SNA), SNA supplemented with 1% (v/v) indicated lipids as the sole carbon source. A section of agar with mycelia of the WT and disruption strains was inoculated on the media for 7 days at 28^{o}C. All mutant strain exhibited a normal growth in lipid containing media like the WT strain.

3.4.4. *LR* family is dispensable for fungicide resistance

In order to examine the susceptibility of the mutant strains to antifungal drugs, one piece of mycelium of all *LR* mutants was tested in complete media with the presence of the commonly used fungicide tebuconazole. It was shown that at the concentration of 0.2 µg/ml and 0.4 µg/ml tebuconazole, no mutant strain was different from the WT (Figure 30).

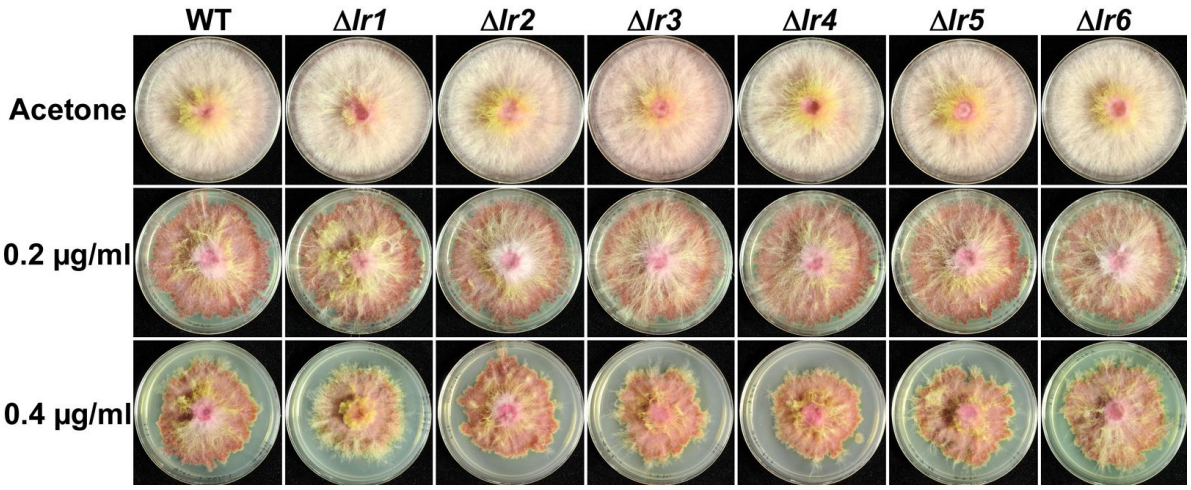


Figure 30: Growth of disruption strains on complete medium supplemented with 0.2 µg/ml and 0.4 µg/ml tebuconazole. A section of agar with mycelia of the WT and disruption strains was inoculated on the media for 5 days at 28 ^{o}C. All mutant strains exhibited a similar growth in tebuconazole containing media like the WT strain.

3.4.5. *LR1* and *FAR1* transcriptionally regulate expression of secreted lipases, cutinases and peroxisomal genes

Next, the lipase activity of the culture supernatants induced with wheat germ oil (WGO) of these mutant strains was examined. It was found that the extracellular lipase activity of the *LR1* deletion (*Δlr1*) and *LR6* deletion (*Δlr6*) strains was reduced compared with that of the WT and other mutant strains. The lipase activity of the *Δlr1* strain was strongly reduced by 74.6 % compared with WT after 8 h induction. Interestingly, the lipase activity of this mutant strain was restored to WT level after 24 h induction (Figure 31).

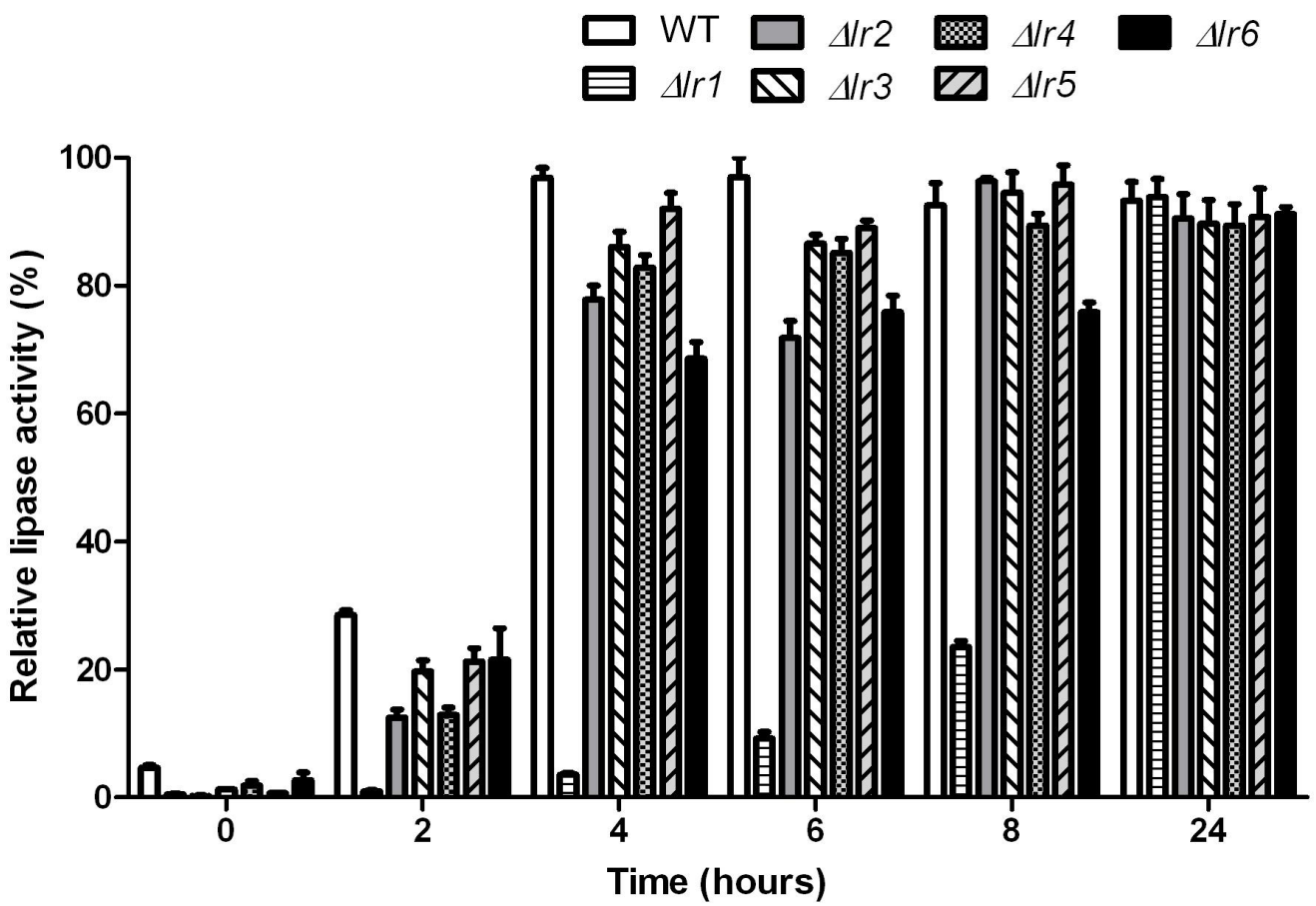


Figure 31: Lipase activity of the lipid induced culture supernatants of the WT, *Δlr1, Δlr2, Δlr3, Δlr4, Δlr5* and *Δlr6* strains was evaluated using pNPP as the substrate. Reduction of lipase activity was observed in the *Δlr1* and *Δlr6* strains. The other mutant strains exhibited a similar lipase activity as the wild-type. Graphs show the mean of two independent assays with triplicates.

