Importance of secreted lipases for virulence of the phytopathogenic fungus *Fusarium graminearum*

Dissertation

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List of figures and tables

ABBREVIATIONS	FULL NAME
%	Percentage
Δ	Delta/mutant
BLAST	Basic Local Alignment Search Tool
bp	base pairs
C	Carbon chain
cAMP	Cyclic Adenosine Monophosphate
cDNA	complementary Deoxyribonucleic Acid
CTF	Cutinase Transcription Factor
CWDE (s)	Cell Wall Degrading Enzyme (s)
cv.	Cultivated variety; cultivar
DIG	Digoxygenin
DNA	Deoxyribonucleic Acid
dNTPs	Desoxynucleotide triphosphate (s)
F_{do}	downstream fragment
DON	Deoxynivalenol
dpi	days post inoculation
dUTP	Desoxyuracil triphosphate
EC	Enzyme Commission
et al.	et alii = and others
Eth	Ethanol
Etp	Ectopic strains
FARE	Fatty Acid Response Element
FDK	Fusarium Damaged Kernel
FFA	Free Fatty Acid
FGL	*Fusarium graminearum* lipase
FHB	Fusarium Head Blight
Fig.	Figure
gDNA	genomic DNA
GFP	Green Fluorescent Protein
Gpmk1	Gibberella pathogenicity MAP kinase 1

hph	Hygromycin B phosphotransferase
kb	kilo bases (= 1000 bp)
kDa	kilo Dalton (= 1000 Da)
KO	Knock-out
LB	Luria-Bertani medium
M	Molar (mol/L)
MAP	Mitogen Activated Protein
MAPK	Mitogen Activated Protein Kinase
MIPS	Munich Information center for Protein Sequences
mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
NIV	Nivalenol
OD	Optical Density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
pH	Potentia Hydrogenii
pNP	para-Nitrophenol
qPCR	quantitative PCR
RNA	Ribonucleic acid
rpm	round per minute
RT-PCR	Reverse transcriptase PCR
SDS	Sodium Dodecylsulfate
SNA	Synthetic Nutrient Agar
Tab.	Table
Tm	Annealing Temperature
Tri	Trichothecene synthase gene
Tris	Tris-(hydroxymethyl) aminomethane
F_{up}	Upstream fragment
UV	Ultra violet
v	Volume
v/v	Volume per volume

w/v	Weight per volume
WGO	Wheat Germ Oil
WT	Wild type
YPG	Yeast-extract Peptone Glucose
Zea	Zearalenone

1. Introduction

1.1. Fusarium head blight

Fusarium head blight (FHB), or scab, is a fungal disease affecting many small grain cereals, but especially wheat and barley (Nelson *et al*. 1981). Different *Fusarium* species, including *Fusarium graminearum*, *F. culmorum*, *F. poae*, *F. avenaceum*, *F. sporotrichoides*, and *F. nivale,* were reported to cause FHB (Osborne and Stein, 2007). Several severe FHB epidemics occurred in North America and other parts of the world, causing world-wide losses in food supplies. Yield losses caused by FHB may reach 50 – 60% on infected fields (Miller and Trenholm, 1994; Windels, 2000; Osborne and Stein, 2007). In the USA, yield losses caused by FHB were estimated to be about $ 3 billion in the 90's and $ 220 million in Canada (Windels, 2000). Infected fields were also reported in China: FHB affected up to 7 million ha and 2.5 million tons of grain. In Minnesota, the disease has had an impact on cropping patterns, between 1992 and 1998 the amount of land planted with wheat decreased by 31% (Windels, 2000). FHB reduces kernel weight and consequently grain yield. The germination rate and seedling vigour are reduced when seeds are infected (Bai and Shaner, 1994; Gilbert and Tekauz, 1995; Argyris *et al*., 2003). The fungus digests proteins and starch, and the use of infected kernels causes enzymes and yeast growth to be inhibited by by-products of the fungus that prevent bread production (Becthel *et al*., 1985, Saric *et al*., 1997). Metabolites produced by *Fusarium* species are also the cause of 'gushing' of beer (Zapf *et al*., 2007). However, of primary concern is the contamination of grain with mycotoxins produced by several of the species associated with FHB. Several of these compounds have been shown to be harmful to human and animal health.

1.2. The fungus *Fusarium graminearum*

Fusarium graminearum Schwabe (teleomorph: *Gibberella zeae* (Schwein) Petch) is a phytopathogenic fungus with a major impact on FHB in many cereal crops such as wheat, barley, maize, and rice (Goswami and Kistler, 2004; McMullen *et al*., 1997). The fungus is capable of producing several trichothecene mycotoxins including deoxynivalenol (DON), nivalenol (NIV), and the estrogenic zearalenone (Zea)

(Tanaka *et al*., 1986; Bennett and Klich, 2003), all of which were determined to be harmful for human and animals. Fusarium-damaged kernels (FDK) are commonly contaminated with mycotoxins, especially DON, resulting not only in yield losses but also posing a threat to animal and human health (Bai and Shaner, 1994; Gilbert and Tekauz, 1995).

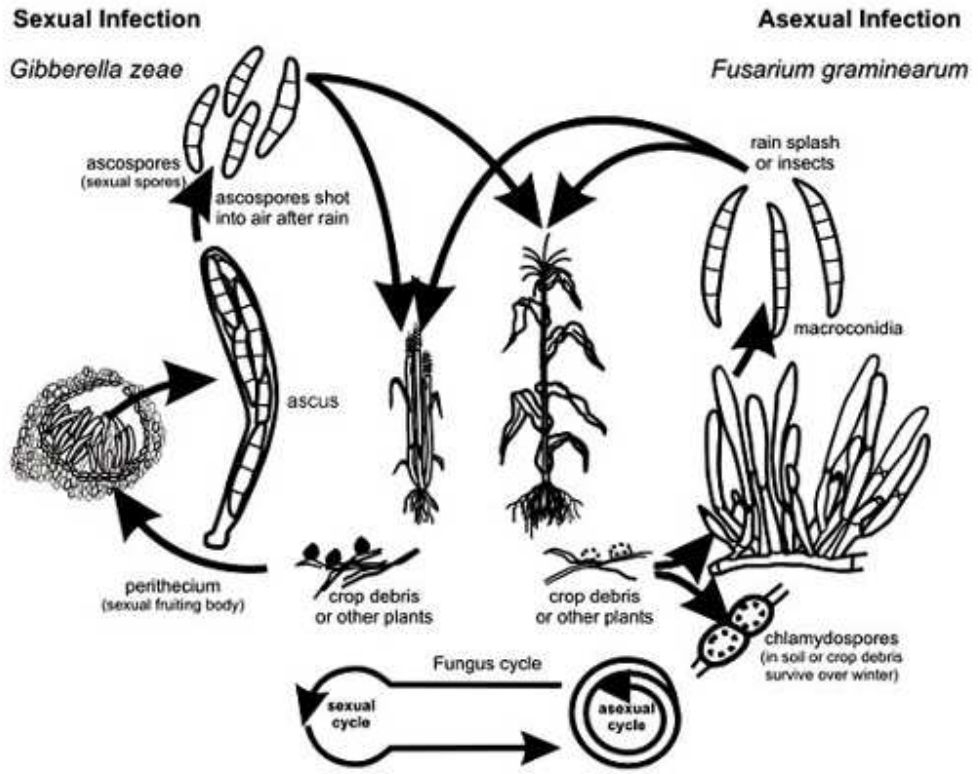

Fig. 1. Life cycle of *Fusarium graminearum*: Occurrence of sexual stage (*Gibberella zeae*) and asexual stage (*Fusarium graminearum*) during plant infection (Sutton, 1982).

1.3. Infection process of *F. graminearum* on wheat

Warm and wet weather are the optimal conditions for infection of wheat heads by conidia of *Fusarium graminearum*. Investigations showed that 100% humidity and a temperature of 25^{o}C resulted in highest infection rates (Osborne and Stein, 2007). Wheat plants are susceptible to *Fusarium* infection from flowering period (anthesis) to the stage of soft dough formation and kernel development. By removing the anthers during *F. graminearum* infection of certain wheat cultivars, Strange and

Smith (1971) report that the incidence of diseased florets was significantly reduced, suggesting that anthers may be the major sites of initial infection. Once *Fusarium* conidia are attached to wheat flowers they infect the exposed anthers. Subsequently, the fungus affects the developing kernels, glumes, and other parts of the head (Bushnell *et al*., 2000). Pristsch *et al*. (2000) show that hyphal penetration appears to occur through glume stomata. They observed two different patterns of colonization, including subcuticular hyphae on inoculated epidermis, and abundant intracellular colonization of parenchymatic tissues. According to Wheeler (1977), subcuticular colonization may allow secondary penetrations and spread of the pathogen. Degradation products of polymeric carbohydrates, which originate from the epidermal cell wall and cutin may serve as energy sources for the pathogen, a process in which cell wall degrading enzymes (CWDE) may participate. Using the green fluorescence protein as a reporter signal, similar growing patterns are also observed when the fungus grows on the epicarps before entering the underlying cells (Jansen *et al*., 2005). Interestingly, the intracellular hyphae attacking the epicarp wheat cells displays multiple appressoria-like structures (Jansen *et al*., 2005), an infection process which was found in leaf infecting fungi e.g. *Cochliobolus heterostrophus, Colletotrichum gloeosporioides* or *Magnaporthe grisea*.

The infection pattern of *F. graminearum* is systematic and is found from floret to floret inside one spikelet and spreads through the other spikelets via vascular bundles in the rachilla and rachis node. A number of studies provide insight into the infection and colonization process of *F. graminearum* on wheat in which GFP-tagged strains were used to follow the infection process (Guenther and Trail, 2005; Jansen *et al*., 2005). In accordance with the findings from Ribichich *et al*. (2000) and Wanjiru *et al*. (2002), Guenther and Trail (2005) have found the systematic colonization of the vascular bundles from the spikelet to the rachis. They also found that the phloem and chlorenchyma of the wheat stem were especially damaged as a result of colonization by the fungus. Jansen *et al*. (2005) found that large amounts of hyphae were seen moving through vascular bundles of xylem and phloem in the rachis at 6 days after infection. Remarkably, they found a thickening of cell walls during infection of *F. graminearum* mutant strain *tri5*-GFP, which is incapable of

synthesizing trichothecenes. The mutant strain is not able to penetrate through the formed barrier in the rachis node. A visible cell wall thickening was also found in the rachis node when colonization of the fungus was impaired by disrupting the virulence gene encoding the extracellular lipase *FGL1* (Voigt *et al*., 2005). These findings implicate a possible potential role of rachis node in wheat plant defense reaction.

1.4. Pathogenicity factors and virulence factors of *F. graminearum*

Fungal pathogens use various ways to attack their host plants. Some fungi, in order to penetrate plant cells, apply a mechanical turgor pressure to break plant surfaces. The rice blast fungus *Magnaporthe grisea* (*Pyricularia oryzae*), for example, upon contact with plant surfaces, creates a special structure, the so-called appressoria, where turgor pressure is generated to break through the cuticula of plants (Clergeot *et al*., 2001). Other fungi produce natural chemicals or substances during the infection process. Various substances including enzymes, toxins, growth regulators, polysaccharides are secreted and used as weapons against host plants. These substances are defined as pathogenicity factors and are the products of respective pathogenicity genes. According to Schäfer (1994), Idnurm and Howlett (2001), pathogenicity genes are genes necessary for disease development, but not essential for the pathogen to complete its lifecycle. However, this definition is not applicable to some cases as previously argued by Oliver and Osbourn (1995). Several factors that are helpful but not essential for induction and development of disease are determined as virulence factors and encoded by virulence genes (Agrios, 2005).

Fusarium graminearum, a plant pathogen, is subject of many investigations. Since its genome has been sequenced and made available to the public, numerous investigations have focused on elucidating pathogenicity and virulence genes as well as focusing on regulatory genes for the pathogenic process of *F. graminearum* during plant infection. Several pathogenicity factors and virulence factors were identified by molecular techniques.

Many cereal and other crops are susceptible to fungal attack either in the field or during storage. These fungi may produce secondary metabolites, a diverse group of

chemical substances known as mycotoxins. Cereal grains infected by *F. graminearum* are normally contaminated with mycotoxins. The fungus is able to secrete several potent toxins, which include trichothecenes, fumonisins, and zearalenone. Trichothecenes are probably the most dangerous mycotoxins, of which deoxynivalenol (DON) and nivalenol (NIV) are the primary toxins normally found in infected grains. There is a gene cluster spanning a region of 26 kb that contains the main trichothecene synthesis genes found in *F. sporotrichioides* and *F. graminearum* (Brown *et al*., 2003; Kimura *et al*., 2003). Using a transformation-mediated gene disruption method, some of trichothecene genes were disrupted for functional analysis. Disruption of *Tri5*, a gene encoding the enzyme trichodiene synthase that catalyzes the first step in the biosynthetic pathway of trichothecenes, resulted in transformants that were unable to produce trichodiene (Proctor *et al.,* 1995; Jansen *et al*., 2005). These *Δtri5* mutants are able to infect inoculated wheat spikelets, but the infection is not completed in wheat spikes (Bai *et al.,* 2001). Investigations indicate that *Δtri5* mutants are still able to infect barley spikes and maize cobs. Hence, *Tri5* is not a general virulence factor (Maier *et al.,* 2006). However, in wheat, the spread of *Δtri5* mutant strain was restricted to inoculated spikelets, suggesting the inhibitory functions of *Tri5* in wheat defense responses (Jansen *et al.,* 2005). Another gene, *Tri14*, was also examined for its role in deoxynivalenol synthesis and in virulence on wheat. *Tri14* deletion mutants synthesized deoxynivalenol on cracked maize kernel medium. However, FHB assays on greenhouse-grown wheat indicate that *Δtri14* mutants cause 50 - 80% less disease than wild type, and do not produce a detectable quantity of deoxynivalenol on plants (Dyer *et al*., 2005). This indicated the involvement of *Tri14* in pathogenicity of *F. graminearum.*

Fungi, like plants and other organisms, use signaling proteins located in membranes that respond to changes in the environment and set off signaling cascades that alter gene expression. The success of a fungal pathogen depends to a high degree on its ability to recognize and perceive the signals generated by host plants. The perceived signals are mediated by a network of signaling genes. Fungal signaling genes include heterotrimeric G-protein coding genes, MAP kinase genes, and cyclic AMP dependent protein kinase genes (Lengeler *et al*., 2000). When one of these genes is

missing, the fungus loses its pathogenicity or exhibits a reduction of several physiological processes (D'Souza and Heitman, 2001; Román *et al*., 2007). With respect to that, *Fusarium graminearum* is not an exception. Initially, disruption of two mitogen activated protein kinases (MAPK) *Mgv1* (Hou *et al.*, 2002) and *Gpmk1*/*MAP1* (Jenczmionka *et al.*, 2003; Urban *et al.*, 2003) revealed their importance in plant infection. The *mgv1* mutant strains showed a reduced virulence and trichothecene production as well. However, the mutant showed a reduced growth rate and a weaker cell wall formation. These additional data suggest that *Mgv1* in *F. graminearum* is not solely involved in regulation of virulence genes. It could also be involved in regulating multiple developmental processes. Studies of the *gpmk1* mutant showed that pathogenisis of *F. graminearum* was regulated by this MAP kinase. Furthermore, the MAP kinase was identified to be responsible for the early induction of extracellular endoglucanases, xylanolytic and proteolytic activities, and for the overall induction of secreted lipolytic activities. These cell wall degrading enzymes are postulated to participate in pathogenicity of *F. graminearum* (Jenczmionka and Schäfer, 2003; Wanjiru *et al*., 2002).

Ras GTPases are small GTP-binding proteins that cycle between GTP-bound and GDP-bound forms. In eukaryotes, Ras GTPases interact with effector molecules to regulate the divergent signaling pathways. The genome of *F. graminearum* contains two putative Ras GTPase-encoding genes (Bluhm *et al*., 2007). The two genes (*RAS1* and *RAS2*) showed different expression patterns under different conditions of nutrient availability and in various mutant backgrounds. *RAS1* was speculated to be essential for the fungal growth, since repeated attempts to disrupt that gene failed. *RAS2* was shown to be dispensable for survival, but the disruption caused significant reductions in virulence on wheat heads and maize cobs. The reduced virulence of *Ras2* mutant (FR-1) on wheat heads is possibly related to the regulation of extracellular enzymes including the virulence lipase *FGL1*. This regulation may occur through the activation of the MAP kinase *gpmk1* (Bluhm *et al*., 2007). However, direct evidence of regulation of MAP kinase *gpmk1* on *FGL1* expression has not yet been found.

Another *F. graminearum* pathogenicity gene, *CPS1*, encoding a protein with two adenosine monophosphate-binding domains, was considered as a general pathogenicity factor (Lu *et al*., 2003). Mutagenesis in the gene of *Cochliobolus heterostrophus* and *Cochliobolus victoriae* led to reduced virulence on maize and oats, respectively. Disruption of its orthologue in *F. graminearum* caused a reduced virulence on wheat. It is notable that the null mutant of *CPS1* in *F. graminearum* produces the same level of toxins as found in the wild type. This suggests that the toxins are not sufficient for the fungus to combat inhibitory stresses produced by host plants, and *CPS1* is, therefore, required to overcome host defense.

1.5. Cell wall degrading enzymes of *F. graminearum*

Unlike other pathogenic fungi, e.g. *Magnaporthe grisea*, *Colletotrichum graminicola,* and *Cochliobolus species*, *F. graminearum* forms no typical appressoria for plant penetration (Pritsch *et al*., 2000). More evidence is supporting the hypothesis that cell wall degrading enzymes are virulence factors of the fungus (Wanjiru *et al*., 2002; Jenczmionka and Schäfer, 2003). In *in vitro* conditions, the fungus secretes various hydrolytic enzymes and cell wall degrading enzymes (CWDE). They were identified in different media as well as during plant colonization (Jenczmionka and Schäfer, 2005; Phalip *et al*., 2005; Paper *et al*., 2007). The secretion of different hydrolytic enzymes could be related to nutrition acquisition of the fungus. That could also be deleterious as the fungus is a plant pathogen. Localization of cellulose, xylan, and pectin by enzyme-gold and immuno-gold labelling techniques showed that host cell walls which were in direct contact with the pathogen surface had reduced gold labelling compared to considerably higher labelling densities of walls distant from the pathogen-host interface or in non-colonized tissues (Wanjiru *et al*., 2002). The reduced gold labeling densities in the infected host cell walls indicated that polysaccharide degrading enzymes might be important pathogenicity factors of *F. graminearum* during infection of wheat spikes. Additionally, studies (Jenczmionka and Schäfer, 2005) identified the pectinolytic, amylolytic, xylanolytic, proteolytic, and lipolytic activities of this fungus in appropriate culture media by enzymatic assays. Identification of extracellular proteins by using proteomic techniques showed that 84 proteins are secreted during culture on hop-cell wall medium (Phalip *et al*., 2005).

Paper *et al*. (2007) identified 289 proteins, many of which are found as secreted ones in different growth media as well as in *planta*. Several secreted proteins of *F. graminearum* were also found during wheat infection. Among the 120 proteins identified from infected wheat spikes, 56 proteins were identified as secreted proteins (Paper *et al*., 2007) some of which were differentially regulated during plant infection of *F. graminearum* (Zhou *et al*., 2005; Paper *et al*., 2007). Nutrient starvation is known to induce genes related to pathogenesis (Trial *et al*., 2003). By changing the carbon source from glucose to plant cell wall material, Carapito *et al*. (2008) detected the significant up-regulation of 23 genes when tested by RT-PCR. These changes include enzymes related to cell wall degradation such as xylanase, endoglucanase, hemicellolase, and polysaccharide deacetylase (Carapito *et al*., 2008). This massive change in gene induction by growing conditions was also reported by Phalip *et al*. (2005). The total amount of protein secreted in medium containing plant cell wall was approximately increased up to 25-fold compared to glucose containing media (Phalip *et al*., 2005).

1.6. Lipases and secreted lipases of *F. graminearum*

Lipases (EC 3.1.1.3) are an extensive family of enzymes, which catalyze the hydrolysis and synthesis of ester bonds. They are ubiquitous in nature and have been used for industrial applications (Jaeger and Reetz, 1998). Because of their broad applications, e.g. in pharmaceutical, cosmetic, and cleaning usages, lipases are the second most important group of enzymes in industry, followed by proteases (Jaeger *et al*., 1999; Pandey *et al*., 1999; Houde *et al*., 2004). One of the most important biological sources for isolation of lipases are fungi. The main sources of commercial lipase production are *Aspergillus*, *Candida*, *Humicola*, *Yarrowia*, *Mucor*, and *Rhizopus* species.

Contrary to applicable industrial attentions, little is known about lipases as virulence factor of pathogens. Nevertheless, there were several reports about lipolytic enzymes and lipases as virulence factors in bacteria and fungi (Ryding *et al*., 1992; Edwards *et al*., 1995; Miskin *et al.,* 1997; Gácser *et al.,* 2007a, 2007b; Mullen *et al.,* 2007). There have been several investigations on fungal lipases as potential

virulence factors of plant pathogens. Generally, plant epidermal cells contain cuticular waxes and a continuous layer of cuticle in outer surface. The cuticle consists of insoluble polyester of C16 and C18 hydroxy fatty acids called cutin (figure 2). Cuticular waxes consist of a soluble complex mixture of long-chain aliphatic compounds such as long-chain fatty acids, aldehydes, alkanes, primary and secondary alcohols, ketones, and wax esters (Juniper and Jeffree, 1983). Lipases have a unique role due to their potential ability to hydrolyze plant cuticular waxes. Besides, various types of lipids occur in all plant cells, the most important being phospholipids and glycolipids, both of which, along with proteins, are the main constituents of all plant cell membranes. Oils and fats are found in many cells, especially in seeds where they function as energy storage compounds. These components may be the target of lipolytic enzymes, such as lipases and phospholipases, which hydrolyze fatty acids from the lipid molecule.

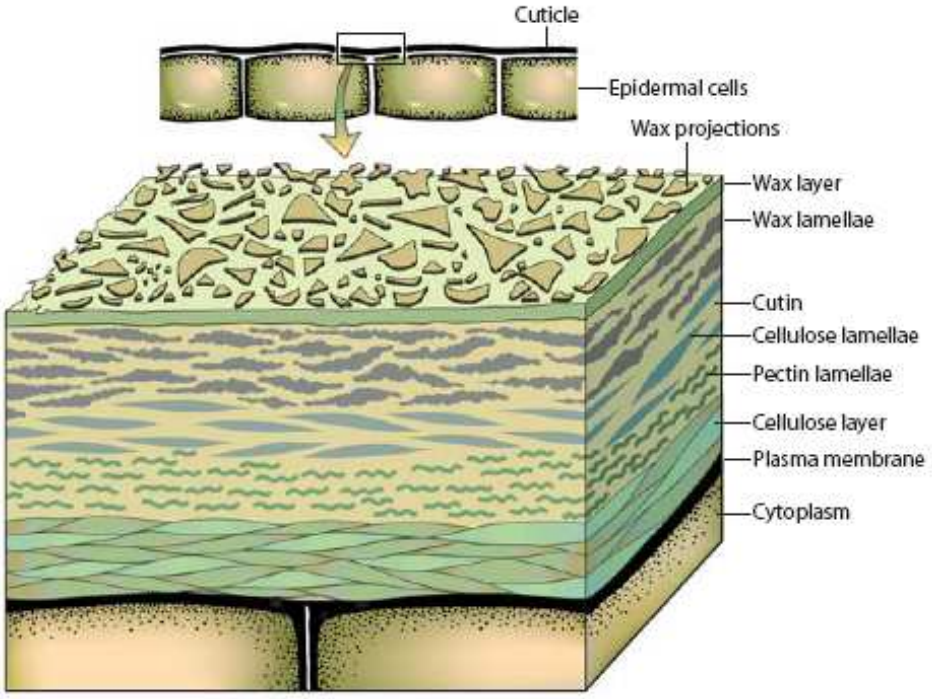

Fig. 2. Schematic representation of the structure and composition of the cuticle and cell wall of foliar epidermal cells (Agrios, 2005).

Involvement of extracellular lipases in fungal pathogenesis has been reported by Comménil *et al.*, (1998) in pathogenic fungi. A lipase, *Lip1* from *Botrytis cinerea*,

which causes grey mould on various plants, was thought to be required for fungal penetration and infection. Lesion formation on inoculated tomato leaves caused by *B. cinerea* was completely suppressed when anti-lipase antibodies were added to a conidial suspension prior to inoculation (Comménil *et al.*, 1998). Interestingly, the anti-lipase antibodies generated from a *B. cinerea* lipase successfully cross-reacted to suppress formation of black spot lesions of *A. brassicicola* on cauliflower leaves. This suggests that a lipase encoded by *A. brassicicola* is important for the fungal infection (Berto *et al*., 1999). However, Reis *et al*. (2005) found that disruption of the *Botrytis cinera* lipase did not prevent the fungus from infecting the plant. Possibly, the anti-lipase antibodies were less specific than originally assumed. Other fungal lipases were also suggested to be involved in pathogenicity. An extracellular lipase, *NhL1*, has been cloned from the pea pathogen *Nectria haematococca* mating population VI (*Fusarium solani* f. sp. *pisi*), and its expression was induced during infection of pea plants (Nasser-Eddine *et al.*, 2001). *Burkholderia glumae* is an emerging rice pathogen in several areas of the world (Cottyn *et al*., 1996; Tsushima *et al*., 1996). Pathogenesis of *B. glumae* is regulated by quorum sensing with acyl homoserine lactone (AHL). It was determined that acyl homoserine lactone (AHL) quorum sensing regulated pathogenicity through a secreted lipase, *LipA*. *B. glumae* AU6208 *lipA* mutants were no longer pathogenic to rice, indicating that the lipase is an important virulence factor (Devescovi *et al*., 2007).

The genome of *Fusarium graminearum* contains a significant number of putative lipase genes, especially for secreted lipases. Gene disruption was used to show that the extracellular lipase *FGL1* of *F. graminearum* was required for successfull colonization during infection of wheat and maize (Voigt, *et al*. 2005). In growth culture, *FGL1*-deficient strains showed strongly decreased lipolytic activity in early time points of wheat germ oil induction.

In *F. graminearum*, MAP kinase *gpmk1* was known to regulate the pathogenicity process of the fungus. As stated above, *Gpmk1* disruption mutants were apathogenic on wheat (Jenczmionka *et al*., 2003). Determination of secreted lipolytic activity of the ∆*gpmk1* mutants showed that *Gpmk1* was responsible for the overall induction of secreted lipolytic activities in culture (Jenczmionka and Schäfer, 2005),

and most likely regulates the onset of lipase gene *FGL1*. 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 (Feng *et al*., 2005). Therefore, *Fusarium graminearum* secretes lipases which are used for both nutrient acquisition and virulence on host plant. As determined in culture, *FGL1*- and *LIP1*-deficient mutant strains still secreted lipolytic activity at later stages. This indicates that other lipases are also expressed and might be used as a nutrition means and virulence weapons against its various host plants.

1.7. Aim of this study

The first aim of this study is to determine the impact of the secreted lipases on virulence of *F. graminearum*. In this study, the results of gene disruptions, and virulence evaluation of lipase genes *FGL2* (fg01240), *FGL4* (fg03209), *FGL5* (fg03583), *FGL9* (fg03687), *FGL12* (fg03012), *FGL13* (fg03095), and *FGL14* (fg05935) are presented. Potential role of the secreted lipases in nutrition acquisition of the fungus is also part of this study. Additionally, lipolytic enzyme of *FGL2*, *FGL3*, *FGL4*, and *FGL5* genes was characterized by heterologous expression. Finally, preliminary results of molecular study of *FGL1* promoter and localization of *FGL1* are presented.

2. Material and Methods

2.1 Material

2.1.1 Enzymes and chemicals

Restriction enzymes, DNA-modifying enzymes, and Taq polymerase were obtained from Fermentas (St. Leon Roth, Germany), with the exception of *AsiS*I which was purchased from NEB (New England Biolabs, USA). Chemicals used in culture media and buffers were obtained from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany), and Sigma-Aldrich (Steinheim, Germany). Chemicals used in Southern and Northern blots were obtained from Roche (Mannheim, Germany).

2.1.2. Microbial strains and culture media

Escherichia coli strain TOP10F (Invitrogen, Carlsbad, CA, USA) was used for DNA plasmid propagation and during DNA cloning procedures. Heterologous gene expression was performed by using the *Pichia pastoris* strain KM71 (*arg4 his4 aox1::ARG4*) and the expression vector pGAPZα (Invitriogen). *Fusarium graminearum* strain 8/1 was obtained from T. Miedaner (Miedaner *et al*., 2000) and preserved on SNA plates (Nirenberg, 1981). Induction of conidiation was performed by placing a mycelial plug (0.5 cm^2) or pipetting a drop of conidia suspension on SNA plates and incubation for 2 weeks at 18^oC 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. For long time storage, conidia were kept in water, and the suspensions were stored at –70^oC.

Additionally, genomic DNA isolated from *F. graminearum* strain PH-1 was used for cloning *FGL10* as a positive control.

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 antibiotics Ampicilin (100 μg/ml) were 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. The following are compositions of these media:

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 (100x): 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; 0.5 g Casein, hydrolyzed by acid degradation.

To prepare for 1 l CM medium, 10 ml of solution A was added to 869 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, the complete solution E, and 10 ml of the suspension F were added to the medium. For selection of the transformants 100 µg/ml Hygromycin B were added to the solid medium.

YPG medium (Sambrook *et al*., 1989): 1% Yeast extract; 2% Pepton; 2% Glucose. To prepare a solid agar plate, 16% granulated agar was added before autoclaving. YPG medium containing 1 M sorbitol (YPGS) were used for transformation of *Pichia pastoris* KM71 strain.

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).

Media prepared for lipolytic plate assays: SNA medium was used by adding either 2% wheat germ oil (WGO), 2% Triolein, 2% Olive oil, or 2% Tributyrin as the sole carbon source.

2.1.3. Primers

All oligonucleotide primers used in this study were designed by using PrimerSelect program (DNASTAR software, USA). PCRs were performed by employing non-proofreading Taq DNA Polymerase (Fermentas), 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 *et al*., 1988).

List of oligonucleotide primers used in this study. (F): forward primer; (R) reverse primer; (up) upstream flanking region; (do) downstream flanking region; (n) nested primer. All primers are listed in 5´-3´-direction.

Note: upF = primer 1; upR = primer 2; upnF = primer 5; doF = primer 3; doR = primer 4; donR = primer 8 in figure 3.

