MOLECULAR CHARACTERIZATION OF A *FUSARIUM GRAMINEARUM* LIPASE GENE AND ITS PROMOTER

A Thesis Submitted to the College of
Graduate Studies and Research
In Partial Fulfillment of the Requirements
For the Degree of Doctor of Philosophy
In the Department of Plant Sciences
University of Saskatchewan
Saskatoon

By
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ABSTRACT

A triglyceride lipase gene *FgLip1* was identified in the genome of *Fusarium graminearum* strain PH-1. Yeast cells overexpressing FgLip1 showed lipolytic activity against a broad range of triglyceride substrates. Northern blot analyses revealed that expression of *FgLip1* was activated *in planta* during the fungal infection process and under starvation conditions *in vitro*. *FgLip1* expression was strongly induced in minimal medium supplemented with wheat germ oil, but only weakly induced by olive oil and triolein. Saturated fatty acids were the strongest inducers for *FgLip1* expression and this induction was proportionally suppressed by the presence of unsaturated fatty acids. To determine the potential function of *FgLip1*, gene replacement was conducted on strain PH-1. When compared to wild-type PH-1, Δ*FgLip1* mutants showed greatly reduced lipolytic activities at the early stage of incubation on minimal medium supplemented with either saturated or unsaturated lipid as the substrate, indicating that *FgLip1* encodes a secreted lipase for exogenous lipid hydrolysis. The Δ*FgLip1* mutants exhibited growth deficiency on both liquid and solid minimal media supplemented with the saturated triglyceride tristearin as the sole carbon source, suggesting that *FgLip1* is required for utilization of this substance. No variation in disease symptoms between the Δ*FgLip1* mutants and the wild-type strain was observed on susceptible cereal hosts including wheat, barley and corn. To delineate the promoter region responsible for the specific regulation of *FgLip1* expression, a series of deletions of *FgLip1* 5' upstream region were fused with the open reading frame of a green florescent protein (GFP) gene and the constructs were introduced into *F. graminearum*. GFP expression in the resulting transformants indicated that a 563-bp *FgLip1* promoter sequence was sufficient to

regulate expression of the *FgLip1* gene and regulatory elements responsible for gene induction were located within the 563-372 bp region. To further investigate the regulatory elements, putative *cis*-acting elements within the 563-372 bp region were mutated using a linker-scanning mutagenesis approach. A CCAAT box, a CreA binding site, and a fatty acid responsive element (FARE) were identified and confirmed to be responsible for *FgLip1* basal expression, glucose suppression and fatty acid induction, respectively.

ACKNOWLEDGEMENTS

Sincerely thanks to my co-advisors Drs. Geoff Hughes, Pierre Hucl and Yangdou Wei for their guidance throughout the project. All my research was conducted with Dr. Wei, who has supported my work through the last 3.5 years, and pushed me in every aspect of my life. Without him I would not have been able to complete this research. Dr. Hughes was the person who introduced me to the scientific area. I always feel deeply grateful and cherish the memory of my eight year experience with him. He encouraged me to initiate my PhD study and helped me to find the most amazing environment to explore my interests. Although Dr. Hucl has only served as my advisor since the retirement of Dr. Hughes, his support of this project was equal to the others. His advices were always concise and to the point.

I wish to thank my committee members Drs. Pierre Fobert, Curtis Pozniak and Bruce Coulman for the advice and help given by them during this project. My thanks are due also to Dr. Guosheng Liu, David Greenshields and all other members of Dr. Wei's Lab for their useful discussion and friendly cooperation. Many thanks also to Dr. Gopalan Selvaraj and Ms. Rozina Hirji from the Plant Biotechnology Institute, Saskatoon, for their help including primer synthesis and DNA sequencing. I have been the beneficiary of many people who allowed me to discuss my ideas with them including Dr. Gary Peng, Dr. Susan Kaminskyj and Dr. Jitao Zou.

I gratefully acknowledge the financial support received from Canadian Wheat Board, Vanterpool Prize from the Department of Biology, Rene Vandeveld Scholarship and Alan McLean Scholarship from the College of Agriculture and Bioresources.

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LIST OF ABBREVIATIONS

ABBREVIATION	FULL NAME
BTF	Basal Transcription Factors
BLAST	Basic Local Alignment Search Tool
BMMY	Buffered Methanol-complex medium
bp	base pairs
CWDE	Cell Wall Degrading Enzyme
DPE	Downstream Promoter Element
EC	Enzyme Commission
f. sp.	formae speciales
FA	Fatty Acid
FARE	Fatty Acid Responsive Element
FDK	Fusarium Damaged Kernel
FGSC	Fungal Genetics Stock Center
FHB	Fusarium Head Blight
GFP	Green Florescent Protein
hai	hours after inoculation
Hsp	Heat shock protein
Inr	Initiator
IPTG	Isopropyl-β-D-thiogalactoside
IUPAC	International Union of Pure and Applied Chemistry
LB	Luria-Bertani medium
LCFA	Long Chain Fatty Acid
LSM	Linker Scanning Mutagenesis
MAP	Mitogen-Activated Protein
NCBI	National Center for Biotechnology Information
NRRL	Northern Regional Research Laboratories
nt	nucleotide
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar

PEG	Poly Ethylene Glycol
Pol II	Polymerase II
SAGE	Serial Analysis of Gene Expression
TAF	TATA binding protein Associated Factor
TBP	TATA Binding Protein
TFII	RNA polymerase II Transcription Factor
WGO	Wheat Germ Oil
YNB	Yeast Nitrogen Base
YPD	Yeast-extract Peptone Dextrose

1. INTRODUCTION

Plant pathogenic fungi secrete an array of extracellular hydrolytic enzymes capable of degrading components of the plant cell wall during infection (Walton, 1994). Global identification and transformation-mediated disruption of genes encoding these enzymes have been important objectives during the last decade in studying fungal pathogenicity. Among these enzymes, lipase (EC 3.1.1.3) and cutinase (EC 3.1.1.74), with their potential function in plant surface wax and cuticle hydrolyzation, may contribute to pathogen infection when the fungus comes in contact with the plant. Preliminary studies showed that secreted lipolytic activity was commonly present among a range of plant pathogenic fungi (Fig. 1.1). A triglyceride lipase gene *FGL1* (the nomenclature throughout this thesis follows that of the author(s) who first reported the corresponding gene) has been reported to be critical for *Fusarium graminearum* pathogenesis on cereal hosts (Voigt *et al.*, 2005). However, the biochemical and molecular mechanism of *FGL1* in fungal pathogenicity remains unknown. Other studies have suggested that lipases secreted by pathogenic fungi can hydrolyze the plant surface wax during the infection process (Comménil *et al.*, 1999; Nasser-Eddine *et al.*, 2001). Lipases are used extensively in industries, including detergents and degreasing formulations, food processing and degradation of fatty waste and polyurethane (Schmid and Verger, 1998; Pandey *et al.*, 1999; Masse *et al.*, 2001; Takamoto *et al.*, 2001). Utilization of lipases or lipase-producing microorganisms in these processes requires the lipase gene to be expressed at specific times and in defined conditions, which is ultimately controlled by the promoter. Thus, clarifying the function of the lipase gene

promoter is important. Furthermore, for a lipase involved in fungal pathogenesis, knowledge of control of its expression is critical for understanding the interaction between the host plant and the pathogen.

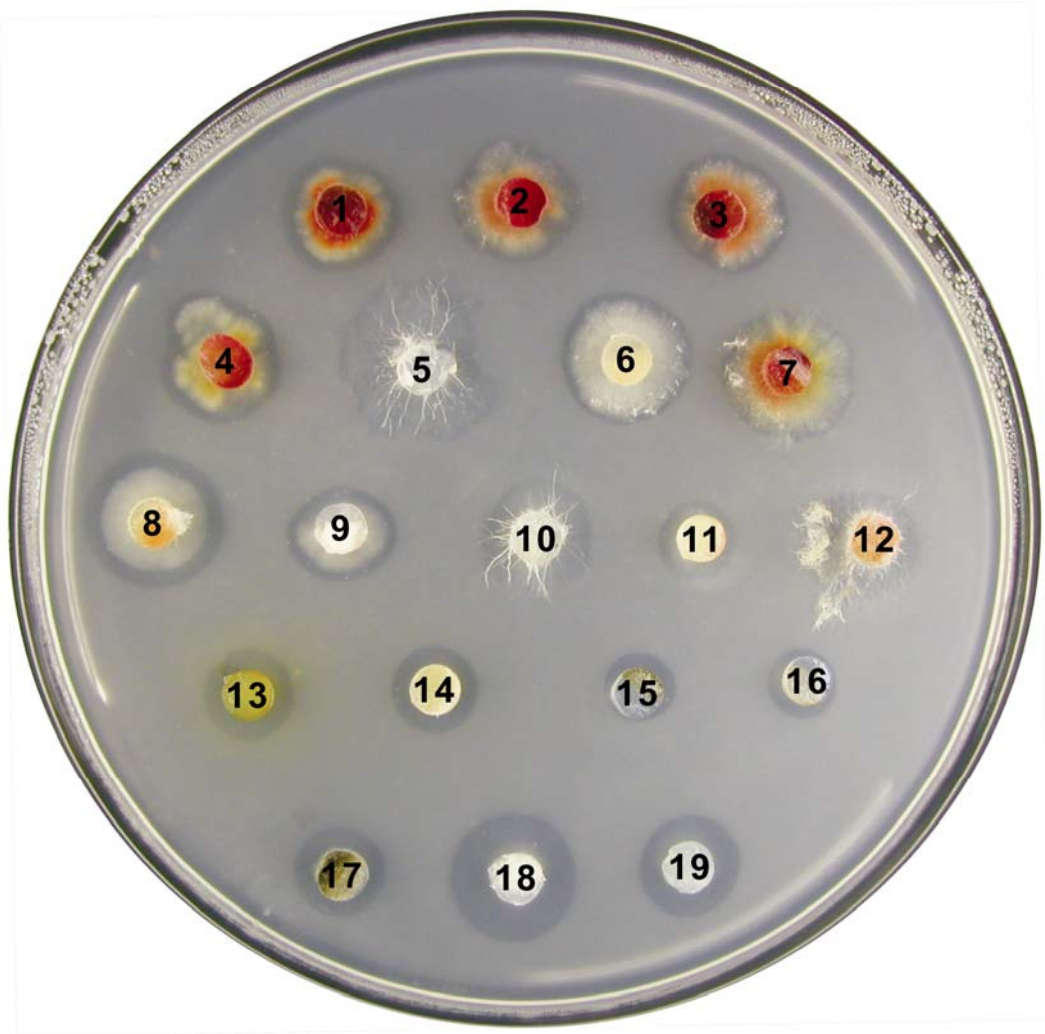

Fig. 1.1. Lipolytic activity of various plant pathogenic fungi. Potato Dextrose Agar **(**PDA) culture plugs of different fungal species were inoculated on 1.5% (w/v) agar supplemented with 1% (w/v) tributyrin as the sole carbon source for 24 h. Lipolytic activity is indicated by the production of clear zone around the colonies. Fungal species are: 1-5, *F. graminearum* strain PH-1 (1) and strains isolated from Saskatchewan (2), Manitoba (3), Alberta (4) and an unknown source (5); 6-19, *F. pseudograminearum* (6), *F. culmorum* (7), *F. poae* (8), *F. equiseti* (9), *F. avenaceum* (10), *F. acuminatum* (11), *F. sporotrichioides* (12), *Pyrenophora tritici-repentis* (13), *Stagonospora nodorum* (14), *Colletotrichum gloeosporioides* f. sp. *malvae* (15 and 16), *Botrytis cinerea* (17), and *Aspergillus flavus* (18-19) isolated from Saskatchewan.

Previous work conducted in Wei's lab (Yangdou Wei, unpublished data) identified a triglyceride lipase gene (*BgLip1*) from the wheat powdery mildew fungus *Blumeria graminis* f. sp. *tritici*. The expression profile of this gene was found to be correlated temporally and spatially with the fungal infection process on wheat leaves. The recombinant BgLip1 exhibited potent activity to hydrolyze leaf epicuticular waxes. This process was found to be important for fungal pathogenicity because pretreatment of the wheat leaf surface with recombinant BgLip1 severely compromised the development of the powdery mildew fungus on subsequently inoculated leaves. To further study the function of lipase on fungal biology and pathogenicity, this project was conducted to investigate the mechanism of homologous genes present in the *Fusarium graminearum* genome. Searching the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) database with the deduced amino acid sequence of BgLip1 as the query resulted in twelve putative lipases that share varying identities with BgLip1. Of these twelve, the one that showed the highest identity with BgLip1 was named FgLip1 and selected for this research project.

There are several scientific reasons for selecting *F. graminearum* as the subject for this study. During the last decade, Fusarium head blight (FHB) caused by this fungus has become a serious disease threatening wheat and barley production, especially in North America (Gilbert and Tekauz, 2000). As a result, the biology and pathogenicity of *F. graminearum*, the major causal agent of FHB, have been extensively investigated. The genome of *F. graminearum* strain PH-1 (FGSC 9075, NRRL 31084) was sequenced and subsequently released to the public in 2003 by the Whitehead Institute, Center for Genome Research (Cambridge, MA; http://www.broad.mit.edu/annotation), and thus

promoted access to all the corresponding putative proteins for this organism. *F. graminearum* is amenable to both classical and molecular genetic manipulation.