Voigt et al. showed that lipolytic activity was strongly reduced in *Δfgl1* at all examined time points (Voigt et al., 2005). Here, it was also indicated that lipolytic activity was strongly reduced in *Δlr1* strain. To test whether *LR1* regulates *FGL1* expression, the level of *FGL1* transcripts in mycelium grown under inducing conditions was determined by RT-PCR with the gene-specific primer pair FGL1rtF-R (see primer table 2.3) (Figure 32A). Northern blot analysis was also used to determine the effect of the *Δlr1* mutation on *FGL1* expression. Total RNA obtained from mycelia of the strains grown either in YPG for 3 days or in YPG for 3 days, then induced for 4 h with WGO was hybridized with the *FGL1* DNA probe (Figure 32B). Transcript level of *FGL1* was clearly reduced in *Δlr1* strain, while unchanged in ECT strains with both methods used in WGO inducing conditions. No signal was detected in YPG medium consistent with no *FGL1* transcript in uninduced medium (Voigt et al., 2005).

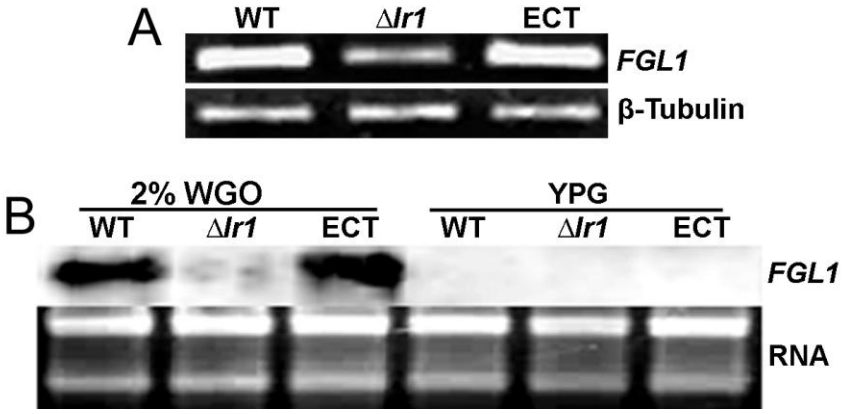
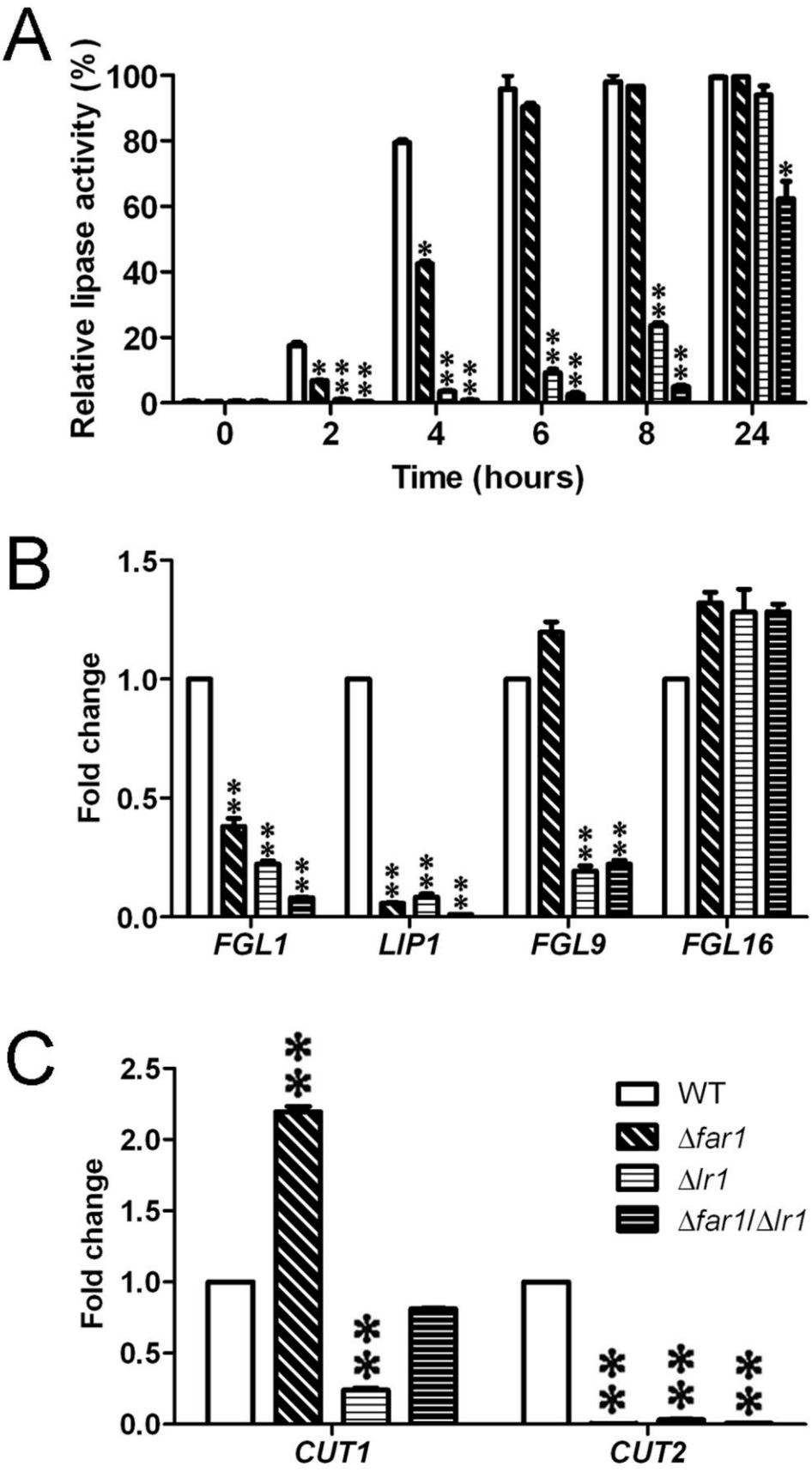


Figure 32: Expression of *FGL1* in wild-type, *Δlr1* and ECT strains. A) Semi-quantitative PCR visualizing the expression of *FGL1* at 4h-WGO induction (24 cycles). B) Northern analysis for *FGL1* transcript accumulation in YPG with 4h-WGO inducing. Upper panel: the filter hybridized with the *FGL1* probe. Lower panel: total RNA.

As mentioned above, it was also shown that deletion of the *FAR1* gene reduced the extracellular lipase activity of the fungus. However, the lipase activity of the *FAR1* deletion strain was reduced after 4 h, suggesting that the total lipase activity of the fungus is regulated by several factors. Thus, the *LR1* and *FAR1* double deletion strain was generated. The data shown that the lipase activity of the *Δfar1/Δlr1* strain

was further reduced compared with either *Δfar1* or *Δlr1* strains, indicating that *FAR1* and *LR1* both contribute to the regulation of lipase expression. The lipase activity of the *Δfar1/Δlr1* strain was reduced by 95 % and 37.4 % after 8 and 24 h induction, respectively (Figure 33A). A previous study showed that the extracellular lipase activity of a lipase deletion mutant strain (*FGL1*) was also significantly reduced after 4 h induction with WGO (Voigt et al., 2005). Thus, the transcriptional expression of this lipase and other lipases in the *Δlr1, Δfar1, and Δfar1/Δlr1* strains was tested at 4 h induction. It was found that the reduced lipase activity of these mutant strains correlates with decreased expression of several lipases such *FGL1 and LIP1* (Figure 33B). Moreover, the transcriptional expression of two putative cutinases was also reduced in *LR1* deletion strain (Figure 33C). Additionally, the expression of *PEX11* and *FOX2* which are important genes for beta-oxidation of fatty acids was tested. However, the expression of these two genes was not significantly reduced in *Δlr1* strains, albeit a slight reduction was observed (Figure 33D). Thus, it was concluded that *LR1* and *FAR1* act in concert to regulate secreted lipases and cutinases which are the major secreted enzymes to hydrolyze lipids at least under the conditions tested. Furthermore, in contrast to *FAR1, LR1* is not important for regulation of genes in the beta-oxidation process.

Virulence of *F. graminearum* is influenced by the production of mycotoxin and secreted lipases. Thus, DON production of *FAR1* and *LR1* deletion strains in culture was measured. The data was shown that deletion of *FAR1* did not alter the DON production, but deletion of *LR1* increased the production of DON (Figure 33E).