Internal HYG primers used in fusion PCR

(Note: YgF = primer 7; HyG = primer 6 in figure 3)

YgF: GTTGGCGACCTCGTATTGG

HyR: CTTACCACCTGCTCATCACCT

Set of primers for generation of *FGL2* disruption construct

(Note: Lower case letters are overlapping oligonucleotides of HYG)

FGL2upF: GCGTTATGCTCCCTCTCAAG

FGL2upR: agatgccgaccgaacaagagctgtcccccGTAAACGCGGCTGTAGACG

FGL2upnF: CCGGTTTCACTAGCCTTCAG

FGL2doF: caatgctacatcacccacctcgctcccccACCAATAGGCATCCCGTGT

FGL2doR: GAATCCACGAACCTGGATG

FGL2donR: TTTAGCAACTCACTTACCAGCT

Set of primers for generation of *FGL3* disruption construct

FGL3upF: TTGGCGAGCATGAAGTTGAG

FGL3upR: agatgccgaccgaacaagagctgtcccccCACACGGCAGTATCAGCTCA

FGL3upnF: GCAGGAACATAGGTGACAACA

FGL3doF: caatgctacatcacccacctcgctcccccTTAGCAGCAAACACCACACT

FGL3doR: CATTTACTGCACGAGGGGTC

FGL3donR: CGCGGATCTCTGAAGCAG

Set of primers for generation of *FGL4* disruption construct

FGL4upF: TGTGTACCTTTTCCGTTCTTG

FGL4upR: agatgccgaccgaacaagagctgtccccTGTACAGCACTGATGGAGATG

FGL4upnF: CGAAAAGATTAGGACACAGGA

FGL4doF: caatgctacatcacccacctcgctcccccCTGAGCCAATCTTATCGACC

FGL4doR: 5 - AAGGATATCCTGTTGCTCCC

FGL4209R: CGCATGCGACTAATCTTTG

Set of primers for generation of *FGL5* disruption construct

FGL5upF: GCATATTTTGGAGCGAGTTG

FGL5upR: agatgccgaccgaacaagagctgtccccCACTTTTAAATCCGGTATGTCC

FGL5upnF: TGTCTCATTCAACCAAACGC

FGL5doF: caatgctacatcacccacctcgctcccccATGGACGGACTTTATGCAGA

FGL5doR: CGTTTGTCTCGCTCCACTC

FGL5donR: GGCCACCACCACAGTTGA

Set of primers for generation of *FGL9* disruption construct

FGL9upF: ACATCGGAGAAATACCCGG

FGL9upR: agatgccgaccgaacaagagctgtccccGGTACTTTTGTCTTGGGAGGG

FGL9upnF: GACATGTTGGGTCATTACGG

FGL9doF: aatgctacatcacccacctcgctcccccGATTTGTTCTGTCTTCCTCCC

FGL9doR: GTTATTCCAGATCCTTGCCC

FGL9donR: CACTTCCCTACACCAACGC

Set of primers for generation of *FGL12* disruption construct

FGL12upF: TCGCATTCGACTTTTGTGAG

FGL12upR: agatgccgaccgaacaagagctgtccccTACAGGACGGTTAGATAGTGGC

FGL12upnF: ATTAATGATGTCGTACTGCGG

FGL12doF: caatgctacatcacccacctcgctcccccTGAACTGGAAGCTATTATGTGC

FGL12doR: GCTTCTCGACTTCTTCATTCC

FGL12donR: CAGACCATCTCCTTCCTGCT

Set of primers for generation of *FGL13* disruption construct

FGL13upF: ACAGACAGCCAGAACATCCC

FGL13upR: agatgccgaccgaacaagagctgtcccccCACGAATGCCAAGACTATCAG

FGL13upnF: ACGAATTCATACCAGAGGAGG

FGL13doF: caatgctacatcacccacctcgctccccGAGAAATATTAAGGCCATCAGG

FGL13doF: GAAGCTTCGGACTTGAGAGG

FGL13donR: ACTCTTTCCGTTTTATCCGTC

Set of primers for generation of *FGL14* disruption construct

FGL14upF: ATATCACAAAGCCCAGCTCTC

FGL14upR: agatgccgaccgaacaagagctgtcccccAATGGAAGACAGGACATGAGG

FGL14upnF: TGTGACCTTTGACGAGTTGG

FGL14doF: caatgctacatcacccacctcgctccccGCCCTTCCATAGACATACCTC

FGL14doR: GCAACACTAACATCTCCTCTCG

FGL14donR: TGGTCTATGATGGCGTCTG

Primers for expression analysis by RT- PCR

β-TubF: TGCTGTTCTGGTCGATCTTG

β-TubR: ATGAAGAAGTGAAGTCGGGG

Fgl1RtF: CACCCCTTGACATCTACACCTAC

Fgl1RtR: GCGGCCTGGCATGAGTCTTGATA

Fgl2RtF: GAAAATGTTATCGTCACCACG

Fgl2RtR: CTTGATATCGTCCTCTACCCC

Fgl3RtF: CGACACTATCAAGCAATTCCC

Fgl3RtR: AGAACCCACCAACCTGAGC

Fgl4RtF: TATCTCCACAACATCTCGCC

Fgl4RtR: CTTCCTGATTTCGTCCTTCTC

Fgl5RtF: CGGAACCAACACAAACGAG

Fgl5RtR: CGCTAAAAACTTGCTCAACC

Fgl6RtF: AAGCCAGTATCCCGAAGTG

Fgl6RtR: AATTGCAGTGCTGTTTCTCG

Fgl7F: TCAAAGAGGGATCAACACGA

Primers for expression analysis by RT- PCR (continued)

Fgl7R: TTCCATCAAAGTTGGGGTC

Fgl8F: GACCGACGCTACAACATCAT

Fgl8R: GTTGGGAGACTCGAACAGG

Fgl9rtF: AGGAATCAACACCACCGAG

Fgl9rtR: CAGCAGTGTAGCCAAGTCCA

Fgl10rtF: TTGCTAAAGCCTATCCCGA

Fgl10rtR: TTTTCGACCGTGCTACTGAG

Fgl11rtF: TGCCATCAACCACCATACC

Fgl11rtR: ACAACTCCCTTGCTACCGC

Fgl12rtF: AATCCTGAGCGCATCACC

Fgl12rtR: AACATCTTGGTCCGGTCC

Fgl13rtF: GATCCAGAAAACTCAAGCCC

Fgl13rtR: CTTTCGTGACGAGACCTCTG

Fgl14rtF: ATGTCACTTCAGCCCAAGTC

Fgl14rtR: AGTCTGATTGCTCCTCCGTC

Fgl15rtF: TTTGCAAAGATTCCCATCC

Fgl15rtR: CTTAACTGTTGAGCATGGCG

Fgl16rtF: CACATTCCCGACGCTGAC

Fgl16rtR: TGCACAACAGGATACTTGGG

LIP1rtF: CCAACGTGACCACTACATCC

LIP1rtR: CTGTTCTTATTCGCCGTCTC

Primers used in heterologous expression in *Pichia pastoris*

(Note: underlined oligonucleotides are recognition sites for respective enzymes)

FGL2xF: GGTACCCTCAGTAATCCTGGATCATCATC (*Kpn*I)

FGL2xR: TCTAGAAGTTCCTCAGTATCTGGGCC (*Xba*I)

FGL3xF: GGTACCGTTCCTACCGACGCAACC (*Kpn*I)

FGL3xR: TCTAGAAACCCACCAACCTGAGCA (*Xba*I)

FGL4xF: CCGCGGACACCACATACTGGAGAACACTAC (*Sac*II)

FGL4xR: TCTAGATTAAGTCTAGGAGCTCTCTCTGGG (*Xba*I)

FGL5xF: GAATTCAGCCCAACAATCAAGGGC (*EcoR*I)

FGL5xR: GGTACCACAGCAAATCCATTCTGAACAC (*Kpn*I)

Primers used for sequencing of *FGL2*, *FGL3*, *FGL4* and *FGL5*

M13F: GTAAAACGACGGCCAG

M13R: CAGGAAACAGCTATGAC

FGL2seqF: GCATGGTGATAGCGGTGAC

FGL4seqF: ACTACCGCGTCAATTCCCT

FGL4seqR: CCAGGGCAGATATAAGCGTAG

FGL5seqF: CAACTTGCACAGAACTGGGT

FGL5seqR: GAGGCTTAAGCTGGTCTGG

Primers used for molecular study of promoter *FGL1*

(Note: underlined oligonucleotides are recognition sites for respective enzymes; lowercase letters are overlapping region of promoter *FGL1*)

Fgl1ProF: ACGGGAAAGGGGTAGAGGAC

Fgl1ProR: GACAGGAGACGCATCTTGACA

Fgl1XbaIF: GCTCTAGAGCCAAACTCGATGCACCTGACTC (*Xba*I)

Fgl1promGfpF: cccttgtctgtcaagatgcgtctcctgtcaGTGAGCAAGGGCGAGGAG

GfpR: AAGCTAGCGAATTCGTGGATC

GfpHindIIIR: CCCAAGCTTGGGCGAGGTCGACGGTATCGATA (*Hind*III)

Primers used for localization of *FGL1*

(Note: underlined oligonucleotides are recognition sites for respective enzymes; lowercase letters are overlapping region of *FGL1*; underlined and bold letters are synthetic linker sequence)

FGL1GfpF: GCACAAACCCATCACCAACTC

FGL1GfpR: GATGAGCGGTTGGCGTGAG

FGL1GfpnF: CTCTAGACGCTACAGACCCTACACGC (*Xba*I)

Gfp5aaF: cacgccaaccgctcatca**GGTGCTGGTGCTGGT**GTGAGCAAGGGCGAG GAG

Primers used for sequencing of GFP fusion constructs

FustestGPFrev: GAAGCACTGCACGCCGT

2.2. Methods

2.2.1. Lipase sequence analyses and alignments

All manually annotated lipase DNA sequences whose deduced protein sequences contain a putative lipase domain of five amino acids GXSXG, were retrieved from MIPS *Fusarium graminearum* Genome Database (http://mips.gsf.de/). Further analysis was done by comparing MIPS annotated sequences with those retrieved from *Fusarium graminearum* Database (http://www.broad.mit.edu/). Each lipase gene was designated according to that of *FGL1*, in which *FG* stands for *Fusarium graminearum* and *L* stands for lipase. These annotated DNA sequences were further aligned with known lipase DNA sequences of various sources through Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) (BLAST, NCBI) in order to find the sequence similarity and putative catalytic domain. The phylogenetic tree was constructed from deduced amino acid sequences by, firstly, using the alignment software ClustalX 1.83 and, secondly, viewed by TreeView 1.6.6. The deduced amino acid sequence of each lipase was used to analyze the possibility of secretion by using SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The results were considered in cloning steps of signal peptide-truncated DNA sequences of the genes for heterologous expression in *P. pastoris*.

2.2.2. Heterologous expression of lipase genes *FGL2*, *FGL3*, *FGL4*, and *FGL5* in *Pichia pastoris*

2.2.2.1. Cloning gDNA and cDNA sequences of lipase genes

To prepare mycelia for DNA and RNA isolation, *F. graminearum* conidia were cultured in CM or YPG medium at 28^{o}C by shaking at 150 rpm for 2 days. Mycelia were filtered with a 200 µl diameter sieve, medium was washed out, and used for DNA isolation. DNA was isolated by using CTAB solution (2% CTAB, 1 M Tris HCl, 5 M NaCl, 0.5 M EDTA) as described by Kidwell and Osborn (1992). To prepare mycelia for RNA isolation, the washed mycelia was either directly isolated or inoculated with 2% (v/v) wheat germ oil in 50 ml pure water at 28^{o}C by shaking at 150 rpm (Voigt *et al*. 2005), and samples were taken at different time points as

indicated in the result part. The induction was terminated after 24 h. Total RNA was isolated by using RNeasy Kit (Qiagen, Hilden, Germany), and single stranded cDNA was synthesized by using RevertAid™ First Strand cDNA Synthesis Kits, and oligo dT_{18} as the primer (Fermentas). *FGL2*, *FGL3*, *FGL4* and *FGL5* open reading frames (ORF) were amplified by PCR from gDNA as well as from cDNA as templates with the use of its specific primer pairs. The PCR program consisted of an initial denaturation step for 4 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at a gradient (Eppendorf Mastercycler) Tm = 54 – 58°C for 45 s, and an appropriate elongation time, 1 min 30 s for *FGL2*, 1 min for *FGL3*, and 2 min for *FGL4* and *FGL5*, at 72°C. The final elongation step was performed for 10 min at 72°C. The DNA sequences from gDNA as well as from cDNA were subcloned into pGEM-T (Promega), and then sequenced with M13F/R primers (Invitrogen) and primers shown in section 2.1.3.

2.2.2.2. Expression of lipase genes 2, 3, 4, and 5 in *Pichia pastoris*

Various steps for generation of recombinant plasmids were followed. The signal peptide-truncated coding sequence of *FGL2* (*tFGL2,* 1155 bp), *FGL3* (*tFGL3*, 867 bp), *FGL4* (*tFGL4*, 1608 bp), and *FGL5* (*tFGL5*, 1539 bp) were amplified from genomic DNA with the primer pairs FGL2xF/R, FGL3xF/R, FGL4xF/R, and FGL5xF/R. These primers carry various restriction sites (see 2.1.3). The PCR-amplified ORF sequences of *FGL2*, *FGL3*, *FGL4*, and *FGL5* were ligated into pGEM-T (Promega) to generate pGEM-tFGL2, -tFGL3, -tFGL4, and -tFGL5 plasmids. These recombinant plasmids were then excised at the introduced restriction sites to release respective DNA fragments. These gel-purified fragments (purified by using Qiagen Gel extraction Kit, Qiagen, Germany) were then ligated into expression vector pGAPZαA (Invitrogen, USA), which contains an α-signal peptide and the constitutive expression promoter GAP, to generate pGAPZαA-tFGL2, -tFGL3, -tFGL4, and -tFGL5 plasmids. Therefore, the secretion of the lipases is led by the α-signal peptide, and the amino acid sequences were also tagged with 6x histidine at C - terminus.

2.2.2.3. Transformation of *P. pastoris* cells by electroporation

Pichia pastoris strain KM71 was used for heterologous protein expression. Following the manufacturer's instruction the pGAPZαA-tFGL2, pGAPZαA-tFGL3, pGAPZαA-tFGL4, pGAPZαA-tFGL5, and the original pGAPZαA (control) plasmids were linearized in GAP region by enzyme *Pag*I (Fermentas). 10 µg of each linearized plasmid were introduced into the *Pichia pastoris* cells by electroporation as recommended by the manufacturer (Electroporator II, Invitrogen). Zeocin-resistant *Pichia pastoris* colonies were preliminarily selected, and the further screening was performed in YPGS (1% Yeast extract; 2% Pepton; 2% Glucose; 1 M Sorbitol) agar with increasing concentrations of Zeocin as recommended in the *Pichia* expression manual (Invitrogen).

2.2.2.4. Verification of *P. patoris* transformants

DNA of the *P. pastoris* transformants was isolated by the glass-bead based method as described by Harju *et al*. (2004) and used for PCR screening with pGAP forward and 3'AOX1 primers to select the right integration. The resulting transformants KM71-tFGL2, KM71-tFGL3, KM71-tFGL4, and KM71-tFGL5 were inoculated in 50 ml of YPG media and expressed for 5-7 days at 30^{o}C with shaking at 180 rpm. The expression supernatant was clarified by centrifugation at 5000 rpm for 10 min and used for lipolytic activity assays.

2.2.2.5. Heterologous expression of lipases

Overexpression of lipase was conducted by *Pichia pastoris* strain KM71 in YPG medium for a period of 6 days. Initially, for each lipase, three recombinant *P. pastoris* colonies were cultured overnight in 5 ml of YGP to reach OD_{600} = 1.5 - 2. For overexpression, 50 ml of fresh YPG medium prepared in a 100 ml flask were inoculated with 500 µl of the overnight culture (final concentration 1% v/v). The expression lasted for 6 days at 30^{o}C by shaking at 180 rpm. To determine extracellular lipolytic activity of *P. pastoris* clones, samples were taken out after 4, 5, and 6 days, and centrifuged at 5000 rpm for 10 min. The clarified supernatants were subsequently assayed for lipase activity with synthetic substrates of para-

nitrophenyl-palmitate (Sigma, St Louis, MO, USA). Expression supernatant of *P. pastoris* strain carrying original pGPAPαA was simultaneously used for control.

The same procedures were also conducted to prepare lipase solutions for temperature, pH optima or substrate specificity tests. However, a bigger volume of cultures was needed due to the low lipase activity of 6 days old expression cultures. Thus, each culture was prepared in a 2 l flask, in which 1% (v/v) freshly prepared-overnight culture was inoculated in 500 ml YPG. Prior to lipase assays, the expression cultures were clarified by centrifugation as described above and supernatants were concentrated by using 10 kDa cut-off membrane (Milipore, Schwalbach/Ts., Germany).

2.2.3. Lipolytic activity assays and substrate specificity

Lipase activity was determined in 1 ml plastic cuvette according to Kok *et al*. (1993) using p-nitrophenyl esters (Sigma-Aldrich) as substrates. To prepare the 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 reaction was terminated after 1 hour of incubation at 37^{o}C, the absorbance (optical density - OD) was read in a spectrophotometer at wavelength of 410 nm. The concentrated supernatant of KM71-pGAPZαA was used as control. Relative lipolytic activity was determined as the differences of OD value between samples and control.

This lipase assay was also applied for the measurement of lipolytic activity from various *F. graminearum* culture supernatants. For these experiments, wheat germ oil (WGO) -induced supernatant was prepared as described by Voigt *et al*. (2005) and assayed with p-nitrophenyl palmitate as described above.

2.2.4. Effect of temperature and pH on lipolytic activity

In order to investigate the effect of temperature, the concentrated supernatant of each lipase was incubated with its optimal substrate (resulted from substrate specificity assay) in Bis-Tris-Propane buffer (pH = 7) at different temperatures

ranging from 4 to 70^{o}C (indicated in result section). Lipolytic activity was also conducted as described above.

Effect of pH values on lipolytic activity was determined by using the following buffer systems: 100 mM Citric phosphate buffer (pH values: 2 to 4), 50 mM MES (pH values: 5 and 6), 50 mM Bis-Tris-Propane buffer (pH values: 7 to 9), and 50 mM Na_2CO_3 (pH values: 10 to 12). Due to the affect of different pH values on the formation of colour of the liberated *p*-nitrophenol, all reactions were adjusted to pH 7.5 by either 1 N NaOH (for lower pH values than 7.5) or 1N HCl (for higher pH values than 7.5) before measurement (Zivkovic, 2003).

2.3. Gene disruption methods in *F. graminearum*

2.3.1. Gene disruption by split-selection marker recombination technique

To improve the rate of homologous integration, the split-selection marker technique was used, in which a selectable marker gene is split up into two overlapping, non-functional parts (Fairhead *et al*., 1996). A hygromycin resistant gene hph (HYG) containing selection marker cassette was used. For disruption of a gene by this method, two DNA fragments are required. Each fragment contains a flanking region of the target gene and an overlapping region of the selection marker cassette (figure 3). In general, three steps of PCR were established for the whole synthesis of one disruption construct. (1) Approximately 600 - 900 bp DNA 5'-end and 3'-end flanking fragments of each lipase gene were amplified from genomic DNA (gDNA) by polymerase chain reaction (PCR) with the use of its specific primer pairs upF/R and doF/R (section 2.1.3), respectively. One of the two primers is a fusion primer, which contains an overlapping region with HYG. (2) 5' and 3' flanking DNA fragments were fused with *Sma*I-excised HYG fragment (pGEM-T containing HYG cassette was provided by Dr. Lösch, University Hamburg) in two separate PCR reactions. No primer was used in these PCRs as the fragments acted as primers and templates for the elongation (figure 3). The fused DNA fragments are named up-HYG and HYG-do. (3) Amplification of DNA fragments was achieved by using nested primers. Nested forward primers (primer 5) and HyR (primer 8) were used to amplify the up-HY fragments; YG-do fragments were amplified by using nested reverse primers

(primer 8) with YgF primer (primer 7) (section 2.1.3). The up-HY and YG-do have a 1049 bp overlapping region called “Y”, and each was truncated in HYG cassette, a 347 bp fragment and a 365 bp fragment, respectively. To disrupt one lipase gene, a mix of 20 µg PCR products (both fragments) was directly used for transformation.

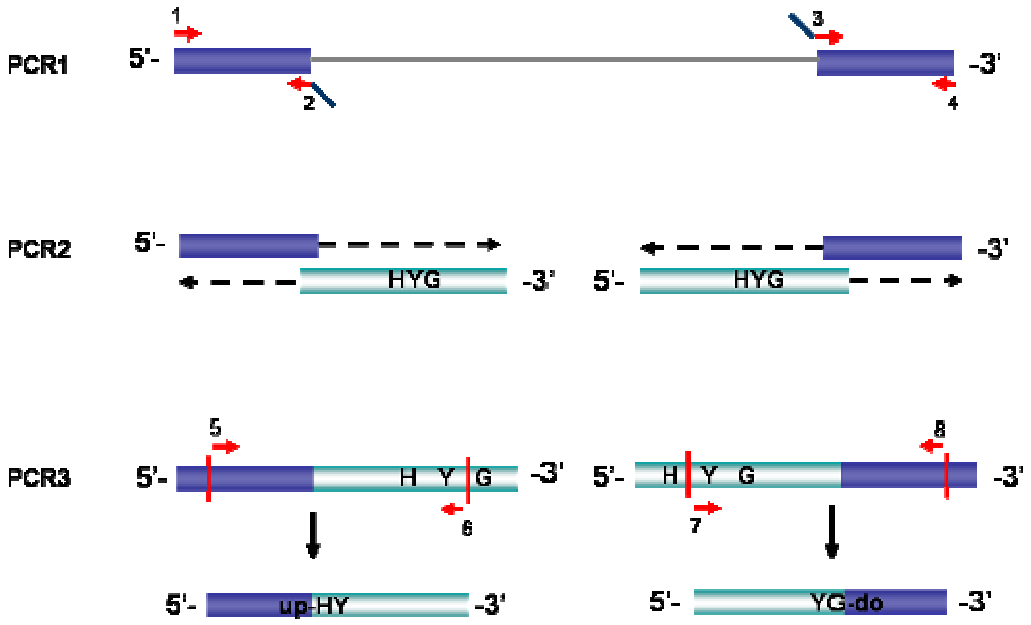

Fig. 3. Strategies to generate a split-selection marker construct by PCR. PCR1: two flanking fragments of a target gene are amplified with the use of primer pair 1 and 2 for 5’ homologous fragment, and primer pair 3 and 4 for 3’ homologous fragment. PCR2: two flanking fragments are fused with HYG fragments in two separate PCR reactions. PCR3: propagation of a fused fragment by the use of nested primer pair 5 and 6 for up-HY fragment, primer pair 7 and 8 for YG-do fragment. (up-HY fragment consists of 5’ homologous fragment of the target gene and 1414 bp fragment of HYG; do-YG consists of 1396 bp fragment of HYG and 3’ homologous fragment of the target gene).

All PCRs were carried out by using Taq DNA polymerase (Fermentas) with the following conditions: 1 x PCR buffer; 2.5 mM $MgCl_2$; 0.2 mM dNTPs; 0.4 µM specific primers; 0.25 U Taq DNA Polymerase; 50 – 100 ng genomic DNA. With some modified conditions in PCR2: 150 ng purified DNA fragments (upstream, downstream) were used with an equal amount of HYG fragment, and in PCR3: PCR products of PCR2 were used as templates.

Program of PCR1:

Cycles	Thermal settings
1x	94°C, 4 min
35x	94°C, 0.5 min; 52°C to 58°C, 0.75 min; 72°C, 1 – 1.5 min
1x	72°C, 10 min

Program of PCR2:

Cycles	Thermal settings
1x	94°C, 4 min
20x	94°C, 0.5 min; 60°C, 2 min; 72°C, 3.5 – 4 min
1x	72°C, 10 min

Program of PCR3:

Cycles	Thermal settings
1x	94°C, 4 min
35x	94°C, 0.5 min; 52°C to 58°C, 0.75 min; 72°C, 3.5 – 4 min
1x	72°C, 10 min

2.3.2. Gene disruption by double homologous recombination

In this study, four split-selection marker constructs were synthesized to disrupt *FGL2*, *FGL3*, *FGL4*, and *FGL5*. The same procedures were also used in synthesis of double homologous recombination constructs for gene disruption, in which a selection marker cassette (HYG cassette) was flanked by two homologous or flanking fragments (figure 4). In order to generate the constructs, an equal amount of two gel-purified flanking fragments generated from PCR1 (150 - 200 ng) were mixed in one PCR reaction with a double amount (300 - 400 ng) of *Sma*I-excised HYG fragment. Nested primers were also used to propagate the desired PCR products, and then ligated into cloning vector pGEM-T. Before fungal transformation, the recombinant pGEM-T plasmids were digested with appropriate enzymes to release the disruption construct. A solution of 50 – 100 µl of 30 µg DNA was used for *F. graminearum* transformation.

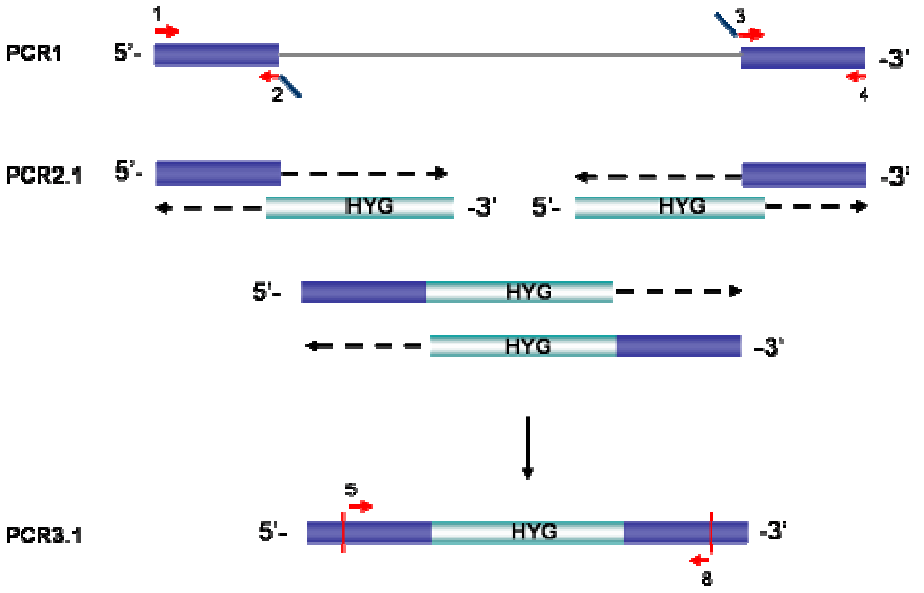

Fig. 4. Modified strategies to generate a double homologous recombination construct (disruption construct). PCR1: Described in figure 3. PCR2.1: Two flanking fragments amplified from PCR1 (figure 3) are fused with HYG fragment in one PCR reaction. In first PCR cycles, each flanking fragment is fused with HYG fragment, which results in up-HYG and HYG-do fragments. These fragments contain the same HYG, which then anneal each other in later PCR cycles and elongated. PCR3.1: propagation of disruption constructs by the use of nested primers 5 and 8. PCR conditions described for synthesis of split marker construct are applied. Only longer elongation times are changed according to the expected size of each construct.

2.3.3. Construction of the expression system of the green fluorescence protein driven by lipase 1 promoter

To generate a DNA construct in which the *FGL1* promoter is used to regulate the expression of the green fluorescence protein (GFP) gene, a fusion polymerase chain reaction (PCR) approach was employed. Initially, a 2473 bp upstream fragment included the first 15 nucleotides in the 5' end of *FGL1* open reading frame (ORF) was cloned from genomic DNA by PCR with the use of primer pair Fgl1ProF/R. This fragment was then fused with an 1146 bp fragment containing GFP ORF, which was also synthesized by PCR with the use of primer pair Fgl1promGfpF/GfpR and pICPAPA plasmid (Lee *at al*., 2003) as templates. A fusion PCR approach with two steps was used. Firstly, the *FGL1* promoter and GFP ORF fragments were fused by

a fusion PCR with the following program: 94^{o}C for 3 minutes, following 20 cycles of 94^{o}C for 30 seconds, 56^{o}C for 2 minutes and 72^{o}C for 3.5 minutes, and 10 minutes for final extension at 72^{o}C. No primer was used in this PCR (see 2.3.1). Secondly, these PCR products were used as templates for PCR amplification with the use of nested primers Fgl1XbaIF and Fgl1HindIIIR containing restriction sites for *Xba*I and *Hind*III, respectively. Finally, a 2160 bp fragment was produced. This DNA fragment was ligated into *Xba*I and *Hind*III sites of fungal transformation vector pAN7.1 to generate pAN7promFGL1-GFP.

2.3.4. Construction of the expression system of the fusion protein of lipase 1 with the green fluorescence protein

A similar PCR strategy was also used to generate a fusion construct of lipase gene 1 with GFP ORF. In this construct, the 5'end-truncated *FGL1* gene was tagged with GFP gene via a linker containing 15 nucleotides (GGTGCTGGTGCTGGT). To generate the construct, the following steps were used. Firstly, an 883 bp fragment of *FGL1* gene and an 1149 bp fragment containing GFP gene were amplified from genomic DNA of wild type *F. graminearum* and pPICPAPA plasmid by using primer pairs FGL1GfpF/R and Gfp5aaF/GfpR, respectively (primers, see 2.1.3). In the 883 bp fragment, the stop codon (TAG) of *FGL1* gene was eliminated. The 1149 bp fragment has an overlapping region with 3' end of the truncated *FGL1* gene, and the starting codon (ATG) of GFP gene was also eliminated. Secondly, these DNA fragments were fused in a fusion PCR with the following program: 94^{o}C for 3 minutes, following 20 cycles of 94^{o}C for 30 seconds, 56^{o}C for 2 minutes and 72^{o}C for 2 minutes, and 10 minutes of final extension at 72^{o}C. To achieve the fused fragments, nested primers FGL1GfpnF and GfpHindIIIR, which have restriction sites for respective enzymes *Xba*I and *Hind*III, were used. The amplified fragments (1828 bp) were then ligated into *Xba*I and *Hind*III sites of vector pAN7.1 to produce pANt.FGL1::GFP.

2.4. Transformation-mediated gene disruption

In this study, various gene disruption constructs were generated, which included four spit marker homologous recombination constructs for *FGL2*, *FGL3*, *FGL4*, and

FGL5, four double homologous recombination constructs for *FGL3*, *FGL9*, *FGL12*, *FGL13*, and *FGL14* (pGEM-FGL3, -9, -12, - 13, and -14), one construct of promoter region of *FGL1* in fusion with GFP gene (pANpromFGL1-GFP), and one construct for expression of FGL1::GFP fusion protein (pANtFGL1::GFP). These constructs, which all contain hygromycin resistant cassette hph as selective marker, were used to transform wild type *F. graminearum* strain 8/1 via protoplast transformation method.