In this study, the triglyceride lipase gene *FgLip1* was cloned and molecularly characterized with the hypothesis that *FgLip1* is a pathogenicity factor of *F. graminearum.* However, when it was found that *FgLip1* was not essential for pathogenesis but was important for fungal utilization of saturated fatty acids, promoter regions controlling the specific expression of *FgLip1* were investigated. The results from this research will help understand the plant-pathogen interaction in this pathosystem, the adaptation of the fungus to its environment especially when lipid is present, and the mechanism underlying the expression profiles of lipase genes. Furthermore, the lipase gene, its promoter or both could be used in industrial processes where strong and well-characterized lipase expression is needed.

2. LITERATURE REVIEW

2.1. *Fusarium graminearum* as the pathogen of Fusarium head blight (FHB)

2.1.1. Fusarium head blight on wheat

The recent increase in prevalence and severity of Fusarium head blight (FHB) on wheat in Canada and elsewhere has caused hardship and economic loss to producers and the grain industry (Gilbert and Tekauz, 2000). FHB is more common in warm and humid areas of the world (Parry *et al.*, 1995). Several severe FHB epidemics have occurred in North America with losses in excess of one billion US dollar a year (McMullen *et al.*, 1997). Grain yield is reduced because of shriveled grain, which either is blown away during harvest, or has a lighter test weight. Infected grain germinates poorly, resulting in seedling blight and a poor stand when it is used as seed (Bai and Shaner, 1994). *Fusarium* damaged kernels (FDK) are often contaminated with mycotoxins that are toxic to humans and livestock when used as food or feed (Sutton, 1982; Bennett and Klich, 2003). The common mycotoxins present in FDK are trichothecenes, a group of sesquiterpenes, including deoxynivalenol (DON) and nivalenol (NIV), and zearalenone (ZEA), an oestrogenic mycotoxin (Bennett and Klich, 2003). The rapid global re-emergence of FHB along with mycotoxin contamination of grain has promoted basic research on its causal agents. As a result, *Fusarium graminearum* quickly has become one of the most intensively studied filamentous fungal species.

Fusarium graminearum (Schwabe) is the principal head blight pathogen in Canada and most other countries (Gilbert and Tekauz, 2000). Its sexual state, *Gibberella zeae*

(Schweinitz) Petch, is a homothallic ascomycete in which alternative forms (idiomorphs) of the mating type (MAT) locus, normally found in separate nuclei in heterothallic fungi, are juxtaposed at the same locus (Yun *et al.*, 2000). *F. graminearum* is also associated with stalk and ear rot of corn and may cause root rot of cereals (McMullen *et al.*, 1997). Two natural populations of *F. graminearum*, referred to as group 1 and group 2, have been described (Francis and Burgess, 1977). Isolates in group 1 were normally associated with crown diseases and did not form perithecia in culture, whereas those in group 2 were associated with diseases of aerial plant parts and readily formed perithecia. Recent studies demonstrated that these two groups are separate biological and phylogenetic species, each producing its own characteristic sexual state. The former group 1 is now known as *F. pseudograminearum* and has a sexual stage called *G. coronicola* (Aoki and O'Donnell, 1999). The monophyletic *F. graminearum* species complex now refers only to the former group 2 that consists of at least nine phylogenetic species, some of which are localized on particular continents or geographic regions. These species have now been formally named with *F. graminearum sensu stricto* retained for the species most commonly associated with *Fusarium* head blight worldwide (O'Donnell *et al.*, 2000, 2004).

In Canada, *F. graminearum* was first identified in Manitoba in 1923, but no serious outbreaks were reported until 1984. According to Clear and Patrick (2000), there is compelling evidence that *F. graminearum* has recently spread westward from Manitoba, causing increasing economic losses from the effects of FHB. Surveys in western Canada conducted by the Canadian Grain Commission's Grain Research Laboratory in 2006 found that *F. graminearum* has reached northern British Columbia.

In wheat, five types of resistance to FHB have been proposed (Mesterházy 1995): type I is resistance to initial infection; type II prevents spread within the head following infection; type III is resistance to kernel infection; type IV is tolerance whereby yields are maintained despite the presence of disease; and type V is ability of hosts to degrade the toxins produced. Most research has been conducted on type II resistance, in which both polygenic (Bai and Shaner, 1994) and oligogenic (Singh *et al.*, 1995; van Ginkel *et al.*, 1996) inheritance have been reported. There appears to be three main sources of unrelated resistant germplasm to FHB: genotypes of Chinese/Japanese origin, e.g. Sumai-3 (Ban and Suenaga, 2000); genotypes of Brazil, e.g. Frontana (van Ginkel *et al.*, 1996); and numerous lines from central Europe (http://www.scabusa.org). Despite considerable breeding efforts, no highly resistant cultivars of wheat have been developed to date (Bai and Shaner, 2004).

2.1.2. Infection process of *Fusarium graminearum*

Wheat plants are susceptible to *F. graminearum* infection from the flowering period up to the soft dough stage of kernel development (Sutton, 1982). Atanasoff (1920) found that disease was more severe when plants were inoculated with spores of *F. graminearum* during anthesis than other growth stages. Strange and Smith (1971) observed that removal of anthers in certain cultivars significantly reduced the incidence of diseased florets, suggesting that anthers may be the major sites of initial infection. Spores infect the exposed anthers at flowering and then grow into the developing kernels, glumes, or other parts of the head (Bushnell *et al.*, 2003). Pritsch *et al.* (2000) observed that hyphae may also grow subcuticularly along the stomatal rows on the surface of the glume. Such subcuticular growth was thought to serve as a mechanism for fungal spread

(Bushnell *et al.*, 2003). Similar subcuticular growth was observed when the fungus grew on inoculated epicarps after entering the floret and before penetrating the epicarp cell wall (Jansen *et al.*, 2005). Subcuticular growth of hyphae suggests the utilization of plant cuticle or surface wax by the fungus for nutrients, a process in which cell wall degrading enzymes may participate. Involvement of such enzymes in the infection of wheat by *F. culmorum* has been reported by Kang and Buchenauer (2000, 2002) and Jenczmionka and Schäfer (2004). Utilization of degradation products of the epidermal cell wall and the cutin as energy sources has been observed in other plant pathogenic fungi (Wagner *et al.*, 1988; Koga, 1995). In addition to entering the floret by hyphal growth, the fungus can also infect the host through stomatal openings or directly penetrate the adaxial surface of the floral bracts and the base of the wheat glumes where the epidermis and parenchyma are thin-walled (Bushnell *et al.*, 2003).

Upon contact with the developing kernel, *F. graminearum* infects through the epicarp, successively destroying the layers of the fruit coat and finally the starch and protein accumulating endosperm (Jansen *et al.*, 2005). The principal mode of fungal spread in wheat from floret to floret inside a spikelet and from spikelet to spikelet is through the vascular bundles in the rachis and rachilla (Ribichich *et al.*, 2000). The fungus has been observed to have a brief biotrophic relationship with the host before switching to the necrotrophic phase (Goswami and Kistler, 2004). However, Jansen *et al.* (2005) found that infected cells appeared to be living only as long as the fungus grew between the cuticle and the walls of the epicarp. As soon as the fungus entered the cytosol of the epicarp cells, cell death was observed in both barley and wheat. Thus, an initial biotrophic phase of *Fusarium* in head blight could not be identified.

2.1.3. Pathogenicity or virulence factors of *Fusarium graminearum*

Fungal pathogens employ various pathogenicity genes to infect their host plants. Pathogenicity genes are genes necessary for disease development but not essential for the pathogen to complete its life cycle (Idnurm and Howlett, 2001). This definition was developed based on the loss of the ability to cause disease of the mutant after the gene has been knocked out by gene replacement or random gene disruption. However, as Oliver and Osbourn (1995) argued, this definition cannot apply to obligate parasites. Thus, identification of pathogenicity genes from obligate biotrophic fungi is a more complicated process since more effort is required to differentiate between the fitness and the pathogenicity of the gene disruption mutants.

As a necrotrophic fungal pathogen, *F. graminearum* has recently been extensively studied to uncover its pathogenicity genes. This was due not only to its economic importance, but also to the availability of the whole genome sequence and the amenability of this fungus to genetic transformation. However, only few pathogenicity genes have been found. Instead, some genes identified were demonstrated to be virulence rather than pathogenicity factors. Virulence is a component of pathogenicity and can be defined as the relative ability of a pathogen to cause damage on the host (Harris *et al.*, 1999).

Numerous investigations were conducted to verify whether or not the mycotoxins produced by *F. graminearum* are pathogenicity factors. By using a transformation-mediated gene knockout approach, Proctor *et al.* (1995) disrupted the *Tri5* gene encoding trichodiene synthase which catalyzes the first step in trichothecene biosynthesis. The mutant strains exhibited reduced virulence on wheat and rye seedlings.

A later study showed that the Δ*Tri5* mutants were able to infect the inoculated spikelet, but infection of the complete wheat spike did not occur (Bai *et al.*, 2001), suggesting that *Tri5* is a virulence rather than a pathogenicity factor. Based on investigation of *in planta* development of a Δ*Tri5* strain labeled with the green florescence protein (GFP) gene, Jansen *et al.* (2005) concluded that *Tri5* could not be considered a virulence factor because the mutant and the wild type strain showed the same infection pattern in the seed coat tissues of the barley and wheat lines investigated. Nevertheless, in wheat, the mutant strain was prevented from moving into the rachis by the development of cell wall fortification in the rachis node, suggesting that *Tri5* does function to inhibit host defense responses. The different investigation methods employed in these studies might affect the results obtained from pathogenicity tests. Jansen *et al.* (2005) conducted tests on detached spikes whereas Proctor *et al.* (1995) and Bai *et al.* (2001) tested seedlings and heads in the greenhouse or the field. Bai *et al.* (2001) found that under field and greenhouse conditions, the wild type strain produced significantly more head blight symptoms and reduced yields to a greater degree than the mutant strain in all wheat cultivars tested. Under field conditions, Harris *et al.* (1999) also found that trichothecenes act as virulence factors to enhance the spread of *F. graminearum* on maize. Another gene *Tri14*, which is associated with trichothecene synthesis but does not share similarity with any previously described genes in the fungus, has been reported to be required for virulence and DON production on wheat, but not for DON synthesis *in vitro* (Dyer *et al.*, 2005).

In plant pathogenic fungi, recognition of the plant and expression of genes necessary for infection are related to signal transduction pathways. It has been

demonstrated that mitogen-activated protein (MAP) kinase-mediated signal transduction pathways play important roles in the pathogenicity of many filamentous fungi (Xu, 2000). The MAP kinase encoded by *MPS1* in the rice blast fungus *Magnaporthe grisea* is important for maintaining fungal cell wall integrity and essential for plant penetration (Xu *et al.*, 1998). When the orthologue of *MPS1* in *F. graminearum*, *Mgv1*, was deleted, the mutants were still able to infect wheat spikes, although virulence was reduced (Hou *et al.*, 2002). Δ*Mgv1* mutants produced conidia and DON mycotoxin normally, however, vegetative growth rates were reduced *in vitro* and perithecia failed to form. Therefore *Mgv1* can be considered to be contributing to virulence but is not essential for pathogenicity. Another MAP kinase gene, denoted as *Gpmk1* (Jenczmionka *et al.*, 2003) or *MAP1* (Urban *et al.*, 2003), was independently studied by two groups. This gene is the orthologue of a *Saccharomyces cerevisiae* gene belonging to the extracellular signal-regulated kinase 1 subgroup of MAP kinases, whose main function is the transduction of extracellular signals (Kültz, 1998). Both groups observed loss of pathogenicity in the gene disruption mutant and concluded that this gene was a pathogenicity factor.

Production of plant cell wall degrading enzymes by fungi may be regulated by signal transduction pathways (Xu, 2000). For example, it was shown that an endopolygalacturonase gene is under the transcriptional control of the Kss1 MAP kinase pathway in *S. cerevisiae* (Madhani *et al.*, 1999). Regulation of cell wall-degrading enzyme production via the MAP kinase pathway is also possible in *F. graminearum*. Jenczmionka and Schäfer (2004) found that *Gpmk1* regulated the early induction of extracellular endoglucanase, xylanolytic and proteolytic activities. Furthermore, the Δ*Gpmk1* mutant completely lost lipolytic activities. Since the Δ*Gpmk1* mutant showed

non-pathogenic phenotype (Jenczmionka *et al.*, 2003), these results suggested that cell wall degrading enzymes played an important role during fungal pathogenesis.

Secreted proteases from *F. graminearum* might contribute to fungal virulence. El-Glendy *et al.* (2001) suggested that fungal proteases play an important role by helping to overcome the barrier of structural proteins produced in wheat cell walls as a defensive response following infection by *F. graminearum*. During infection of barley, *Fusarium* species including *F. graminearum* produce alkaline proteases. The protease activity detected in inoculated grains was found to be proportional to the virulence of the infecting *Fusarium* species, implying that protease is associated with pathogenicity as a general virulence factor (Pekkarinen *et al.*, 2003).

Since pathogens can produce a multitude of enzymes that work together to decompose the host plant structures (Walton, 1994; Oliver and Osbourn, 1995; Knogge, 1996), one particular enzyme is generally not critical for fungal invasion. However, a triglyceride lipase, FGL1, has been reported to be essential for *F. graminearum* pathogenesis on cereal hosts (Voigt *et al.*, 2005). Besides the role of degrading the plant cell wall, the possible function of this gene in fungal growth and development has not been investigated.