A
Relative lipase activity (%)
100
80
60
40
20
0
0
2
4
6
8
24
Time (hours)
B
Fold change
1.5
1.0
0.5
0.0
FGL1
LIP1
FGL9
FGL16
C
Fold change
2.5
2.0
1.5
1.0
0.5
0.0
CUT1
CUT2
WT
Δfar1
Δlr1
Δfar1/Δlr1

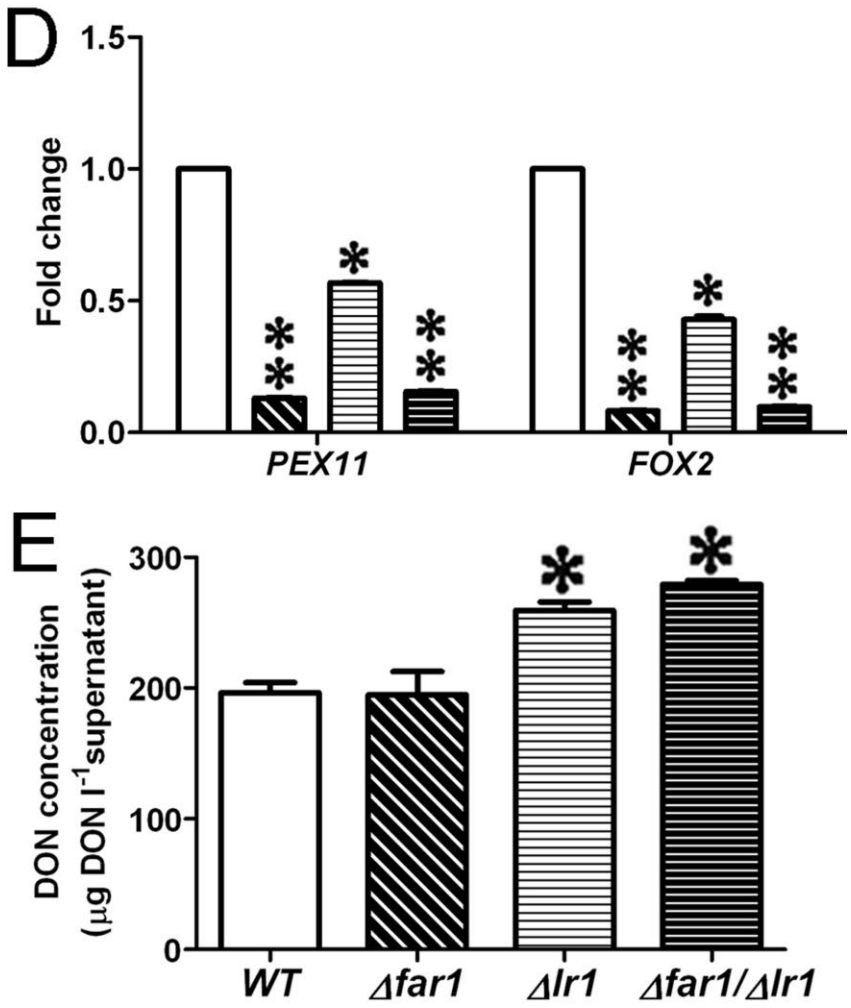


Figure 33: Reduction of extracellular lipolytic activity and transcript expression of different genes involved in lipid hydrolysis and fatty acid oxidation in the *FAR1* and *LR1* or *FAR1/LR1* disruption strains. A) Lipase activity of the lipid induced culture supernatants of the WT, *Δfar1, Δlr1*, and *Δfar1/Δlr1* strains was evaluated using pNPP as the substrate. Lipase activity of *Δfar1* and *Δfar1/Δlr1* strains was significantly reduced at early induction time points. B) Transcription level of lipases *FGL1, LIP1, FGL9, FGL16*. C) Transcription level of cutinases *CUT1, CUT2*. D) Transcription level of peroxisomal genes *PEX11, FOX2*. Expression of these genes was assessed by real-time quantitative PCR. Transcription expression of *FGL1, LIP1, CUT1, PEX11, FOX2* in the *Δfar1, Δlr1*, and *Δfar1/Δlr1* strains was normalized to its expression in the wild type. Experiments were repeated twice with triplicates. ∗P< 0.01; ∗∗P< 0.001. E) DON production of the mutant strains was measured in inducing conditions with 5mM $(NH_4)_2SO_4$ 24 h post inoculation. DON assays were repeated with similar results.

3.4.6. Butyrate inhibits the expression of *LR1* and *FAR1*

As shown above, sodium butyrate (SB) inhibits the expression of *FAR1* and *FAR2*. It was also shown that these transcription factors regulate the expression of extracellular enzymes such as lipases and cutinases. Thus, the inhibitory effect of SB to *LR1* and other secreted lipases of the fungus was tested, and it was found that transcriptional expression of these genes was significantly down-regulated (Figure 34A). Next, extracellular lipase activity in the presence of SB in inducing and non-inducing condition with wheat germ oil (WGO) was examined. The data showed that SB significantly inhibited the extracellular lipase under WGO induced conditions (Figure 34). Deletion of *FGL1, FAR1* or *LR1* did not reduce the expression of lipase after 24 and 48h of induction with WGO. In contrast, the effect of SB inhibition was also observed after 48h under these conditions which indicates that SB exerts a broad spectrum of genes related to expression of secreted lipases. Thus, the effect of SB on inhibition of secreted lipase activity was also tested in several fungi such as *F. oxysporum* and *F. solani* which have been shown to produce extracellular lipase activity. Consistent with results from *F. graminearum* lipase activity inhibition, SB has a strong effect on secreted lipase activity in these two fungi, albeit with less effect on *F. solani* lipase production after 48 h. Nevertheless, these data showed for the first time that the short chain fatty acid butyrate inhibits the production of secreted lipases from different fungi and SB has an inhibitory effect on the expression of *LR1* and *FAR1* which are the regulators of lipase expression.