In the beginning of this thesis, the transformation protocol described by Jenczmionka (2004) was used to transform *FGL2*, *FGL3*, *FGL4*, and *FGL5* disruption constructs. In the later transformation protocol, which was used to transform *FGL9*, *FGL11* to *FGL14*, pANpromFGL1-GFP, and pAN-FGL1::GFP, some modifications for protoplastation were applied as follows: (1), 3.10^4 conidia were cultured in 50 ml CM medium in a 100 ml Erlenmeyer flask and incubated over-night at 28^{o}C and 150 rpm. (2), the overnight culture was added to 150 ml fresh CM. This culture was then blended in a 1 l Waring-blender three times, each repetition lasted for 10 seconds. (3), 50 ml of this blended culture were cultivated in 150 ml fresh CM medium in a 500 ml Erlenmeyerflask overnight at 24^{o}C, 150 rpm. Protoplast preparation followed the steps as described by Jenczmionka (2004).

2.5. Southern blot analysis

Genomic DNA of wild-type and knock-out (KO) strains were isolated and restricted overnight with appropriate enzymes. The digested DNA was then separated on 0.8% agarose gel by electrophoresis with a voltage of 80 – 100 V. 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 upstream regions of the target genes when split marker constructs were used, or from the coding sequence of the gene when double homologous recombination constructs were used. Detection and visualization procedures were followed according to the manufacturer's manual (Roche).

Otherwise noted, re-hybridization with Hyg probe was also conducted to select single copy of Hyg- vector integrated strains. In some transformations, due to the presence of vector pGEM as the backbone of construct in the transforming DNA solutions, PCR was used to confirm its non-occurrence in the genome.

2.6. Pathogenicity tests of lipase disruption strains

2.6.1. Virulence assays on wheat heads

Susceptible spring wheat Nandu *c.v*. (Lochow-Petkus, Bergen-Wohlde, Germany) was used for virulence assay. 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, 2005). The inoculated spikes were enclosed in small plastic bags misted with water during the first 3 days and monitored up to three weeks post-inoculation. For each disrupted lipase gene strain, three independent mutant strains were inoculated. Wild type conidia with the same concentration were used for virulence comparison. Spikes were inoculated with 20 µl pure water as negative control.

Wheat spikes inoculated with the wild type and lipase knock-out strains prepared for RNA isolation at different time courses (as indicated in the result section) were similarly carried out.

2.6.2. Virulence assays on maize cobs

Maize inbred line A188 (Green and Philips, 1975) was grown in a green house (temperature: 26^{o}C - 30^{o}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 x 10^4 conidia per ml of *F. graminearum* wild type strain and lipase disruption strains. 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 inoculation lasted for five weeks. Disease severity assessments were determined as described by Reid and Hamilton (1995).

2.7. Phenotypic assays *in vitro*

2.7.1. Detection of extracellular lipolytic activity of lipase disruption strains

For pre-cultivation of *F. graminearum* wild-type and lipase disruption strains, 50 ml YPG medium were inoculated with 3.10^5 conidia and incubated for 2 days at 28^{o}C and 150 rpm. The mycelia were collected by filtering with 200 µm diameter sieve and were washed out by rinsing three times with double-distilled water. Afterwards the mycelia were transferred into flasks containing 50 ml water supplemented with 2% (v/v) of wheat germ oil. Induction was conducted at 28^{o}C and 150 rpm. 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). 100 µl of the clarified supernatants were mixed with 900 µl of the reaction buffer (as described in 2.2.3). The assay was carried out in 1 ml cuvette at 37^{o}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 amount of lipase transcripts in the presence of glucose were similarly performed. Pre-cultures of wild type mycelia were prepared as described above. The washed mycelia were inoculated one additional day in water with the supplementation of either 2% (v/v) wheat germ oil, or 1% glucose, or 1% glucose plus 2% (v/v) wheat germ oil. Samples were taken after 4 hours of induction for RNA isolation. Lipolytic activity of samples taken after every 4 hours of induction were assayed described in 2.2.3.

2.7.2. Growth assays

The growth rate of lipase disruption strains was determined by placing a mycelial plug (approximately 0.5 cm^2) on agar plates of CM and YPG medium as well as in SNA medium supplemented with either 2% (v/v) wheat germ oil (WGO), or 2% Triolein, or 2% Olive oil, or 2% Tributyrin as the sole carbon sources. The cultures

were incubated at 28^{o}C for 5 days. Diameter of mycelial colonies was manually measured by using a technical ruler.

2.7.3. Easily wettable phenotype assays

To determine the hydrophobicity of aerial hyphae of lipase disruption strains, 5 days old mycelial cultures grown on CM agar were placed with water drops on the surface of mycelia. The retention was monitored for 30 min, and pictures were taken by a Canon digital camera. When water drops were sucked into the mycelial layer the strain was considered as easily wettable phenotype (Nakayashiki *et al*., 2005).

2.8. Expression analysis

2.8.1. Expression analysis by reverse transcription (RT) PCR and semi-quantitative RT-PCR

To study the expression of these lipase genes *in vitro* and *in planta*, RNA from various samples was isolated by using Qiagen RNAeasy Kit (Qiagen, Germany). To verify the expression of 16 lipases at transcriptional level, wild type *F. graminearum* RNA was isolated from wheat germ oil-induced mycelia after 12 and 24 hours of induction. Single stranded complementary DNA (cDNA) was then synthesized by using ReverseAid M-MuLV Reverse Transcriptase and oligo $dT_{(18)}$ as primer (Fermentas, St. Leon-Rot, Germany). Semi-quantitative PCR was carried out for evaluation of relative amount of the lipase transcripts from synthesized cDNA. The PCR program consisted of an initial denaturation step for 3 min at 94^{o}C, followed by 25 cycles of denaturation at 94^{o}C for 30 s, annealing at 55^{o}C for 45 s, and extension at 72^{o}C for 35 s, the final elongation step was performed at 72^{o}C for 10 min. In these PCR reactions, the specific primers were used (see 2.1.3). Expression of a housekeeping gene beta-tubulin (β-tub) was used as control.

2.8.2. Northern Blot

Wheat spikes inoculated with *Δfgl2* strain for three days were implemented with 2 µl of 0.1% (v/v) palmitic acid (16:0) (Merck, Darmstadt, Germany); 2 µl of 0.1% (v/v) γ-linolenic acid (18:3) (Sigma), and 2µl ethanol (control). Infection was kept for one additional day. Total RNA was isolated by using NucleoSpin® RNA Plant Kit

(Macherey-Nagel, Düren). Detection of RNA transcripts of *FGL1* were carried out by using *FGL1* DNA probe, which was generated by PCR. Hybridization and detection steps were carried out as described in the manufacturer manual (Roche).

2.9. Microscopic analysis

Induction of promoter *FGL1* in *promFGL1-GFP* strains was carried out by using wheat germ oil, triolein, and tributyrin (see 2.1.2). Mycelia expressing the green fluorescence protein (GFP) were examined under a stereo fluorescence microscope Leica MZ FLIII using the filter set Leica GFP2 (excitations 480/40 nm, barrier filter 510 nm). A wild type strain expressing GFP was used as control.

2.10. Accession numbers

Sequence data of lipase genes *FGL2*, *FGL3*, *FGL4*, and *FGL5* from *F. graminearum* strain 8/1 have been deposited in Genbank under the following accession numbers: ***FGL2*****:** EU191902**;** ***FGL3*****:** EU139432**;** ***FGL4*****:** EU191903**;** ***FGL5*****:** EU402385**.**

3. Results

3.1. Characterization of lipases

3.1.1. Computer-based identification of secreted lipase genes

Identification of lipase gene sequences was based on the annotated sequences of *F. graminearum* from the MIPS *Fusarium graminearum* Genome Database (FGDB: http://mips.gsf.de/genre/proj/fusarium/). Initially, the key word ‘’lipase’’ was used to search for maximal lipase entries in the FGDB database. A list of 74 entries was retrieved, including groups of phospholipases, hormone sensitive lipases, lipases, and triacylglycerol lipases. The two latter groups consisting of 39 entries were manually selected. Further analyses of these entries based on following criteria were applied: presence of a leading sequence for secretion, bearing a putative lipase domain GXSXG. To identify the leading sequence, deduced amino acid sequence was analyzed by using SignalP 3.0 Server, an online-software used for the prediction of signal peptides of proteins (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen *et al*., 2004; Emanuelsson *et al*., 2007). To identify the lipase domain, multiple alignments of the lipase amino acid sequences were carried out by using the CluxtalX program. The sequence of five amino acids, which matched the above criteria, was manually selected. Among them 17 entries were identified. Similar to two investigated lipases *FGL1* (Voigt *et al*., 2005) and *LIP1* (Feng *et al*., 2005), all identified lipase sequences have a leading sequence and a putative lipase domain (figure 3). The 15 newly identified lipase sequences were designated according to the name of *FGL1* (Voigt *et al*., 2005) from *FGL2* to *FGL16* (table 1).

To confirm the accuracy of annotated sequences, these selected sequences were compared with the ones retrieved from the Fusarium Group Database (http://www.broad.mit.edu/). No difference was found in the annotated sequences between the two databases. Furthermore, the lipase amino acid sequences were also compared with known lipases and computed sequences of fungal lipases by using BLAST tool (BLAST, NCBI). The results showed that identity from 38% to 68% with other fungal lipases was found. This result was also consistent with the data

shown in the SIMAP database (FGDB). This result reflected the diversity of sequences among lipases (Fischer and Pleiss, 2003).

The relevant information of lipase sequences is represented in FGDB. All the identified lipases are predicted to belong to different lipase classes, class I, II, III and V. In the genome, these lipase genes are distributed at different locations and chromosomes. Hence, this indicates that these lipases do not belong to a family. Table 1 is the collective information of the 17 lipases from FGDB.

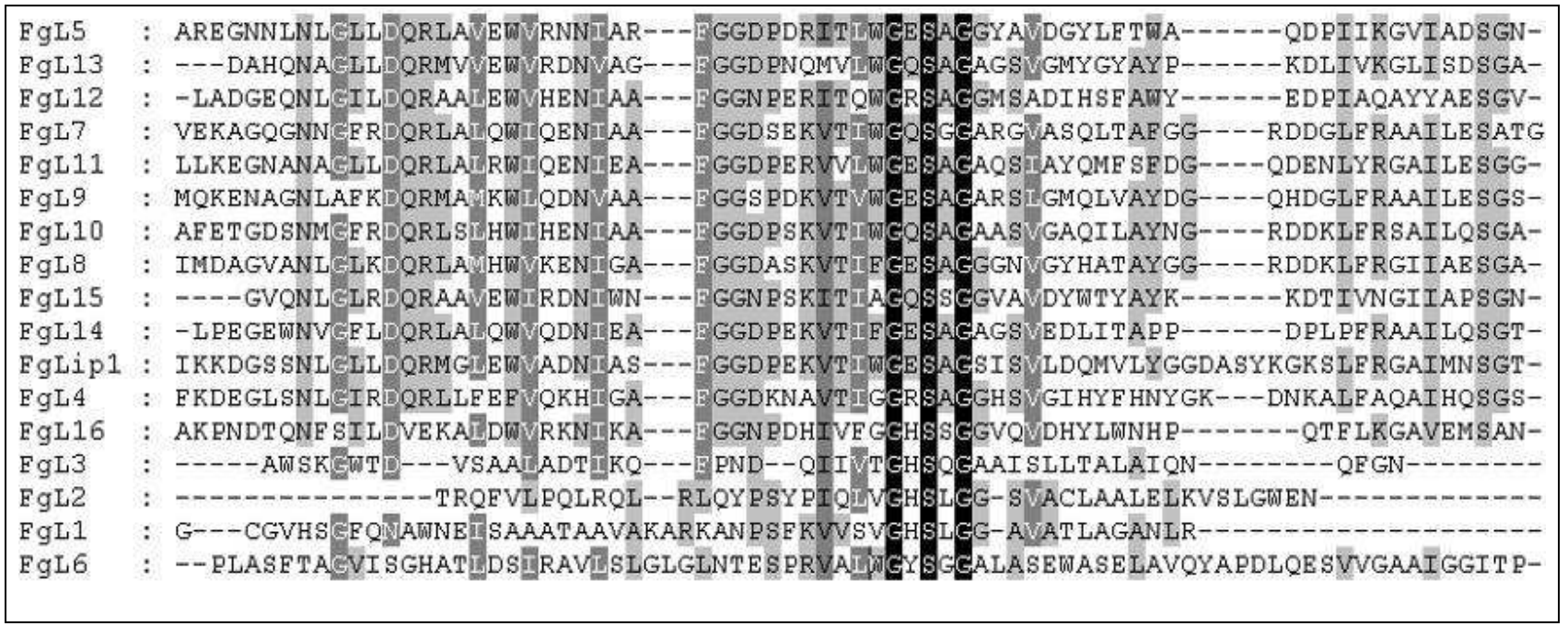

Fig. 5. The representative sequence of 17 identified lipases containing a putative lipase motif. The deduced amino acid sequences of the 17 lipases were aligned using the ClustalX program. All the 17 lipases had the same predicted domain (highlighted in dark shadow), which consisted of pentapeptides GXSXG, where G is glycine; S is serine; and X is among A (alanine), E (glutamic acid), G, H (histidine), L (leucine), Q (glutamine), R (arginine), S, and Y (tyrosine). The order of lipases showed the homologous degrees among the lipase sequences (detail information is shown in figure 6).

Table 1: List of 17 putatively secreted lipases identified from genomic sequence of *F. graminearum* strain PH-1. Some common features of the 17 lipases were called in FGDB such as access entry, lipase class, genome coordinates, and number of base pairs (bp)

Name	FGDB entry	Lipase class	Genome Coordinate	Length (bp)	Comment
FGL1	Fg05906	III	40254 - 39095 (C)	1160	Published by Voigt *et al*. 2005
FGL2	Fg01240	III	42206 - 43432 (W)	1227	**This study**
FGL3	Fg04818	III	30037 - 31119 (W)	1083	**This study**
FGL4	Fg03209	II	45730 - 47400 (W)	1671	**This study**
FGL5	Fg03583	V	46904 - 48511 (W)	1608	**This study**
FGL6	Fg03846	I	79390 - 78050 (C)	1341	Being characterized
FGL7	Fg09181	II	40179 - 41849 (W)	1671	Being characterized
FGL8	Fg03243	II	63550 - 66083 (W)	2534	Being characterized
FGL9	Fg03687	V	45359 - 43483 (C)	1877	**This study**
FGL10	Fg10713	V	216573 - 214773 (C)	1801	**This study**
FGL11	Fgd406-620	V	164221 - 162494 (C)	1728	Being characterized
FGL12	Fg03012	V	133250 - 134964 (W)	1715	**This study**
FGL13	Fg03095	V	6965 - 5295 (C)	1671	**This study**
FGL14	Fg05935	V	108726 - 110401 (W)	1676	**This study**
FGL15	Fg09099	V	164827 - 166450 (W)	1624	Being characterized
FGL16	Fg11386	II	137438 - 139216 (W)	1779	Being characterized
LIP1	Fg01603	V	35522 - 37288 (W)	1767	Published by Feng *et al*. 2005

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

3.1.2. Lipase sequence homology

Although lipases share several common features such as structural fold, identical catalytic machinery, they appear to be variable in amino acid sequence. Construction of the dendrogram of the 17 deduced amino acid sequences revealed three major branches of lipases (figure 6). The first branch consists of *FGL6*; it is classified as lipase class 1 (table 1). Particularly, it was shown that *FGL6* has 56% sequence identity with *Tri8*, a gene encoding an esterase that adds or removes ester groups in the biosynthetic pathway of trichothecenes (McCormick and Alexander, 2002). The second branch includes *FGL1*, *FGL2*, and *FGL3*. Closely related to the previously examined *FGL1* is *FGL2*; these two lipases have the same putative lipase domain GHSLG. In comparison with other fungal lipases, *FGL2* has 48.1% identity with the *Aureobasidium pullulans* lipase (access No.: EU117184); *FGL3* shares 39% identity with a secreted lipase *Lip1* (XP_001732204) from the lipophilic fungus *Malassezia globosa*, whose lipase family was supposed to be important for harvesting host lipids and, thereby, important for pathogenesis (Xu *et al*., 2007). These lipases are classified as class III among the 17 putatively secreted lipases in the *F. graminearum* genome. The third branch includes the remaining lipases. They belong to either class II or V (table 1). Among them, *FgLIP1* or *LIP1* is a characterized lipase. Sharing the highest similarity with *LIP1*, a non-virulence factor, is *FGL4*. In the genome, *FGL4* is located near to a putative cutinase transcription factor (fg03207). This branch also included *FGL5, FGL7*, *FGL8*, *FGL9*, *FGL10*, and *FGL11*, *FGL12*, *FGL13*, *FGL14*, *FGL15*, and *FGL16* (figure 6). Besides *LIP1*, none of the lipases of this group has been investigated previously.

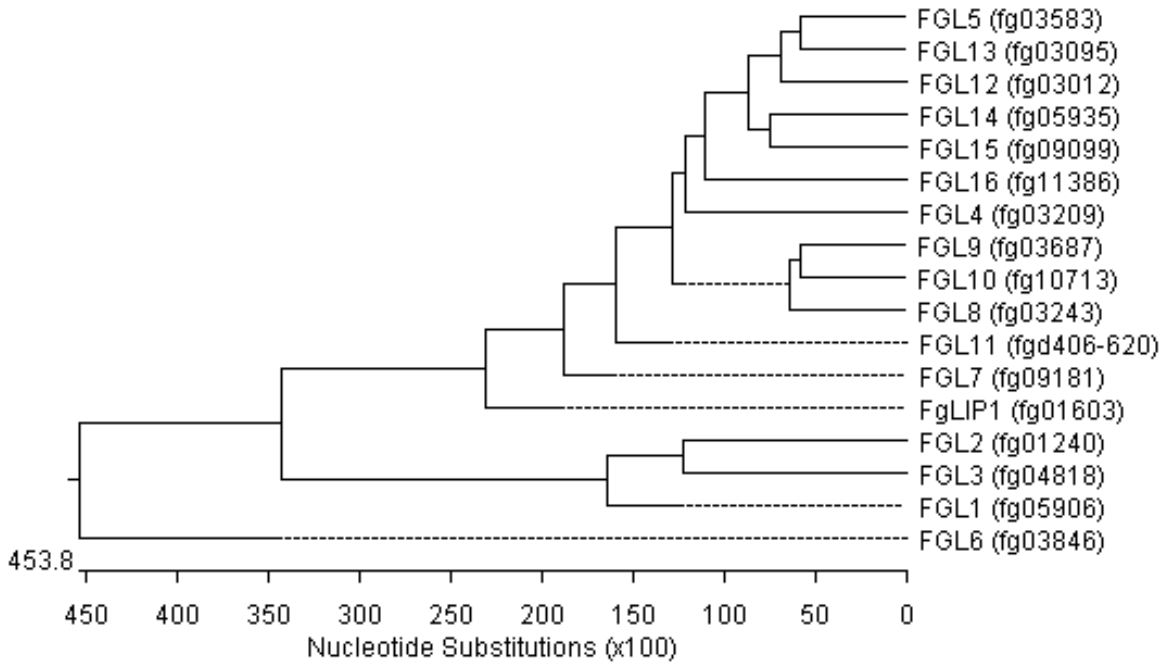

Fig. 6. Phylogenetic relationship of the 17 putative lipases in *F. gramineaum* PH-1 strain. The deduced amino acid sequences of the lipases were retrieved from FGDB as follows: *FGL1* (fg05906); *FGL2* (fg01240); *FGL3* (fg04818); *FGL4* (fg03209); *FGL5* (fg03583); *FGL6* (fg03846); *FGL7* (fg09181); *FGL8* (fg03243); *FGL9* (fg03687); *FGL10* (fg10713); *FGL11* (fg09832); *FGL12* (fg03012); *FGL13* (fg03095); *FGL14* (fg05935); *FGL15* (fg09099); *FGL16* (fg11386); *LIP1* (fg01603, alternative: fg13207). The sequences were aligned by ClustalX 1.83 and then viewed by TreeView 1.6.6. The 17 lipases formed three branches: branch 1: *FGL6*; branch 2: *FGL1* to *FGL3*; branch 3: *FGL4* to *FGL16*, and *LIP1*.

3.1.3. Verification of lipase genes in *F. graminearum* strain 8/1

The presence of the 16 lipases was verified by PCR from genomic DNA using their specific primer pairs (section 2.1.3). As shown in figure 7, only one PCR product was amplified in each reaction and as expected the size of the DNA fragments was similar. This result indicated that these lipase genes were specifically amplified and present in *F. graminearum* 8/1. Remarkably, amplification of *FGL10* gave no PCR product. Thus, a new specific primer pair was designed. In these experiments, genomic DNA of *F. graminearum* 8/1 as well as PH-1 strain (FGSC 9075, NRRL 31084) was simultaneously tested. In agreement with the above result, strain 8/1 gave no PCR product, whereas the PH-1 strain was positive with one specific PCR band (data not shown). The consistent results were also found when single stranded cDNA was used as

template (figure 8 and 9). This indicates that the *FGL10* is likely absent in *F. graminearum* 8/1 strain.

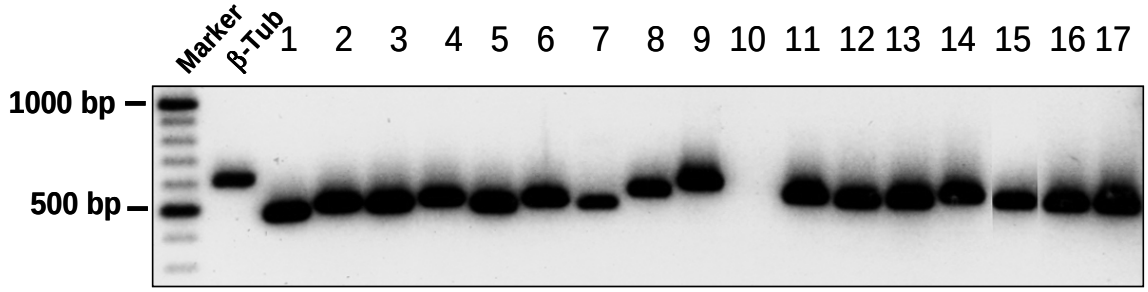

Fig. 7. Verification of the existence of the 16 lipase genes in *F. graminearum* strain 8/1. PCR products of the 16 lipases were specifically amplified from gDNA of *F. graminearum* strain 8/1, except lipase 10. All the PCR reactions were performed with the same conditions as follows: 50 ng of gDNA was used in 25 µl PCR reactions as templates with the same components as listed in section 2.3.1. PCR program was conducted as follows: 94°C for 3 minutes, following 30 cycles of 94°C for 30 seconds, 55°C for 45 seconds and 72°C for 35 seconds. 10 minutes of extension at 72°C was used to complete the synthesis. 10 µl of PCR solution was loaded on 0.8% agarose (w/v) gel. Amplification of beta-tubulin was used as a positive control. Marker: GeneRuler™ Mix (Fermentas).

3.1.4. Expression of lipases *in vitro* with induction of wheat germ oil

To study the expression of the lipases *in vitro*, transcriptional expression of lipases was examined. Following the procedure that was used to analyze expression of *FGL1* (Voigt *et al*., 2005), wheat germ oil-induced culture of the wild type *F. graminearum* was prepared. Total RNA isolated from the mycelia was used for synthesis of single stranded cDNA. Semi-quantitative PCR reaction was used to evaluate the relative expression level of the lipases. As shown in figure 6, the relative amount of PCR products is significantly different. This suggests that the lipases are expressed at differential amounts in the same growing conditions. Significantly, the lipases *FGL1*, *FGL6*, *FGL8*, *FGL9*, and *LIP1* are strongly expressed in comparison with *FGL4*, *FGL12*, *FGL13*, *FGL14*, and *FGL15*. Transcription of these genes is comparable to transcription of the housekeeping gene beta-tubulin after 12 and 24 hours of induction (figure 8). Interestingly, whereas most of the lipases show increased amounts of transcripts for longer culture time (24h), transcripts of *FGL5* are clearly decreasing.

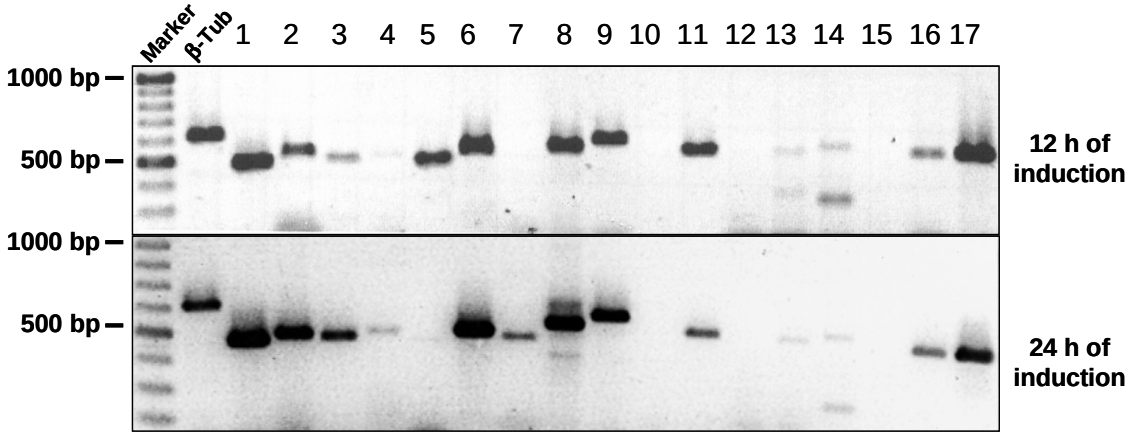

Fig. 8. Detection of lipase transcripts *in vitro*. Total RNA from wheat germ oil-induced culture of wild type *F. graminearum* was isolated after 12 and 24 hours. Single stranded cDNA was generated and used as templates in RT-PCR. The same cDNA amount and numbers of elongation cycles were used in semi-quantitative PCR reactions. 10 µl of the PCR solution was loaded on 0.8% (v/v) agarose gel. Lanes 1 to 17 correspond to *FGL1 -16* and *LIP1*. β-Tub: housekeeping gene beta-tubulin used as control. Marker: GeneRuler^{TM} Mix (Fermentas).

3.1.5. Lipase transcript detection during colonization of wheat heads

To examine the transcriptional expression of extracellular lipases during plant infection, relative amount of RNA transcripts was measured at different time points of infection (dpi) on wheat spikes (indicated in figure 9). Examinations of lipase gene expression were performed at the following time points during wheat infection: germinated conidia (1 dpi); colonization of the inoculated spikelet (3 dpi); spreading within the spikelets (7 dpi); complete colonization of the spike (14 dpi). Total RNA was isolated and cDNA was synthesized. cDNA derived from infected wheat spikelets 1, 3, 7, and 14 days after infection was used as templates in RT-PCR. Similar PCR conditions that were applied as described in section 3.1.3 were used. In agreement with the result from *in vitro* expression analysis, transcript of *FGL10* is not detected. Most of the remaining lipases are detectable 3 dpi. As shown in figure 9, relative amounts of transcript of the lipases differ from each other. This suggests a different expression pattern of each lipase during wheat infection. Significantly, *FGL1*, *FGL2*, *FGL3*, *FGL6*, *FGL16*, and *LIP1* are amongst the highest expressed lipases at all infection time points. In general, the expression increases at later stages (7 and 14 days) of fungal infection with the exception of *FGL9*, whose transcriptional expression is

dramatically decreased at later infection stages. In comparison of relative lipase transcripts, expressions of *FGL5*, *FGL7*, and *FGL11* to *FGL15* are lower in all infection stages (figure 9).

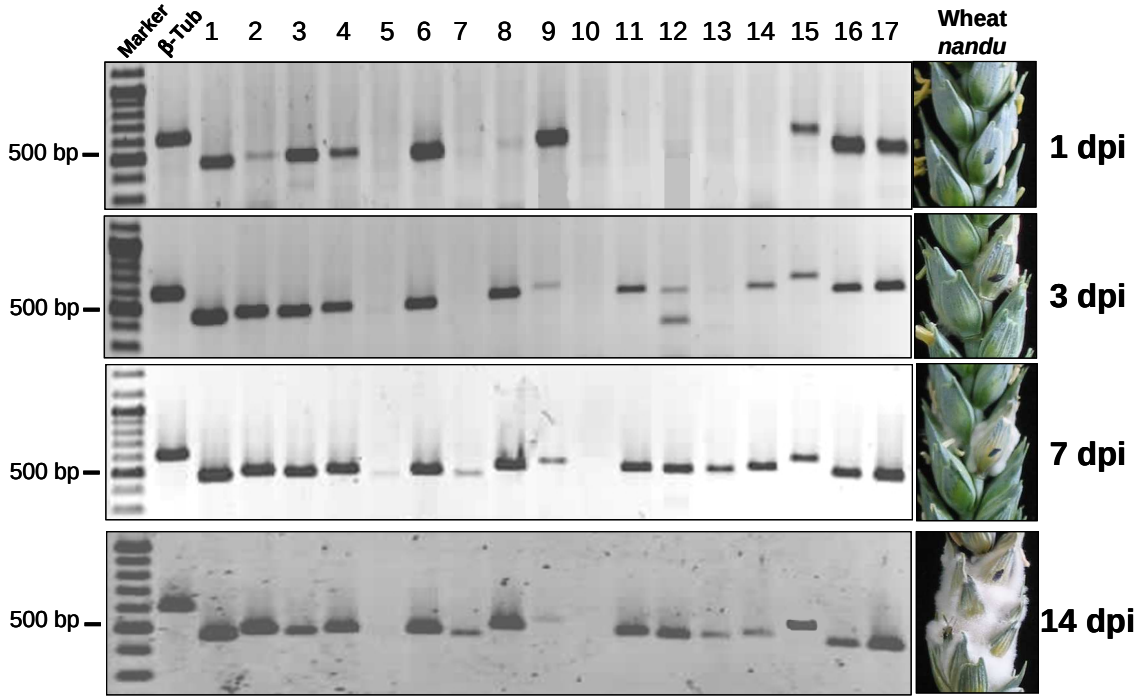

Fig. 9. Detection of lipase transcripts *in planta*. Total RNA from inoculated wheat spikes was isolated. Single stranded cDNA was generated and used as templates in RT-PCR. Equal amounts of cDNA were used as template in PCR reactions. The same elongation cycles were applied in the PCR reactions (25 cylces). 10 µl of PCR solution was loaded on 0.8% (w/v) agarose gel. Dpi: day post infection. Lanes 1 to 17 correspond to *FGL1* to *FGL16* and *LIP1.* β-Tub: housekeeping gene beta-tubulin used as control. Marker: GeneRuler^{TM} Mix (Fermentas).