Other genes that have been reported to be associated with *F. graminearum* pathogenicity include *CPS1*, which encodes a protein with two adenosine monophosphate (AMP)-binding domains and is a general fungal virulence factor found in *F. graminearum*, *Cochliobolus victoriae* and *C. heterostrophus* (Lu *et al.*, 2003), and *FgFSR1*, which encodes a putative protein involved in cell differentiation and ascocarp development (Shim *et al.*, 2006). Rapid advances in fungal genomic resources and

technologies have led, at an unprecedented rate, to the identification of novel candidate virulence genes. For example, Goswami *et al.* (2006) constructed three cDNA libraries by suppression subtractive hybridization using wheat heads inoculated with a highly aggressive strain and either water or a less aggressive strain of *F. graminearum*. Eighty-four fungal genes expressed during initial disease development were identified from one of these libraries. Of the four selected genes, deletion of *Rrr1* caused reduced disease incidence on wheat heads.

2.2. Lipases and lipase genes

2.2.1. Characteristics of lipases

Lipases (EC.3.1.1.3, triacylglycerol acylhydrolases) are a group of esterases, which have the ability to hydrolyze long-chain acyl glycerols and show a dramatic increase in activity at an oil-water interface (Schmid and Verger, 1998). This phenomenon, called interfacial activation, is believed to be controlled by the conformation of a lid domain composed of an amphiphilic α helix peptide sequence (Schmid and Verger, 1998). Lipases contain a hydrogen-bond network in the active site consisting of the triad of serine (S), aspartic acid (D) or glutamic acid (E), and histidine (H) (Brady *et al.*, 1990; Schrag *et al.*, 1991). A three-dimensional picture of lipase 2 from *Candida rugosa*, whose crystal structure has been analyzed at a resolution of 1.97 Å (Mancheño *et al.*, 2003), is presented in Figure 2.1.

Lipases are widely present in microorganisms, plants and animals (Schmid and Verger, 1998). They catalyze not only the reactions of ester hydrolysis, but also those of ester synthesis and transesterification (Benjamin and Pandey, 1998; Schmid and Verger,

1998). These reactions are widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics (Pandey *et al.*, 1999). Lipases can also be used to accelerate the degradation of fatty wastes and polyurethane (Masse *et al.*, 2001; Takamoto *et al.*, 2001).

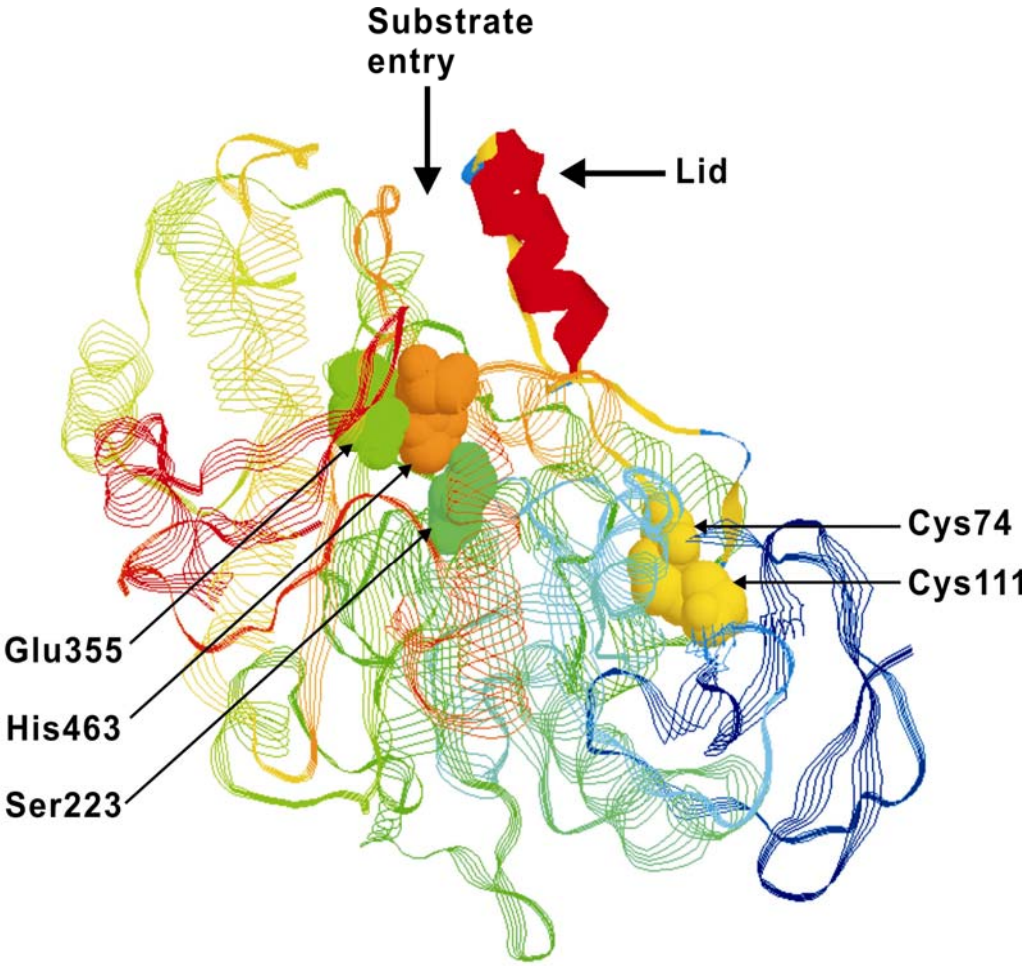

Fig. 2.1. Three-dimensional structure of lipase 2 from *Candida rugosa*. The lipase catalytic triad Ser223, Glu355 and His463, as well as the two cysteines (Cys74 and Cys111) embracing the lid region are indicated. The amino acid sequence (GenBank accession no. P32946) was analyzed for three-dimensional structure with 3D-JIGSAW version 2.0 (http://www.bmm.icnet.uk/servers/3djigsaw/) and the result was presented using RasMol version 2.6 (http://www.umass.edu/microbio/rasmol/getras.htm).

There are four features common to most lipases: an active triad, interfacial activation, a lid domain, and the ability to hydrolyze long-chain acyl glycerols. However, none of these alone is an appropriate criterion to classify an enzyme into the lipase family. Some other enzymes such as serine protease (Kraut, 1977; Craik *et al.*, 1987) and cutinase (Martinez *et al.*, 1992; Longhi and Cambillau, 1999) also possess the S-D-H active triad. A number of lipases, including pancreatic lipase from coypu (Thirstrup *et al.*, 1994) and lipases from *Pseudomonas glumae* (Noble *et al.*, 1993) and *Candida antarctica* (Uppenberg *et al.*, 1994) do not show interfacial activation, although each of them possesses a lid domain. Schmid and Verger (1998) suggested that the most appropriate way to classify an enzyme as a lipase is whether or not it could hydrolyze long-chain acyl glycerols. However, exceptions exist. The cutinase from *F. solani*, for example, can hydrolyze both short- and long-chain triacylglycerols (Mannesse *et al.*, 1995). These exceptions have made the classification of lipases difficult and some researchers consider cutinases as the link between esterases and lipases (Egmond and van Bemmel, 1997). Protein databases, such as GenBank (http://www.ncbi.nlm.nih.gov/Genbank), The Brookhaven Protein Databank (http://www.ch.cam.ac.uk/cil/SGTL/Brookhaven.html), The Protein Information Resource (http://pir.georgetown.edu), Swiss Protein Sequence Database (http://www.expasy.ch/sprot), and The Lipase Engineering Database (http://www.led.uni-stuttgart.de), have proven to be powerful tools for the identification and characterization of lipases. Multiple alignments between amino acid sequences can easily be conducted and the putative function of the protein under investigation can be inferred through comparison with well-studied orthologues from related organisms.

The lid domain is a critical structure for lipase function. Among the five lipase isoforms identified from *C. rogusa*, greater divergence at the amino acid level was found in the lid regions than in other regions (Benjamin and Pandey, 1998). The varying amino acid sequences within lid regions were believed to be responsible for the different activities and specificities of the isoforms (Akoh *et al.*, 2004). The X-ray structure of *C. rugosa* lipases indicated that the catalytic sites were clearly buried beneath the amphipathic helical lid which arises from a long external loop between two β strands (Grochulski *et al.*, 1993). This is known as the 'closed' structure of the enzyme, and is believed to be its structure in aqueous solution in the absence of an interface, where lipases display very low or no catalytic activity because the triad is not accessible to the substrate (Lawson *et al.*, 1994). The most favored hypothesis to explain interfacial activation involves a conformational change in the enzyme when contacting the interface occurs, essentially a displacement caused by the lid rotating around its hinge region to expose the active site (Desnuelle *et al.*, 1960). The large hydrophobic patch created by this movement would likely be stabilized by interaction with a non-polar interface, such as micelles or bilayers, where the enzyme will adopt a catalytically active or 'open' conformation (van Tilbeurgh *et al.*, 1992; Cygler and Schrag, 1997). This structural rearrangement is supported by the finding that crystal structures of lipases are covalently complexed with hydrophobic inhibitors such as alkyl phosphonates, cycloalkyl phosphonates, or alkyl sulfonates (Brzozowski *et al.*, 1991; van Tilbeurgh *et al.*, 1993; Hermoso *et al.*, 1997).

Lipase belongs to the α/β hydrolase fold family (Ollis *et al.*, 1992). The α/β hydrolase family includes lipases, esterases, proteases, peroxidases, lyases and others

(Schrag and Cygler, 1997). Lipases can be classified into two groups, the mammalian lipases and the microbial lipases (Svendsen, 2000). Within the microbial lipases, there are two main groups, the bacterial lipases and the fungal lipases, each containing several families (Svendsen, 2000). The fungal lipases can be further classified into the *Rhizomucor miehei* lipase family and the *C. rugosa* lipase family (Schmidt-Dannert, 1999). This classification of lipases is based mainly on sequence homology and differences in molecular mass and protein function (Nagao *et al.*, 1994; Schmidt-Dannert, 1999).

2.2.2. Lipases from plant pathogenic fungi

Necrotrophic and hemibiotrophic phytopathogenic fungi secrete an array of extracellular hydrolytic enzymes capable of degrading components of the plant cell wall during invasion of the host (Walton, 1994). Generally, the cell wall consists of a cellulose microfibril-xyloglucan framework embedded in a matrix containing pectin, β-glucans and glycoprotein (McNeil *et al*., 1984). The outer surface of the epidermal cell is covered by epicuticular waxes and the cuticle. The cuticle is a continuous layer of lipid material that consists of insoluble polymeric material called cutin. Epicuticular 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 epicuticular wax.

Botrytis cinerea, which causes grey mould on many plant species, requires a lipase, Lip1 for penetration of the intact plant host cuticle and infection (Comménil *et al.*, 1998). The expression of this lipase is induced by components of the cuticle of the grape fruit

(Comménil *et al.*, 1999). When specific anti-lipase antibodies were added to a conidial suspension of *B. cinerea*, lesion formation on inoculated tomato leaves was completely suppressed (Comménil *et al.*, 1998). Another plant pathogenic fungus, *Alternaria brassicicola*, possesses a lipase that contributes to the early stage of fungal pathogenesis (Berto *et al.*, 1997). This lipase cross-reacted with the anti-*B. cinerea* lipase antibody which, when added to a conidial suspension of *A. brassicicola*, caused a 90% reduction of black spot lesions on intact cauliflower leaves, but not on leaves from which the surface wax had been removed (Berto *et al.*, 1999). These results suggested that both lipases play an important role during fungal pathogenesis. However, Reis *et al.* (2005) recently found that disruption of *Lip1* did not prevent the fungus from penetrating intact plant tissue.

Involvement of lipase in fungal pathogenesis has been reported also in other plant pathogenic fungi. Triacylglycerol lipase activity in *M. grisea* significantly increased during appressorium development (Thines *et al.*, 2000). A gene encoding an extracellular lipase, *NhL1*, has been cloned from the pea pathogen *Nectria haematococca* mating population VI (*F. solani* f. sp. *pisi*) and its expression was induced during infection of pea plants (Nasser-Eddine *et al.*, 2001). Yu *et al.* (2003) showed that the lipase gene *lipA* from *Aspergillus parasiticus* and *A. flavus* was induced by lipid substrates and its expression was correlated with aflatoxin production. In the biotrophic fungus *Ustilago maydis*, lipase activity was involved in the switch from budding to filamentous growth, a process critical for completion of the fungal life cycle and infection of maize plants (Klose *et al.*, 2004). Serial analysis of gene expression (SAGE) in the barley powdery mildew fungus *B. graminis* f. sp. *hordei* revealed that a

gene encoding a lipase was among the 20 most expressed genes in germinated conidia and appressoria and, of the 20 genes, this gene was the only one encoding a hydrolytic enzyme (Thomas *et al.*, 2002). The orthologue of this lipase gene was cloned from the wheat powdery mildew fungus *B. graminis* f. sp. *tritici* and its role in the pathogenicity of this biotrophic fungus has been further studied (Yangdou Wei, unpublished data).

2.2.3. Lipases from *Fusarium graminearum*

Among all lipase genes identified from plant pathogenic fungi, only the study of *FGL1* from *F. graminearum* has provided direct evidence that a lipase gene is a pathogenicity factor (Voigt *et al.*, 2005). The authors disrupted this lipase gene by genetic transformation and found that the mutant strains showed reduced virulence on both wheat and corn. *FGL1* encodes a 352-amino-acid protein with a molecular mass of 35 kDa. This is in contrast to well-characterized lipases from *C. rugosa* and *B. cinerea*, which encode proteins with a mass around 60 kDa (Benjamin and Pandey, 1998).