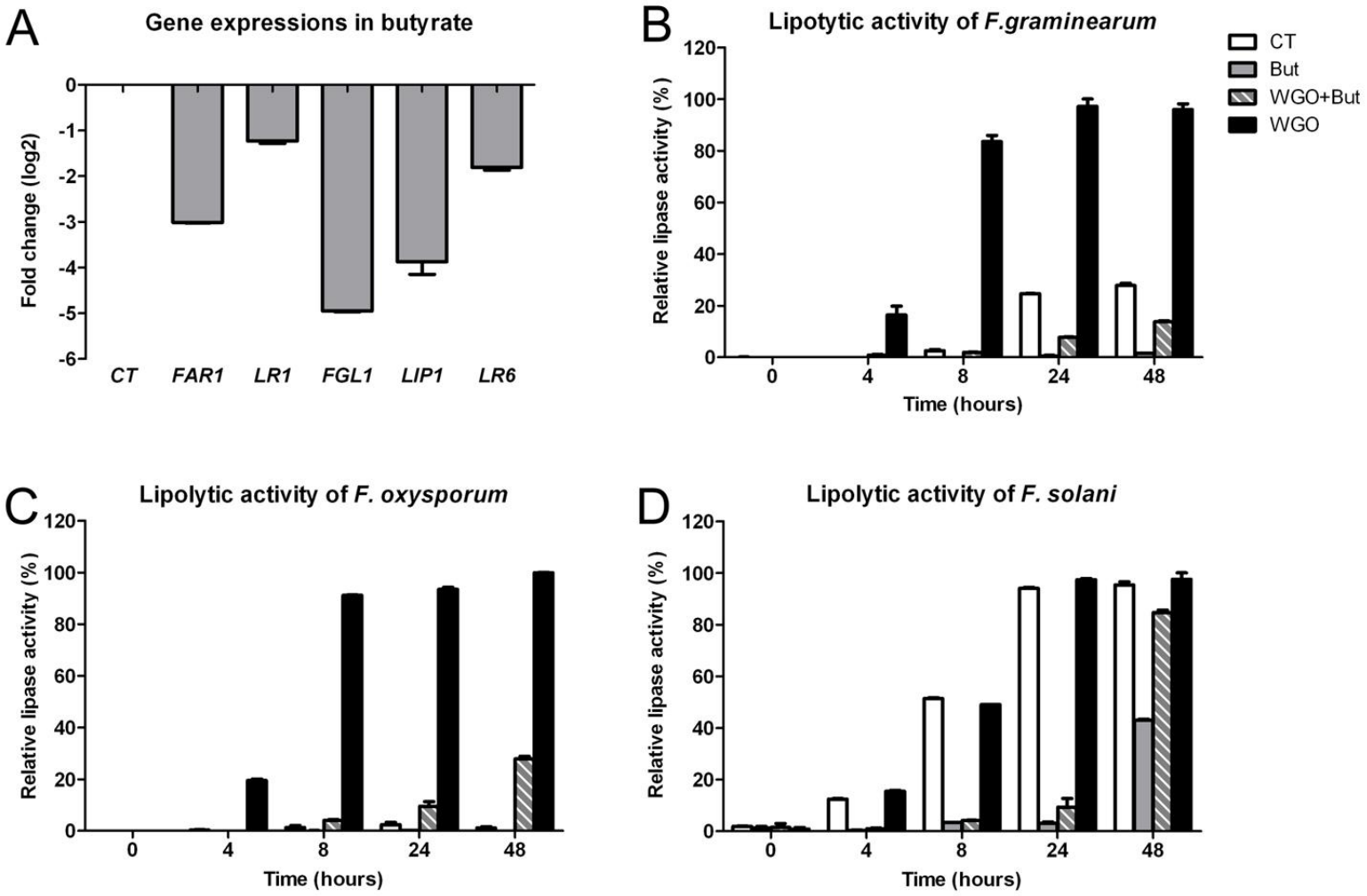


Figure 34: Butyrate inhibits expression of fungal extracellular lipolytic activity. A) SB inhibits the transcriptional expression of *LR1, LR6, FAR1* and lipases. SB inhibits the extracellular lipase activity of *F. graminearum* (B), *F. oxysporum* (C), and *F. solani*. CT: water alone, SB: 50 mM SB, WGO+SB: 2% wheat germ oil and 50 mM SB, and WGO: 2% wheat germ oil. The medium was sterile water. Experiments were repeated twice with triplicates. Error bars indicate standard deviation.

3.4.7. *LR* family is dispensable for virulence on wheat cv Nandu

In the current study, it was observed that deletion of *LR1* did not reduce the fungal virulence on wheat cv Nandu (Figure 35A). Thus, the number of infected per non-infected spikelets of wheat Nandu infected with the *LR1* mutant was counted. However, no difference compared to the wild type and ectopic strains was observed (data not shown). Deletion of both *FAR1* and *LR1* did not alter the virulence of the fungus tested in wheat cv Nandu either. Moreover, a similar virulence of the mutant strains on maize cobs compared to the wild type was also observed (Figure 35B). Thus, it is concluded that *LR1* is dispensable for fungal virulence at least under the tested conditions. Additionally, the virulence of the other *LR* mutant strains on wheat cv Nandu was also examined and no reduced virulence was found (Figure 35C). In summary, the *LR* genes are not essential for fungal virulence on wheat cv Nandu.

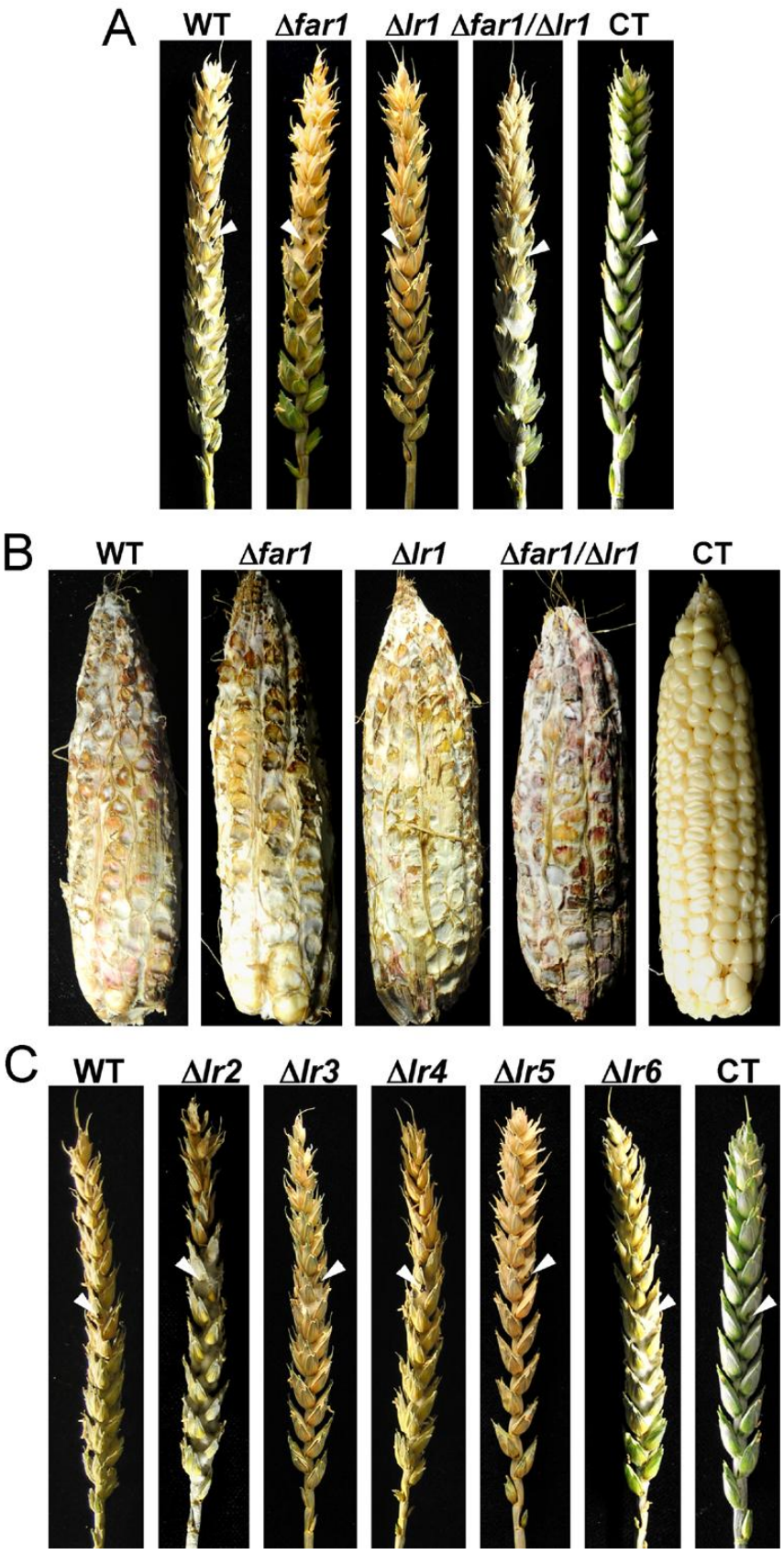

A
WT
Δfar1
Δlr1
Δfar1/Δlr1
CT
B
WT
Δfar1
Δlr1
Δfar1/Δlr1
CT
C
WT
Δlr2
Δlr3
Δlr4
Δlr5
Δlr6
CT

Figure 35: Infection of wheat cv Nandu with *F. graminearum* wild-type PH1, *Δfar1, Δlr1*, and *Δfar1/Δlr1* strains. A) Two central spikelets were each inoculated with 200 wild-type conidia (wt), 200 conidia of each *Δfar1, Δlr1*, and *Δfar1/Δlr1* strains. The figure shows spikes 3 weeks post infection. Pathogenicity tests were repeated fifteen times for every strain. B) Infection of *Δfar1, Δlr1*, and *Δfar1/Δlr1* strains in maize cobs. The infection was monitored after 5 weeks of inoculation by injecting 4 ml of conidial suspension (4×10^4 conidia x ml^{-1}) of wild-type (WT), *Δfar1, Δlr1* and *Δfar1/Δlr1* strains into the silk channel. Maize cob infection was repeated three-times with the same results. C) Infection of wheat Nandu with *LR2-6* deletion strains. Pathogenicity tests were repeated two times with 5 replicates with the same results.