3.2. Lipase characterization by heterologous gene expression

3.2.1. Lipase 2, 3, 4, and 5 encode triglyceride lipases

To confirm that the cloned genes encode lipases, heterologous gene expression in *Pichia pastoris* was employed. The successful expressions of *F. graminearum* lipase genes in *P. pastoris* are shown by Voigt *et al*. (2005) and Feng *et al*. (2005). Therefore, the similar procedures were used to confirm the encoded gene products of *FGL2*, *FGL3*, *FGL4*, and *FGL5*.

The open reading frame of *FGL3* contains a small intron of 16 bp, which interrupts the putative signal peptide. Thus, to clone the signal peptide-truncated sequence of the *FGL3* gene genomic DNA was used. The remaining lipase

genes contain no intronic sequence. Hence, signal peptide-truncated sequence of *FGL2*, *FGL4*, and *FGL5* were also cloned from genomic DNA for the heterologous gene expression. Section 2.2.2 described the procedures that were used to generate expression plasmids pGAPZαA-FGL2, -FGL3, -FGL4, and -FGL5. These plasmids were used to transform *P. pastoris* strain KM71 by electroporation (2.2.2.3). Initially, more than 30 primary transformants were received from each transformation. 10 fast growing colonies were selectively picked and tested by PCR with the use of pGAP forward and 3'AOX primers (Invitrogen). All PCR reactions were positive, suggesting the integration of recombinant plasmids (data not shown). A small expression scale was set up to screen the lipolytic activity of these recombinant *P. pastoris* strains (section 2.2.2.5). Finally, two transformants which have lipolytic activity are selected: KM71-FGL2.2 and KM71-FGL2.5 for *FGL2*; KM71-FGL3.14 and KM71-FGL3.17 for *FGL3*; KM71-FGL4.1 and KM71-FGL4.2 for *FGL4*; and KM71-FGL5.9 and KM71-FGL5.17 for *FGL5*. To measure extracellular lipolytic activity of lipases secreted by recombinant strains, culture supernatant was used to assay with the synthetic substrate of para-nitrophenyl palmitate (pNPP, C16). As shown in figure 10, the lipolytic activity is detected in the supernatant of *P. pastoris* strains carrying *FGL2*, *FGL3*, *FGL4*, and *FGL5* genes. Culture supernatant of a *P. pastoris* strain carrying pGAPZαA was used as control. These results indicate that the investigated genes encode lipolytic enzymes.

Since lipases have a wide spectrum of substrates, substrate specificity of these lipases was defined. Various synthetic para-nitrophenyl esters were assayed with culture supernatant (figure 10). *FGL2* and *FGL3* show their highest activity against C10 (para-nitrophenyl decanoate), whereas *FGL4* and *FGL5* have maximal activity on C12 (para-nitrophenyl laurate) and C8 (para-nitrophenyl caprylate), respectively (figure 10).

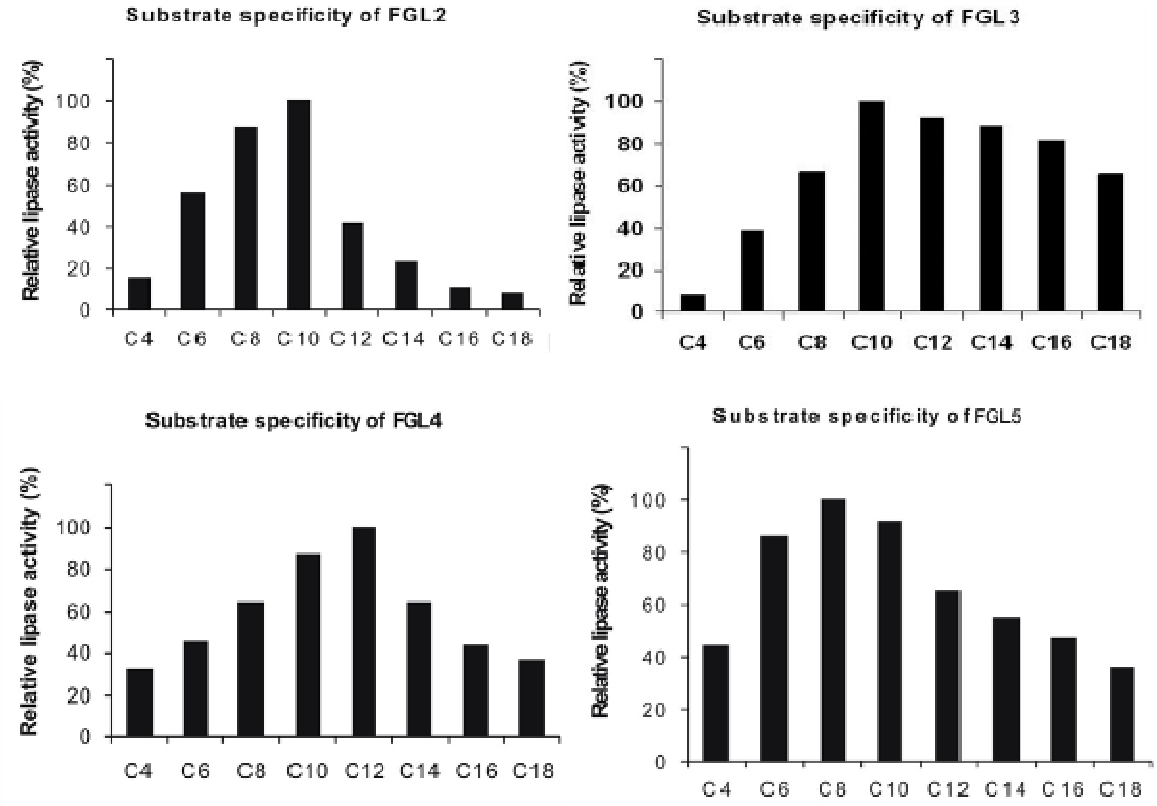

Fig. 10. Substrate specificity of *FGL2*, *FGL3*, *FGL4*, and *FGL5*. Expression culture of *P. pastoris* KM71 carrying lipase genes *FGL2*, *FGL3*, *FGL4*, and *FGL5* were assayed with lipase substrates of para-nitrophenyl esters. The *P. pastoris* strain carrying pGAPZαA was used as a control. C4: para-nitrophenyl butyrate, Mw = 209.2; C6: pNP-hexanoate (caproate), Mw = 237.25; C8: pNP-octanoate (caprylate), Mw = 265.30; C10: pNP-decanoate (caprate), Mw = 293.4; C12: pNP- dodecanoate (laurate), Mw = 321.42; C14: pNP- tetradecanoate (myristate), Mw = 349.5; C16: pNP- hexadecanoate (palmitate), Mw = 377.52; C18: pNP- octadecanoate (stearate), Mw = 405.57. Assays were conducted as described in section 2.2.3.

3.2.2. Temperature and pH optima of *F. graminearum* lipase genes 2, 3, 4, and 5

Following the determination of their substrate specificity, optimum temperatures and pH values of each lipase against its preferred substrate were also measured. pNP-decanoate was used to assay *FGL2* and *FGL3* activity; pNP- dodecanoate and pNP-octanoate were used to assay *FGL4* and *FGL5* activity, respectively. All assays for temperature optima were conducted in 50 mM Bis-Tris-Propane buffer system at pH 7. All four lipases show the highest activity against its optimum substrates at 37^{o}C (figure 11). Similar results are also found in the activity of *F. graminearum FGL1* and *N. haematoccoca NhL1* (Zivkovic, 2003). As also shown

in figure 12, these lipases are the most active at a pH value of 7 when measured at 37°C.

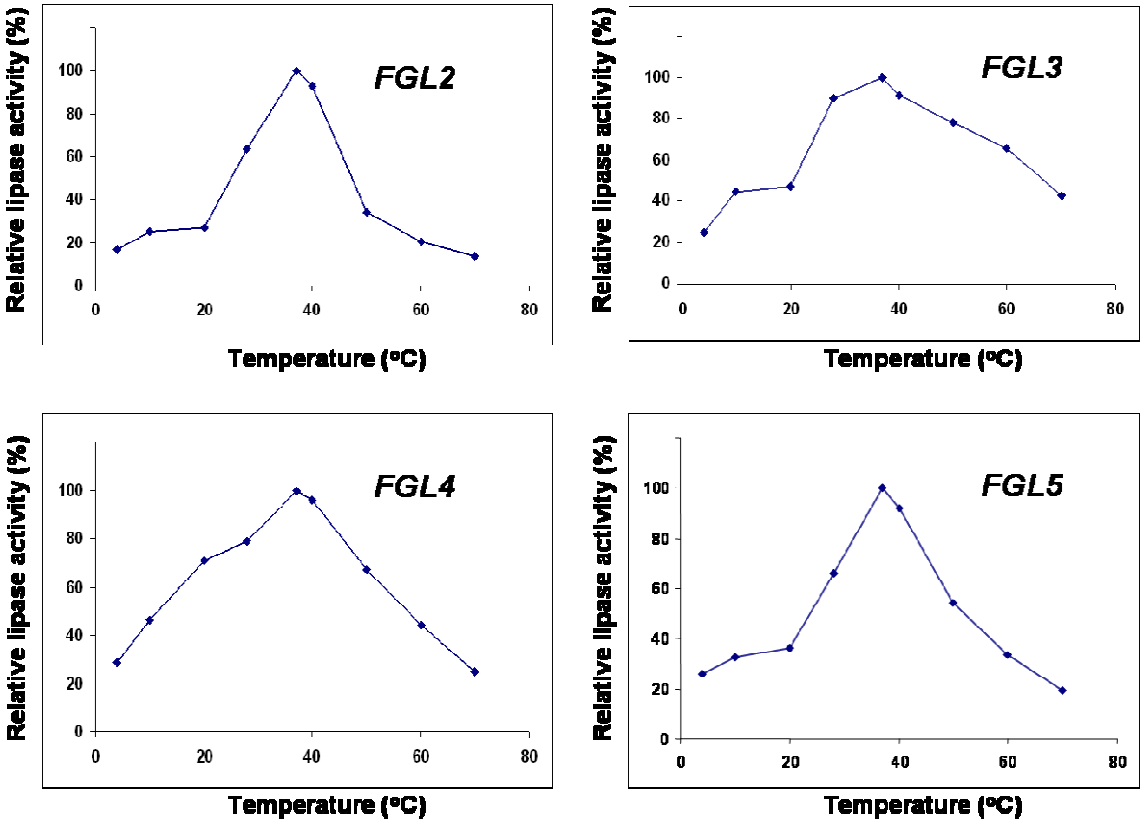

Fig. 11. Optimum temperatures of *FGL2*, *FGL3*, *FGL4*, and *FGL5*. *Pichia pastoris* culture supernatants of *FGL2*, *FGL3*, *FGL4*, and *FGL5* were incubated in Bis-Tris-Propane buffer (pH = 7) for 1 h. Activity was measured with a photometric assay using their optimum substrates at indicated temperatures ranging from 4 to 70°C. All four lipases show a similar spectrum of optimum temperatures.

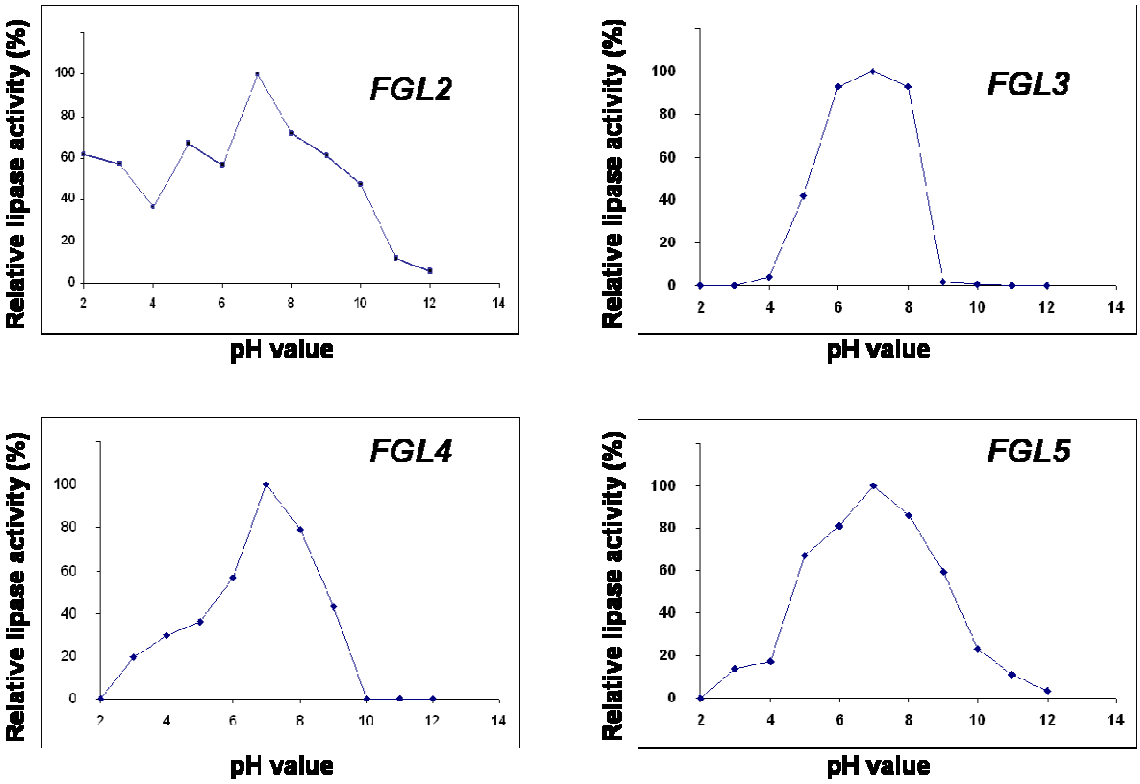

Fig. 12. Optimum pH values of *FGL2*, *FGL3*, *FGL4*, and *FGL5*. *Pichia pastoris* expression supernatant of *FGL2*, *FGL3*, *FGL4*, and *FGL5* were incubated in different buffer systems established by Zivkovic (2003) at 37°C for 1 h. Lipolytic activity was assayed with using their optimum substrates (defined in section 3.2.1, figure 10) at indicated pH values ranging from 2 – 12. Assays were performed as described in section 2.2.3.

3.3. Characterization of lipase genes 2, 3, 4, 5, 9, 12, 13, and 14

3.3.1. Disruption of lipase genes 2, 3, 4, 5, 9, 12, 13, and 14

In the beginning of this study, homologous split-marker recombination (Fairhead *et al*., 1996) was used to disrupt the lipase genes 2, 3, 4, and 5. For lipase gene 3, no disrupted mutant was found, although repeated transformations were carried out. Disruption of lipase 2, 4, and 5 was carried out as described below. Introduction of disruption constructs (PCR products) into *F. graminearum* via protoplast-mediated transformation resulted in four transformants for *FGL2*, six transformants for *FGL4,* and six transformants for *FGL5*. Genomic DNA isolated from single conidia-derived mycelia of these transformants was tested by PCR with the use of their appropriate specific primer pairs. For *FGL2*, two primer pairs FGL2upF/HyR and FGL2doR/YgF were used in two PCR reactions. FGL2upF and FGL2doR, respectively, anneal to 5' and 3' homologous regions outside of

disruption constructs. HyR and YgF anneal to HYG cassette. Due to the fact that two DNA fragments were used for transformation, expected lipase gene disruption strains require positive results in both PCR reactions. With regard of *FGL2*, all four transformants for *FGL2* show positive amplifications in both PCR reactions (data not shown). The mutants were further analyzed by Southern blot (figure 13). gDNA samples isolated from the four transformants and the wild type strain were digested with *Kpn*I for Southern blot examinations. This enzyme has one restriction site in the 5' homologous region outside of up-HY fragment (figure 3) and another site in the end of 3' region of HYG (belong to YG-do fragment) as well as one in 3' homologous region of the wild type sequence (figure 13.A). This digestion results in a 6884 bp sequence from the native sequence (wild type) and a 4809 bp sequence from the mutants when hybridized with a specific probe, which is synthesized by PCR from 5' homologous flanking fragment (figure 13.A). As shown in figure 13.B, a 4809 bp fragment is present in four transformants, but not in the wild type strain. This result indicates that *FGL2* open reading frame is completely disrupted in the four transformants (figure 13.B).

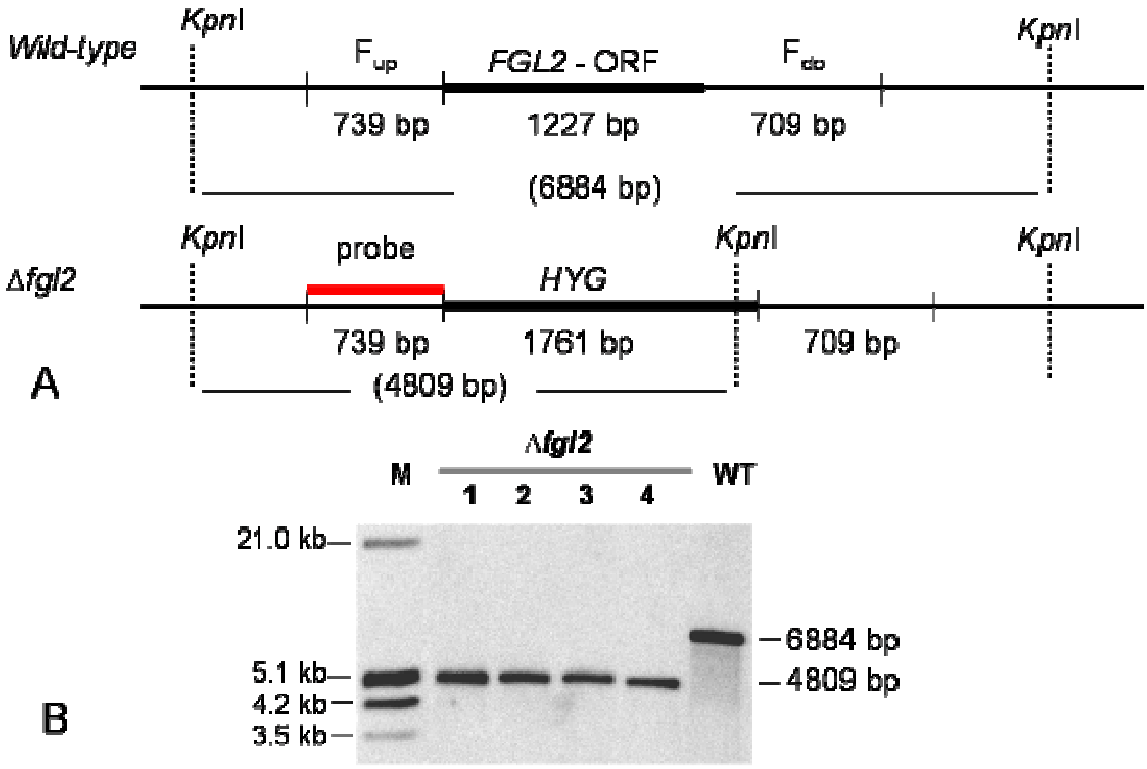

Fig. 13. Knock-out strategy (A) and Southern blot (B) analysis of *Δfgl2*. Enzyme *Kpn*I was used for genomic DNA digestion of wild type and Δ*fgl2* strains; the Southern blot probe was amplified by PCR from 5' homologous fragment (F_{up}) (dark red line). 1 to 4: *FGL2* disrupted mutants *(Δfgl2);* WT*:* wild type, M: dig-labeled marker (Roche).

Similar PCR and Southern blot strategies were also employed for analyses of *FGL4* and *FGL5* transformants. Regarding *FGL4*, *Spe*I was used to digest DNA samples of the transformants and the wild type as well. This digestion resulted in a 2852 bp fragment from the mutants and an 8894 bp from the wild type (figure 14.A). Among six transformants tested, four of them are the desired mutant strains, one transformant carries most likely a double integration of the disruption construct, and the other transformant is likely a false positive one (figure 14.B).

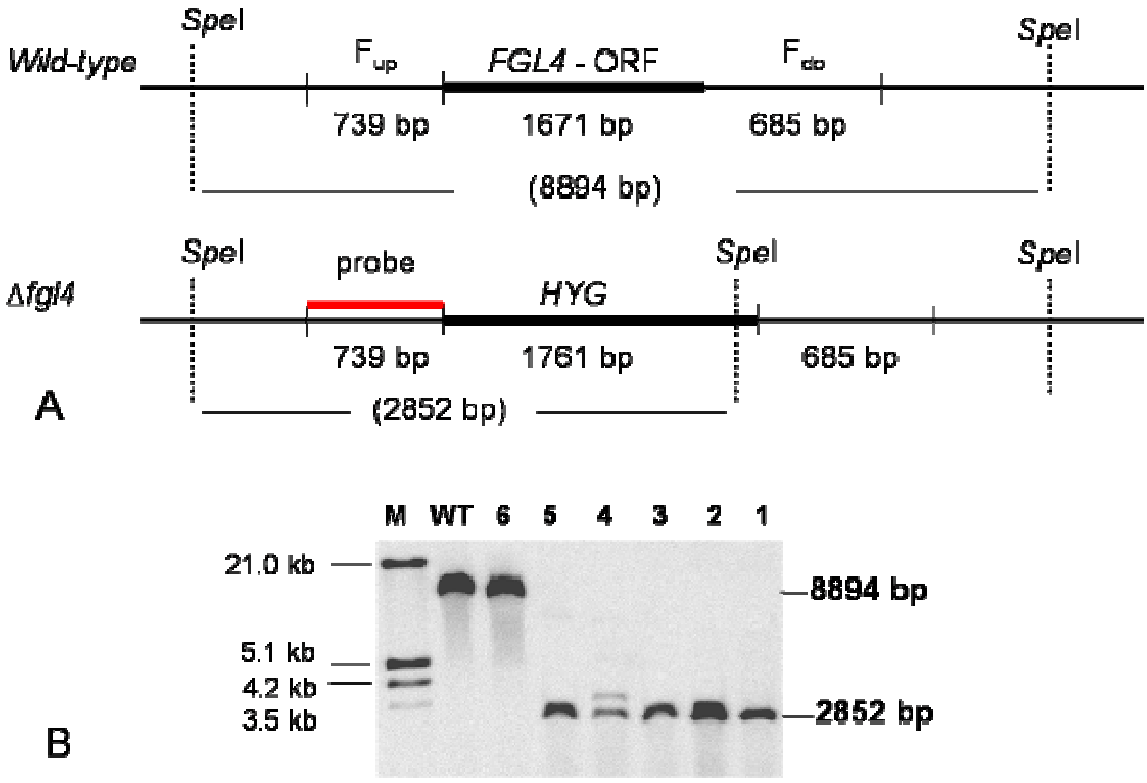

Fig. 14. Knock-out strategy (A) and Southern blot (B) analysis of *Δfgl4*. Enzyme *Spe*I was used for genomic DNA digestion of wild type and Δ*fgl4* strains; the Southern blot probe was amplified by PCR from 5' homologous fragment (F_{up}) (dark red line). 1, 2, 3, and 5: *FGL4* disrupted mutants (Δ*fgl4*). 4: double HYG integration; 6: false positive transformants, respectively. WT*:* wild type, M: dig-labeled marker (Roche).

With regards to *FGL5*, enzyme *Kpn*I was used to digest the isolated DNA of the transformants and the wild type as described for the case of *FGL2*. This digestion resulted in a 4428 bp fragment from the transformants and an 8070 bp fragment from the wild type (figure 15.A). Southern blot analysis reveals the successful gene replacement in four transformants 1, 2, 3, and 5 (figure 15.B). Transformants 4 and 6 are most likely mutant strains contaminated with the wild type strain and a false positive strain, respectively.

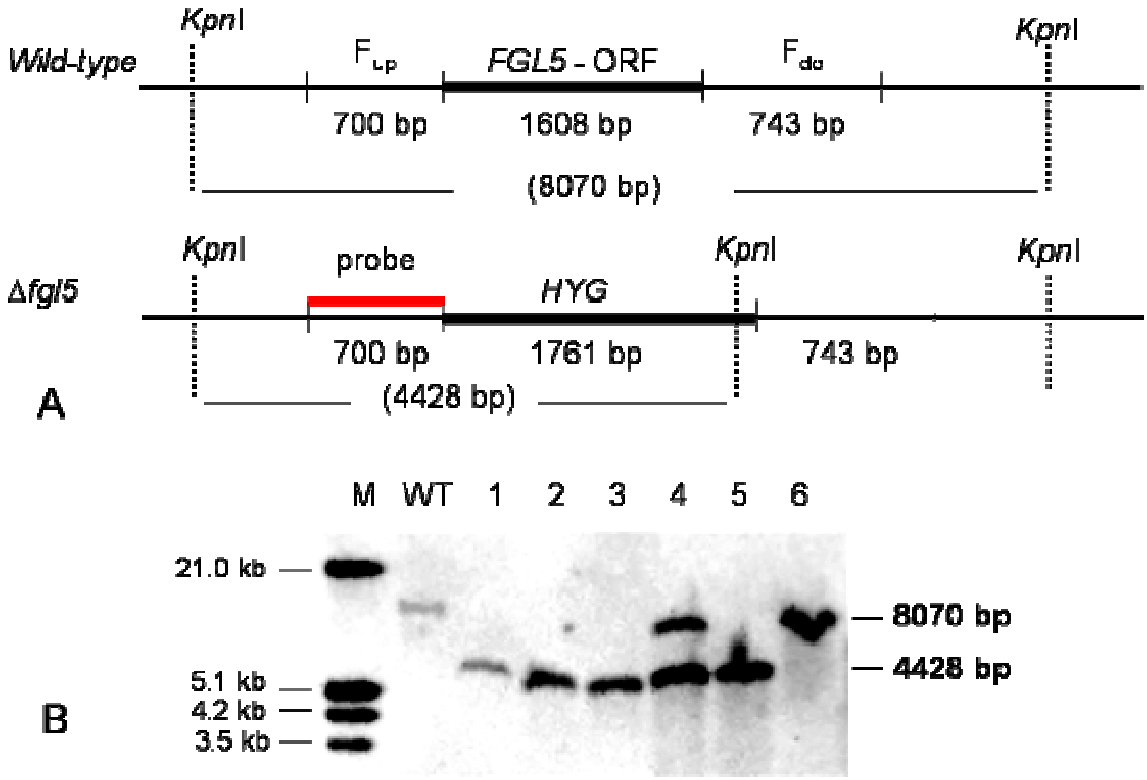

Fig. 15. Knock-out strategy (A) and Southern blot (B) analysis of *Δfgl5*. Enzyme *Kpn*I was used for genomic DNA digestion of wild type and Δ*fgl5* strains; the Southern blot probe was amplified by PCR from 5' homologous fragment (F_{up}) (dark red line). 1, 2, 3, and 5: *FGL5* disrupted mutants *(Δfgl5);* 4: wild type-contaminated mutant strain; 6: false positive transformant. WT*:* wild type, M: dig-labeled marker (Roche).

The split marker recombination technique is a sufficient method to disrupt a gene. The transformants resistant to HYG require the proper recombination of two split fragments of HYG. As experienced through above transformations, however, no ectopic transformant is achieved. Therefore, later gene disruptions of this study use double homologous recombination constructs (figure 4).

Generation of a double homologous recombination construct is illustrated in figure 4 (see 2.3). To generate the construct for disruption of *FGL9*, the following steps were taken: A 989 bp upstream fragment (F_{up}) of *FGL9* was amplified from genomic DNA with the primers FGL9upF and FGL9upF (see 2.1.3). Similarly, a 1004 bp downstream fragment (F_{do}) of *FGL9* was amplified by using FGL9doF and FGL9doR (see 2.1.3). These two fragments were purified from a 0.8% (w/v) agarose gel by using the Qiagen Gel extraction Kit (Qiagen). To prepare HYG fragment, the pGEM-HYG (section 2.3.1) was excised by *Sma*I. Two modified

steps of section 2.3.1 for fusion PCR approach were used. First, the upstream and downstream fragments were fused with the HYG fragment without using oligonucleotide primers in one PCR. Second, this PCR product was used as template for propagation of the desired products with the use of nested primers FGL9upnF and FGL9donR (see 2.1.3). The resulting PCR fragment (3535 bp) was ligated into pGEM-T to create pGEM-FGL9. Similar strategies were also used to generate pGEM-FGL12, pGEM-FGL13 and pGEM-FGL14 plasmids.

To study the possible roles of *FGL9* in virulence of *F. graminearum*, the double disruption constructs was used for transformation. Transformation of the wild type with the disruption construct pGEM-FGL9, which was previously digested in the pGEM backbone with *Not*I and *Pae*I, resulted in 50 transformants. In this transformation, a step of enzymatic digestion of hyphae in preparation of protoplasts was changed to 37^{o}C instead of 28^{o}C. Genomic DNA samples isolated from single conidia-derived mycelia was tested by PCR. In order to examine the presence of *FGL9* in the genome of transformants primer pair FGL9rtF/R was used (section 2.1.3). As the result from analysis of 12 randomly selected transformants, two of them (transformants 4 and 7) give no PCR product. This result indicates that *FGL9* gene is disrupted from the genome of the two transromants (figure 16).

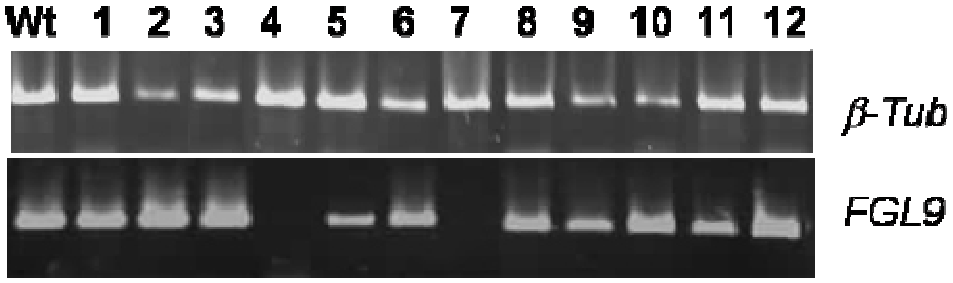

Fig. 16. Screening of *Δfgl9* strains. Genomic DNA samples isolated from 12 transformants were examined by PCR with the use of primer pair FGL9rtF/R. Two transformants (4 and 7) show the absence of *FGL9*. Wt: wild type; transformants 1 - 12: twelve HYG-resistant transformants. *β-Tub*: beta-tubulin gene used as control.