2.3. Gene transcription regulation

Transcription is the process by which a DNA sequence is enzymatically copied by an RNA polymerase to produce a complementary RNA, i.e. the transfer of genetic information from DNA into RNA. For protein-coding DNA, transcription is the first step that ultimately leads to the translation of the genetic code into a functional peptide or protein. In both prokaryotes and eukaryotes, gene transcription can vary during the lifetime of cells and this allows the cells to respond to changes in their environments. Transcription is mostly controlled by the region of DNA upstream of a gene, which usually contains important regulatory signals. This region is referred to as the promoter.

2.3.1. Eukaryotic core promoter and basal transcription

There are at least two features common to most eukaryotic promoters of protein-coding genes, the core promoter and the sequences bound by transcriptional regulators (Novina and Roy, 1996). The core promoter, which includes DNA elements that can extend approximately 35 bp upstream and/or downstream of the transcription initiation site, is responsible for initiation and basal transcription by interacting directly with components of the basal transcription machinery (Smale and Kadonaga, 2003). The core promoter is found in all protein-coding genes and can contain both TATA and initiator (Inr) elements (composite), either element alone (TATA- or Inr-directed), or neither element (null). Composite promoters are found primarily in viral genes whereas most cellular protein encoding genes contain TATA-directed promoters and a more limited number contain Inr-directed promoters. Most null promoters have multiple transcription start sites, suggestive of imprecise initiation (Geng and Johnson, 1993). Inr-directed promoters often contain a downstream promoter element (DPE) which acts in conjunction with the Inr element for binding of transcription factor IID (Kutach and - Kadonaga, 2000; Butler and Kadonaga, 2002).

The TATA box was the first core promoter element identified in eukaryotic protein-coding genes (Breathnach and Chambon, 1981) and has a consensus sequence of TATAAA. It is a common, but not absolute, component of core promoters and is generally located 25 to 30 bp upstream in higher eukaryotes and 40 to 120 bp upstream in yeast (Struhl, 1995). Database analyses of *Drosophila* genes revealed that the TATAAA consensus sequence, or a sequence with one mismatch from the consensus, was present in 43% of 205 (Kutach and Kadonaga, 2000) and 33% of 1,941 (Ohler *et al.*,

2002) promoters. Among 1,031 human genes investigated, more than 300 contained the TATA box (Suzuki *et al.*, 2001).

The Inr element encompasses the transcription start site and is defined as a discrete core promoter element that is functionally similar to the TATA box. In an analysis of the lymphocyte-specific terminal transferase promoter, it was demonstrated to function independently of a TATA box (Smale and Baltimore, 1989). Analyses of randomly generated and specifically targeted Inr mutants by *in vitro* transcription and transient transfection led to the functional consensus sequence YYANWYY (Degenerate nucleotides are designated according to the IUPAC code, Appendix I) in mammals (Javahery *et al.*, 1994; Lo and Smale, 1996) and TCAKTY in *Drosophila* (Hultmark *et al.*, 1986; Purnel *et al.*, 1994).

The initiation of transcription requires the interaction between the core promoter and basal transcription factors (BTFs), typically defined as the minimal complement of proteins necessary to reconstitute accurate transcription from a core promoter (Lee and Young, 2000). These BTFs are distinct from regulatory transcription factors, which generally bind to sequences farther away from the initiation site and serve to moderate levels of transcription. BTFs consist of RNA polymerase II (pol II) and additional proteins necessary for correct initiation and elongation, including TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (Hampsey, 1998). The initiation and early steps of transcription require the assembly of all BTFs into a preinitiation complex at the promoter to form an open complex, followed by synthesis of the RNA transcript. Initially, the TATA binding protein (TBP) or TBP-containing TFIID complex binds sequence-specifically to the TATA box to establish a nucleoprotein recognition site for

pol II on the DNA. Then TFIIB functions as an adaptor to promote selective binding of pol II to TFIID at the promoter by binding specifically to both polymerase and the TBP subunit of TFIID to form the Dbpol II complex. TFIIF performs dual roles in the assembly of the preinitiation complex, (a) binding to and strongly stabilizing the Dbpol II intermediate, and (b) recruiting TFIIE and TFIIH into the complex. Following formation of the fully assembled preinitiation complex, DNA helicase activity associated with TFIIH catalyzes ATP-dependent unwinding of the DNA template at the transcriptional start site to form the open complex, prior to initiation and synthesis of the RNA transcript by pol II (Conaway and Conaway, 1993; Roeder, 1996; Smale and Kadonaga, 2003).

2.3.2. *Cis*-acting elements and *trans*-acting factors

Gene expression in eukaryotes is also controlled by complex interactions between *cis*-acting elements within the regulatory regions of the DNA and *trans*-acting factors that generally are proteins encoded by alternative genes. The common *cis*-acting elements include the enhancer, the silencer and the insulator, whereas the common *trans*-acting factors include the activator and the repressor.

The enhancer and the silencer are clusters of DNA-binding sites for transcriptional regulators that influence transcription and can be located upstream, downstream, or even within the protein coding regions of the genes they control (Blackwood and Kadonaga, -1998). The enhancer upregulates whereas the silencer downregulates the corresponding genes. They act independent of their orientation and can occur at distances as great as 85 kb from the start site (Blackwood and Kadonag, 1998). The terms UAS (upstream activation sequence) and URS (upstream repression sequence) are typically used in yeast

to refer to the enhancer and silencer to describe elements that influence transcription from nearby start sites (Guarente, 1988). Insulators are stretches of DNA located between the enhancer(s)/silencer(s) and the promoter of adjacent genes. Their function is to prevent a gene from being influenced by the activation or repression of neighboring genes (Bell and Felsenfeld, 1999).

Activators and repressors are proteins that activate or repress the gene expression. Activators typically consist of two domains: one that binds enhancers and one that recruits or stimulates the activity of the BTF (Triezenberg, 1995; Ptashne and Gann, 1997). A single activator is frequently used to activate multiple genes in a genome, thus providing a mechanism for coordinated control of those genes. Individual genes can be regulated through the action of multiple activators, thereby providing a mechanism for cooperative control.

2.3.3. Mechanism of activation and repression

The current view of transcriptional regulation is based primarily on the concept of "recruitment" (Ptashne and Gann, 1997). According to this view, various BTFs participating in transcription initiation are recruited to activated promoters via protein-protein interactions with the enhancer-bound activator. At least two arguments support this model. First, many researchers have observed direct binding between activators and components of the BTFs *in vitro* (Burley and Roede, 1996; Barberis and Gaudreau, 1998; Hampsey, 1998). Second, if the role of the activators is to bind and recruit the BTFs, then fusions of DNA-binding domains to components of BTFs should bypass the requirement for an activation domain. This prediction was confirmed in experiments with artificial activators, where the fusion protein could substitute for the genuine

activator *in vivo* (Barberis *et al.*, 1995; Chatterjee and Struhl, 1995; Gonzalez-Couto *et al.*, 1997). Activators can also recruit chromatin-modifying complexes to promoters (Blanco *et al.*, 1998; Cosma *et al.*, 1999; Ikeda *et al.*, 1999). The importance of chromatin modification in transcriptional regulation is supported by the observation that histone acetylases are components of many transcriptional coactivators, which dock on transcription factors, allowing effective transcription to take place. Compelling evidence for the role of certain activators in recruiting chromatin-modifying complexes *in vivo* comes from the study of *HO* gene activation in yeast, where the transcriptional activator Swi5 recruits the Swi/Snf chromatin modification complex and the SAGA histone acetylase prior to association of a second activator, SBF, which recruits the transcription initiation apparatus (Cosma *et al.*, 1999). Furthermore, certain activators increase the overall elongation rate of the polymerase, possibly by stimulating the rate of promoter escape or polymerase II processing (Yankulov *et al.*, 1994; Krumm *et al.*, 1995; Brown *et al.*, 1998).

Repressors can be divided into two classes, general and gene-specific. Many general repressors function via interactions with the TBP (Lee and Young, 1998). For example, binding of repressor NC2 to the basic repeat domain of TBP in yeast can prevent the RNA polymerase II holoenzyme or its TFIIB subunit from assembling into an initiation complex (Meisterernst and Roeder, 1991; Inostroza *et al.*, 1992). Gene-specific repressors function by binding to activators or by competing for DNA binding sites of activators. For example, the Gal80 protein represses the Gal4 activator by binding to a portion of its activation domain in yeast (Ma and Ptashne, 1987; Leuther and Johnston, 1992). The chaperone heat shock protein Hsp90 binds to the heat shock

transcription activator Hsf1, preventing the formation of Hsf1 trimers required for binding of the DNA element on mammalian Hsp genes (Zou *et al.*, 1998). Repressors can also compete with activators by binding to overlapping sites in DNA, as illustrated by the Acr1 repressor and the ATF/CREB activator in yeast (Vincent and Struhl, 1992).

Cis-acting elements are often located far away from the core promoters. To date, four different hypotheses have been proposed to explain how the bound *trans*-acting factor makes contact with the BTFs bound to the core promoter. The most accepted is the chromatin looping model in which a *trans*-acting factor binding on the *cis*-acting element approaches a basal transcription factor at the core promoter by looping the intervening DNA (Wang and Giaever, 1988; Rippe *et al.*, 1995; Ptashne and Gann, 1997). The tracking or scanning model was hypothesized by Tuan *et al.* (1992). In this model the *trans*-acting factor linearly tracks along chromatin until it encounters a competent promoter. The facilitated tracking model incorporates aspects of both the looping and the tracking models: a *cis*- and *trans*-acting complex migrates along the chromatin until it encounters the cognate promoter and the intervening chromatin between the complex and then the promoter 'reels out' through the complex and forms a loop, which is progressively enlarged during tracking (Blackwood and Kadonaga, 1998). The linking model proposes that the binding of a *trans*-acting factor between a *cis*-acting element and its cognate gene defines the activated domain and mediates transcription-regulating activity (Bulger and Groudine, 1999).

2.4. Green florescence protein (GFP) as a tool to study fungal promoters

2.4.1. The wild type GFP

The discovery of green fluorescent protein (GFP) occurred in 1955 when Davenport and Nicol (1955) noticed that green fluorescence can be emitted from the light-producing cells of the jellyfish *Aequorea victoria* when the animals were irradiated with long-wave ultraviolet light. Shimomura *et al.* (1962) extracted a protein from the jellyfish which could produce the same fluorescence. Thirty years later, Prasher cloned a cDNA for the *GFP* gene from *A. victoria* (Prasher *et al.*, 1992) and, along with others, showed that *Escherichia coli* and *Caenorhabditis elegans* exhibited bright green fluorescence when transformed with the *GFP* gene under the control of native promoters (Chalfie *et al.*, 1994). Since then, *GFP* has been heterologously expressed in many organisms including yeast, mammals, *Drosophila*, plants and filamentous fungi (Lorang *et al.*, 2001; Schmid and Neumeier, 2005).

GFP has been used as a reporter marker due to the fact that it provides a visual means to identify genetically engineered cells (Lippincott-Schwartz and Patterson, 2003). Cells transformed with *GFP* exhibit bright green fluorescence under ultraviolet or blue light. Compared to other reporter genes, such as *uidA* (β-glucuronidase, GUS) (Jefferson *et al.*, 1987) or *lacZ* (β-galactosidase) (Nielsen *et al.*, 1983) from *E. coli* which confer blue color to the transformed cells, and the luciferase gene from firefly or bacteria (Devine *et al.*, 1993) which produces luminescence, *GFP* has several advantages in terms of sensitivity and manipulation. Firstly, GFP protein can be detected without destroying the tissue. Secondly, it is highly stable and shows very little photobleaching, e.g., it remains active up to 65°C and at pH 5 to 11, or in the presence of 1% sodium

dodecyl sulfate (SDS) or 6 M guanidinium choloride (Lorang *et al.*, 2001). Thirdly, fluorescence from GFP requires only oxygen and no other substrates for visualization.

GFP is a small protein of 28 kDa with a barrel-like structure composed of 11 β-sheets slightly twisted around the central axis, designated as a β-can structure (Ormö *et al.*, 1996; Yang *et al.*, 1996). The fluorescence is caused by three cyclized and oxidized amino acids located in the center of the molecule (Fig. 2.2). The process of fluorophore formation and maturation requires oxygen for the generation of oxidized intermediate states of these amino acids. For this reason, GFP can only be expressed under aerobic conditions, but as soon as GFP maturation is completed, oxygen is no longer needed for fluorescence. The wild-type GFP has a major absorption maximum at 397 nm and a minor excitation peak at 475 nm (Yang *et al.*, 1996).

2.4.2. Modified GFPs

Several variations and improvements have been made in the wild type *GFP* gene, including higher fluorescence coefficients, improved expression characteristics in eukaryotic cells, and altered spectral properties. The *sGFP* (S65T) incorporates a threonine amino acid substitution in place of serine at position 65, which results in 'red-shifted' fluorescence characteristics (Heim *et al.*, 1995) while the mGFP5-ER incorporates several changes directed towards improved and correct expression in transgenic plants (Haseloff and Siemering, 2006). A cryptic intron, that has been shown to prevent GFP expression in tobacco (Reichel *et al.*, 1996) and *Arabidopsis* (Haseloff *et al.*, 1997), was removed in mGFP5-ER. Elliott *et al.* (1999) found that the fluorescent signal of sGFP (S65T) was more easily detected than mGFP5-ER under blue light excitation in transgenic plant cells.