3.4.8. Transcriptional expression of secreted lipases, cutinases, and peroxisomal genes in *LR1* deletion strains *in planta*

The expression of several lipases such as *FGL1, LIP1*, or cutinases such as *CUT1* and *CUT2*, and *PEX11* and *FOX2* was tested *in planta*. It was found that transcriptional expression of these lipases was reduced in the *FAR1* deletion strain and further reduced in *LR1* deletion strains (Figure 36A). Expression of *CUT1* was not significantly altered in the *FAR1* or *LR1* deletion strains, but expression of *CUT2* was markedly reduced in *LR1* deletion strains (Figure 36B). Interestingly, it was found that expression of *PEX11* and *FOX2* was slightly reduced in *FAR1* deletion strains, but significantly increased in *LR1* deletion strains. Thus, deletion of both *FAR1* and *LR1* led to unchanged expression of these two peroxisomal genes (*PEX11* and *FOX2*) compared to the wild type levels (Figure 36C).

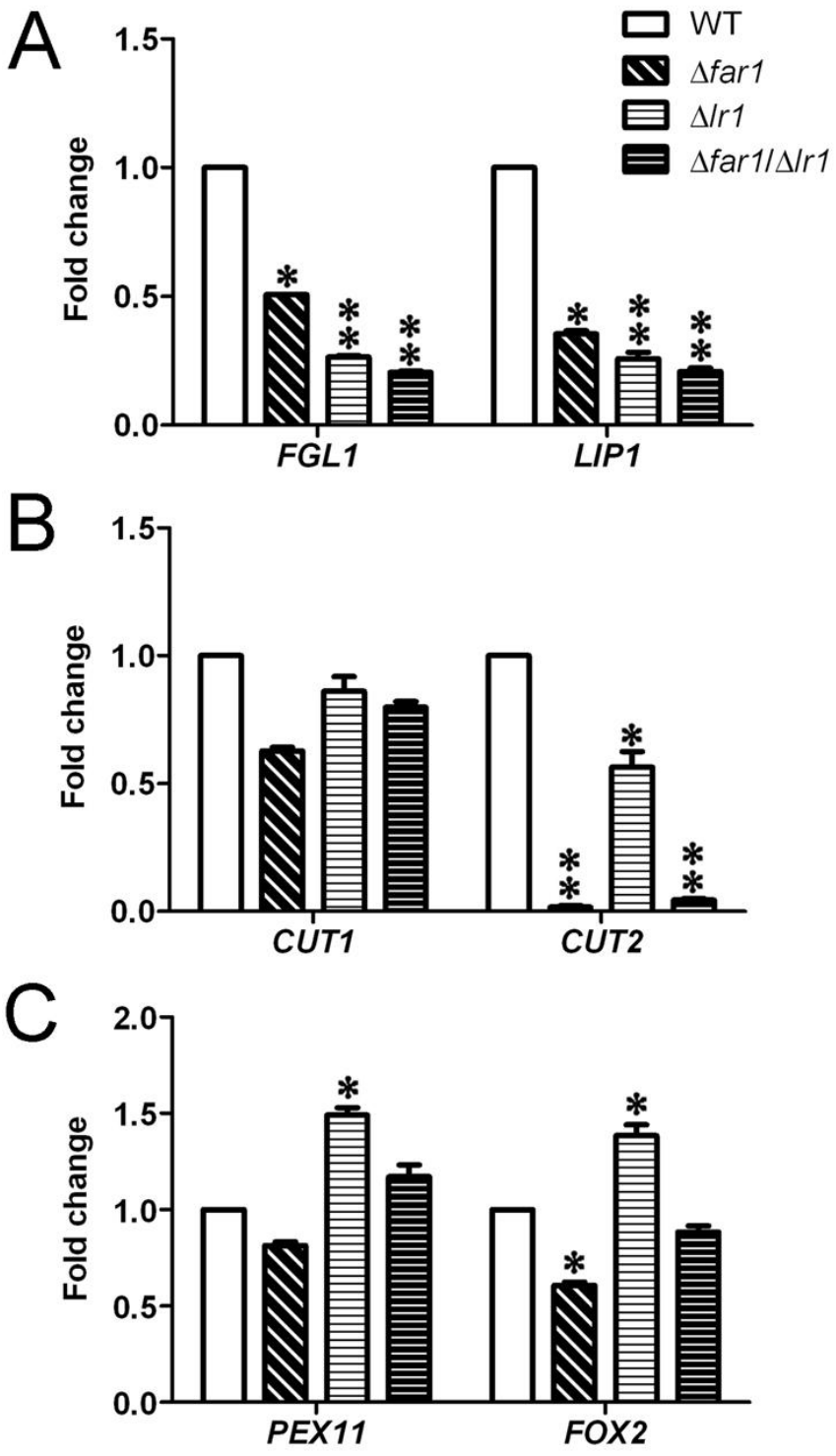


Figure 36: Transcript expression of lipases, cutinases, *FOX2*, and *PEX11* in the *FAR1* and *LR1* deletion strains *in planta*. Transcriptional expression of lipases *FGL1* and *LIP1* (A), cutinases *CUT1* and *CUT2* (B), and *PEX11* and *FOX2* (C) 5 days post infection. Experiments were repeated twice with triplicates. ∗P<0.01; ∗∗P<0.001.

4. Discussion

It has been postulated that phytopathogenic fungi produce an array of extracellular hydrolytic enzymes that facilitate the penetration and infection of the host tissue. These fungal enzymes are collectively called cell wall degrading enzymes (CWDEs). They may contribute to pathogenesis by degrading wax, cuticles and cell walls, thus aiding tissue invasion and pathogen dissemination. Furthermore, they can act as elicitors of host defence reactions and may also play a nutritional role during certain stages of the fungal life cycle (Feng et al., 2005; Kikot et al., 2009). Evidences have been shown that lipid degrading enzymes of *F. graminearum* play an important role during infection, suggesting that lipid or fatty acid acquisition from plant tissues is related to pathogenicity of the fungus. To further dissect the role of the enzymes in this pathway, the aim of this study was to identify and characterize the functions of proteins which regulate this process. In this study, a family of transcription factors of the Zn2Cys6 finger protein type was identified and characterized. Among 8 genes which have a conserved domain of a zinc finger protein, two transcription factors were identified as regulators of fatty acid catabolism and at least one transcription factor involved in regulation of lipid hydrolysis was also identified.

4.1. Fatty acid regulators

Several fungi can use fatty acids as the sole carbon sources. Thus, in these fungi there must be enzymes which function in fatty acid utilization. It has been shown in several pathogenic fungi that the gene regulators of fatty acid utilization are involved in pathogenesis. As several studies revealed the importance of this pathway in pathogenesis of *F. graminearum*, this study aimed at investigating the role of these transcription factors in lipid metabolism and virulence of the fungus. In the current study, the functions of the transcription factor family *FAR1* and *FAR2* in the phytopathogenic fungus *F. graminearum* were characterized. By comparing the annotated protein sequences from different fungi, it was evident that the *FAR1* and *FAR2* gene family are conserved, especially in filamentous fungi. Analysis of the protein sequences showed that the conserved domain CysX2CysX6CysX5–

12CysX2CysX6–8Cys, typical for zinc finger proteins, is present in the *FAR1* and *FAR2* orthologs. Interestingly, however, it was found that while *FAR1* and *FAR2* are apparently present in many filamentous fungi, only one of its orthologs is present in yeast species (e.g. Ctf1 in *C. albicans* or Oaf1/Pip2 in *S. cerevisae*). No *FAR1* ortholog has been reported in yeast species and apparently this gene has been replaced by the Ctf1 or Oaf1/Pip2. The difference might reflect the differentiation of the gene family responsible for fatty acid and lipid metabolism across fungal species. In yeasts, the well-known Oaf1/Pip2 from *S. cerevisae* or recently described Ctf1 from *C. albicans* have been shown to regulate the lipid metabolism by controlling the expression of genes important to fatty acid oxidation for energy production. In filamentous fungi, the FarA and FarB of the *A. nidulans* were also recently characterized as playing a similar role in fatty acid metabolism regulation. In the current study, it was also evident that *FAR1* and *FAR2* have several conserved functions as found in other fungi such as *A. nidulans* or in the yeasts *C. albicans* and *S. cerevisae*. The evidence of this study proves that *FAR1* and *FAR2* in *F. graminearum* regulate the expression of genes involved in beta-oxidation. It was also demonstrated that these transcription factors regulate the expression of genes necessary for exogenous lipid breakdown, thus establishing the global regulatory functions of the transcription factors from assimilation of exogenous lipid sources to energy production in peroxisomes. More importantly, it was demonstrated that by regulation of fatty acid and lipid metabolism, the transcription factors regulate the pathogenesis of the fungus. Previous studies have described the cutinase transcription factors from *F. solani* (Li and Kolattukudy, 1997; Li et al., 2002). It has recently been shown that this transcription factor family is involved in regulation of fatty acid metabolism by controlling the expression of sets of genes involved in beta oxidation in the peroxisomes or mitochondria (Hynes et al., 2006; Poopanitpan et al., 2010; Ramirez and Lorenz, 2009). Thus, the conserved function of this gene family in several fungi was demonstrated.