Similarly, lipase genes *FGL12*, *FGL13*, and *FGL14* are also disrupted. Transformations were carried out with the use of pGEM-FGL12, pGEM-FGL13, and pGEM-FGL14 plasmids. Prior to transformation, pGEM-FGL12, pGEM-FGL13, and pGEM-FGL14 were all digested in the plasmid backbone with *Pae*I

and *Not*I (see pGEM-T manual, Promega). Two transformations were carried out, which resulted in 22 transformants for *FGL12*, 12 transformants for *FGL13*, and 15 transformants *FGL14*. A similar PCR screening strategy as described for the analysis of *FGL9* transformants was employed. Genomic DNA samples isolated from above transformants was examined by PCR with the specific primer pairs FGL12rtF/R, FGL13rtF/R, and FGL14rtF/R (section 2.1.3) for the presence of *FGL12*, *FGL13*, and *FGL14* gene, respectively. The results show that 4 of 10 *FGL12* transformants, 5 of 12 *FGL13* transformants and 5 of 12 *FGL14* transformants tested give negative results in PCR screenings (data not shown). These results indicate the successful disruption of lipase genes *FGL12*, *FGL13*, and *FGL14* genes in the transformants.

Throughout this study, a HYG cassette is used that comprises a 1761 bp DNA cassette driven by the gpdA promoter (*Cochliobolus heterostrophus*) and terminated by the trpC terminator (*Aspergillus nidulans*). Because of the similar length of HYG cassette sequence and *FGL12*, *FGL13*, and *FGL14* genes, results of gene disruptions are not easily discriminated by single hybridization. Therefore, a Southern blot strategy with two hybridizations was employed to analyze the disrupted strains (figure 17). Firstly, a gene-specific probe was hybridized in order to detect the native gene (figure 17.A). Secondly, the Southern blot was re-hybridized with a HYG-specific probe to locate the HYG cassette (figure 17.B).

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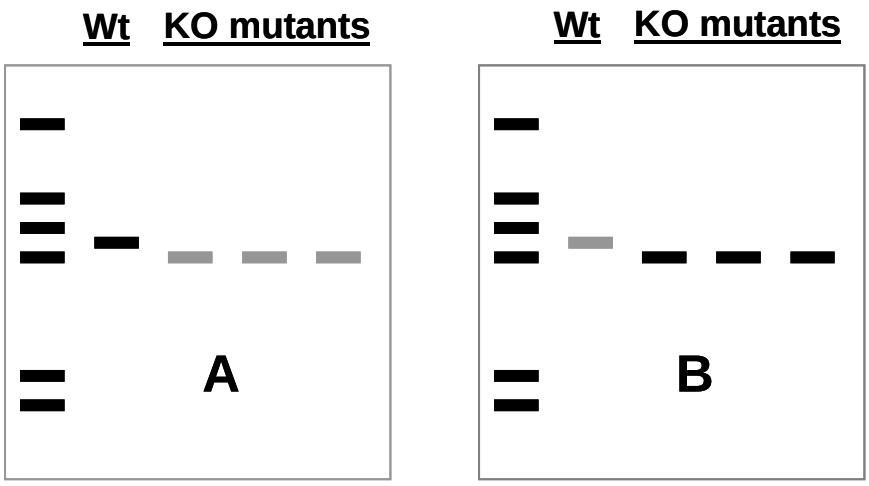

Fig. 17. Illustration of Southern blot strategies. A: a gene-specific probe is used for detection of the native gene in the wild type (Wt), and disrupted strains (KO mutants); B: a HYG-specific probe is re-hybridized to detect the presence of HYG in the same locus of the expected KO mutants. (Dark black lines indicate the positive hybridization; faint grey lines indicate the negative hybridization).

With respect to *FGL12*, enzyme *Kpn*I was used to digest gDNA isolated from the transformants and the wild type. The digestion resulted in a 3468 bp DNA fragment for the expected disrupted mutants and a 3996 bp DNA fragment for the wild type (figure 18). The first hybridization with lipase gene *FGL12* probe that is synthesized by PCR is used to confirm the complete elimination of the *FGL12* gene. As shown in the picture 19.A, among the 10 transformants analysed 7 show negative hybridization with the probe (transformants 1 - 4, 6, 9, and 10), whereas three transformants (transformants 5, 7, and 8) and the wild type show positive hybridization. This result indicates the successful elimination of the *FGL12* gene. The re-hybridization of the HYG-specific probe was used to confirm the replacement of *FGL12* gene by the HYG cassette. As shown in figure 19.B, the transformants 1 - 4, 6, 9, and 10 have the right replacement of the HYG cassette, indicating the successful disruption of the *FGL12* gene in the transformants.

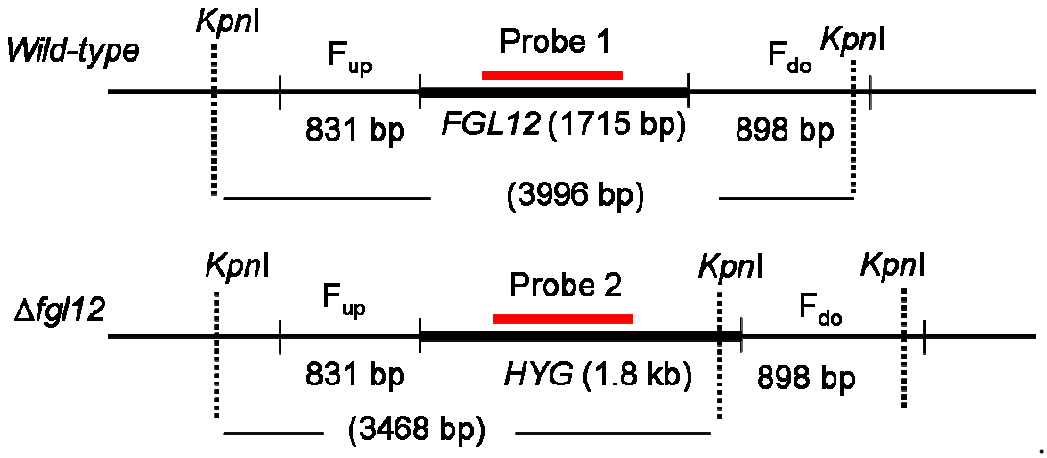

Fig. 18. Schematic illustration of Southern blot analysis of *Δfgl12* strains. Enzyme *Kpn*I was used to digest genomic DNA samples isolated from the wild type and expected *FGL12* gene disrupted (*Δfgl12*) mutants as well as ectopic strains (Etp). First hybridization with the *FGL12* gene specific probe (probe 1) was used to detect the native *FGL12* gene. The second hybridization used the 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 FGL12rtF/R and wild type DNA as template. Probe 2 was synthesized by PCR with the use of primers HYGF/R and plasmid pGEM-HYG as template. F_{up}: upstream flanking fragment; F_{do}: downstream flanking fragment. bp: base pair.

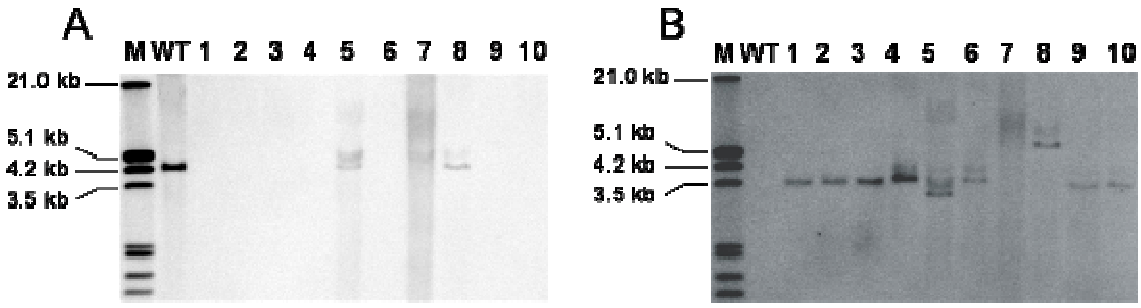

Fig. 19. Southern blot analysis of *Δfgl12* strains. A: *FGL12* gene-specific probe (Dig-labelled DNA probe amplified by PCR) was hybridized with DNA of the wild type (WT), and 10 DNA samples of *FGL12* mutants (transformants 1 - 10). Disrupted mutants show negative hybridization. B: Re-hybridization of HYG-specific probe (Dig-labelled DNA probe). Disrupted mutants show positive hybridization and the expected location. M: Dig-labelled DNA marker (Roche).

Similar strategies were used for Southern blot analysis of *FGL13* and *FGL14* disrupted mutants. Genomic DNA samples isolated from *FGL13* and *FGL14* transformants were digested by *Xba*I and *Bgl*II, respectively. For *FGL13*, *Xba*I digestion of DNA samples of transformants as well as the wild type resulted in a 5299 bp DNA fragment and a 5327 bp DNA fragment (figure 20), respectively. In

the first hybridization with the *FGL1*3-specific probe it was shown that seven transformants (transformants 1, 3, 4, 6, and 8 - 10) have the native gene eliminated. The remaining transformants as well as the wild type give positive results with the probe (figure 21.A). However, the re-hybridization with the HYG-specific probe show that only four transformants (transformants 6, 8, 9, and 10) are the desired disruption strains (figure 21.B).

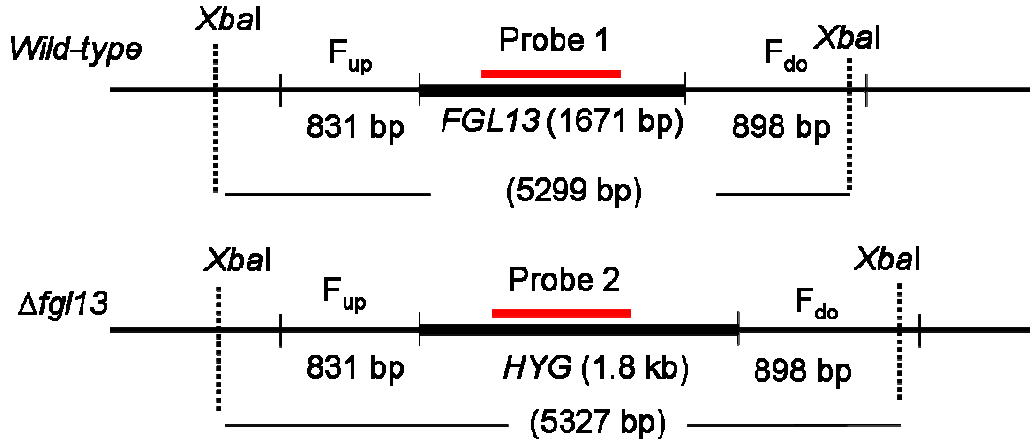

Fig. 20. Schematic illustration of Southern blot analysis of *Δfgl13* strains. Enzyme *Xba*I was used to digest genomic DNA samples isolated from the wild type and expected *FGL13* gene disrupted mutants (*Δfgl13*) as well as ectopic strains (Etp). First hybridization with *FGL13* gene specific probe (probe 1) was used to detect the native *FGL13* gene. The second hybridization used HYG-specific probe (probe 2) to locate HYG cassette in the mutant strains. Probe 1 is synthesized by PCR with the use of primers FGL13rtF/R and wild type DNA as templates. Probe 2 is synthesized by PCR with the use of primers HYGF/R and plasmid pGEM-HYG as templates. F_{up}: upstream flanking fragment; F_{do}: downstream flanking fragment. bp: base pair.

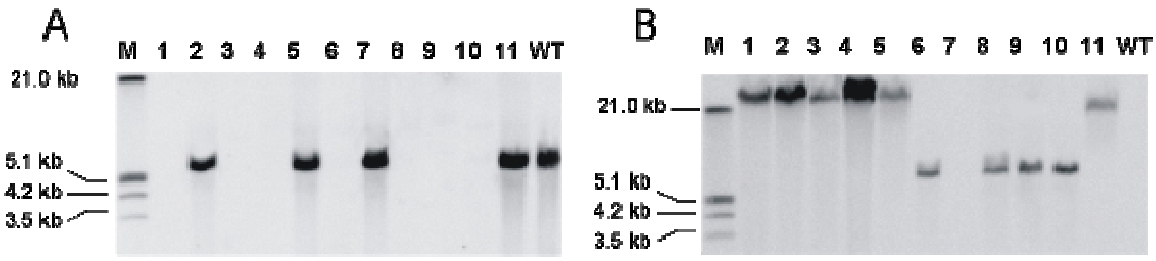

Fig. 21. Southern blot analyses of *Δfgl13* strains. A: *FGL13* gene-specific probe (Dig-labelled DNA probe amplified by PCR) was hybridized with DNA of the wild type (WT), and 11 DNA samples of *FGL13* mutants (transformants 1 - 11). Disrupted mutants show negative hybridization. B: Re-hybridization of HYG-specific probe (Dig-labelled DNA probe). Disrupted mutants show positive hybridization and the expected location. M: Dig-labelled DNA marker (Roche).

For *FGL14*, *Bgl*II digestion resulted in a 5011 bp fragment from the transformants and a 5118 bp fragment from the wild type (figure 22). As shown in the first hybridization, among twelve transformants tested seven of them (transformants 1 - 6 and 8) show a negative result when the *FGL14*-specific probe was used (figure 23.A). The re-hybridization with the HYG-specific probe was performed to detect the HYG cassette. As shown in figure 23.B, five transformants (transformants 1 - 3, 5, and 8) are the expected disrupted mutants.

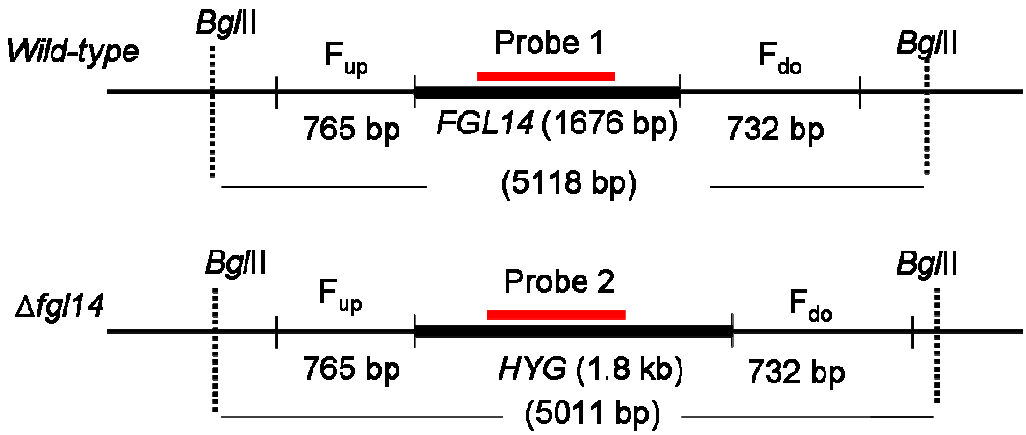

Fig. 22. Schematic illustration of Southern blot analysis of *Δfgl14* strains. Enzyme B*gl*II was used to digest DNA samples isolated from the wild type and expected *FGL14* disrupted mutants as well as ectopic strains (Etp). First hybridization with the *FGL14* gene specific probe (probe 1) was used to detect the native *FGL14* gene, and the second hybridization using 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 FGL14rtF/R and wild type DNA as template. Probe 2 was synthesized by PCR with the use of primers HYGF/R and plasmid pGEM-HYG as template. F_{up}: upstream flanking fragment; F_{do}: downstream flanking fragment. bp: base pair.

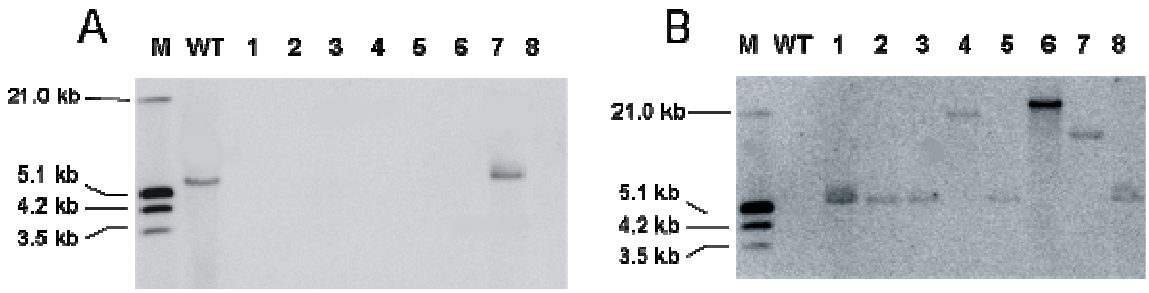

Fig. 23. Southern blot analyses of *Δfgl14* strains. A: *FGL14* gene-specific probe (Dig-labelled DNA probe amplified by PCR) was hybridized with DNA of the wild type (WT), and 8 DNA samples of *FGL14* mutants (transformants 1 - 8). Disrupted mutants show negative hybridization. B: Re-hybridization of HYG-specific probe (Dig-labelled DNA probe). Disrupted mutants show positive hybridization at the expected location. M: Dig-labelled DNA marker (Roche).

3.3.2. Phenotypic characteristics of transformants

3.3.2.1. Growth behaviour and utilization of lipids

Lipase gene 2, 4, and 5 were disrupted by the split-marker recombination method. Therefore, the resulting mutant strains (*Δfgl2*, *Δfgl4*, and *Δfgl5* strains) were examined simultaneously. To test whether the loss of *FGL2*, *FGL4*, and *FGL5* genes affected the growth behaviour of *F. graminearum*, the null mutants were grown on nutrient-rich media CM and YPG. The result shows that the deletion of *FGL4* does not affect the fungal growth in both media. However, deletion of *FGL2* or *FGL5* results in decreased aerial mycelia of the fungus when grown in YPG medium (recipe shown in section 2.1.2) (figure 24).

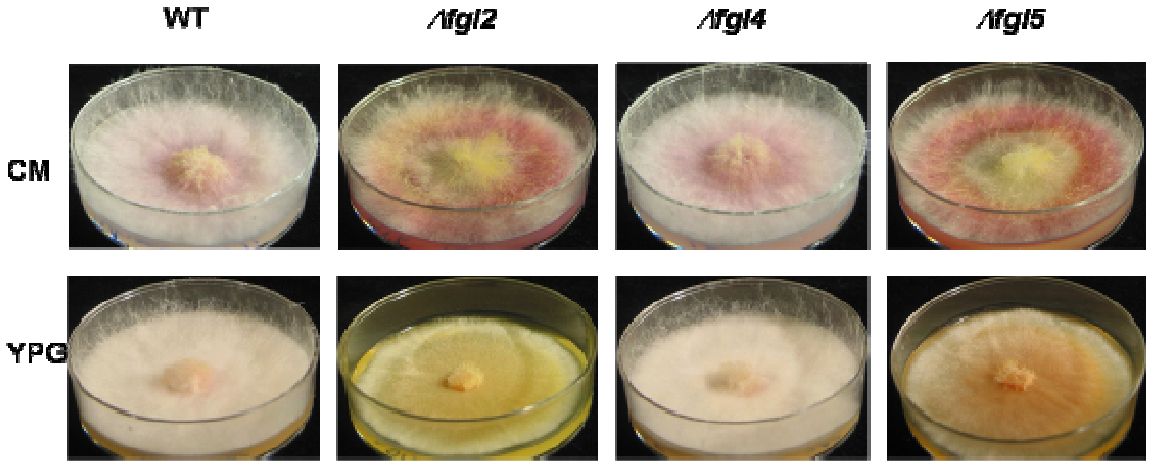

Fig. 24. Growth behaviour of lipase 2, 4, and 5 disrupted strains cultivated on nutrient-rich media (CM and YGP media). A plug (0.5 cm^2) of mycelia was inoculated on 92 mm Petri plates for 5 days at 28°C. *Δfgl2*, *Δfgl4*, and *Δfgl5* strains show a normal wild type growth on CM. However, *Δfgl2* and *Δfgl5* strains produce less aerial mycelia on YPG. Pictures were taken by a digital camera (Canon PowerShot S40). Experiments were repeated thee times with similar results.

To determine whether lipase 2, 4, and 5 are required for nutrition utilization of lipids, Δ*fgl2,* Δ*fgl4*, and Δ*fgl5* strains were cultivated on solid SNA medium (section 2.7.2) supplemented with 2% (v/v) wheat germ oil (WGO), olive oil, triolein, and tributyrin as the sole carbon source. After five days of incubation with a plug of mycelia (0.5 cm^2), the rate of radial growth and the colony morphology of Δ*fgl2,* Δ*fgl4*, and Δ*fgl5* strains, and the wild type strain are almost similar in size, when wheat germ oil, olive oil and triolein were used (figure 25). However, the growth rate of Δ*fgl2* and Δ*fgl5* strains significantly increase in comparison to

that of Δ*fgl4* strains and the wild type strain on medium supplemented with 2% tributyrin. In this medium, the mutants produce more aerial mycelia when grown in media containing wheat germ oil, olive oil and triolein (figure 25).

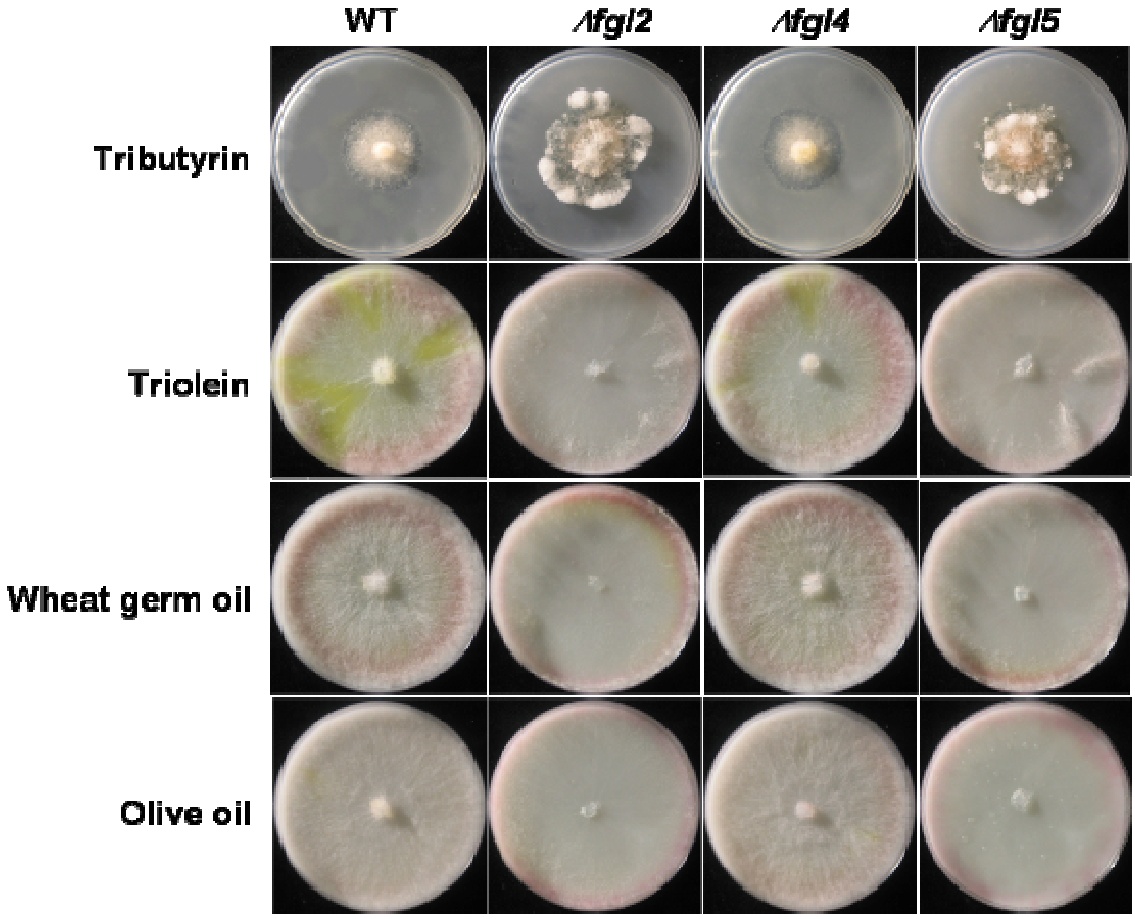

Fig. 25. Growth behaviour of lipase 2, 4, and 5 disrupted strains cultivated on media with different oils as the sole carbon sources. Small mycelial plugs from cultures of wild type, *Δfgl2, Δfgl4*, and *Δfgl5* mycelia were inoculated for 5 days on agar plates of minimal media supplemented with 2% (v/v) of wheat germ oil, tributyrin, triolein, and olive oil. Experiments were repeated three times with three independent strains of each lipase-deficient strain. Pictures were taken by a digital camera (Canon PowerShot S40).

To study the potential functions of lipase 9, 12, 13, and 14 in nutrient uptake, and growth of the fungus *F. graminearum*, these null mutants (*Δfgl9, Δfgl12*, *Δfgl13*, and *Δfgl14* strains) as well as the wild type strain were grown on CM medium and SNA medium supplemented with oils such as wheat germ oil and tributyrin as the sole carbon source. One plug (0.5 cm^2) of mycelia was inoculated on agar plates at 28^{0}C for 5 days. When grown on CM medium all *Δfgl9, Δfgl12*, *Δfgl13*, and *Δfgl14* strains as well as the ectopic strains show the similar colony size and morphology as the wild type. The disrupted mutants grow as fast as the wild type,

and produce as much aerial mycelia as the wild type in this medium. When grown in different oils as a sole carbon source, *Δfgl9*, *Δfgl12*, and *Δfgl14* strains show the same growth rate as observed in the ectopic strains (not shown) and the wild type. However, the growth rate of *Δfgl13* strains on these media is decreased in comparison with its ectopic counterpart (not shown) and the wild type. The growth rate of *Δfgl13* strains on tributyrin as the sole carbon source is impaired significantly (figure 26), with just small colonies formed after 5 days of incubation. Tributyrin is also called glycerol tributyrate, a lipid which is constituted from one glycerol with three molecules of butyric acid. This short chain carbon lipid is normally a preferred substrate for esterases (Pérez *et al*., 1990). A slightly impaired growth of *Δfgl13* strains is also observed in SNA medium plus 2% wheat germ oil (figure 26). These results indicate the involvement of lipase 13 in nutrient uptake of *F. graminearum*. Lipase 9, 12, and 14 are dispensable for the fungal growth.

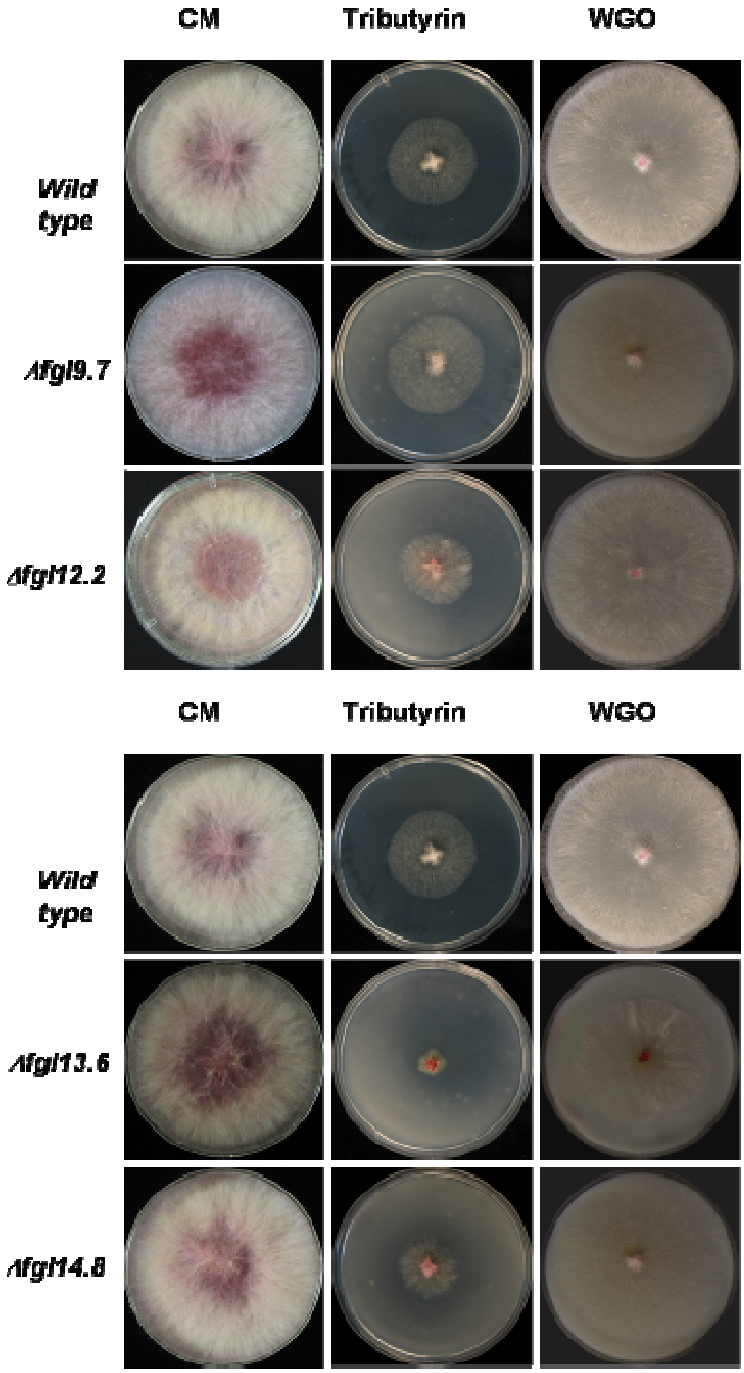

Fig. 26. Growth behaviour of lipase 9, 12, 13, and 14-disrupted strains cultivated on different media. Small mycelial plugs of wild type, *Δfgl9.7, Δfgl12.2, Δfgl13.6*, and *Δfgl14.8* strains as well as their ectopic counterparts were inoculated for 5 days on agar plate of CM medium and SNA medium supplemented with either 2% (v/v) of wheat germ oil or 2% tributyrin. Experiments were repeated three times and gave the same results. Pictures were taken by a digital camera (Canon PowerShot S40).

3.3.2.2. Extracellular lipolytic activity of lipase 2, 4, 5, 9, 12, 13, and 14-disrupted strain

Lipolytic activity of lipase 2, 4, and 5-disrupted strains has already been characterized (Fehrholz, 2007). It is restated that total lipase activity of the fungus *in vitro* is not changed when lipase 2, 4 or 5 is deleted.