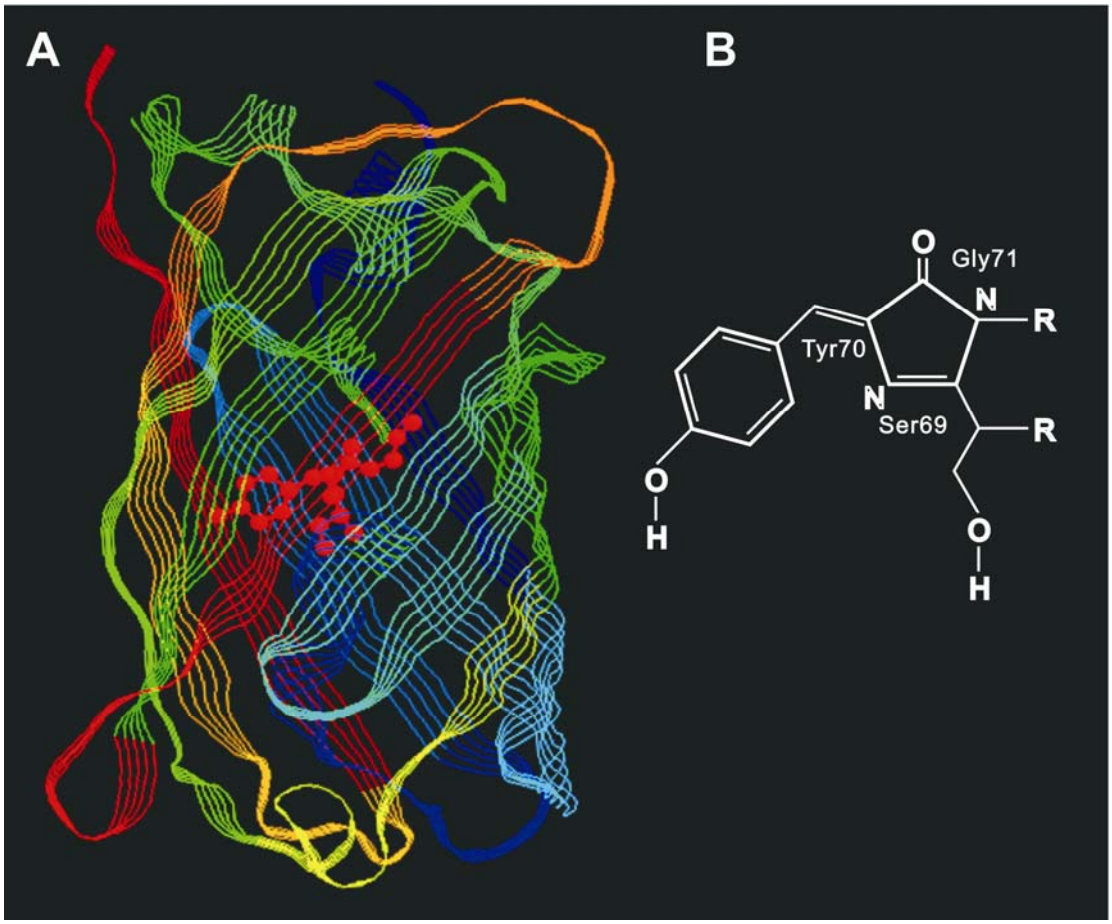

Fig. 2.2. Structure of mGFP. **A.** Three-dimensional structure of mGFP. The amino acid sequence (GenBank accession no. AAF65344; Hajdukiewicz *et al.*, 1994) was analysized for three-dimensional structure with 3D-JIGSAW version 2.0 (http://www.bmm.icnet.uk/servers/3djigsaw) and the result was presented using RasMol version 2.6 (http://www.umass.edu/microbio/rasmol/getras.htm). The chromophore is located within a helix inside the 11-strand barrel. **B.** The chromophore consists of a cyclized tripeptide containing Ser69, Tyr70, and Gly71.

Another significant breakthrough was made in the finding and generation of new fluorescent proteins that emit light of different colors (Heim *et al.*, 1994). A red fluorescent protein (RFP/DsRed) has been identified in coral and its fluorescent property studied (Heikal *et al.*, 2000). By substituting amino acids in the chromophore of the wild-type GFP, proteins which yield blue and red fluorescence have been developed (Schmid and Neumeier, 2005). The availability of multiple colors of fluorescent proteins

led to the development of complex technologies, such as fluorescence resonance energy transfer, which can be used to detect protein-protein interactions *in vivo*.

2.4.3. Applications of GFP in studies on fungal genes and promoters

GFP has been extensively used in studies on fungal biology, including visualizing hyphal invasion during pathogenesis, investigating cell dynamics, protein localization, protein-protein interaction, locating important genes by promoter trapping, and delineating the composition of fungal promoters (Lorang *et al.*, 2001). *U. maydis* was the first filamentous fungus in which successful expression of GFP was reported (Spellig *et al.*, 1996), and subsequently in *Aureobasidium pullulans* (Vanden Wymelenberg *et al.*, 1997) and *Aspergillus nidulans* (Suelmann *et al.*, 1997; Fernández-Ábalos *et al.*, 1998). In *F. graminearum*, GFP has been used to visualize fungal infection patterns in barley (Skadsen and Hohn, 2004; Jansen *et al.*, 2005), wheat (Miller *et al.*, 2004; Guenther and Trail, 2005; Jansen *et al.*, 2005) and *Arabidopsis* (Skadsen and Hohn, 2004; Chen *et al.*, 2006). GFP has also found application in characterization of genes important for metabolism or pathogenicity. Replacement of the wild type *MAT1-2* gene with *GFP*, followed by observation of GFP expression in outcross strains clarified the function of the mating type genes *MAT1-1* and *MAT1-2* in *F. graminearum* (Lee *et al.*, 2003). Tagging genes with *GFP* provides a more precise method to study the function of the encoding proteins, as exemplified by the cellular localization of the ferrichrome transporter protein FgSit1 (Park *et al.*, 2006). By fusing a promoter-less GFP with a sequence from the open reading frame (OPF) of a *F. graminearum* hydroxymethyl-glutaryl CoA reductase gene, Seong *et al.* (2006) found that the sequence possessed a cryptic promoter activity.

GFP can be used to identify and characterize fungal promoters. When linked to a specific promoter, GFP will be expressed in place of the native protein, allowing temporal and spatial expression of gene of interest to be determined. Most importantly, the expression of GFP can be easily detected using microscopy. By fusion of *GFP* with the promoter of the endopolygalacturonase gene *CLPG2* from *Colletotrichum lindemuthianum*, the expression pattern of this gene during bean infection was investigated (Dumas *et al.*, 1999) and a subsequent promoter deletion study using *GFP* as the reporter identified *cis*-acting elements responsible for gene induction under various conditions (Herbert *et al.*, 2002). By fusion of the 5' fragments of the *U. maydis* pheromone response factor gene *Prf1* with *GFP* under the control of a minimal promoter P*mfa1,* Hartmann *et al.* (1999) identified enhancer sequences responsible for *Prf1* induction. Using the same approach, Garrido *et al.* (2004) demonstrated that expression of the *Crk1* gene, which encodes a novel MAP kinase, was required for enhancer-dependent transcriptional activity of the *Prf1* promoter. *GFP* has also been used in a promoter deletion study of the *mig1* gene that is extensively upregulated during maize infection by *U. maydis* (Basse *et al.*, 2000). Promoter analyses with *GFP* have been reported in fungi other than plant pathogens, e.g., the manganese peroxidase gene *mnp1* from the lignin-degrading fungus *Phanerochaete chrysosporium* (Ma *et al.* 2004) and the galectin encoding gene *cgl2* transcribed during fruiting body formation in *Coprinopsis cinerea* (Bertossa *et al.*, 2004). However, there are no reports of analysis of *F. graminearum* promoters using *GFP* as the reporter gene.

3. CHARACTERIZATION OF A TRIGLYCERIDE LIPASE GENE *FgLip1* FROM *FUSARIUM GRAMINEARUM*

3.1. Introduction

During the initial association with their hosts, plant pathogens encounter the epicuticular waxes and cuticle covering host epidermal cells. The cuticle is a continuous layer of lipid material that consists of insoluble polymeric material called cutin (Juniper and Jeffree, 1983). The cutin polymer matrix with embedded waxes forms an efficient barrier against desiccation and decreases the vulnerability of plants to pathogen attack by providing both mechanical disease resistance and cellular signals for resistance responses (Kolattukudy, 1985).

Plant pathogenic fungi produce an array of extracellular hydrolytic enzymes that enable penetration and infection of the host tissue (Kolattukudy, 1985; Oliver and Osbourn, 1995; Knogge, 1996), and are collectively called cell wall degrading enzymes (CWDEs). These enzymes may contribute to pathogenesis by degrading wax, cuticle and cell walls, thus aiding in spore germination and infection. Furthermore, they can act as elicitors of host defense reactions and may also play a nutritional role during certain stages of the fungal life cycle. Early studies suggested that fungal cutinases played an important role in the penetration of plant surfaces. However, contradictory evidence as to the importance of cutinases in disease establishment has emerged from disruption studies of individual cutinase-encoding genes in several pathosystems (Stahl and Schäfer, 1992; Sweigard *et al.*, 1992; Crowhurst *et al.*, 1997; van Kan *et al.*, 1997). It has been proposed that cutinolytic activities expressed by pathogenic fungi during the infection process could also be caused by other enzymes (Comménil *et al.*, 1995, 1998, 1999; van

Kan *et al.*, 1997; Nasser-Eddine *et al.*, 2001). Upon contact with the host surface, plant pathogenic fungi often produce an extracellular matrix underneath the fungal germling, a phenomenon of the prepenetration process that determines the success of infection and disease development. Production of lipolytic activities associated with the extracellular matrix has been reported for the biotrophic powdery mildew (Roberts and Mims, 1998) and rust pathogens (Deising *et al.*, 1992), as well as the hemibiotrophic *Colletotrichum* (Pain *et al.*, 1996) and necrotrophic *Botrytis* species (Doss, 1999). Thus, it is likely that secreted fungal lipolytic enzymes play an important role in the infection processes.

Fusarium head blight (FHB) has emerged as a major disease of wheat, barley, and other small grains in North America in recent years (McMullen *et al.*, 1997). *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schw.) Petch) is the major pathogen responsible for this disease, but other *Fusarium* species, especially *F. culmorum*, also play a role (Gilbert and Tekauz, 2000). *Fusarium* species can cause head blight and/or root rot on many other plant species (Sutton, 1982).

The importance of *F. graminearum* as a plant pathogen is highlighted by the recently coordinated efforts in the *F. graminearum* genome project. Through the USDA/NSF (United States Department of Agriculture/National Science Foundation) microbial genome sequencing program, a 36 mb assembly has been released based on ~10x genome coverage from shotgun sequencing of the *F. graminearum* genome (http://www.broad.mit.edu/annotation/fungi/fusarium). The rapid availability of the genome sequences of *F. graminearum* and other fungal species will immediately promote functional identification of many fungal genes, particularly for those fungi in which gene disruption is feasible.

Based on the unique catalytic property of fungal lipase and its possible role in pathogenesis, this research was undertaken to clone the secreted lipase gene *FgLip1* and to determine its function on fungal development and pathogenicity. The polymerase chain reaction (PCR) was used to amplify *FgLip1* from PH-1 genome and expression levels of *FgLip1* were examined *in vitro* and *in planta*. The function of *FgLip1* was investigated by heterologous expression of the recombinant protein and gene replacement studies.

3.2. Materials and methods

3.2.1. Chemicals

All restriction enzymes were purchased from New England Biolabs (Beverly, MA). Agar, potato dextrose agar (PDA), corn meal agar, tryptone, peptone, yeast nitrogen bases without amino acids and without ammonium sulphate (YNB) were purchased from Difco (Detroit, MI). β-D-glucanase and Driselase were purchased from Interspex Products (Foster City, CA). Other chemicals, unless stated otherwise, were purchased from Sigma-Aldrich (St. Louis, MO).

3.2.2. Fungal materials and culture conditions

F. graminearum strain PH-1 (FGSC #9075, NRRL 31084), which was used for whole genome sequencing, was obtained from the University of Kansas Medical Center (Kansas City, KS). The fungus was routinely maintained on 4% PDA and a long-term stock was kept at -80°C as a spore suspension in 15% glycerol. For inoculum production, the fungus was cultured on 1.7% corn meal agar for at least seven days before making a conidial suspension using the method described by Groth *et al.* (1999). For the growth

assay, PH-1 and transformants were cultured at 24°C on minimal medium (Czapek-Dox without sugar, 0.2% $NaNO_3$, 0.05% KCl, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.001% $FeSO_4 \cdot 7H_2O$) supplemented with 2% agar (for plates only) and/or substrate as required. All media were autoclaved at 15 psi/121°C for 20 min. When needed, the lipid substrate was added into the autoclaved media and the media were emulsified for at least 30 min using a Branson 2200 ultrasonic water bath (Bransonic, Danbury, CT).