Yeast species and filamentous fungi can utilize different fatty acids as the carbon source. This process requires the functions of enzymes in the glyoxylate cycle and enzymes involved in beta oxidation in the peroxisomes. In yeasts, the zinc cluster

proteins Oaf1/Pip2 family have been known to regulate the metabolism of fatty acids. It is found that Oaf1/Pip2 regulates the expression of genes of peroxisomes. Transcriptional regulatory networks of fatty acid metabolism seem to be rewired in yeast species. For example, the yeast *C. albicans* does not contain the Oaf1/Pip2 orthologs, instead it contains a single homolog of *CTF1* as found in filamentous fungi (Ramirez and Lorenz, 2009). In filamentous fungi such as *A. nidulans* and *F. solani*, it has also been found that the Oaf1/Pip2 orthologs, initially known as cutinase transcription factor *CTFs*, regulate the utilization of fatty acids as the sole carbon source. The *A. nidulans* FarA and FarB have been found to regulate the metabolism of fatty acids. It is also known that FarA and FarB play an overlapping function in regulation of very short chain fatty acids (Hynes et al., 2006). The current study has found that *F. graminearum* has both FarA and FarB orthologs which retain the Zinc2Cys6 domain in their sequences. It was shown that the functions of these transcription factors also regulate the metabolism of short chain and long chain fatty acids as described for its orthologs in *A. nidulans*. Initially, evidence showed that *FAR1* and *FAR2* were differentially expressed in response to different carbon sources. Expression of *FAR1* was induced by long chain fatty acids and oils. Short chain fatty acids had a negative impact on expression of both genes, with more effect in expression of *FAR2*. Furthermore, it was evident that *FAR1* and *FAR2* might play a distinct role in regulation of very short chain and long chain fatty acids rather than having an overlapping function as reported for *A. nidulans* FarA and FarB. *F. graminearum FAR1* plays the major role in regulation of long chain fatty acids, while *FAR2* has an impact on the utilization of very short chain fatty acids of the fungus. This finding is supported by the data of a double *FAR1* and *FAR2* mutant in this study, in which the double mutants have overlapping phenotypes from the single deletion mutants. Interestingly, it was found that the *FAR2* deletion strain had a growth defect in acetate, propionate, and butyrate-containing medium as the sole carbon source, indicating the possible function of the gene in regulating the utilization of different carbon sources. However, it was then demonstrated that growth of this mutant was also affected in rich media in the presence of propionate and butyrate, implicating the inhibitory effect of these short chain fatty acids on the

growth of the mutant strain. These short chain fatty acids, especially butyrate, also inhibited the conidia germination of the *FAR2* deletion strain, indicating the important role of *FAR2* in detoxification. Intriguingly, it was also found that the *FAR2* deletion strain was more sensitive to several fungicides. Thus, these data indicate the differential regulative roles of *FAR2* compared to *FAR1* in *F. graminearum*. Nevertheless, it was evident that *FAR2* retains its conserved function by regulation of a set of genes such as lipases, cutinases, *PEX11,* and *FOX2* which are the targets of *FAR1* regulation. These data again suggest that the regulatory networks of fatty acid metabolism have been redefined in fungal species (Lavoie et al., 2009).

Although there are certain functional differences of these transcription factors from yeasts to filamentous fungi, they both function in the regulation of a common set of genes responsible for fatty acid degradation, thereby suggesting the divergent functions of these transcription factors during evolution. Oaf1/Pip2 or CTF1 in yeasts is the positive regulator of peroxisomal genes in response to fatty acids in the growth media. In this respect, the *A. nidulans* FarA and FarB govern the expression of genes involved in shuttling of acetyl-CoA between peroxisomes and mitochondria (*acuJ* and *acuH*). They also regulate expression of the genes encoding both mitochondrial and peroxisomal beta-oxidation enzymes, suggesting the global regulatory functions of these transcription factors to fatty acid or lipid turnover in fungi. In *S. cerevisae*, the zinc cluster proteins Oaf1 and Pip2 have been extensively characterized and shown to mediate the response to oleate by binding as heterodimers to oleate responsive elements (consensus: CGGNNNTNA (N9-12) CCG) found in the promoters of beta-oxidation genes (Karpichev and Small, 1998). However, the binding consensus sequence of FarA or FarB of *A. nidulans* was CCTCGG or the complement CCGAGG which was found upstream of a number of peroxisomal proteins such as *PEX1, PEX3, PEX11*, or the key enzyme in the glyoxylate cycle *ICL1* (isocitrate lyase) (Hynes et al., 2006). The consensus sequence was not found in the peroxisomal genes in *S. cerevisae*. Thus, the functions of these transcription factors (Oaf1/Pip2 vs. FarA/FarB) might be divergent in fungal species. In fact, our sequence analysis data showed that most of filamentous fungi contain the *FAR1* (FarA) and *FAR2* (FarB) orthologs, whereas

yeasts have a single ortholog of *FAR1*. Thus, the *FAR2* may have been lost in the yeast species. It was also evident that, although the sequences of *FAR1* orthologs in several filamentous fungi are highly conserved, the Zn2Cys6 motif is not found in these sequences. Therefore, these *FAR1* orthologs may have lost their conserved function in these fungi and *FAR2* orthologs play similar roles as the Oaf1/Pip2 complex in yeasts. Although in this study the regulatory functions of *F. graminearum FAR1* and *FAR2* to peroxisomal gene expression are conserved as described in *A. nidulans* orthologs, the distinct roles of these transcription factors in regulation of short chain or long chain fatty acid metabolism were evident. It was shown that *FAR1* only regulates the metabolism of long chain fatty acids. Thus, its functions in regulation of short chain fatty acids may have been lost. Consequently, it remains an interesting question whether the function of *FAR1* in other fungi is conserved.

Filamentous fungi such as *F. graminearum* and *F. oxysporum* secrete a repertoire of lipolytic enzymes such as lipases and cutinases for external carbon source utilization from lipids or cuticles, respectively. It is reported that the conserved CCTCGG motif is present in the promoter region of the cutinase genes in *M. grisea* and *F. solani* as well as *A. nidulans,* indicating that these genes are also the target of regulation by the transcription factor *FAR1* and *FAR2* family. It was observed that expression of the transcription factor *FAR1* and its target genes such as *PEX11* and *FOX2* as well as several new targets such lipases are simultaneously up-regulated by induction with long chain fatty acids. These data implicate that these genes are related to each other in the same pathway. Furthermore, data have shown that deletion of *FAR1* or *FAR2* led to a significant reduction of lipolytic activity in culture supernatant. This result was supported by the finding that transcription of several lipases and cutinases is also down-regulated in the *FAR1* and *FAR2* deletion strains. Deletion of both genes led to more profound reduction of gene expression as well as enzymatic activity. Indeed, the consensus sequence CCTCGG is present in lipase and cutinase genes. These data indicate that *FAR1* and *FAR2* also regulate the expression of secreted enzymes which are important for degradation of lipid compounds. In humans, it is known that the transcription factors of the PPAR family are the regulators of fatty acid metabolism by regulation of peroxisomal genes. It is also

known that these transcription factors regulate the expression of adipose triglyceride lipase which is important for lipid hydrolysis (Kershaw et al., 2007). Thus, functions of *FAR1* and *FAR2* in fungi and PPARs in humans might be descendants from a common ancestor (Naar and Thakur, 2009; Phelps et al., 2006).