Similarly, examination of extracellular lipolytic activity of lipase 9, 12, 13, and 14-disrupted strain was carried out. To examine whether the loss of lipase 9, 12, 13, and 14 affects the secretion of lipolytic activity, the null mutant strains as well as the wild type were grown for two days in YPG medium. Mycelia were then washed out by pure water (see 2.2.3). For induction of lipase, 2% (v/v) wheat germ oil was inoculated with the mycelia for one additional day. Supernatants taken every four hours were assayed for lipolytic activity with para-nitrophenyl palmitate (pNPP, C16) as a substrate. As shown in the figure 27, no significant difference of lipolytic activity was found between lipase 9, 12, 13, and 14-disrupted strains and the wild type. These results suggest that lipase 9, 12, 13, and 14 are dispensable for total extracellular lipase activity of the fungus *in vitro*. Although lipase 9 is found as a secreted protein in growth media (Paper *et al*., 2007), its deletion does not diminish total extracellular lipase activity of the fungus. Deletion of lipase 13 seems to affect fungal growth. However, the extracellular lipase activity still remains the same as the wild type level.

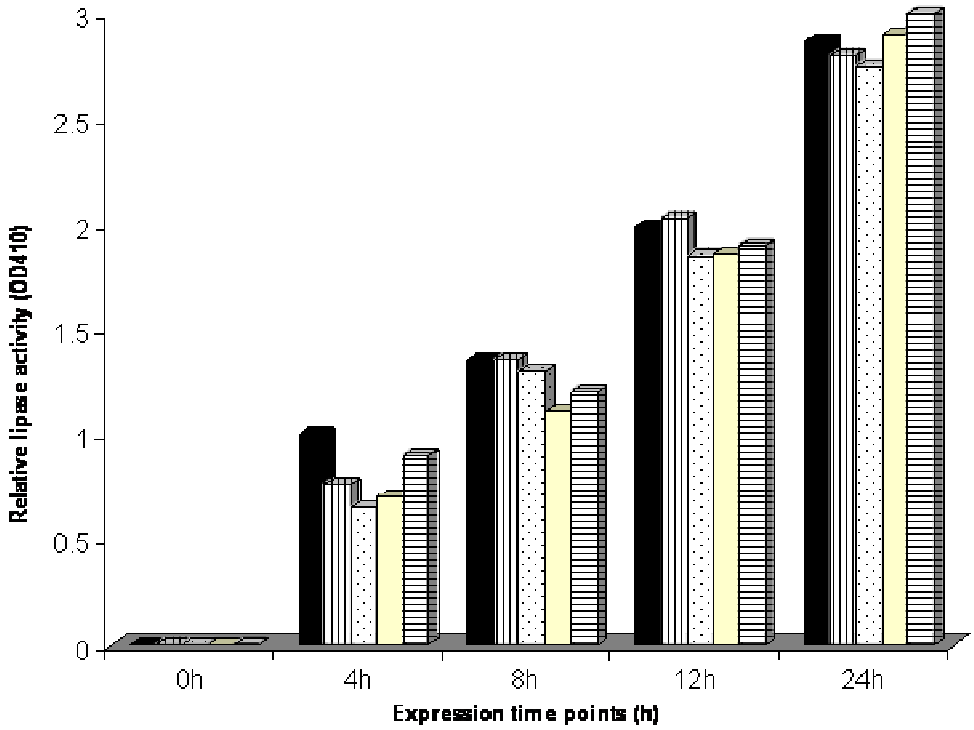

Fig. 27. Extracellular lipolytic activity of lipase 9, 12, 13, and 14-disrupted strain. Expression supernatants at indicated time points of *Δfgl9.7, Δfgl12.2*, *Δfgl13.6*, and *Δfgl14.1* strains were assayed with para-nitrophenyl palmitate. Relative lipase activity was displayed as OD differences between samples and control. Black bars: wild type; vertical bars: *Δfgl9.7*strain; dotted bars: *Δfgl12.2* strain; blank bars: *Δfgl13.6* strain; horizontal bars: *Δfgl14.1* strain. h: hour. Experiments were repeated two times and gave similar results.

3.3.2.3. Easily wettable phenotype of lipase 1, 2, 4, and 5-disrupted strains

As reported by Stringer *et al*. (1991), secreted hydrophobic proteins, so-called hydrophobins, are involved in formation of aerial structures in *Schizophyllum* and *Aspergillus*. Genetic and morphological evidence from the studies suggested that the hydrophobins are morphogenetic proteins that allow or cause hyphae to emerge off the substrate and into the air. Aerial production of lipase 2 and 5-disrupted strains in YGP is reduced. Thus, to gain more knowledge about the behaviour of these lipase-deficient strains, the wettability (hydrophilic phenotype) of hyphae was characterized by the easily wettable assay, in which a water drop was placed on top of 5 days old mycelia. In the Δ*fgl5* strains, the water drop was sucked under the agar layer after 1 hour of retention. Mycelia of Δ*fgl1,* Δ*fgl2*, and Δ*fgl4* are as hydrophobic as the wild type mycelia when none of the water drops

was sucked away (figure 28). This result implies a change in hydrophobicity of mycelia of Δ*fgl5* strains.

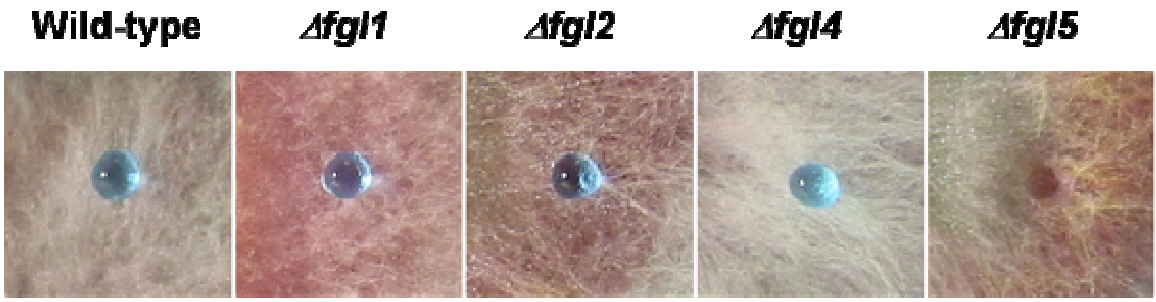

Fig. 28. Easily wettabe phenotype of Δ*fgl1*, Δ*fgl2*, Δ*fgl4*, and Δ*fgl5.* Wettability of mycelia was tested by placing a drop of water on the aerial hyphae. After one hour of retention, the water drop was sucked away in Δ*fgl5* strain. Water was coloured by mixing with a trace amount of Calcofluor White Stain (Fluka/Sigma). The assay was repeated three times. Pictures were taken after 1 h of retention. Δ*fgl1* strain was received from Voigt (University of Hamburg).

3.4. Pathogenicity assays

To determine the involvement of lipase 2, 4, and 5 in virulence of *F. graminearum*, wheat heads and maize cobs were inoculated with conidia of the wild type strain and three independent lipase 2-disrupted strains (strains Δ*fgl2-1, -2, -3),* lipase 4-disrupted strains (strains Δ*fgl4-1, -2, -3*), and lipase 5-disrupted strains (strains Δ*fgl5-1, -2, -3*). Water inoculations were used as negative controls. Since these transformations resulted in no ectopic transformant, the virulence assays were controlled by comparing with the infection of the wild type and the use of three independent mutant strains.

In growth chamber conditions, wheat spikes inoculated with conidia of the wild type strain are totally blighted three weeks post inoculation (figure 29.A). Similar to the wild type, Δ*fgl4* strains rapidly colonize the inoculated spikelets and spread to cause severe symptoms in the same spikes within three weeks. However, the spreading to lower spikelets by Δ*fgl4* strains is slightly delayed when compared to that of the wild type. In some cases, the lower part of spikes infected by Δ*fgl4* strains remains green within three weeks of infection. However, a longer infection period completely bleached the whole spike (figure 29.A). Harvested kernels from wheat spikes inoculated with wild type and Δ*fgl4* strains show similar results. They are shrivelled, brownish and flattened, indicating the typical symptoms of

FHB (figure 29.B) (Goswami and Kistler, 2004). Thus, it is clear that lipase 4 is not required for wheat infection by *F. graminearum.*

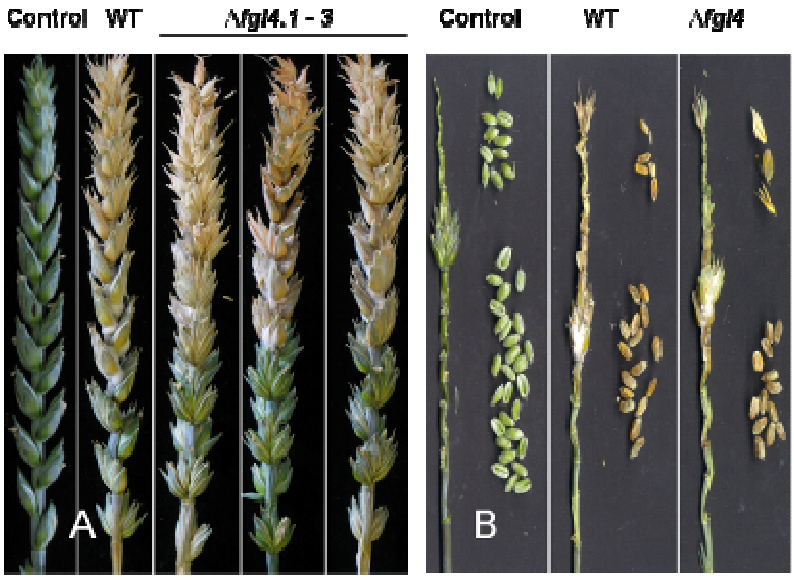

Fig. 29. Pathogenicity tests of Δ*fgl4* on wheat spikes. Two central spikelets of wheat spikes were each inoculated with a suspension of 200 conidia of wild type (WT) and three Δ*fgl4* strains (strains 1, 2, and 3). The infection was monitored for three weeks post inoculation (A). Then, wheat kernels were harvested (B). Pathogenicity tests were repeated 10 times with three independent Δ*fgl4* strains. Control: pure water-inoculated spike.

In contrast to the results achieved from deletion of lipase 4, the wheat spikes inoculated with lipase 2 and 5-disrupted (Δ*fgl2* and Δ*fgl5*) strains show a drastic reduction in FHB symptoms. With respect to Δ*fgl2* strains, the mutant strains are not able to infect adjacent spikelets after three weeks of infection (figure 30.A). Although the inoculated spikelets are strongly bleached, the neighbouring spikelets and stem between the two directly inoculated spikelets are not infected. The disease progression is only observed in directly inoculated spikelets, which results in complete bleaching in these sites (figure 30.A). As a result of reduced infection, only kernels of inoculated spikelets are completely destroyed, the remaining kernels of the same spike appear healthy like those of water inoculations (figure 30.B). The infection patterns of Δ*fgl5* strains are quite similar to those of Δ*fgl2* strains. Although there are mycelia growing outside on the surface of the inoculated spikelets, the mutants are unable to spread to neighbouring ones (figure 31.A). The infected spikelets become flattened, and no soft dough remains. As a result of the limited colonization, only the kernels of directly inoculated spikelets are damaged, the remaining kernels of the same

spike develope as normally found in uninfected wheat spikes (figure 31.B). Similar observations are also reported for Δ*fgl1* strains (Voigt *et al*., 2005) and the *Ras2* mutant (Bluhm *et al*., 2007), where the infections are restricted to inoculated sites. These results implicate the involvement of lipase 2 and 5 in virulence of *F. graminearum.*

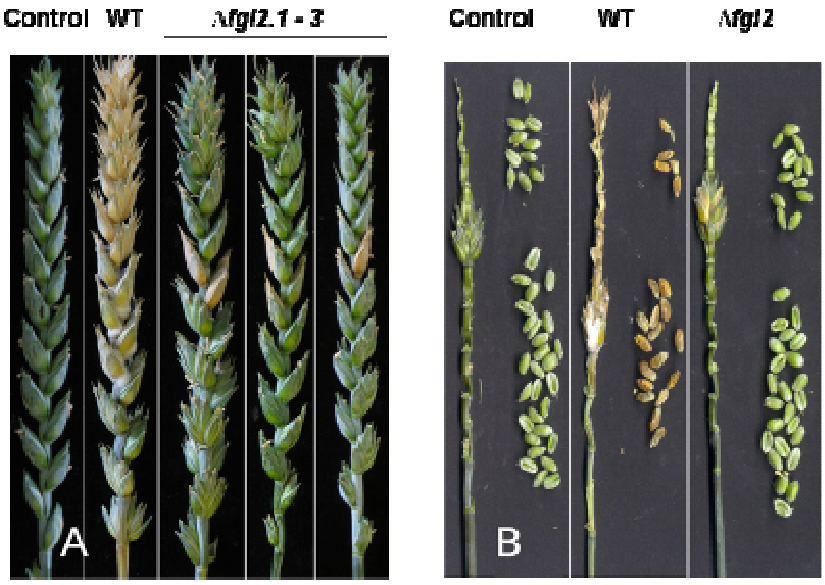

Fig. 30. Pathogenicity tests of Δ*fgl2* on wheat spikes. Two central spikelets of wheat spikes were each inoculated with a suspension of 200 conidia of the wild type (WT) and three Δ*fgl2* strains (strains 1, 2, and 3). The infection was monitored for three weeks post inoculation (A). Then, wheat kernels were harvested (B). Pathogenicity tests were repeated 10 times with three independent Δ*fgl2* strains. Control: pure water-inoculated spike.

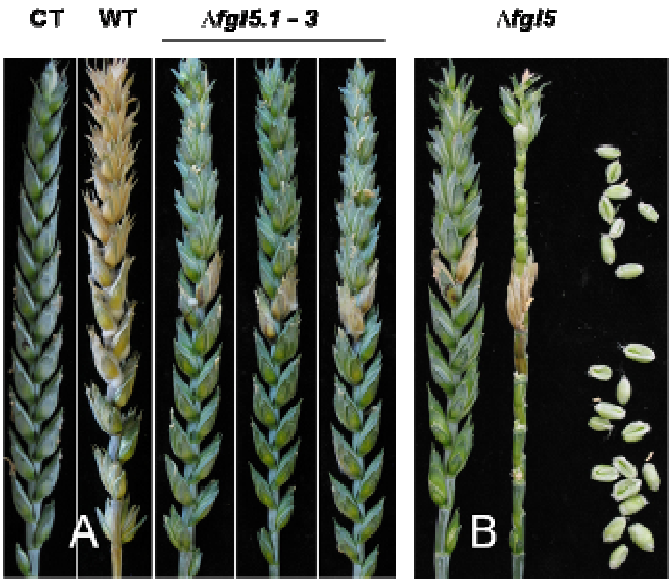

Fig. 31. Pathogenicity tests of Δ*fgl5* on wheat spikes. Two central spikelets of wheat spikes were each inoculated with a suspension of 200 conidia of the wild type (WT) and three Δ*fgl5* strains (strains 1, 2, and 3). The infection was monitored for three weeks post inoculation (A). Then, wheat kernels were harvested (B). Pathogenicity tests were repeated 10 times with three independent Δ*fgl5* strains. CT: pure water-inoculated spike.

The colonization of wheat heads by Δ*fgl2* and Δ*fgl5* strains is limited to the inoculated spikelets with less mycelium forming on the surface outside of the spikelets. To examine the infection patterns of Δ*fgl2,* Δ*fgl4*, and Δ*fgl5* strains, longitudinal sections of the stem between two inoculated spikelets were carried out. Observation of the sectioned tissues indicates that the stems connecting the two inoculated spikelets are green and not bleached when wheat spikes are inoculated with Δ*fgl2* and Δ*fgl5* strains. In contrast, the stems colonized by Δ*fgl4* and wild type are completely bleached (figure 32). It is concluded that the lipase 2 and lipase 5, but not lipase 4, are required for infection of wheat by *F. graminearum.*

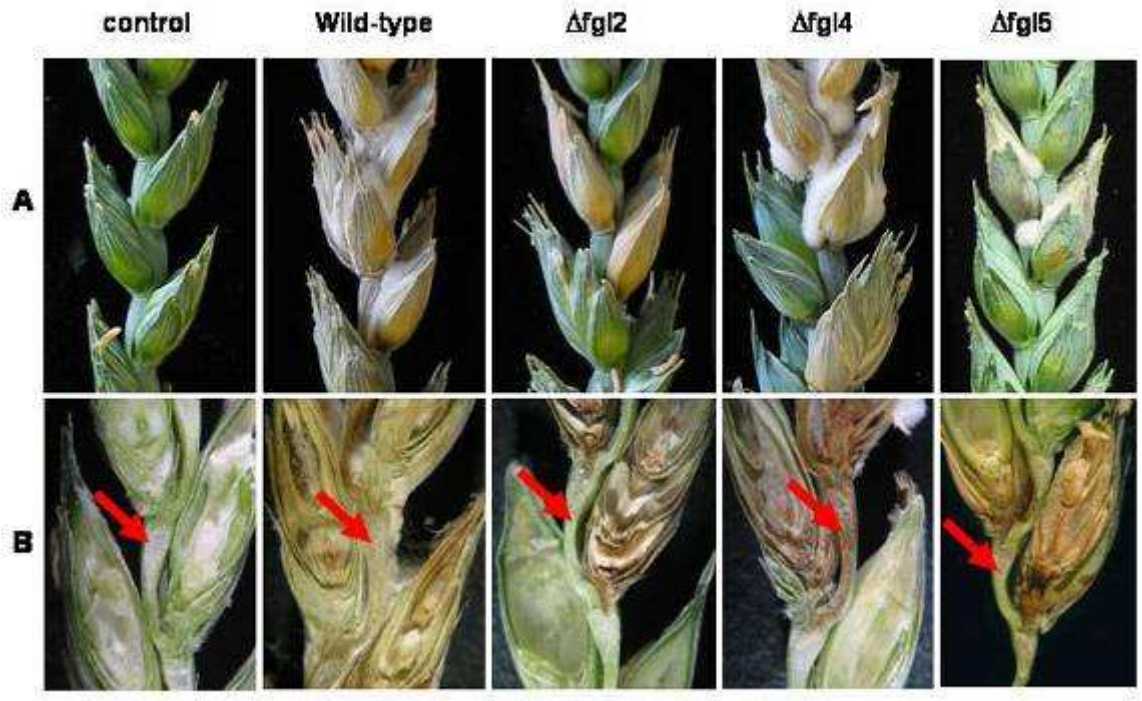

Fig. 32. Close view (A) and longitudinal sections (B) of infected wheat spikelets by Δ*fgl2*, Δ*fgl4*, and Δ*fgl5*. Close views show that lipase 2 and 5-disrupted (Δ*fgl2* and Δ*fgl5*) strains are restricted at inoculated spikelets. Lipase 4-disrupted (Δ*fgl4*) strains remained able to spread on wheat heads. Longitudinal sections of stems and rachis of the two inoculated spikelets reveal the failure of spreading of Δ*fgl2* and Δ*fgl5* strains. Arrow indicates differences of Δ*fgl2* and Δ*fgl5* strains in comparison with wild type and Δ*fgl4* infections. Control: water inoculation.

Examination of virulence behaviours of Δ*fgl2,* Δ*fgl4,* Δ*fgl5* strains on maize cobs was also carried out. Maize cobs were inoculated with a conidial suspension of the Δ*fgl2,* Δ*fgl4,* Δ*fgl5* strains and the wild type strain. The infections were monitored for 5 weeks under green house conditions. In comparison to that of maize cobs inoculations by the wild type, maize infections of Δ*fgl2* and Δ*fgl5* strains are greatly reduced after 5 weeks of inoculation (figure 33). Infected and healthy kernels were manually counted. Disease severity was estimated following the rating scales established by Reid and Hamilton (1995). As shown in the picture below, while mycelia of the infection are clearly visible and spreading over some parts of inoculated maize cobs, Δ*fgl2* and Δ*fgl5* strains fail to cause severe symptoms. As a result, the disease ratings are between 4 - 10%. In contrast, Δ*fgl4* strains and the wild type strain cause similarly severe symptoms in

inoculated maize cobs (figure 33). The disease ratings were estimated between 70 -100%. Hence, it is concluded that lipase 2 and lipase 5 are indispensable for infection of maize cobs by *F. graminearum*. Lipase 4 is, on the contrary, not essential for the fungus in infection of maize.

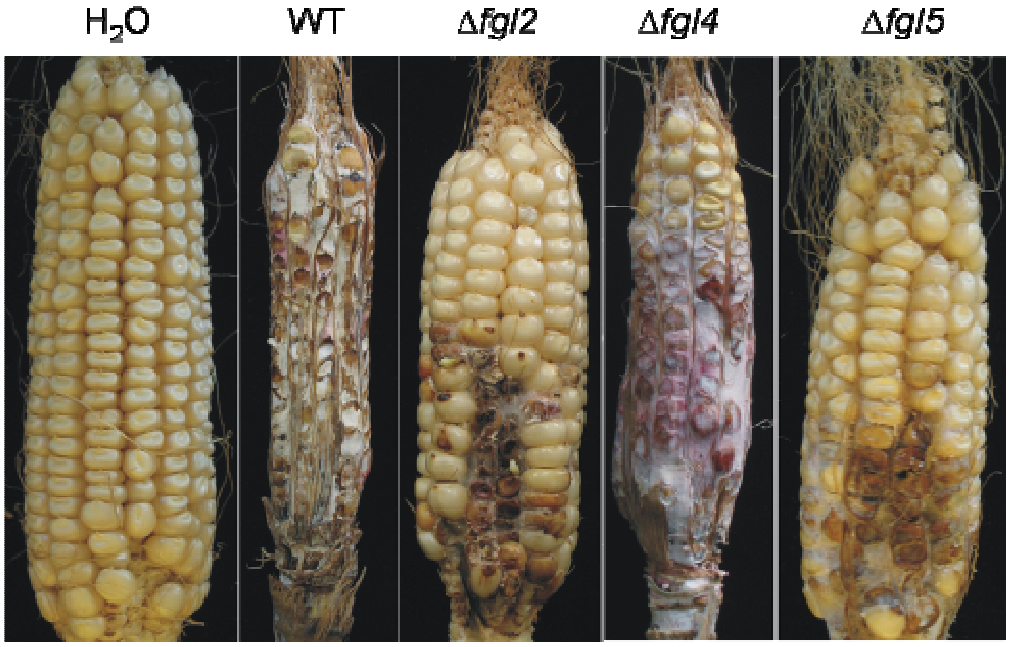

Fig. 33. Maize cobs after five weeks inoculated with Δ*fgl2*, Δ*fgl4*, and Δ*fgl5* strains and the wild type strain. 4 ml of conidia suspension with a concentration of 40 conidia per µl of the wild type strain (WT), Δ*fgl2,* Δ*fgl4,* and Δ*fgl5* strains were injected into the maize cobs at the stage of early kernel formation. Water inoculation was used as a control (CT). Pathogenicity tests were repeated 5 times with three independent strains of each lipase-deficient strain.

Summarizing the above results, it is concluded that lipase 2 (*FGL2*) and lipase 5 (*FGL5*) are novel virulence factors of *F. graminearum*. They are not required by the fungus for its growth, but required for at least wheat heads and maize cobs infection. Lipase 4 (*FGL4*) is, in contrast, essential for neither wheat and maize infection nor fungal growth.

The potential roles of lipase 9, 12, 13, and 14 for fungal infection were also examined. The disrupted strains were inoculated on wheat spikes. Similarly, 200 conidia were directly injected into each of two central wheat spikelets at flowering stage. Observation over 21 days of infection indicated that no difference in disease symptoms is found between *Δfgl9, Δfgl12, Δfgl13*, and *Δfgl14* strains, the corresponding ectopic strains, and the wild type (figure 34). Under greenhouse conditions, all these lipase-disrupted strains develope mycelia at the inoculated

spikelets, and vertically spread to the neighbouring spikelets after 7 days of infection. The upper part of the wheat head above the inoculation points of wheat head is, firstly, observed to be bleached after 12 – 14 days of infection, and then, secondly, the fungus progresses to completely bleach the whole wheat spike after 21 days of infection (figure 34). The wheat scab caused by each of the lipase-disrupted strains is comparable to that of ectopic mutants as well as the wild type. These results indicate that lipase 9, 12, 13, and 14 are not required for virulence of *F. graminearum*.

Fig. 34. Wheat heads infected by lipase 9, 12, 13, and 14-disrupted (*Δfgl9*, *Δfgl12*, *Δfgl13*, and *Δfgl14*) strains. Two central wheat spikelets were each inoculated with 200 conidia of *Δfgl9.4* and 7, *Δfgl12.2* and 3, *Δfgl13.6* and 8, and *Δfgl14.1* and 8 strains as well as the corresponding ectopic strain (Etp) and the wild type (WT). The infection was monitored for three weeks under greenhouse conditions. The assay was repeated five times. The pictures were taken 21 days after infection.

3.5. Enhancement of virulence by addition of free fatty acids to fungal inoculum

It has been shown by quantitative RT-PCR analysis that the relative transcript amounts of *FGL1* are decreased in *Δfgl2* strains (Fehrholz, 2007). Therefore, it is hypothesized that *FGL2* is needed for transcriptional activation of *FGL1*. To find out the possible role of *FGL2* in activation of *FGL1* in planta, exogenous free fatty

acids were used to implement the infection of a *Δfgl2* strain (*Δfgl2.1* strain). As shown in figure 35, infection of the *Δfgl2* strain is enhanced by adding gamma-linolenic acid (18:3), a polyunsaturated long chain fatty acid (PUFA), but not by addition of ethanol (control). Evaluation of *FGL1* transcript by Northern blot of total RNA isolated from wheat spikelets implemented with fatty acids reveals that gamma-linolenic (18:3) acid induce the expression of *FGL1* during wheat infection of *Δfgl2*. In contrast, addition of polysaturated long chain palmitic acid (16:0) and ethanol do not induce the expression of *FGL1*. This result shows that polyunsaturated long chain free fatty acids induce expression of *FGL1*.

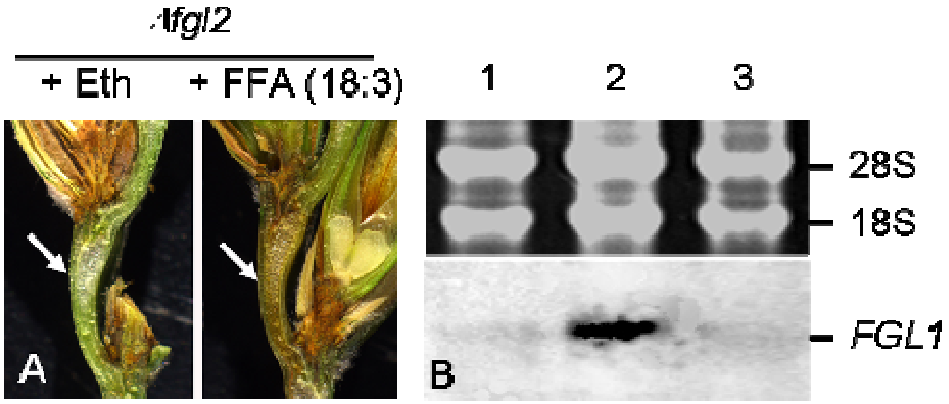

Fig. 35. Implementation of exogenous gamma-linolenic acid increases the infection of *Δfgl2* via induction of *FGL1*. A: wheat stem turned brown when 2 µl of 0.1% (v/v) gamma-linolenic acid was added to spikelets inoculated with the *Δfgl2* strain. Arrows show the differences between addition of ethanol and gamma-linolenic acid. B: The amount of *FGL1* transcripts is detected by Northern blot in spikelets inoculated with *Δfgl2* + 2 µl of ethanol (1), *Δfgl2* + 2 µl of 0.1% (v/v) gamma-linolenic acid (2), and *Δfgl2* + 2 µl of 0.1% (v/v) palmitic acid (3).

3.6. Enhancement of virulence by over-expression of lipase gene 1 in lipase gene 2 disruption mutant

Expression of lipase gene *FGL1* is impaired in the infection process of *Δfgl2* strains (Fehrholz, 2007). Also, exogenous fatty acid (linolenic acid) enhances the infection of the *Δfgl2* strain and the expression of *FGL1*. It seems possible that the reduced *FGL1* transcription is responsible for the reduced virulence of *Δfgl2* strains. To reveal this possible involvement, *FGL1* is constitutively expressed in *Δfgl2* strains. Examination of wheat spikes infected with *Δfgl2gpdFGL1* strain shows that the enhanced infection is observed after 21 days of inoculation (figure 36.A). However, the infection is not as strong as the wild type infection,

particularly at early infection time points. To clarify the impact of lipase 1 on virulence of *Δfgl2* strain, its transcription expression was analysed. It reveals that the expression of *FGL1* is increased in the *Δfgl2gpdFGL1* strain. However, this expression is weaker than that of the wild type after 3 days of inoculation. The expression of *FGL1* in the *Δfgl2gpdFGL1* strain is constitutively increased after 7 days of inoculation (figure 36.B), indicating an accumulative increase of *FGL1* transcripts.

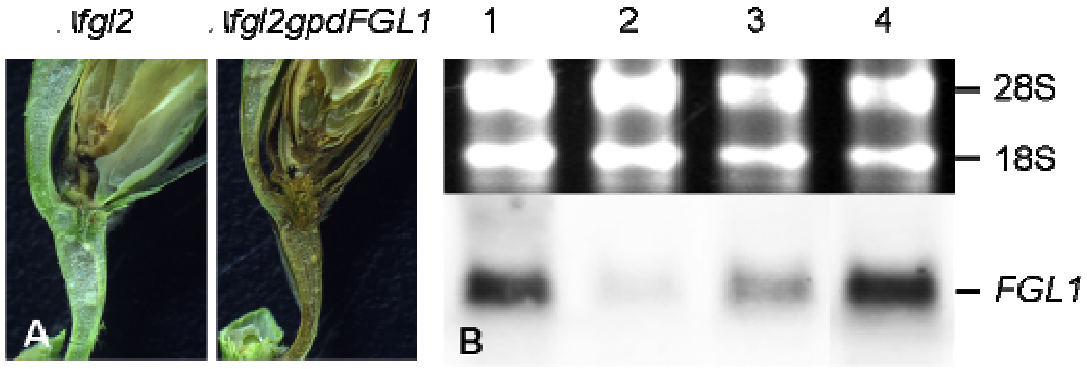

Fig. 36. Over-expression of lipase 1 in the lipase gene 2 disrupted strain (*Δfgl2*). The Coding sequence of lipase 1 (*FGL1*) regulated by the gpd promoter (Provided by Voigt, University of Hamburg) was cloned in the vector pII99, which carries the nptII marker gene for geneticin resistance. The resulting vector pII99gdpFGL1 is linearized in *FGL1* region by *BstX*I (Fermentas) and transformed into a lipase 2-disrupted strain (*Δfgl2.1*). Achieved transformants (*Δfgl2gpdFGL1*) were assayed for pathogenicity on wheat. A: wheat spikelets were inoculated with *Δfgl2.1*and *Δfgl2gpdFGL1* strain 21 dpi. B: Northern blot analysis of lipase gene 1 (*FGL1*) transcripts in the wild type (1) and *Δfgl2* (2) 3dpi, and *Δfgl2gpdFGL1* strain 3 dpi (3) and 7 dpi (4).