3.2.3. Plant materials

The spring wheat (*Triticum aestivum* L.) cultivar 'CDC Teal', which is highly susceptible to *F. graminearum*, was used in this study for extracting total RNA from fungal infected and healthy spikes. For testing the pathogenicity of transformants, spikes of CDC Teal, spring wheat 'Infinity' (DePauw *et al.*, 2006), winter wheat 'CDC Clair' and winter barley (*Hordeum vulgare* L.) 'McDiarmid', as well as the silk of corn (*Zea mays* L.) 'Seneca 60', were used. Plants were grown in a growth chamber with a 24°C day/18°C night temperature and a 16 h photoperiod.

3.2.4. Database search and computational analysis

Amino acid sequences of lipases from other fungi were obtained using BLAST (Basic Local Alignment Search Tool) at NCBI with the deduced amino acid sequence of FgLip1 as the query. Alignment of FgLip1 with other lipases was performed with ClustalW (http://www.ebi.ac.uk) and presented with Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html). A phylogenic tree was constructed based on multiple alignment using the Seqboot-Protdist-Neighbor-Consense method included in the Phylip 3.6 phylogeny package (http://evolution.genetics.washington.edu/phylip.html). The robustness of the phylogenic

tree was determined by Seqboot on the basis of 1,000 random samples taken from the multiple sequence alignment. The phylogenic tree was presented by Treeview software (Page, 1996). Signal peptides of lipase proteins were predicted using SignalP 3.0 (http://www.cbs.dtu.dk). The three-dimensional image of FgLip1 was constructed with 3D-JIGSAW software (http://www.bmm.icnet.uk). Other analyses of FgLip1 were performed using appropriate software accessed from http://ca.expasy.org. Analysis of restriction sites from DNA sequences was performed using the pDRAW32 DNA analysis software available at http://www.acaclone.com.

3.2.5. DNA and RNA manipulation

Most DNA and RNA manipulations were performed according to Sambrook and Russell (2001). Plasmid DNA was isolated by the alkaline lysis method and, when necessary, was additionally purified by the phenol/chloroform purification method. DNA fragments were purified from agarose gels using either a home-made glassmilk method (Boyle and Lew, 1995) or a Qiagen mini column purification kit (Qiagen, Chatworth, CA) following recommended procedures.

Isolation of fungal DNA for polymerase chain reaction (PCR) followed a protocol modified after Liu *et al.* (2000). A small piece of mycelium taken from a PDA plate was incubated in 670 µl lysis buffer (400 mM Tris-HCl pH 8.0, 60 mM EDTA, 150 mM NaCl and 1% sodium dodecyl sulfate) in a 1.5-ml Eppendorf tube for 10 min and then mixed with 200 µl of potassium acetate (60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml distilled water for 100 ml). After centrifugation at 14,000 rpm for 1 min, the supernatant was transferred to a new tube and centrifuged again. The supernatant was then transferred to a new tube, mixed briefly by inversion with an equal

volume of isopropanol and centrifuged at 14,000 rpm for 2 min. The supernatant was discarded and the DNA pellet was washed in 300 µl of 70% ethanol and dissolved in 50 µl H_2O. For PCR, 0.5 µl of the purified DNA was used in 25-50 µl of PCR mixture.

3.2.6. Cloning of *FgLip1* and other putative lipase genes from *F. graminearum*

Three genomic sequences of *FgLip1* and the open reading frame of other putative lipase genes were amplified from *F. graminearum* by PCR using *Pfu* and *Taq* polymerases (Stratagene, La Jolla, CA) and specific primer pairs (Tables 3.1 and 3.2). The PCR reaction was carried out on a MJ Thermocycler (Bio-Rad, Richmond, CA) with a initial denaturation for 5 min at 94°C followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 30 s at 62°C and extension for 2-5 min at 72°C.

F_{Lip1-1}/R_{Lip1-1} were used to amplify *FgLip1* including its coding region and 5'- and 3'-flanking sequences. F_{Lip1-2}/R_{Lip1-2} and F_{Lip1-3}/R_{Lip1-3} were used to amplify the *FgLip1* coding region with and without the predicted signal peptide, respectively. The 4,532-bp fragment amplified by F_{Lip1-1}/R_{Lip1-1} was cut with SacII/ApaI and cloned into the same restriction site of pBluescript II KS^+ (Stratagene). The other two fragments as well as the open reading frames of other lipase gene members were cloned into the TA vector (pBluescript II KS^+). The resulting plasmids that contain *FgLip1* were named pFgLip1, pFgLip1ORF and pFgLip1ORF-SP and transformed into *E. coli* strain DH5α. The transformants of DH5α were selected on LB agar plates (1% NaCl, 1% tryptone, 0.5% yeast extract, 2% agar) containing 100 µg/ml ampicillin. All PCR products were confirmed by sequencing (Plant Biotechnology Institute, Saskatoon, Canada).

Table 3.1. Oligonucleotide primers used in characterization of the *FgLip1* gene

Primer	Sequence (5'→3')	Restriction site*
F_{Lip1-1}	GCCGCGGCCCTCTCAATAGATGCGTTACTTAC	SacII
R_{Lip1-1}	CGGGCCCCGATACATGAACCTCAGTCTGAGCC	ApaI
F_{Lip1-2}	CGGCCCAGCCGGCCACGATGAGATTCTCTGGTTTCGTC	SfiI
R_{Lip1-2}	CGGGCCCTACCACCAAAGCACCGGCATTCGCC	ApaI
F_{Lip1-3}	CGGCCCAGCCGGCCGAGTCCAGCTGCCTTTCCTGC	SfiI
R_{Lip1-3}	GTCTAGAACCACCAAAGCACCGGCATTCGC	XbaI
F_{Lip1-4}	CCATGGACGGCGATGGCTTCCTCTTCT	
R_{Lip1-4}	CGTGGTGGATGGTTAGCGGGTTGAG	
F_{GPDH}	ATGGCTCCCATCAAGGTCGGCATC	
R_{GPDH}	TTACTTGGAGGCATCGACCTTGGC	
$F_{Cellulase}$	ATGACGGCCTACAAGCTTTTCC	
$R_{Cellulase}$	TTACAGGAAAGAGGGGTTGGCG	

* Incorporated restriction site sequences are underlined.

Table 3.2. Oligonucleotide primers used in the *FgLip* gene family study

Gene (GenBank accession no.)	**Forward Primer (5'→ 3')**	**Reverse Primer (5'→ 3')**
FGL1 (AY292529)	ATCTTGTCACAGCTTGTCTATCTA	TCACTTTATCATGCTTGCACCAGA
FgLip2 (EAA72690)	ATGGCTAGCAAGGACATCATGGA	AGAACCTCTTTGGAAGCATTGTAC
FgLip3 (EAA71500)	TTCAGTGATGAGATGCAGAAGGAGA	AAAGGTTTCAGGCTTGCCCTCGAAT
FgLip4 (EAA77021)	ATGGAGTTCAGCTGTTGAGAAAGCA	AACTCAGCCGAAGCTTTCGAGACT
FgLip5 (EAA70335)	TGTACGACTCTGGAAGGCCGAGAAA	AAAGAACCAGGCCTAGTCTCTGTA
FgLip6 (EAA77685)	CAGAATTTCAGTCTTCTTAAAG	CATGACTATCATATTGAGGGCTT
FgLip7 (EAA76763)	TGGCAAGGAGATCAAGGAGGAT	GTTGTGGACAAAGCTGATCCAGT
FgLip8 (EAA78222)	AAATAGAGGCGGCTGGCGTATCTA	TGTTTCTCCTTCAGGTTAGGCTC
FgLip9 (EAA78149)	TGCAAGATGTACTTGGCTTATTG	TTTCTACACGGAAGTTGAAAGAG
FgLip10 (EAA72835)	ATAGTCTTGGCATTCGTGGCAAT	AACTCTCATGCCTTGCTACATTG
FgLip11 (EAA71242)	TGTGTCACCATGAAGCTTCTTAC	TTGCCTTACTTGTTAAGTCTAGG
FgLip12 (EAA73165)	TTAACGTTGCCATGCAGTCGGTA	TCGTATCTCATAACAAGTCCGCTG

3.2.7. Heterologous expression in yeast and lipolytic activity assay

To construct the vectors for overexpressing FgLip1 in yeast *Pichia pastoris*, plasmid DNA from pFgLip1ORF and pFgLip1ORF-SP was double-digested with SfiI/ApaI and SfiI/XbaI, and the resulting fragments containing the *FgLip1* sequence were inserted into the same restriction sites of EasySelect *Pichia* expression vectors pPICZA and pPICZαA (Invitrogen, Carlsbad, CA), respectively. The difference between pPICZA and pPICZαA is that the latter contains a native *S. cerevisiae* α-factor secretion signal, which allows for efficient secretion of most proteins from *P. pastoris* (Cregg *et al.*, 1993). The constructed vectors were named pPICZA-FgLip1ORF and pPICZαA-FgLip1ORF-SP, respectively. Both vectors, as well as pPICZαA, were transformed into *P. pastoris* X-33 cells as recommended by the manufacturer. The transformants were selected on YPD medium (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) containing 50 μg/ml zeocin. For the lipolytic activity assay, X-33 cells were grown on BMMY medium (1% yeast extract, 2% peptone, 1.34% YNB, $4x10^{-5}$ % biotin, 100 mM potassium phosphate pH 6.0, 2% agar) and recombinant FgLip1 expression was induced by adding 0.5% methanol to the medium. Secreted lipolytic activity was detected using both the emulsified tributyrin assay (Rapp and Backhaus, 1992) and the rhodamine-B assay (Kouker and Jaeger, 1987). Rhodamine-B plates were supplemented with 1% (w/v) tributyrin, triolein, tristearin, olive oil, wheat germ oil or canola oil.

3.2.8. Transcript induction of *FgLip1*

CDC Teal wheat spikes were inoculated with the PH-1 strain of *F. graminearum* by the spore droplet method (Jenczmionka *et al.*, 2003). Healthy spikes and severely infected spikes were collected five days after inoculation. For *in vitro* induction tests,

PH-1 was shaken (130 rpm) overnight in YPG medium (0.3% yeast extract, 1.0% peptone, 2% glucose) at 30°C. Young mycelium was washed twice with sterile distilled water and transferred to minimal medium or minimal medium supplemented with 1% (w/v) glucose, sucrose, tributyrin, triolein, olive oil, wheat germ oil, wheat cell wall material or apple pectin as the sole carbon source. Wheat cell wall material, used as a potential inducer of CWDE gene expression, was prepared from leaves of CDC Teal by the method described by Lehtinen (1993). After shaking (130 rpm) at 30°C for 6-72 h as required, the mycelium was collected and subjected to RNA extraction.

3.2.9. Construction of gene replacement vector

To construct the gene replacement vector pΔFgLip1, a 3.2-kb cassette containing the *E. coli* hygromycin-B phosphotransferase gene (*hygR*), that had been rendered suitable for fungal expression by being fused to a *Glomerella cingulata* promoter and an *A. nidulans* terminator, was cut from the pGC1-1 vector (Rikkerink *et al.*, 1994; Appendix II) by SalI/HindIII and inserted into the same site of the pFgLip1 backbone. As a result, the hygromycin resistance gene *hygR* replaced the *FgLip1* coding region and was flanked by border sequences from the wild-type genomic locus. The left and right flanking sequences were 1.2 kb (SacII-SalI fragment) and 1.0 kb (HindIII-KpnI fragment), respectively (Fig. 3.1).

3.2.10. Fungal transformation

To produce transformants, *F. graminearum* mycelium from the surface of one 7-day old PDA plate (10 cm) was cultured in 50 ml YPG medium in 150-ml bottles. The culture was shaken for 40 h at 150 rpm at 30°C, and then the mycelium was collected by filtering through 200-μm nylon mesh (VWR, Cat 47746-104). After washing twice with

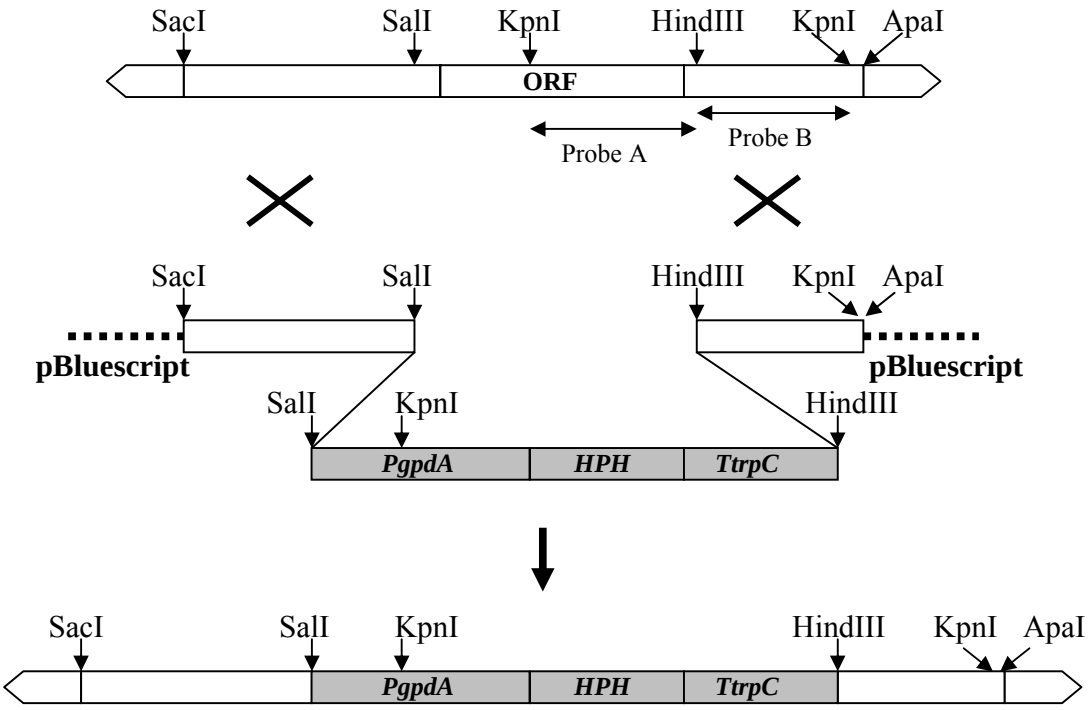

Fig. 3.1. Diagram of pΔFgLip1 and the homologous gene replacement event. Locations of probe A (1.6 kb) used in northern analysis and probe B (0.9 kb) used in Southern analysis are indicated. *PgpdA*, *Glomerella cingulata gpdA* promoter; *HPH*, *E. coli* hygromycin phosphotransferase (hygromycin resistance) gene; *TtrpC*, *Aspergillus nidulans trpC* terminator.