The importance of lipid metabolism for fungal virulence has been researched in several fungi such as *M. grisea* (Wang et al., 2007), *Cryptococcus neoformans* (Shea et al., 2006), and *C. albicans* (Lorenz and Fink, 2001). *F. graminearum* also deploys secreted lipases as virulence determinants during plant infection (Voigt et al., 2005). It is also evident that lipid metabolism is important for *F. graminearum* colonization and sexual development in wheat (Guenther et al., 2009). As shown in here, deletion of either *FAR1* or *FAR2* mildly attenuated the virulence of the fungus. It was observed that the reduced virulence of the mutant strains was dependent on the wheat host's cultivar and varied among the wheat spikes in the same cultivar (Figure 7). The data indicate that *FAR1* or *FAR2* are indeed involved in, but not crucial for *F. graminearum* virulence. The contribution of both *FAR1* and *FAR2* to virulence of the fungus was demonstrated by the even more reduced virulence in the double deletion strains. These results are in unison with findings by Ramirez and Lorenz, who found that the *C. albicans CTF1* deletion strains was also mildly reduced in virulence (Ramirez and Lorenz, 2009). Thus, these data demonstrated that some genes regulating lipid metabolism are involved in virulence, albeit it in a subtle way. Although the mechanism of reduced virulence regulated by *FAR1* and *FAR2* is not known, the study provided preliminary evidence that these regulators control the expression of other cascades of genes which might be important for virulence such as lipases, cutinases, or genes which are associated with peroxisomal functions such as *PEX11* and *FOX2*. It is also noted that deletion of genes involved in glyoxylate or peroxisomal functions led to reduced virulence which is comparable to the *CTF1* null mutant in *C. albicans*, implicating that indeed *CTF1* regulates *C. albicans* virulence via controlling expression of these genes (Lorenz and Fink, 2001; Ramirez and Lorenz, 2009). The reduced virulence of *FAR2* deletion strains might be related to the reduced expression of genes which are important for mycotoxin production or drug resistance. It is also remarkable that *FAR2* deletion

strains were reduced in utilization of SCFAs, which might implicate a malfunction of mitochondria. In the future, it will be interesting to investigate the mechanism of reduced virulence in *FAR2* deletion strains.

4.2. Lipase regulators

Lipases are an important class of enzymes for application in industrial purposes. This enzyme family is ubiquitously present in fungi. Previously, it has been shown that secreted lipases are important enzymes for virulence of *F. graminearum*. However, little is known about the regulation of these hydrolytic enzymes in this fungus and other fungi. This study identified and revealed the role of six transcription factors of the zinc finger protein family in *F. graminearum*.

Disruption of each transcription factor did not affect the growth of the fungus in complete media or minimal media supplemented with fatty acids or lipids, suggesting that these genes were not involved in the regulation of fungal growth and fatty acid utilization. However, it was found that expression of extracellular lipase activity in the *LR1*, but not other *LR* mutant strains, was strongly reduced. This data correlated with the reduced lipase activity of the *FGL1* mutant strain, suggesting that *LR1* might regulate expression of *FGL1*. Thus, the transcriptional expression of *FGL1* was examined and it was found that its expression was dramatically reduced in the *LR1* mutant strain. Additionally, expression of *FGL9* and *LIP1* in the *LR1* mutant strain was reduced. These data suggest that *LR1* transcriptionally regulates expression of these lipases. The expression of two putatively secreted cutinases in the *LR1* mutant strain was also evaluated. Expression of these cutinases was also significantly reduced. As shown above, *FAR1* and *FAR2* co-ordinately regulate fatty acid utilization. *FAR1* also plays a minor role in regulation of secreted lipase activity. It was also found an essential role of *FAR1* in regulation of peroxisomal genes. These data suggest that *FAR1* and *FAR2* are major regulators of fatty acid catabolism of *F. graminearum*. We also examined the possible regulatory role of *LR1* towards peroxisomal genes, but it seems that *LR1* is not involved in regulation of these genes. To further characterize the role of *FAR1* and *LR1* in lipase expression regulation, the lipase activity in the *FAR1/LR1* double deletion strain was examined

and it was found that deletion of *FAR1* in the *LR1* strain further reduced lipase activity. These data suggest that *FAR1* is also involved in regulation of lipase expression, but with a minor role compared to *LR1*. Previously, Rocha et al. have found that a cutinase transcription factor (Ctf1) was also involved in regulation of secreted lipases and cutinases (Rocha et al., 2008). Here, it was found that *LR1* and Ctf1 share a significant homology sequences and both these transcription factors have a conserved Zn2Cys6 domain. Thus, it is proposed that *Fusarium* strains have a conserved transcription factor which is essential for regulation of secreted lipases and cutinases. The secreted lipases and cutinases in several *Fusarium* strains are important for fungal pathogenesis. Thus, identification of a regulator of these enzymes could help uncover other virulence factors in this pathway. The data presented in this study also revealed the role of other transcription factors called *LR2-LR6*. Although, these transcription factors are functionally redundant in regulation of lipase expression as well as the virulence of the fungus under the test conditions of this study, they might be involved in other regulatory processes.

As the study showed the regulatory role of *LR1* in expression of lipases, the virulence of the *LR1* deletion strain was examined. Unexpectedly, the virulence of the *LR1* strain was not reduced compared to that of WT strain. Double deletion of *FAR1* and *LR1* also did not reduce virulence of the fungus in wheat cv Nandu. This result indicates that *LR1* is dispensable for virulence at least in wheat cv Nandu. As *LR1* is the positive regulator of *FGL1* expression *in vitro*, examination of *FGL1* expression *in planta* and it was found that the transcriptional expression of this lipase was reduced to about 73.5% compared to its expression level in the WT. Expression of *FGL1* in the *FAR1/LR1* double mutant strain was reduced to 79.5%. Nevertheless, the reduced expression level of *FGL1* in these mutant strains was insufficient to decrease the virulence of the fungus. Although previous studies of *FGL1* deletion strains showed reduced virulence, this and another study using a *FGL1* promoter deletion showed that down-regulation of *FGL1* is insufficient to control the virulence of the fungus. Interestingly, it was also observed that deletion of *FGL2* or *FGL5* decreased transcriptional expression of *FGL1* and this reduced expression of *FGL1* correlated with the reduced virulence of *FGL2* or *FGL5* strains.

Thus, there might be multiple levels of regulation of *FGL1*. It would be interesting to investigate the regulation of *FGL1* at post-transcriptional levels in the future.

4.3. Effect of butyrate on expression of lipase and fatty acid regulators

Butyrate is a short chain fatty acid which has been shown to have inhibitory effects on growth and proliferation of various cell types. In mammalian cells, butyrate exerts its effect by inhibition of histone deacetylase which is essential for the removal of the acetyl moiety from histones prior to gene expression. Butyrate was found to inhibit the chitin formation and germination in Candida strains (Hoberg et al., 1983; Smith and Edlind, 2002). However, the mechanism of inhibition of fungal growth by butyrate was not known. It was found that butyrate inhibits the growth of WT *F. graminearum* and deletion of *FAR2* enhanced the susceptibility of the fungus to butyrate. However, no evidence of inhibitory effects of butyrate on histone deacetylase activity in the mutant strain was found. By examination of gene expression of *LR1* and *FAR1* genes, it was found that butyrate represses transcriptional expression of these genes. A reduced expression of lipases in the presence of butyrate was also observed. Thus, it is possible that butyrate inhibits the extracellular lipase production via inhibition of *LR1* and *FAR1* in fungi. It also inhibits *Fusarium* growth and germination. This study also showed that butyrate synergistically exerts its activity with fungicides. Thus, this short chain fatty acid could be used as an antifungal compound.