3.7. Molecular study of lipase gene 1 promoter

3.7.1. Expression of the green fluorescence protein under regulation of lipase gene 1 promoter

To produce the fungal strains expressing the green fluorescence protein that is regulated by lipase 1 promoter, pANpromFGL1-GFP was transformed into the wild type *F. graminearum*. Prior to transformation, the plasmid was digested by *Kpn*I in the promoter to promote the insertional recombination. The resultant mutant strains carry two copies of the *FGL1* promoter. The native promoter regulates the GFP gene, and the synthetic, truncated promoter regulates the *FGL1* gene (figure 37).

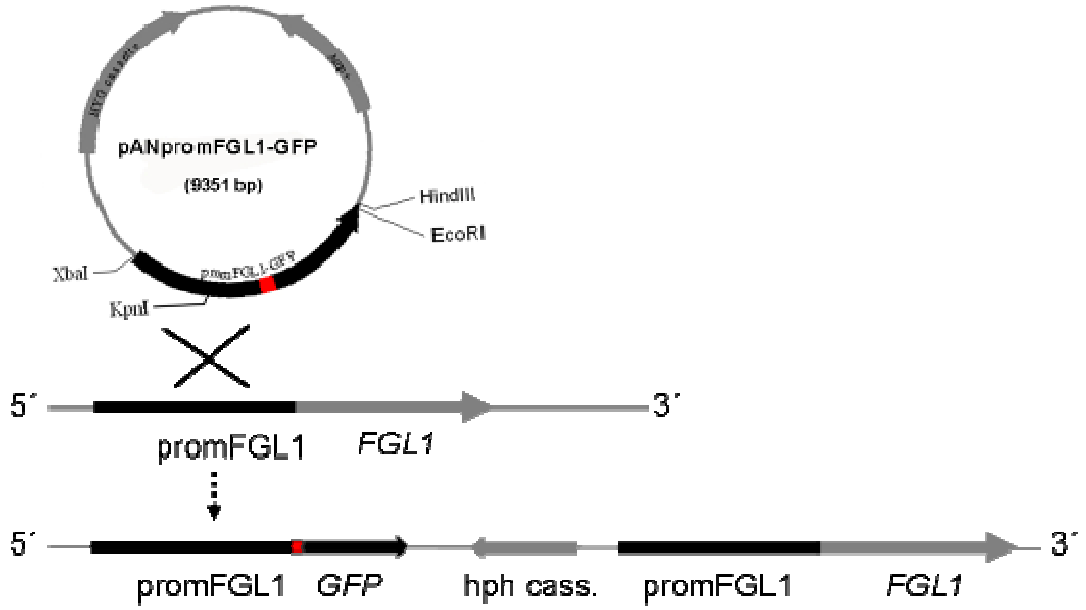

Fig. 37. Strategy for generation of *F. graminearum* strain expressing the green fluorescence protein (GFP) that is regulated by *FGL1* promoter. Homologous integration of *Kpn*I-linearized pANpromFGL1-GFP in the promoter of *FGL1* resulted in *promFGL1-GFP* strains. The transgenic strains contain two copies of promoter *FGL1* (promFGL1), the native promoter regulating the GFP gene, and the synthetic promoter regulating the native *FGL1* gene. Red bars: 15 nucleotides of the *FGL1* gene.

One transformation was carried out, which resulted in 50 hygromycin-resistant transformants. Genomic DNA samples isolated from 14 transformants were subject to Southern blot analysis. Genomic DNA samples of the transformants and the wild type were digested overnight by *Hind*III. This resulted in a 3231 bp and an 8645 bp fragment in the transformants and a 2520 bp fragment in the wild type when hybridized with a probe amplified from *FGL1* promoter (figure 38.A). As shown in figure 38.B, the two expected bands are present in the transformants. The wild type DNA shows a single hybridization band. These results indicate the successful integration of the promFGL1-GFP construct into the original promoter region of lipase 1.

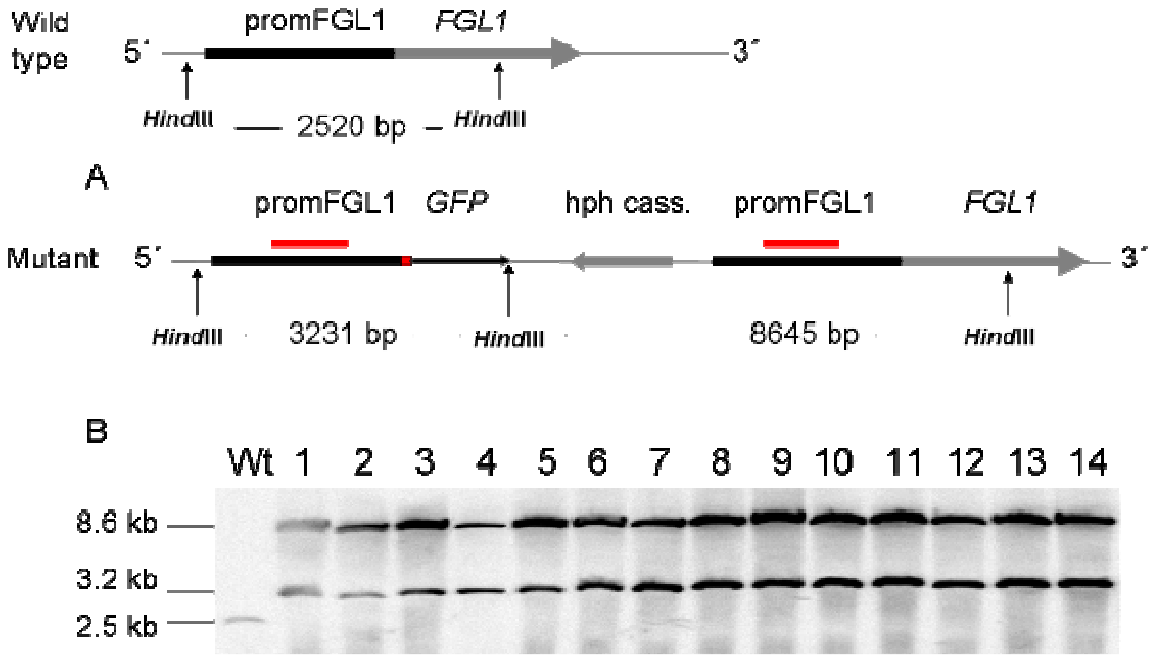

Fig. 38. Southern analysis of *promFGL1-GFP* strains. A: Enzyme *Hind*III was used for digestion of genomic DNA isolated from the wild type strain (Wt) and *promFGL1-GFP* strains. B: Hybridization of a Dig-labeled probe (red bars) amplified from promoter *FGL1* region. From 1 to 14: *promFGL1-GFP* strains.

3.7.2. Expression of lipase 1 is induced by different oils and hindered by the presence of glucose

Transcriptional expression of lipase *FGL1* is induced by different oils such as wheat germ oil (Voigt *et al*., 2005; Feng *et al*., 2005). To examine the inducer role of oils for the lipase 1 promoter, the *promFGL1-GFP* strains were grown on SNA medium plus wheat germ oil, triolein, and tributyrin as the sole carbon sources. GFP activity was examined by a fluorescence microscope. As shown in figure 39, GFP activity is intensively expressed in mycelia grown in cultures containing 2% wheat germ oil, triolein, and tributyrin as the sole carbon source. A faint GFP signal is also detected in mycelia grown in SNA medium (control), which contained 0.02% (w/v) glucose. These results indicate that lipase 1 promoter is inducible with wheat germ oil, triolein, and tributyrin. It also indicates the basal expression of lipase 1 promoter in SNA medium without inducers (oils).

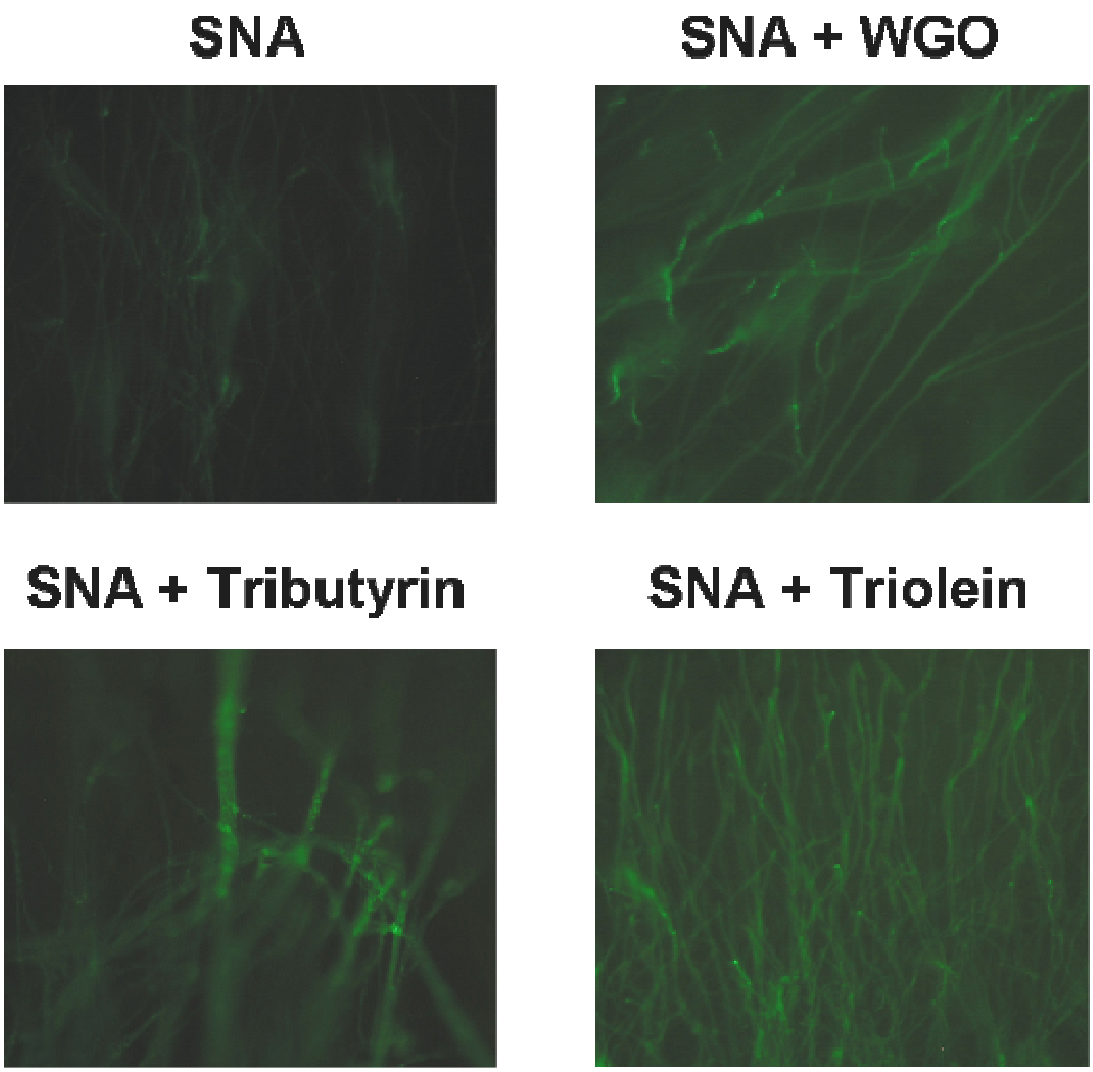

Fig. 39. GFP activity is induced by oils. *promFGL1-GFP* strains were grown for four days on SNA medium plus indicated oils as the carbon sources. GFP signal was visualized by a stereo fluorescence microscope Leica MZ FLIII using the Leica GFP2 filter set (excitations 480/40 nm, barrier filter 510 nm).

3.8. Localization of lipase 1

3.8.1. Generation of fungal strains expressing fusion protein of lipase 1 with the green fluorescence protein

To produce the fungal strains which express lipase 1 in fusion with the green fluorescence protein, the plasmid pANt.FGL1::GFP was used to transform wild type *F. graminearum*. Prior to transformation, the plasmid was restricted in *FGL1* by enzyme *Bst*XI to promote the insertional recombination. The integration of the plasmid into the *FGL1* locus results in mutant strains which have the native *FGL1* fused with the GFP gene (figure 40).

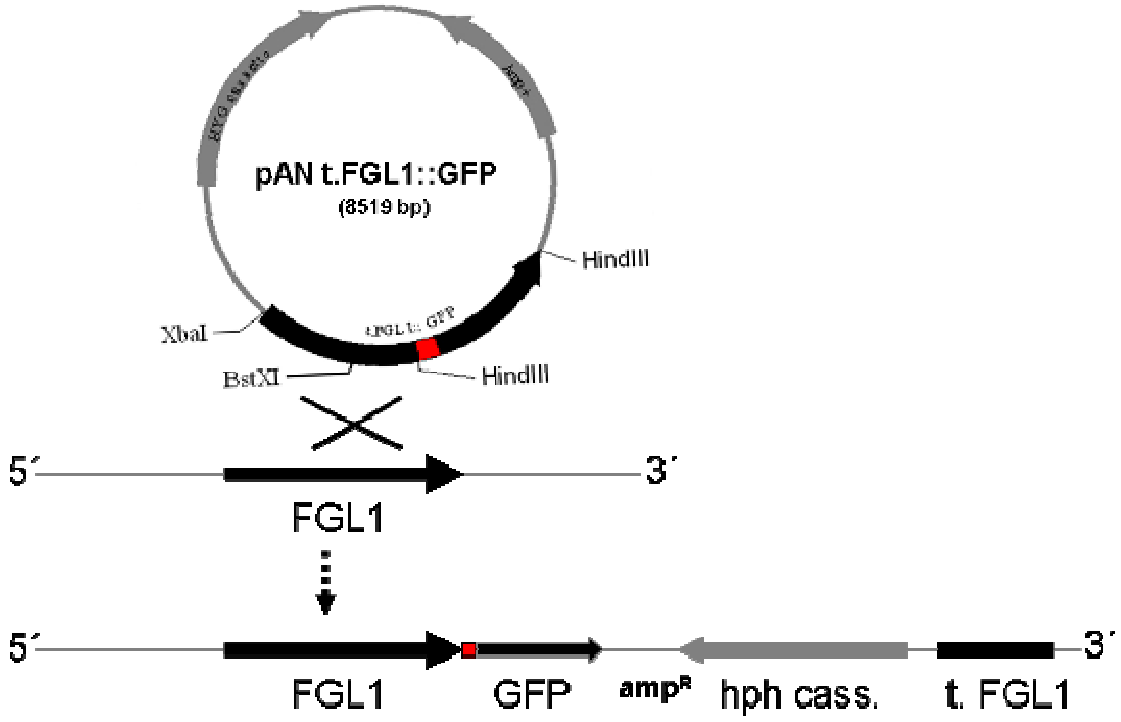

Fig. 40. Strategy for generation of *F. graminearum* strain expressing the fusion protein lipase 1 with the GFP protein. Plasmid pANt.FGL1::GFP was linearized in the truncated *FGL1* gene (t.FGL1) by *BstX*I. Homologous integration of the plasmid results in a transgenic strain, which has the native cassette of lipase gene 1 (promoter + gene) fused with GFP gene, and a truncated version of *FGL1*. The transgenic strain contains only one functional copy of lipase gene 1. Red bars: linker of 15 nucleotides.

Two transformations were carried out, which resulted in three hygromycin-resistant transformants. To analyze the transformants, RT-PCR was used to examine the transcripts of the fusion gene. Total RNA isolated from wheat germ oil induced mycelia was used to synthesize the single stranded cDNA. The primers FGL1GfpnF and FustestGFPrev, which respectively anneal to *FGL1* and GFP gene, were used in a PCR with the cDNA as templates. As shown below, a 991 bp fragment is amplified from the transformants, but not from the wild type (figure 41). This result proves the successful integration of the recombinant plasmid in lipase gene 1 locus.

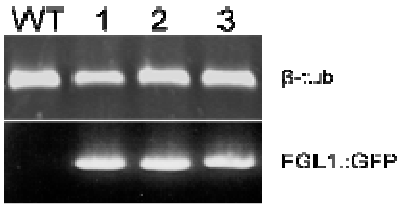

Fig. 41. Reverse transcription polymerase chain reaction (RT-PCR) analysis of *FGL1::GFP* strains. Single stranded cDNA generated from wild type (WT) and three transformants (1, 2, and 3) was examined by PCR for the presence of fusion transcripts (FGL1::GFP). Beta-tubulin (β-tub) was used as positive control.

3.8.2. Lipase 1 is successfully expressed as a fusion protein with GFP *in vivo*

Wheat germ oil is known as a good inducer for the expression of lipase gene 1. To examine the expression of the fusion protein, wheat germ oil was used to induce the lipase gene 1. To induce the expression, the *FGL1::GFP* strain (strain 3) and the WT-GFP strain (control) (Jansen *et al*., 2005) were cultivated on SNA medium plus 2% wheat germ oil. GFP activity was observed by using a fluorescence microscope. As shown in the figure below, the faint GFP activity is found in *FGL1::GFP* strain. This GFP signal is not comparable to that, which is constitutively expressed in WT-GFP strain (figure 42). Nevertheless, the GFP signals indicate the successful expression of fusion protein (lipase 1-green fluorescence protein) in the mutant strain. It is also observed that the GFP activity vanishes after few minutes of exposure.

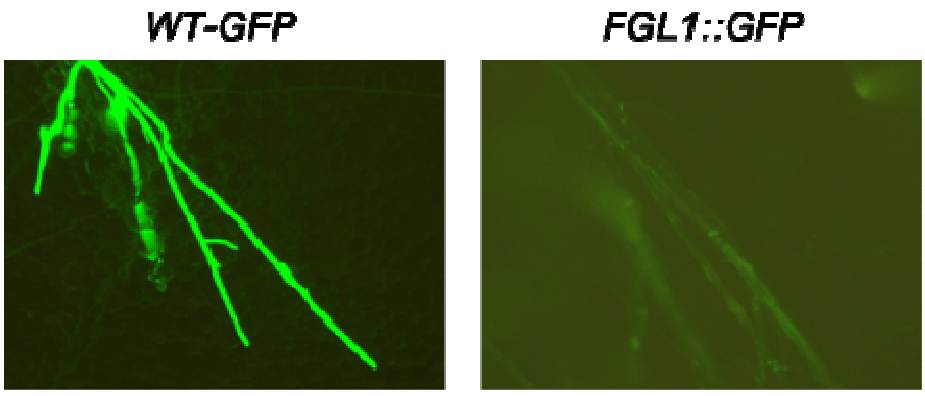

Fig. 42. GFP activity assay of the *FGL1::GFP* strain. Expression of functional fusion protein FGL1::GFP was examined *in vitro* in wheat germ oil induction medium. Three days old mycelia of the *FGL1::GFP* strain and the wild type-like strain expressing GFP (WT-GFP) were examined with a stereo fluorescence microscope Leica MZ FLIII using Leica GFP2 filter set (excitations 480/40 nm, barrier filter 510 nm).

4. Discussion

Fusarium graminearum (teleomorph: *Gibberella zeae*) is the causal agent of the head blight disease of small grain cereals in many regions in the world. It is responsible for significant yield loss and post harvest storage damage. For this reason, many investigations are conducted to study fungal physiology, the infection process, and to elucidate virulence factors as well as pathogenicity factors of the fungus. To accelerate investigations, its genome comprising of four chromosomes has recently been sequenced and this makes gene elucidation easier. To elucidate causal factors involved in infection process of *F. graminearum*, secreted enzymes, especially cell wall degrading enzymes, (CWDEs) are one of the main foci of investigations (Wanjiru *et al*., 2003; Jenczmionka, 2005; Phalip *et al*., 2005; Paper *et al*., 2007). Lipases are widely distributed among organisms (Jaeger and Reetz, 1998; Schmid and Verger, 1998), but not considered traditional cell wall degrading enzymes. A secreted lipase was found to play a role during *F. graminearum* infection of wheat and maize. This prompts new findings towards novel roles of lipases during infection of the fungus. Thus, additional studies to find new virulent lipases are necessary.

4.1. Secreted lipase genes of *F. graminearum*

Fusarium graminearum is able to secrete various hydrolytic enzymes in appropriate growth conditions and during plant infection (Wanjiru *et al*., 2003; Phalip *et al*., 2005; Paper *et al*., 2007). Besides endoglucanase, xylanolytic, proteolytic, and cellulosic enzymes, lipolytic enzymes are also detected in the presence of lipid substrates such as wheat germ oil (Jenczmionka, 2004). Several lipases are detected in culture media by proteomic approaches (Paper *et al*., 2007). However, their function is not yet characterized. When total lipolytic activity is blocked by applying the hydrolytic enzyme inhibitor ebelactone B, the fungal infection on wheat is reduced (Voigt *et al*., 2005). These studies suggest the existence of lipases in the fungus, and their inhibition leads to reduced infection of the fungus.

Recently, the genome of *F. graminearum* strain PH-1 was sequenced and released by the Whitehead Institute, Centre for Genome Research (Cambridge, MA, USA). Sequence annotation is also being constructed by *Fusarium*

graminearum Database (Broad institute, USA) and MIPS *Fusarium graminearum* Genome Database (Munich Information Center for Protein Sequences, Germany). Identification of lipase genes from *F. graminearum* is, thereby, boosted by the completion of the genome sequence. Following the finding of one secreted lipase, *FGL1* (Voigt *et al*., 2005), another secreted lipase, *LIP1*, was also revealed for its function (Feng *et al*., 2005). In this work, 15 phylogenetically related lipases were identified. They all contain a putative lipase domain of five amino acid peptides (pentapeptide) GXSXG as similarly found in *FGL1* and *LIP1*. As characterized in several lipases, this domain is known as the central active sites, in which serine functions as the catalytic residue (Blow, 1990; Davis *et al*., 1990; Lowe, 1992; Shen *et al*., 1998). Also, a putative leading sequence is present in the N-terminus of each lipase. Transcriptional expression of most of the lipases was also found during fungal growth and infection (see 3.1.4 and 3.1.5). This could lead to a “cocktail” expression, provided that they are translated into active proteins. Therefore, a quantitative impact of the lipases for virulence of the fungus is deduced.

Among these lipases, *FGL1* and *LIP1* are characterized. Notably, in the absence of a single lipase *FGL1*, the mutant strains become less virulent to wheat and maize. This raises a question about the function of the other lipases. As determined in the culture supernatants of the two mutant strains (*Δfgl1* and *ΔFgLip1*), the remaining lipolytic activity was significantly high at the later induction stages (Voigt *et al*., 2005; Feng *et al*., 2005). Therefore, it is necessary to reveal the role of the remaining lipases.

To find out the phylogenetic relationship of the lipases, their deduced amino acid sequences were compared. This reveals three main branches of lipases (figure 4). It is found that *FGL1*, *FGL2*, and *FGL3* are closely related to each other. In the MIPS FGDB database, these lipases belong to a group of seven putative proteins that are classified as lipase III family. One of their homologs called PSI-7 (FGDB entry: fg02519) is predicted to encode an autophagic lipase. Its homologs are absent in animals but present in fungi. Deletion of the homologous gene in *Yeast* (Cvt17) caused the accumulation of lipid vesicles (Teter *et al*., 2001). The fungus *Magnaporthe grisea* requires the autophagic cell death of the conidia for initiation of rice blast (Veneault-Fourrey *et al*., 2006). This could suggest a

possible involvement of the *F. graminearum* PSI-7 in nutrition recruitment and virulence. The majority of the identified lipases are assembled in the third group (see 3.1.2). As stated above, *LIP1* is the only one that was characterized in this group (Feng *et al*., 2005). It is reported that lipases *FGL8*, *FGL9*, and *FGL16* are found as secreted proteins in culture media (Paper *et al*., 2007). This suggests that these lipases might be exerted for nutrient acquisition by the fungus. *F. graminearum* secretes mycotoxins during plant infection (Brown *et al*., 2003; Kimura *et al*., 2003; Goswami and Kistler, 2004). These secondary metabolites are the products of a series reaction of the trichothecene biosynthesis pathway. This series of reactions requires a cluster of genes (trichothecene genes) to participate. It is noticed that *FGL6* has a significant sequence homology with *Tri8* (56% identity). Thus, it might play a role in trichothecene biosynthesis.

Analysis of sequence similarity and loci of the 17 lipases indicated that they are not a gene family as found in some *Candida* genera (Hube *et al*., 2000; Bigey *et al*., 2003). Besides, there is another lipase family, the so-called GDSL lipase (Akoh *et al*., 2004) family, which is also present in *F. graminearum*. There are 18 putative lipolytic enzymes which contain GDSL as catalytic motif in *F. graminearum*. The involvement of this lipase family in virulence has not been studied in *F. graminearum*. Nevertheless, the expression of one GDSL lipase (FGDB entry: fg12332) is found in plant cell wall media (Paper *el al*., 2007). The existence of many lipases, proteases or xylanases (Hatsch *et al*., 2006) might be related to the aggressiveness of the phytopathogenic fungus. Therefore, clarification of the identified lipases for their roles in virulence *in planta* is necessary.

4.2. Virulence role of lipase 2 and 5 and their proposed functions

In this study, seven lipase genes are individually disrupted in *F. graminearum*. Lipases from *FGL1* to *FGL5* and *LIP1* encode lipolytic enzymes. The remaining lipase genes are not characterized yet. The discovery that *FGL1* plays a key role in pathogenicity paved a way for elucidating other lipases as virulence factors. Hence, *FGL2* and *FGL5* were also characterized to be essential for the fungal infection of wheat and maize (virulence assays, see 3.4). The involvement in virulence of the two lipases is examined by gene disruption strategy, in which *FGL2* and *FGL5* gene are individually disrupted in the genome of *F.*

graminearum. Pathogenicity assays of wheat and maize reveal the reduced virulence in both mutant strains (*Δfgl2* and *Δfgl5* strains). Infection of *Δfgl2* and *Δfgl5* strains on wheat spikes is restricted to the inoculated spikelets. In the case of *Δfgl2* strains, the infection progresses from inoculated spikelets, but less mycelia is observed outside of the spikelets. Longitudinal sections of infected spikelets reveal that the infection is unable to penetrate through the rachis node. As shown in some cases of virulence factors such as *FGL1* or *Tri5*, the impairment of further colonization in wheat spikes of the corresponding mutant strains *Δfgl1* and *Δtri5* is due to the formation of a barrier or cell-wall thickenings of the host cells at infected areas (Voigt *et al*., 2005; Jansen *et al*., 2005). Since no symptoms were observed in the stem, similar effects occurring in the rachis node on the spreading of *Δfgl2* strains could be possible. With regard of *FGL5*, the infection pattern of *Δfgl5* strains is quite similar to that of *Δfgl2* strains and previously described mutant strains (*Δfgl1* and *Δtri5*). In some cases, mycelia growing outside of the inoculated spikelets are observed. However, no spreading signs are found in the neighbouring spikelets. Longitudinal sections reveal that the rachis node and internodes of the two infected spikelets are not infected. It is notable that the loss of one of the virulence lipases such as *FGL1*, *FGL2* or *FGL5* brings about reduced fungal infection. Most likely, the local changes at rachis node form the barriers to prevent further colonization of the lipase-disrupted strains in wheat spikes. However, to study the fungal-host interactions, histological investigations of the lipase-deficient strains with constitutive expression of green fluorescence protein could provide the knowledge of whether the infection is restricted to the rachis region.

Lipase *FGL2* shares a significant sequence homology (34% identity) with lipase *FGL1*. Furthermore, they have the same putative motif (GHSLG). These suggest similar functions of the two lipases. Among the lipase-synthetic substrates of para-nitrophenyl esters, *FGL1* prefers p-nitrophenyl myristate (C14) as the optimum substrate (Zivkovic, 2003), whereas *FGL2* has the strongest activity against p-nitrophenyl caprate (C10). It is observed that the phenotypic characteristics of lipase 1 and 2-disrupted strains are quite similar in terms of growth and infection patterns. It is also found that loss of *FGL2* gene caused the impaired expression of *FGL1* gene (Fehrholz, 2007). Thus, a possible activation

of *FGL1* by diacylglycerol or fatty acids liberated from activity of lipase 2 is surmised. In this study, it is shown that gamma-linolenic acid (18:3) but not palmitic acid (16:0) induces the expression of lipase gene 1, thus enhancing the infection of the *Δfgl2* strain. In studies of mammalians, polyunsaturated fatty acids (PUFAs) have been implicated as natural ligands for transcription factors in gene regulation (Göttlicher *et al*., 1992: Kliewer *et al*., 1997; Jump, 2004; Sampath and Ntambi, 2004). Fatty acids interact with the genome through several mechanisms. They and their various metabolites bind directly to specific transcription factors to regulate gene transcription. Several of these transcription mediators have been identified. They include the nuclear receptors peroxisome proliferator-activated receptor (PPAR), hepatocyte nuclear factor (HNF)-4alpha, and liver X receptor (LXR) and the transcription factors sterol-regulatory element binding protein (SREBP) and nuclear factor-kappaB (NFkappaB). Their interaction with PUFAs has been shown to be critical for the regulation of several key genes of lipid metabolism (Jump, 2004; Sampath and Ntambi, 2005). In yeast, fatty acid regulation of genes seems to occur through the two fatty acid–responsive transcription factors Oaf1p/Pip1p and Oaf2p/Pip2p (Black *et al*., 2000). Oaf1p and Oaf2p have been shown to form a heterodimer in response to exogenous fatty acids and bind to a cis element in the DNA promoter, termed the oleate responsive element (ORE). The element is the sequence of CGGNNNTNA(N9–12)CCG (Black *et al*., 2000; Sampath and Ntambi, 2005). As identified in *FGL1* promoter sequence there are several elements, which supposedly contribute to the basal expression of the gene. A regular TATA-box within 200 bp of the start codon is found in the *FGL1* promoter. Additionally, in the *FGL1* promoter RNA enhancing binding protein (*REB1*) region seems to play a similar effect (Jäger, 2008). In yeast, *Reb1p* cooperates with *Gal4p* to exclude nucleosomes from the transcription initiation site, thus modulating the basal transcription (Morrow *et al*., 1993; Angermayr and Bandlow, 2003). Therefore, these identified elements might be responsible for the basal expression of *FGL1*. In *A. nidulans* a promoter element, termed a fatty acid response element (FARE), is responsible for the induction by fatty acids (Hynes *et al*., 2006). This sequence, CTTCGG, is identified in the promoter region of *LIP1* (Feng, 2007), but not in *FGL1* (Jäger, 2008). This could imply a differentially mediated activation of *FGL1*

via unknown factors. Here, the evidence for activation of *FGL1* by exogenous free fatty acid is provided. However, the direct evidence of activation of *FGL1* expression via products released by *FGL2* activity is still unknown, since the actual substrates of *FGL2* activity are not characterized. Therefore, it is necessary to determine the natural substrates of *FGL2*.