20 ml H_2O and twice with 10 ml 1.2 M KCl, the mycelium was gently shaken (75 rpm) in 10 ml 1.2 M KCl plus 0.5 g D-glucanase (Interspex Products, Cat 0439-1) and 0.5 g Driselase (Interspex Products, Cat 0465-1) for 2-4 h at 30°C. Protoplasts were then harvested by filtering through 200-μm nylon mesh into a 50-ml tube which was centrifuged at 2,000 g for 5 min. The pellet was washed with 50 ml 1.2 M KCl, centrifuged again as above, and the protoplasts were transferred into a 1.5 ml tube with 100 μl STC buffer (1 M sorbitol, 50 mM Tris-HCl pH 7.5, 50 mM $CaCl_2$, sterilized with a 0.22-μm filter). Protoplast concentration was adjusted to 10^7/ml with STC buffer. Ten μg unlinearized pΔFgLip1 DNA was added to 200 μl of protoplast suspension, which

was kept on ice for 20 min, and then 1 ml freshly-prepared PEG (poly ethylene glycol; 30% PEG 4000 in STC buffer, filter sterilized) was gently added. After 30 min at room temperature, the suspension was added to melted (50°C) sterile YPSA (0.1% yeast extract, 0.1% tryptone, 1 M sucrose, 2% agar), and then poured into 11-cm diameter plastic petri plates and incubated at 30°C overnight. The plates were overlaid with 10 ml melted selection media (4% PDA with 100 μg/ml hygromycin B) and incubated at 30°C. When regenerated colonies emerged after 4-6 days, they were transferred onto new plates containing the selection medium (4% PDA with 50 μg/ml hygromycin B).

3.2.11. Pathogenicity tests

Wheat spikes were inoculated at anthesis with the *F. graminearum* strain PH-1 or the transformants using either the spore droplet (Jenczmionka *et al.*, 2003) or the spray method (Schisler *et al.*, 2002). The spore droplet inoculation was performed by placing a droplet (10 μl) of conidial suspension (5 x 10^4 conidia/ml) between the palea and lemma of two basal florets of one spikelet in the middle of the wheat spike being tested. For spray inoculation, approximately 5 ml of conidial suspension (5 x 10^4 conidia/ml) was sprayed on each spike using a 2-oz pump sprayer (Goody Products, Atlanta, GA). After inoculation, the spikes were misted with water and covered with a plastic bag to maintain a moist environment for 48 h. Plants were then moved into a growth chamber with >85% relative humidity and grown under a 16/8 h day/night photoperiod and at a day/night temperature of 24°C/16°C. Symptom development on inoculated spikes was assessed every second day from two to 14 days after inoculation by counting the discolored spikelets. Detached barley spikes were inoculated by the spore droplet method and incubated on misted filter paper. A detached corn silk infection assay was

conducted as described by Seong *et al.* (2005) with the exception that small blocks of PDA culture were used as the inoculum. All tests were conducted at least twice and more than 50 spikes or corn silks were inoculated for each cultivar/strain combination.

3.2.12. Northern and Southern hybridization

Total RNA was extracted from 5 g fresh mycelium grown for 48 h in minimal medium or in minimal medium supplemented with glucose, sucrose, tributyrin, triolein, olive oil, wheat germ oil, wheat cell wall material, apple pectin, stearic acid or linoleic acid. For Southern analysis, genomic DNA extracted from wild-type or mutant mycelium grown for 48 h in YPG medium was digested with KpnI for five hours. After electrophoresis, DNA or RNA was transferred onto a GeneScreen Plus Hybridization Transfer Membrane (PerkinElmer, Wellesley, MA). The probes (Fig. 3.1) were radiolabeled with [^{32}P]dCTP using the Megaprime DNA labelling system (GE Healthcare Bio-Sciences, Piscataway, NJ) following the manufacturer's instructions. Hybridization was conducted at 65°C overnight in QuickHyb solution (Stratagene). After hybridization, membranes were washed twice at room temperature in 2 × SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) for 15 min and once in 0.1× SSC, 0.1% SDS at 65ºC for 30 min, then exposed on X-ray film (Sterlin Diagnostics, Newark, DE).

3.2.13. Confocal microscopy

In the rhodamine-B plate assays, oil droplets produced during hydrolysis of triglyceride lipids by secreted fungal lipases were examined under a Zeiss LSM 510 confocal laser scanning electron microscope (Zeiss, Jena, Germany) using a wavelength of excitation/emission 405 nm/560-615 nm.

3.3. Results

3.3.1. FgLip1 sequence resembles other triglyceride lipases

A gene family of 12 members, which shared high sequence similarities with the putative lipase from *Blumeria graminis* f. sp. *hordei* (AW790713; Thomas *et al.*, 2002) and triglyceride lipases from *C. rugosa* (Longhi *et al.*, 1992; Lotti *et al.*, 1993), was identified from the *F. graminearum* genome database. Among the 12 members, one gene designated *FgLip1* (EAA67628) encodes a predicted protein that shows the highest sequence similarity to the well known lipase family from yeasts *C. rugosa* (Lotti *et al.*, 1993) and *Geotrichum candidum* (Shimada *et al.*, 1990) and to a putative secreted lipase from the plant pathogenic fungus *B. cinerea* (Commenil *et al.*, 1999). In the present study, characterization of the *FgLip1* gene is the major objective.

Multiple alignment was conducted to compare the deduced amino acid sequence of FgLip1 with other fungal lipases, including those which have been well-characterized and those from important plant pathogens (Fig. 3.2). The deduced amino acid sequences suggested that they belonged to the type-B carboxylesterase/lipase family and possessed the highly conserved serine active site signature F-[GR]-G-X-X-X-X-[LIVM]-X-[LIV]-X-G-X-S-[STAG]-G ([…] means any one of the characters enclosed in the brackets could occur in the sequence) and the lipase catalytic triad: a serine, a glutamic acid, and a histidine (Schrag *et al.*, 1991; Cousin *et al.*, 1997). The protein sequences also contained a conserved pattern [EDA]-[DG]-C-L-[YTF]-[LIVT]-[DNS]-[LIV]-LIVFYW]-X-[PQR] (Cousin *et al.*, 1997). The C (Cysteine) in this pattern, with another C located upstream, fixes the lid structure through the formation of a disulfide bond (Cygler and Schrag, 1999). Consistent with the observation for the five *C. rugosa* lipases

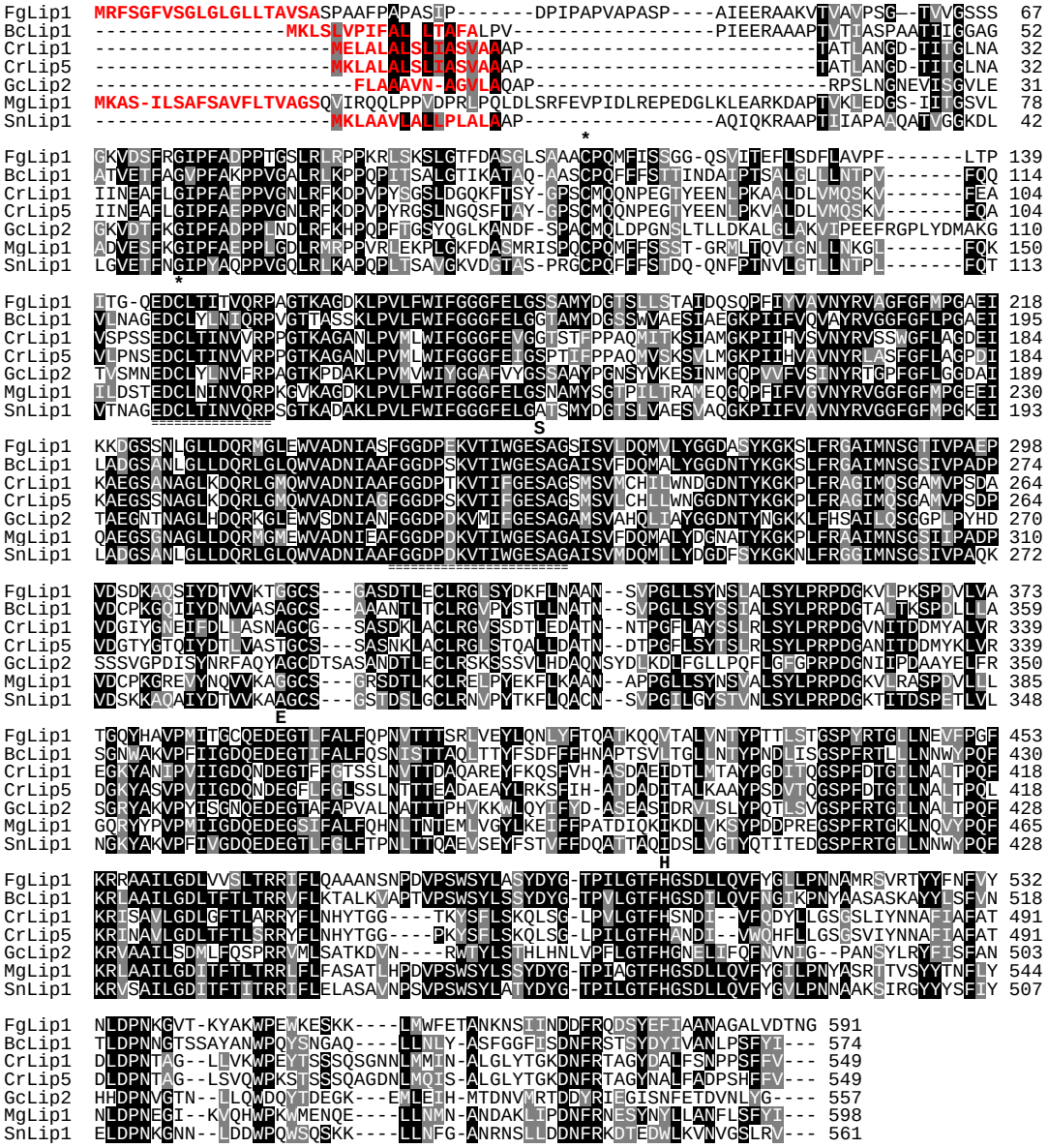

Fig. 3.2. ClustalW alignment of FgLip1 with lipases from other fungi. Alignment includes FgLip1 (EAA67628) and lipases from *Botrytis cinerea* (BcLip1, AAU87359), *Candida rugosa* (CrLip1, P20261; CrLip5, P32949), *Geotrichum candidum* (GcLip2, BAA00603), and putative lipases from *Magnaporthe grisea* (MgLip1, EAA48656) and *Stagonospora nodorum* (SnLip1, EAT82093). Residues that are conserved in at least four of the sequences are highlighted black and residues that are similar are highlighted gray. Consensus residues serine (S), glutamic acid (E), and histidine (H) consisting of the catalytic triad are indicated on the top and the consensus type-B carboxylesterase family signatures are underlined. The two conserved cysteine residues expected to form a disulphide bridge and fix the lid structure are marked with * on the top. Residues making up the predicted signal peptides are shown as red letters.

(Benjamin and Pandey, 1998), the amino acid sequences within the lid region are less conserved than the other regions between FgLip1 and other lipases (Fig. 3.2). A signal peptide was present in the N-terminal of FgLip1, in other lipases included in the multiple alignment (Fig. 3.2) and in 10 other putative lipases from *F. graminearum* (data not shown), suggesting that they are all secreted lipases.

A phylogenetic tree was constructed based on a ClustalW alignment of the deduced amino acid sequences of FgLip1, its closest BLAST hits from other fungal species, and eleven putative *C. rugosa* lipase-like protein of *F. graminearum* (Fig. 3.3). FgLip1 clustered with and showed 61%, 61% and 57% identity to the putative lipase sequences of *M. grisea*, *Stagonospora nodorum* and *B. cinerea*, respectively, suggesting that there are orthologues of the *FgLip1* gene in other fungal genomes. The other eleven putative lipases from *F. graminearum* showed little similarity (< 37%) either to each other or to individuals within the lipase cluster containing FgLip1. At the time of this analysis, none of the putative lipase genes had been investigated.