5. Summary

The importance of secreted lipases of *F. graminearum* was found in previous studies in our lab. However, the mechanism by which the lipases function as virulence factors is not yet known. To better understand the role of lipases, this study focused on the identification of transcription factors which are regulators of lipases expression. In the current study, eight proteins which are putative transcriptional regulators were identified and characterized in the fungus *F. graminearum*. The role of the 8 putative transcription factors in regulation of lipase expression was characterized by gene deletion. Among the putative transcription factors, it was discovered that *FAR1* and *FAR2* are regulators of fatty acid metabolism. It was found that these two transcription factors control fatty acid catabolism by regulating expression of several enzymes, e.g. peroxisomal proteins. Additionally, these transcription factors participate in the regulation of expression of secreted lipases. In this study, an essential lipase regulator named *LR1* was discovered. It was demonstrated that *LR1* regulates expression of several secreted enzymes such as lipases and cutinases.

Moreover, it was found that *FAR1* and *FAR2* participate in virulence mechanisms of the fungus. However, they are not the essential regulators of virulence factors. Interestingly, it was shown that *LR1* regulates expression of *FGL1* and other secreted enzymes *in vitro* and *in planta*. However, deletion of this regulator did not reduce virulence of the fungus. Since deletion of *FGL1* has been shown to reduce the virulence of the fungus, it was concluded that indirect down-regulation of *FGL1* is insufficient to decrease virulence. This underlines the complexity of regulation of virulence factors in *Fusarium*.

Additionally, this study reported that butyrate is a strong inhibitor of secreted lipases, perhaps through inhibition of the Zn2Cys6 proteins in *F. graminearum*. This study provided evidence of a potential application of this short chain fatty acid in antifungal treatment of *Fusarium* diseases.

6. Zusammenfassung

Sekretierte Lipase wurde in vorangegangenen Studien als Virulenzfaktor von *F. graminearum* identifiziert. Allerdings blieb der Funktionsmechanismus der Lipasen als Virulenzfaktoren bisher weitgehend ungeklärt. In dieser Arbeit lag der Fokus auf der Untersuchung von Transkriptionsfaktoren, die die Expression der pilzlichen Lipasen beeinflusen, um die Rolle der Lipasen weiter ausleuchten zu können. Als Lipase regulierende Transkriptionsfaktoren wurden 8 Proteine in *F. graminearum* identifiziert und charakterisiert. Die zugehörigen Gene wurden durch Null-Mutationen untersucht. Dabei wurde herausgefunden, daß die Transkriptionsfaktoren *FAR1* und *FAR2* als Regulatoren des Fettstoffwechsels fungieren. Es konnte festgestellt werden, daß diese beiden Transkriptionsfaktoren den Abbau von Fettsäuren durch die Regulation der Expression verschiedener Enzyme beinflussen z.B. von peroxisomalen Proteinen. Darüber hinaus sind diese Transkriptionsfaktoren an der Regulation der Expression von sekretierten Lipasen beteiligt. Im Laufe dieser Arbeit wurde auch ein entscheidender Lipase regulierender Faktor, *LR1*, identifiziert. Es konnte gezeigt werden, daß *LR1* die Expression verschiedener sekretierter Enzyme wie Lipasen und Cutinasen reguliert.

Außerdem wurden die Transkriptionsfaktoren *FAR1* und *FAR2* als Effektoren im Virulenzmechanismus von *F. graminearum* identifiziert. Allerdings sind diese Faktoren keine essentiellen Regulatoren der Virulenz. *LR1* reguliert die Expression der Lipase *FGL1* in vitro und in planta. Allerdings führte die Null- Mutationen dieses Faktors nicht zu einer Reduktion der Virulenz. Da aber die Deletion von *FGL1* zu einer dramatischen Reduktion der Virulenz führt wie vorher beschrieben, kann vermutet werden, daß die indirekte Herunterregulation der Expression von *FGL1* nicht ausreichend zur Verringerung der pilzlichen Virulenz ist. Diese Ergebnisse unterstreichen die Komplexität der Regulation von Virulenzfaktoren in *F. graminearum*.

Zusätzlich wurde Butyrat, eine kurzkettige Fettsäure, als Inhibitor von sekretierten Lipasen identifiziert, wahrscheinlich verursacht durch die Hemmung von Zn2Cys6

Proteinen in *F. graminearum*. Butyrat könnte demnach eine Anwendung als Fungizid im Einsatz gegen Fusariosen finden.

7. References

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Acknowledgments

I am deeply grateful to my supervisor **Prof. Dr. Wilhelm Schäfer** for the wonderful opportunity of participating in this study his laboratory with an exciting project, and for his scientific guidance and support.

I am thankful to Prof. Dr. **Christian Axel Voigt** for being the second reviewer of this study.

I would like to thank **Prof. Dr. Michael St. John** for his linguistic assessment of this writing.

Many thanks go to **Cornelia Stärkel** for proofreading my dissertation and her encouragement during my stay in Hamburg.

I would like to thank **Brigitte Doormann** for her help and patience with my immense paper work.

I am especially grateful to my present and former colleagues: Dr. Frank Maier, Dr. Anke Lösch, Dr. Jörg Bormann, Cathrin Kröger, Birgit Hadeler, Jakob Bönnighausen, Thanh Nguyen Sy Le, Thuat Nguyen Van, Mayada Woriedh, Peter Ilgen, Marike Boenisch, Antje Blümke, Katja Schäfer, Kai Flachsbart, and Sven Falke for their help, advice, discussions, coffee breaks, ice cream, good and hard times. I would also like to thank all colleagues and friends from **Molecular Phytopathology and Genetics** as well as from the **Biocenter Klein Flottbek**.

I would like to thank the Deutsche Forschungsgemeinschaft (DFG) for funding this research project.

Last but not least I would like to thank my husband **Long** and my daughter **Linh** for their love and moral support. I would also like to thank **my family,** especially my mother for her understanding, encouragement, and taking care of my daughter during my PhD time and **my friends** for their encouragement.

CURRICULUM VITAE

Personal

Name: Mrs. LE, THI THU GIANG

Date of Birth: August 16th,1979

Nationality: Vietnamese

Education

9/2007 – 11/2010: Ph.D student

Biology Department, University of Hamburg, Germany

Study Project: Characterization of transcription factors important for fatty acids and lipid metabolism in the phytopathogen *Fusarium graminearum*.

Certificate: Doctorate

Speciality: Molecular Biology/Genetics/Phytopathology

7/2003 - 7/2005: Master Course

Master degree program in the institute of Ecology and Biological Recources, Vietnamese Academy of Sciences and Technology

Study topic: Construction of several expression plasmids for co-expression of both genes encoding the lipase and a chaperone from *Ralstonia* sp. M1 in *Escherichia coli* BL21.

Certificate: Master Degree

Speciality: Experimental Biology

9/1997 - 6/2001: Full time student

Biology Department- Hanoi University of Science, Hanoi National University

Study topic: Investigation on biosynthesis of a protein Bt from *Bacillus thuringiensis* for control of insects destroying vegetables and crop plants.

Certificate: Bachelor

Speciality: Microbiology

Language Proficiency

Vietnamese (native), English (good)

Disruption list of transcription factors

Order	KO mutant name	Construct name	Upstream fragment (bp)	Dowstream fragment (bp)	Selection marker	KO number	KO transformant number
1	*⊿far1*	pGEMT-CTF1A	363	431	Geneticin	6	1024.2; 3; 5; 6; 7; 13
2	*⊿far2*	pGEMT-CTF1B	945	905	Hygromycin	3	1128.1; 2; 3
3	*⊿lr1*	pGEMT-9318	910	920	Hygromycin	3	998.15; 32; 38
4	*⊿lr2*	pGEMT-3207	794	819	Hygromycin	2	1025.4; 20
5	*⊿lr3*	pGEMT-2520	988	1017	Hygromycin	1	999.6
6	*⊿lr4*	pGEMT-8034	884	865	Hygromycin	4	1026.4; 6; 7; 8
7	*⊿lr5*	pGEMT-8064	942	1000	Hygromycin	3	1027.2; 6; 16
8	*⊿lr6*	pGEMT-13248	740	772	Hygromycin	2	1166.6; 11
9	*⊿far2*/*⊿far1*	pGEMT-CTF1B/ pGEMT-CTF1A			Hygromycin Genticin	6	1087.30; 32; 33 1088. 6; 17; 25
10	*⊿far1*/*⊿lr1*	pGEMT-CTF1A/ pGEMT-9318			Genticin Hygromycin	2	1161. 12; 19