In this study, lipase *FGL5* is shown to be involved in virulence of the fungus. However, its function in virulence of the fungus is not yet known. It is evident that loss of the lipase gene caused the impaired production of aerial mycelia in the mutant strain. Therefore, a link between this phenotype and that of MAP kinase Gpmk1 deletion strain is possible. The MAP kinase *gpmk1* is involved in pathogenicity and supposedly regulates diverse growth and differentiation processes (Jenczmionka and Schäfer, 2005). One of the possible roles of the MAP kinase is the regulation of secreted products such as enzymes and hydrophobins. It is proposed that the MAP kinase regulates the virulence factor *FGL1* (Voigt, unpublished data). Also, it is possible that the MAP kinase regulates hydrophobins, which play an important role in fungal attachment to the plant cuticle (Jenczmionka, 2004). Hydrophobins are uniquely secreted by filamentous fungi and essential for the development of aerial hyphae (Wessel *et al*., 1991; Lugones *et al*., 1996; Ando *et al*., 2001) and virulence (Talbot *et al*. 1993). Furthermore, an easily wettable phenotype is also found in the *FGL5* deletion strain (see 3.3.2.3). This result is similar to that reported for the Gmpk1-disrupted mutant strain. Therefore, hydrophobin-like roles of *FGL5* are assumed. Additionally, the disruption of *FGL5* caused the decreased expression of *FGL1* (Fehrholz, 2007). If the aforementioned hypothesis of a regulating role of MAP kinase *Gmpk1* for *FGL1* is right, a possible regulation via *FGL5* will be imaginable.

Based on a previous study (Voigt, unpublished data) and results from this study, a model of the activation of *FGL1* is constructed (figure 43). In this model, three steps of the activation process of *FGL1* via the released products of *FGL2* and *FGL5* activity are proposed. In the first step, lipase *FGL2* and *FGL5* catalyze the hydrolysis of plant lipids to liberate free fatty acids (FFAs). In the second step, the released free fatty acids are, in turn, used to induce gene expression. It is unknown whether the released free fatty acids directly induce the expression of

FGL1 gene or regulatory genes of *FGL1*. In the third step, the massive expression of *FGL1* is induced, which leads to the successful infection of plants.

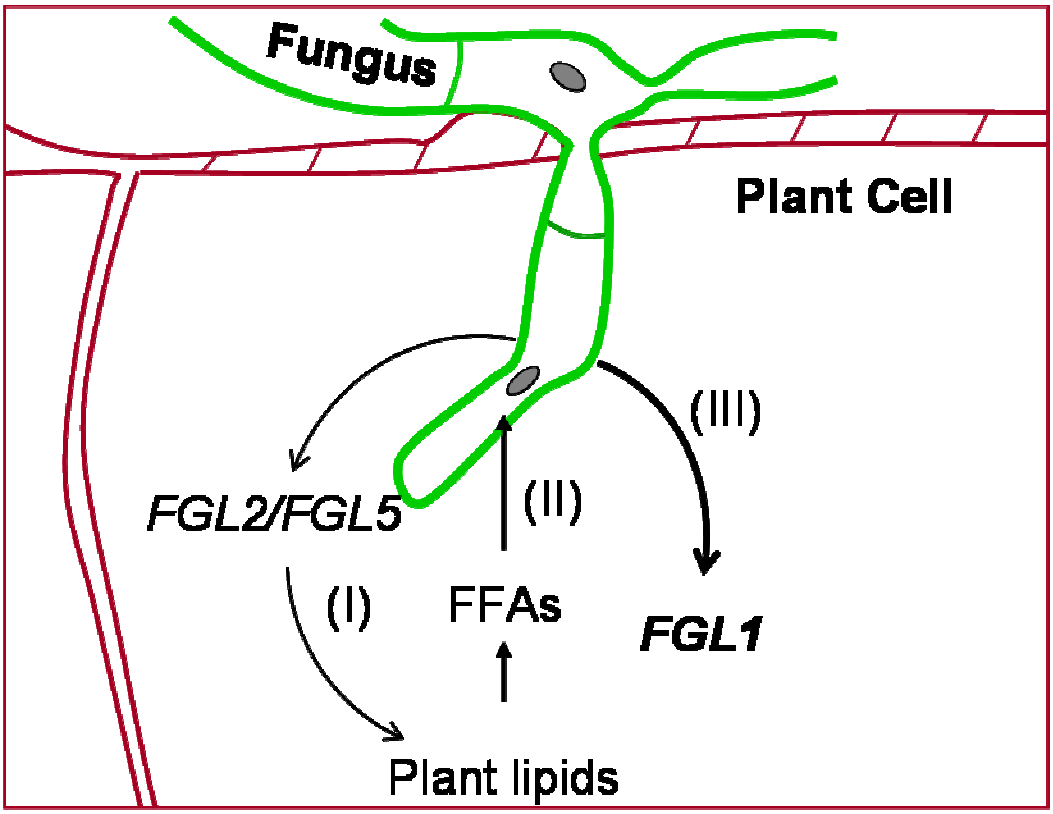

Fig. 43. A model of activation of *FGL1 in planta*. During the infection process, the fungus secretes the lipase *FGL2* and *FGL5* to hydrolyze lipid substances from host tissues. This hydrolysis liberates free fatty acids, which in turn induce the expression of *FGL1*.

Disruption of either *FGL2* or *FGL5* impairs the transcriptional expression of *FGL1 in planta*. Again, *FGL1*-disrupted mutant strains show a great reduction in total lipolytic activity *in vitro*. However, both *FGL2* and *FGL5*-disrupted strains retain their wild type lipolytic activity *in vitro*. Therefore, it is proposed that the possible interactions of the lipases specifically take place *in planta*.

4.3. Possible essential role of lipase gene 3

Repeated transformations were performed to disrupt the *FGL3* gene, which resulted in hygromycin-resistant transformants. However, all transformants are ectopic mutants. Therefore, an essential role of *FGL3* for fungal viability is assumed. The possible vital role of several genes was also reported in this fungus. Bluhm reported that *Ras1*, a Ras GTPase protein, is essential for the fungal viability when repeated disruption attempts failed (Bluhm *et al*., 2007). Additionally, a cutinase transcription factor (*CTF1* alpha) that is the ortholog of

Ctf1 from *F. oxysporum* seems to be essential because its disruption leads to fungal death (unpublished data). *FGL3* gene encodes a triacylglycerol lipase with a putative signal peptide. However, no evidence for a vital role of a secreted lipase is provided. Therefore, the clarification of *FGL3* roles is meaningful. The gene disruption strategy can not be employed for potentially essential genes. Since evidence of RNA interference exists in *F. graminearum* (McDonald *et al*., 2005). Therefore, down-regulating the genes by RNA interference could address this issue.

4.4. Dispensable role of lipase 4, 9, 12, 13, and 14 for fungal virulence

It is clarified that the majority of lipase genes are dispensable for fungal virulence. Here, it is shown that lipase *FGL4*, *FGL9*, *FGL12*, *FGL13*, and *FGL14* are not important for fungal infection. Previously, it is reported that disruption of *LIP1* led to decreased lipolytic activity in the culture supernatant. This indicates that *LIP1* is a secreted lipase. However, *LIP1* is not required for pathogenicity of *F. graminearum* (Feng *et al*., 2005). Although the involvement of the lipases in virulence is not found, the function in nutrition acquisition is essential in some cases. For example, *LIP1* is essential for utilization of saturated fatty acids. Herein, it is likely that *FGL13* is required for lipid utilization. It is shown that the disruption of the gene causes a delayed growth when several oils are provided as the sole carbon source. As shown in 3.3.2.2, however, lipolytic activity of the lipase 13-disrupted mutant strains is not decreased in comparison with the wild type strain. Thus, it is claimed that the lipase is dispensable by the presence of the other lipases or the upregulation of other lipases. The same explanation can be given for *FGL4*, *FGL9*, *FGL12*, and *FGL14* cases.

4.5. Major virulence role of lipase 1 and mechanism of lipase 1 regulation

Lipases (EC 3.1.1.3) are hydrolases, which exert their activity on the carboxyl ester bonds of triacylglycerols and other substrates. The natural substrates of lipases are lipid compounds that are insoluble and prone to aggregation in aqueous solution. Lipases, also called the interfacial enzymes, are activated in the interface of aggregated substrates and aqueous solution. Functionally, lipases are distinguishable from esterases, which are hydrolytic enzymes active on ester bonds of soluble molecules, and their catalytic kinetics follow the

Michaelis - Menten equation. Fungi are mostly saprophytes, and some of them are plant pathogens. The virulence of lipases is investigated in several plant pathogens. Initially, the incubation of specific antibodies against a 60 kDa-secreted lipase with the conidial suspension of *Botrytis cinerea* showed the suppression of lesion formation on tomato leaves (Comménil *et al*., 1998). Similarly, an 80 kDa lipase purified from the washings of ungerminated spores of *Alternaria brassicicola* showed the cross-reactivity with antilipase antibodies from *Botrytis cinerea* (Berto *et al*., 1999). Incubation of the *A. brassicicola* conidia with the antibodies prior to infection led to the reduction of blackspot lesions on cauliflower leaves by 90%. However, disruption of the corresponding *Botrytis* lipase gave a different result in virulence as the lipase-deficient mutants retained full pathogenicity on various host systems (Reis *et al*., 2005). Thus, lipases as virulence factors in *Botrytis cinerea* are being controversially disputed.

Nonetheless, the evidence that lipases are virulence factors in *F. graminearum* is obvious. Initially, disruption of the MAP kipase, *Gpmk1*, led to non-pathogenic phenotypes. Further analysis of the extracellular enzymes of the ∆*gpmk1* strains revealed the reduction of proteolytic, xylanolytic, and particularly lipolytic activity. This suggests the importance of secreted lipases for pathogenicity of *F. graminearum*. This hypothesis is clarified by the disruption of *FGL1*, a secreted lipase. ∆*fgl1* strains show great reduction in lipolytic activity and virulence on wheat and maize (Voigt, *et al*., 2005). Recently, disruption of a GTPase, *Ras2* protein, leads to the reduction in pathogenicity of the fungus. The virulence of FR-1 (disruption of *Ras2* gene) strains is also proven to be related to the reduction of *FGL1*. Herein, it was shown that disrupted lipase genes *FGL2* and *FGL5* caused a reduced infection of the fungus. Again, it is found that the transcriptional expression *FGL1* is decreased in the mutant strains during wheat infection, indicating its involvement in reduced virulence. Additionally, constitutive expression of *FGL1* in a *FGL2*-disrupted mutant strain increases virulence on wheat. It is shown that *FGL1* transcripts are accumulative over time (3.5.2). This accumulation is also consistent with the observed scab symptoms at the later time points. Furthermore, evidence supporting the extensive expression of *FGL1* during fungal infection of wheat spikes are found (Fehrholz, 2007). These

suggest a colonizing role of *FGL1* during colonization. In summary, these results indicate a major role of *FGL1* during fungal infection.

The expression of lipase 1 is induced by different lipids and repressed by glucose. Gene repression by glucose is known as catabolite repression and mainly taking place at the transcriptional level (Gancedo, 1998). In *Saccharomyces cerevisiae*, *Mig1p* is a global repressor protein dedicated to glucose repression. *Mig1p* represses genes either by binding directly to the upstream repression sequence of regulated genes or by indirectly repressing a transcriptional activator, such as *Gal4p* (Verma *et al*., 2005). It is found that the upstream region of *FGL1* contains a binding site for a negative regulator, a homolog of *Mig1p*, for carbon catabolite repression (Jäger, 2008), suggesting the transcriptional regulation of the *FGL1* gene. However, evidence showed that glucose did not hinder the transcription of *FGL1* (Fehrholz, 2007). Recently, a putative cutinase transcription factor, *Ctf1*, was shown to regulate cutinase and lipase genes in *F. oxysporum* (Rocha *et al*., 2008). Particularly, *Ctf1* deletion strains show drastic reduction in lipolytic activity. Though deletion of one of its homologs (*CTF1B*) in *F. graminearum* does not alter the expression of lipolytic activity (unpublished data), this finding could pave a way to elucidating the regulators of lipases.

4.6. Lipases are virulence factors from bacteria to fungi

Bacterial species are among the main sources of lipase production as well as for cloning lipase genes. To date, many lipases cloned from bacteria have been used for various applications (Jaeger *et al*., 1996; Jaeger *et al*., 1998; Jaeger and Eggert, 2002). Most of lipases are cloned or isolated from non-pathogenic species and used for biological applications. However, there are few lipases which are exerted by bacteria as virulence factors. Here, the instances of lipases as virulence factors and as potential virulence factors are summarized. A dermal disease called *acne vulgaris* appearing in humans during puberty and caused by *Propionibacterium acnes* is the first example in this respect. This bacterium produces various enzymes including a lipase during human skin colonization. As a normal inhabitant of human skin, it resides inside the sebaceous glands and produces a lipase that can cleave sebum triglycerides, thereby liberating free fatty acids. It has been showed that the liberated fatty acids supported the

bacterial adhesion and colonization of sebaceous follicles (Ingham *et al*., 1981; Gribbon *et al*., 1993). *Pseudomonas aeruginosa* was probably among the most well-known bacteria exerting lipolytic enzymes as virulence factors. This bacterium is involved in a variety of acute and chronic infectious diseases such as cancer and cystic fibrosis (Döring *et al*., 1993; König *et al*., 1994). Clinical *Pseudomonas aeruginosa* isolates produce both lipase and phospholipase (Berka *et al*., 1981; Chin *et al*., 1988; Jaeger *et al*., 1992; König *et al*., 1996). Pre-incubation of the lipase preparation with human peripheral blood neutrophils and monocytes showed that lipase significantly inhibited the chemotaxis and chemiluminescence of monocyte, but had no or very little effect on neutrophiles (Jaeger *et al*., 1991). Furthermore, the lipase interacts with phospholipase C (PLC) during generation of 12-hydroxyeicosatetraenoic acid (HETE) from human platelets (König *et al*., 1996). Additionally, lipase synergistically interacts with several bacterial PLCs to induce generation of inflammatory mediators, which afterwards activate the inflammatory cells like neutrophils, basophils, monocytes, and platelets. These reactions could lead to initiation of tissue damage and stimulation of inflammatory processes.

Lipids are found ubiquitously on the surface of human skin, which is largely composed of sebum-derived triacylglycerides. It is understandable that most organisms colonizing human skin possess some lipolytic activity, and this is believed to be responsible for the hydrolysis of sebaceous lipids, liberating free fatty acids onto the cutaneous surface. Additional to *S. acnes* isolates, *Staphylococcus epidermidis* is another human skin commensal bacterium, which can become opportunistic when the human immune response is compromised. During colonization of human skin, two lipases were secreted and might be involved in the infection process. However, the mechanism of the lipase activity is not yet known (Longshaw *et al*., 2000).

Burkholderia cepacia complex (Bcc) is a group of Gram-negative bacteria composed of at least nine different species, including *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. vietnamiensis*, *B. stabilis*, *B. ambifaria*, *B. dolosa*, *B. anthina*, and *B. pyrrocinia*. They are recognized as important human pathogens associated with cystic fibrosis in immunocompromised individuals (Mahenthiralingam *et al*., 2002; Mahenthiralingam *et al*., 2005). Among them, *B.*

multivorans and *B. cenocepacia* strains are the most prevalent species in these patients (LiPuma *et al*., 2001; Speert, 2002). Determination of lipase activity of these strains shows that these two strains express more lipase activity than other strains (Mullen *et al*., 2007). Pre-treatment of lung epithelial cells with a commercial *B. cepacia* lipase enhances the invasiveness of the high lipase-producing Bcc strains, suggesting the impact of lipase on cellular invasion. Furthermore, inhibition of lipase activity produced from *B. multivorans* and *B. cenocepacia* strains by lipase inhibitor Orlistat significantly reduced the invasiveness. The effects of the lipase activity on reduction of plasma membrane or tight junction integrity of epithelial cells were experimentally excluded, suggesting the specific lipase effect on the tissue exposure to bacterial infection and bacterial adherence by liberation of fatty acids (Mullen *et al*., 2007). The lipolytic activity of the other human intestinal pathogens, such as *Camplybacter pylori* and *Helicobacter pylori*, is also significant in terms of mucosal lipid degradation, thus facilitating the ulcerative process (Slomiany *et al*., 1989; Kusters *et al*., 2006). Gottlich *et al*. (1995) also demonstrated that the lipolytic activity of the pathogenic fungus *Hortaea werneckii* increases hydrophobic interactions by the liberation of free fatty acids, thereby enabling the fungus to adhere to skin. *Xanthomonas oryzae pv.oryzae* is the causal agent of bacterial leaf blight, a serious disease of rice. Rajeshwari *et al*. (2005) and Jha *et al*. (2007) showed that mutation in a gene that encodes a lipase/esterase (*LipA*) partially affects the virulence. Double mutation of this lipase gene and a gene encoding a cellulase (*ClsA*) strongly reduces virulence (Jha *et al*., 2007), indicating the possible cooperation of the lipase with the cellulase, a cell wall degrading enzyme active during plant infection. The importance of lipase (*LipA*) as a determinant for virulence is also discovered in *B. glumae*, the causal agent of rice grain and seedling diseases (Urakami *et al*., 1994). It is also postulated that the *LipA* lipase plays a crucial role as a cell wall-degrading enzyme, and that this enzyme in co-operation with other cell wall-degrading enzymes breaks down components of cell walls (Devescovi *et al*., 2007).

Fungi are a very important group of pathogens. It is well known that many fungi are saprophytic organisms. During their lifecycles, secreted enzymes are excreted to degrade biomaterials. This fungal activity could be deleterious to

plants, animals and humans in terms of pathogens. Fungal lipases are exploited for various applications in industry and medicine. The latter seems to be important when secreted lipases might be used as virulence factors for infection. Thus, they could be promising targets for antifungal drugs. An increasing number of secreted lipases are discovered to be essential for fungal infection. Two lipases *LIP8* and *CpLIP2* from *Candia albicans* and *Candida parapsilosis*, respectively, have been recently characterized as virulence factors (Gácser *et al*., 2007a; Gácser *et al*., 2007b). The genus *Candida* is known as human opportunistic pathogen, which causes candidosis in individuals with immuno-compromised conditions. Molecular techniques have been successfully established to knock out genes from these diploid organisms. The deficiencies in both alleles of *LIP8* in *C. albicans* and *CpLIP2* in *C. parapsilosis* result in reduced virulence mutants. Particularly, the loss of *CpLIP2* alleles significantly decreases the infection of the fungus in reconstituted human epithelium cells. Additionally, the lipase-negative mutants are vulnerable to phagocytosis by macrophage-like cells (Gácser *et al*., 2007b). These findings could contribute to the synthesis of drugs against candidosis. It is also appropriate to restate that *Fusarium graminearum* infection is inhibited by applying ebelactone B, a general hydrolytic enzyme inhibitor (Voigt *et al*., 2005). Disruption of *FGL1* leads to a similar reduction in virulence as the application of ebelactone B to the wild type *F. graminearum*. The involvement of *FGL2* and *FGL5* in virulence of the fungus is also elucidated. The relationships of *FGL1* with *FGL2* and *FGL5* are implicated. The reduced infection of *Δfgl2* and *Δfgl5* could be related to the diminished expression of *FGL1*. If this is the case, applying an antagonist of *FGL1* could prevent *F. graminearum* from infection. However, this raises the question whether a single lipase, in this case *FGL1*, is a determinant for fungal infection. To clarify this question it is critical to reveal the *FGL1* regulators and regulation mechanism of the lipase.

4.7. Possible applications of lipase 2, 3, 4, and 5 in industry

The main aim of this study is to identify and characterize secreted lipases as virulence factors for *Fusarium graminearum*. Nevertheless, four lipases have been characterized in this study by heterologous expression in *Pichia pastoris,* and their potential applications in industry are not neglectable. Lipases are one of

the most important industrial enzymes due to their versatile applications (Jaeger and Reetz, 1998). Lipases have been extensively used in many applications due to their multiplicity, specificity, and their chemo-, enantio-, and regioselectivity for various enzyme reactions (Jaeger *et al*., 1999; Pandey *et al*., 1999; Houde *et al*., 2004). Fungi are preferred sources for isolation and production of lipases due to their eukaryotic post-translational machinery, which provides means for modifications of primary protein structures (Nevalainen *et al*., 2005). Another fact that many lipases are secreted by fungi to exert exogenous lipid compounds. This makes fungal lipases easy to extract. Lipases *FGL2* and *FGL3* are assigned as class 3. They both have a broad range of substrate specificity and their optimum substrate is 4-nitrophenyl caprate (C10). *FGL2* has a considerable similarity (49% identity) with an extracellular lipase from the plant pathogen fungus *Aureobasidium pullulans*. This lipase gene consists of 1245 bp that contains a lipase putative domain GXSXG and encodes a 47 kDa protein when expressed in *E. coli* (Liu *et al*., 2008). It is reported that this lipase shows highest activity at 35 – 40^{o}C and pH 7 – 8. These conditions are also suitable for *FGL2* activity. The *A. pullulans* lipase is able to hydrolyse several edible oils tested (Liu *et al*., 2008), such as olive oil and peanut oil. This suggests that the lipase has a potential role to digest lipid compounds. *F. graminearum* lipase *FGL2* also has potential for digestion of lipid substrates, since its recombinant form from *P. pastoris* shows a highest activity against 4-nitrophenyl caprate. To clarify *FGL2* activity against natural lipid substrates, lipase assays with wheat germ oil or olive oil need to be performed. *F. graminearum* lipase *FGL3* shares significant identity (39%) with *Lip1*, a lipase secreted by *Malassezia globosa* (Chu *et al*., 2007). However, none of its closed analogues has been characterized for biotechnological interest so far. Nevertheless, an extracellular lipase from a medicinal fungus *Antrodia cinnamomea,* which shares 32% identity with *FGL3,* has been purified and characterized. It is shown that the enzyme is alkaline tolerant (pH 7–10) and has its optimum activity at pH 8.0. The enzyme activity is clarified to be stable within the temperature range of 25 – 60^{o}C, with maximal activity at 45^{o}C (Shu *et al*., 2006). It is interesting that *FGL3* has a wider range of optimum temperatures among four *Fusarium graminearum* lipases characterized in this study, in which its higher activity is recorded at a range of temperatures from 30 – 60^{o}C. *FGL3* also has a broad range of long chain carbon lipase

substrates. However, its activity is found less stable at pH values lower than 4 and higher than 9. It has been shown that many yeast lipases have optimum temperatures ranging from 30 – 50^{o}C (Vakhlu and Kour, 2006). Also, many fungal lipases are proven to be most active at neutral pH (Vakhlu and Kour, 2006). These characteristics are also found in *FGL4* and *FGL5*. These enzymes are most active at 37^{o}C and pH 7. *FGL4* is shown to have its highest activity against p- nitrophenyl dodecanoate (C12), whereas *FGL5* prefers to catalyze the hydrolysis of p- nitrophenyl octanoate (C8). The wild type fungus *F. graminearum* is able to grow in media containing various kinds of oil as the sole carbon source, even when one of its secreted lipases is disrupted (Feng *et al*., 2005; Voigt *et al*., 2005). These data suggest that *F. graminearum* is a potential source of lipolytic enzymes that could be exploited for industrial applications together with the ones that have been characterized in this study and previous studies (Feng *et al*., 2005; Voigt *et al*., 2005).

5. Summary

Fusarium graminearum is a phytopathogenic fungus that causes Fusarium Head Blight (FHB) disease in small cereal crops. It is determined that *F. graminearum* secretes many extracellular enzymes (Wanjiru *et al*., 2003; Jenczmionka and Schäfer, 2005; Phalip *et al*., 2005; Paper *et al*., 2007), which are postulated to play a role in fungal infection. Evidence shows that lipolytic enzymes play a critical role during the infection process of the fungus (Jenczmionka and Schäfer, 2005). This enabled to discover that lipase 1 (*FGL1*) is essential for fungal virulence (Voigt *et al*., 2005). The finding has laid the foundation for this study to elucidate novel lipases as virulence factors. In this study 15 new lipases, which share a common lipase domain with *FGL1*, are identified. Expression analysis *in vitro* and *in planta* shows that most of the lipases are expressed. This could implicate an involvement of the lipases in the infection process. To clarify this question, gene disruption is used to characterize the function of individual lipase. Among them, 7 lipase genes (*FGL2*, *FGL4*, *FGL5*, *FGL9*, *FGL12*, *FGL13*, and *FGL14*) are individually disrupted. It is shown that lipase 2 and 5 are novel virulence factors of the fungus, while lipases 4, 9, 12, 13, and 14 are dispensable for virulence.

Disruption of lipase 1 leads to a great reduction of extracellular lipolytic activity of the fungus at early induction time points (Voigt *et al*., 2005). This indicates that lipase 1 is a secreted lipase and constitutes the majority of onset lipase activity *in vitro*. However, disruption of the aforementioned lipases does not affect the total extracellular lipase activity, suggesting that they are dispensable for total lipase activity *in vitro*. Interestingly, it is found that the disruption of lipase gene 2 and 5 influences the expression of lipase gene 1 *in planta*, thereby prompting the reduction in virulence of the corresponding mutant strains.

Polyunsaturated fatty acids are known as gene regulators. Herein, it is shown that gamma-linolenic acid induces the transcriptional expression of *FGL1*, thus enhancing the virulent behaviour of a lipase 2-disupted strain. Hence, it is interpreted that lipase 2 plays a role in activation of lipase 1 *in planta* by its liberated products. Additionally, it is also shown that overexpression of lipase 1

enhances the virulence of the lipase 2 disruption strain. Therefore, it is concluded that lipase 1 is a major virulence factor.

Localization of lipase 1 with the green fluorescence protein (GFP) is used to gain knowledge of targeted tissues. This study provides preliminary results of functional expression of lipase 1 in fusion with GFP *in vitro*. The expression of lipase 1 is also detected in wheat tissues. This provides direct evidence of involvement of lipase 1 in fungal infection. Understanding the regulation mechanism of lipase 1 is also important. This study provides evidence of oils as inducers of lipase 1 promoter.

Lipases are also known as catalysts for various applications. Lipase genes 2, 3, 4, and 5 are functionally expressed in *Pichia pastoris*. The recombinant lipases encode triacylglycerol lipases. They are able to catalyze different substrates, with optimum pH 7 and temperature 37^{o}C. Hence, this study provides preliminary investigations on the lipases for possible applications in industry.

6. Zusammenfassung

Fusarium graminearum ist ein phytopathogener Pilz, der bei verschiedenen Gräsern Fusarium Head Blight (FHB) hervorruft. Es ist bekannt, daß *F. graminearum* viele Enzyme sezerniert (Wanjiru *et al*., 2003; Jenczmionka and Schäfer, 2005; Phalip *et al*., 2005; Paper *et al*., 2007), die wahrscheinlich eine Rolle bei der Infektion spielen. Es konnte bewiesen werden, daß lipolytische Enzyme eine wichtige Funktion während des Infektionsprozesses von *F. graminearum* einnehmen (Jenczmionka and Schäfer, 2005). Darauf aufbauend wurde entdeckt, daß Lipase 1 (*FGL1*) essentiell für die Virulenz des Pilzes ist. Ziel dieser Studie ist es, die Funktion neuer Lipasen und ihre Rolle bei der Pathogenität von *F. graminearum* aufzuklären. In dieser Arbeit werden 15 Lipasen beschrieben, die eine gemeinsame Domäne mit *FGL1* haben. Expressionsanalyse *in vivo* und *in planta* zeigt, daß die meisten dieser Lipasen exprimiert werden. Dies könnte dafür sprechen, daß die Lipasen am Infektionsprozeß beteiligt sind. Um dieser Frage nachzugehen, wird gene disruption verwendet, um die Funktion der einzelnen Lipasen zu charakterisieren. Sieben Lipasegene werden individuell abgeschaltet (*FGL2*, *FGL4*, *FGL5*, *FGL9*, *FGL12*, *FGL13*, und *FGL14*). Es wird gezeigt, das Lipase 2 und 5 neue Virulenzfaktoren des Pilzes sind, während Lipase 4, 9, 12, 13 und 14 für die Virulenz entbehrlich sind.

Disruption von Lipase 1 führt zu einer großen Reduktion der extrazellulären lipolytischen Aktivität des Pilzes zu Beginn der Induktion (Voigt *et al*., 2005). Daraus kann man schließen, daß Lipase 1 eine sezernierte Lipase und für den Großteil der lipolytischen Aktivität *in vitro* verantwortlich ist. Schaltet man allerdings die oben genannten Lipasen ab, wird die gesamte extrazelluläre lipolytische Aktivität nicht beeinflußt, weshalb diese Lipasen wahrscheinlich für lipolytische Aktivität *in vitro* entbehrlich sind. Interessanterweise beeinflußt das Abschalten von Lipase 2 und 5 die Expression der Lipase 1 *in planta*, so daß die Mutantenstämme in ihrer Virulenz reduziert sind.

Mehrfach ungesättigte Fettsäuren sind als Genregulatoren bekannt. Gamma-Linolensäure induziert die transkriptionelle Expression von *FGL1*, wobei die Virulenz eines Mutantenstammes mit abgeschalteter Lipase 2 erhöht wird.

Dieses Ergebnis kann so interpretiert werden, daß Lipase 2 durch ihre Abbauprodukte eine Rolle bei der Aktivierung von Lipase 1 *in planta* spielt. Außerdem kann man zeigen, daß Überexprimierung von Lipase 1 die Virulenz von Mutantenstämmen mit abgeschalteter Lipase 2 steigert. Daher ist Lipase 1 ein wichtiger Virulenzfaktor. Lokalisation von Lipase 1 mit dem green fluorescent protein (GFP) wird eingesetzt, um die Zielgewebe zu identifizieren. Diese Studie führt zu ersten Ergebnissen zur funktionalen Expression der Lipase 1 in Fusion mit GFP *in vitro*. Außerdem wird die aktive Expression von Lipase 1 in Weizen beschrieben. Diese Ergebnisse liefern Beweise für die Beteiligung von Lipase 1 an der Infektion. Darüber hinaus werden Öle als Induktoren des Lipase 1 Promoters identifiziert.

Lipasen sind außerdem als Katalysatoren für verschiedene Vorgänge bekannt. Die Lipasegene 2, 3, 4 und 5 werden funktionell in *Pichia pastoris* exprimiert. Die rekombinanten Lipasen sind Triacylglycerol Lipasen, die verschiedene Substrate bei einem pH von 7 und einer Temperatur von 37^{o}C umwandeln können. Daher bietet diese Studie vorläufige Ergebnisse für potenzielle industriuelle Anwendungen.

7. References

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