The *F. graminearum* genomic sequence (http://www.broad.mit.edu) located *FgLip1* on contig 1.84. *FgLip1* consists of a 1,827-bp open reading frame, interrupted by a 51 bp intron (beginning at nucleotide 1,762). The predicted mature protein consists of 571 amino acids, with an estimated molecular mass of 61.1 kDa, an overall negative charge at pH 7 and an isoelectric point of 5.27. A Kyte and Doolittle hydropathy plot (Kyte and Doolittle, 1982) of FgLip1 indicated that it has significant hydrophobic regions corresponding to the signal peptide and the open lid that participates in the substrate-binding site and contributes to substrate recognition (Fig. 3.4). A three-dimensional image was constructed based on the FgLip1 amino acid sequence (Fig. 3.5).

It illustrated clearly the lipase catalytic triad located in the center of the protein structure and a unique region representing the lid domain. Although the presence of these structures is not sufficient for an enzyme to be classified in the lipase family, they are commonly observed in most lipases described to date (Wong and Schotz, 2002).

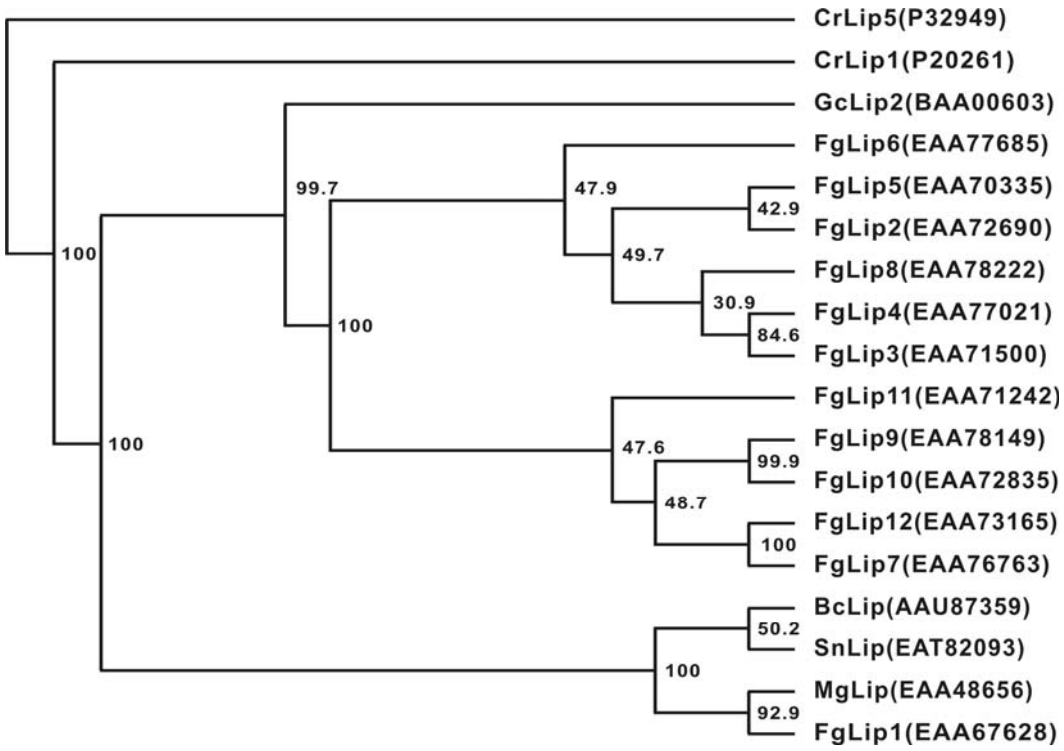

Fig. 3.3. Phylogenetic relationships between FgLip1 and selected lipases from other fungi. GenBank accession numbers are indicated following the protein names. Bootstrap values are shown at nodes as percentages of 1,000 replicates.

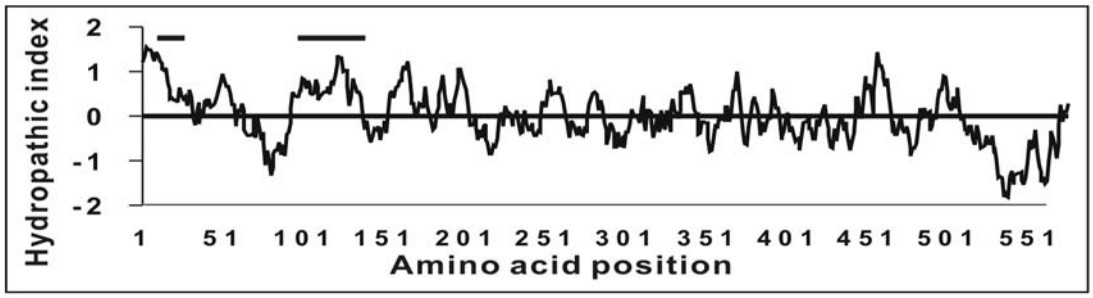

Fig. 3.4. Hydropathy plot of FgLip1. Black bars indicate the predicted signal peptide (left) and the lid region (right).

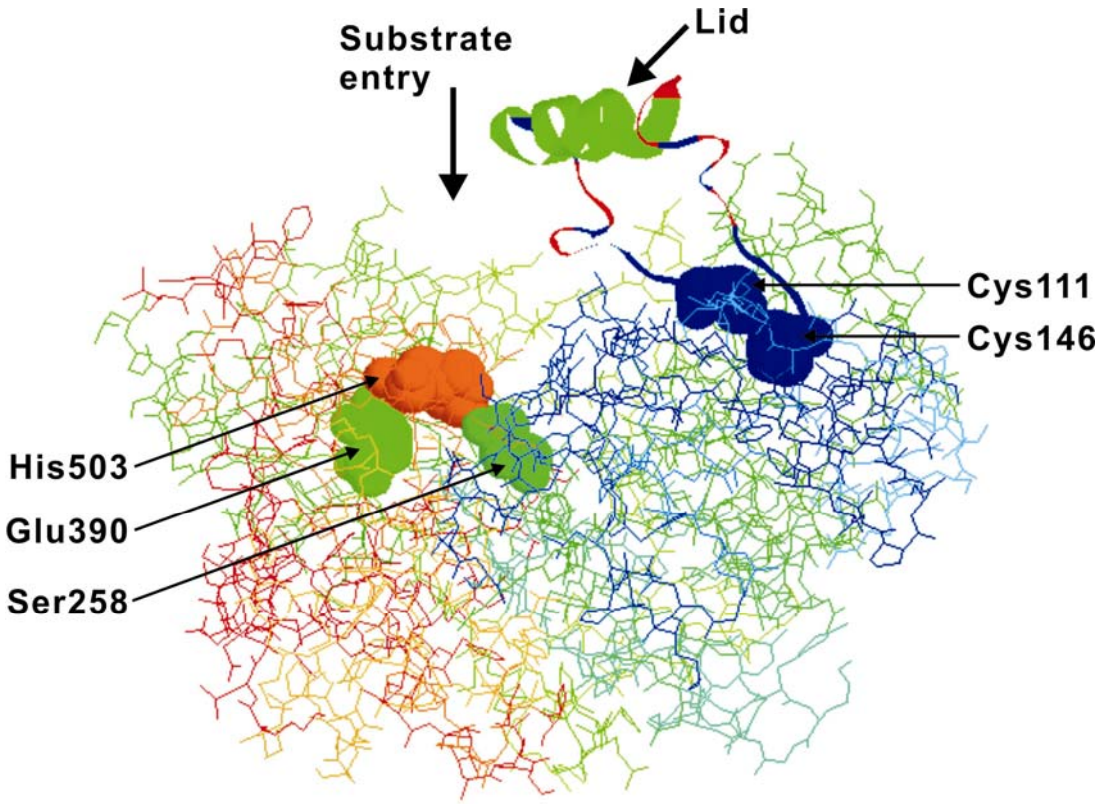

Fig. 3.5. Three-dimensional structure of FgLip1 based on its amino acid sequence. The lipase catalytic triad Ser258, Glu390 and His503, as well as the two cysteines (Cys111 and Cys146) embracing the lid region are indicated. The amino acid sequence was analyzed for three-dimensional structure with 3D-JIGSAW version 2.0 (http://www.bmm.icnet.uk/servers/3djigsaw/) and the result was presented using RasMol version 2.6 (http://www.umass.edu/microbio/rasmol/getras.htm).

3.3.2. *FgLip1* encodes a triglyceride lipase

P. pastoris strain X-33 does not show secreted lipolytic activity on culture plates. The *FgLip1* gene with and without the sequence encoding the signal peptide was introduced into vectors pPICZA and pPICZαA, respectively. Extracellular lipolytic activity of the transformed yeast was determined by the presence of substrate hydrolysis, indicated by production of a clear zone around the colony on tributyrin emulsion plates. Clear zones formed only around colonies of transformants with pPICZαA-FgLip1ORF-

SP (Fig. 3.6A), indicating that *FgLip1* encodes an enzyme that can hydrolyze triglycerides. No clear zone was observed around colonies of yeast transformed with pPICZA-FgLip1ORF or with the empty vector pPICZαA. Therefore, it is likely that the putative FgLip1 signal peptide could not function in the *P. pastoris* strain X-33.

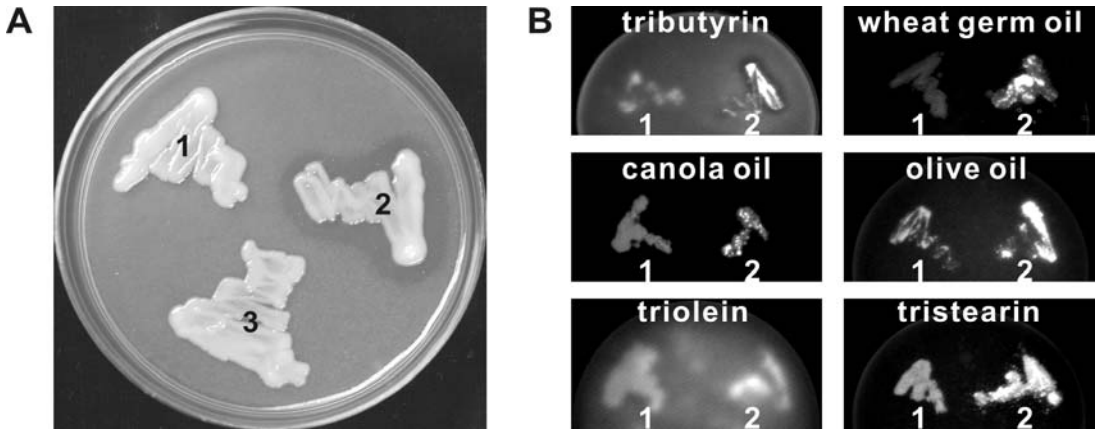

Fig. 3.6. Plate assay to detect FgLip1 lipolytic activity. *P. pastoris* cells were transformed with pPICZαA (1), pPICZαA-FgLip1ORF-SP (2) or pPICZA-FgLip1ORF (3). **A**. Transformants on BMMY plates containing 1% (w/v) tributyrin. **B.** Transformants on BMMY plates containing 0.0001% (w/v) rhodamine-B and 1% (w/v) lipid substrate as indicated.

To determine the substrate specificity of FgLip1, a rhodamine-B staining assay was conducted for yeast strains transformed with the pPICZαA-FgLip1ORF-SP and the empty vector using tributyrin, triolein, tristearin, canola oil, olive oil or wheat germ oil (WGO) as the lipid substrate. Rhodamine-B forms a fluorescent complex with free fatty acids and the hydrolysis of lipids is indicated by orange fluorescence under UV light. Fluorescence was observed in each lipid substrate assay with tributyrin giving the strongest fluorescence. These results demonstrated that yeasts expressing FgLip1 had lipolytic activity on all the lipid substrates assayed (Fig. 3.6B).

3.3.3. *FgLip1* is expressed *in planta* and its *in vitro* expression is regulated by fatty acid substrates

To test whether *FgLip1* expression occurs during the infection process, northern blot analyses were performed using total RNA extracted from CDC Teal wheat spikes five days post-inoculation with *F. graminearum* wild-type strain PH-1 and from uninoculated healthy spikes. *FgLip1* transcripts were detected in the infected but not in healthy spikes (Fig. 3.7A). The induction of *FgLip1* expression was also examined in liquid minimal medium supplemented with various carbon sources (Fig. 3.7B). Northern analysis revealed that *FgLip1* was strongly induced in the medium supplemented with WGO. Expression of *FgLip1* was also detected when the fungus was grown in medium containing olive oil and triolein, but at lower levels. In contrast, no expression was detected from fungal cultures grown in the minimal medium supplemented with glucose, sucrose, tributyrin, wheat cell wall material, or apple pectin as the sole carbon source.

To test whether prepared cell wall material is an efficient inducer of the fungal CWDEs, the same blot was further probed with a *F. graminearum* cellulase gene *FgCel* (EAA73192) amplified by PCR using two specific primers $F_{Cellulase}$/$R_{Cellulase}$ (Table 3.1). Expression of *FgCel* was detected only when the fungus was grown in a medium containing cell wall material prepared from wheat leaves (Fig. 3.7B).

Expression time courses, conducted with minimal medium (starvation conditions) or minimal medium supplemented with either WGO or glucose as the sole carbon source, did not detect expression of *FgLip1* in fungal cultures grown in the minimal medium supplemented with glucose. Induction of *FgLip1* by starvation conditions and WGO commenced 12 h after establishment of the culture and the expression levels increased and decreased progressively over time, respectively (Fig. 3.7C).

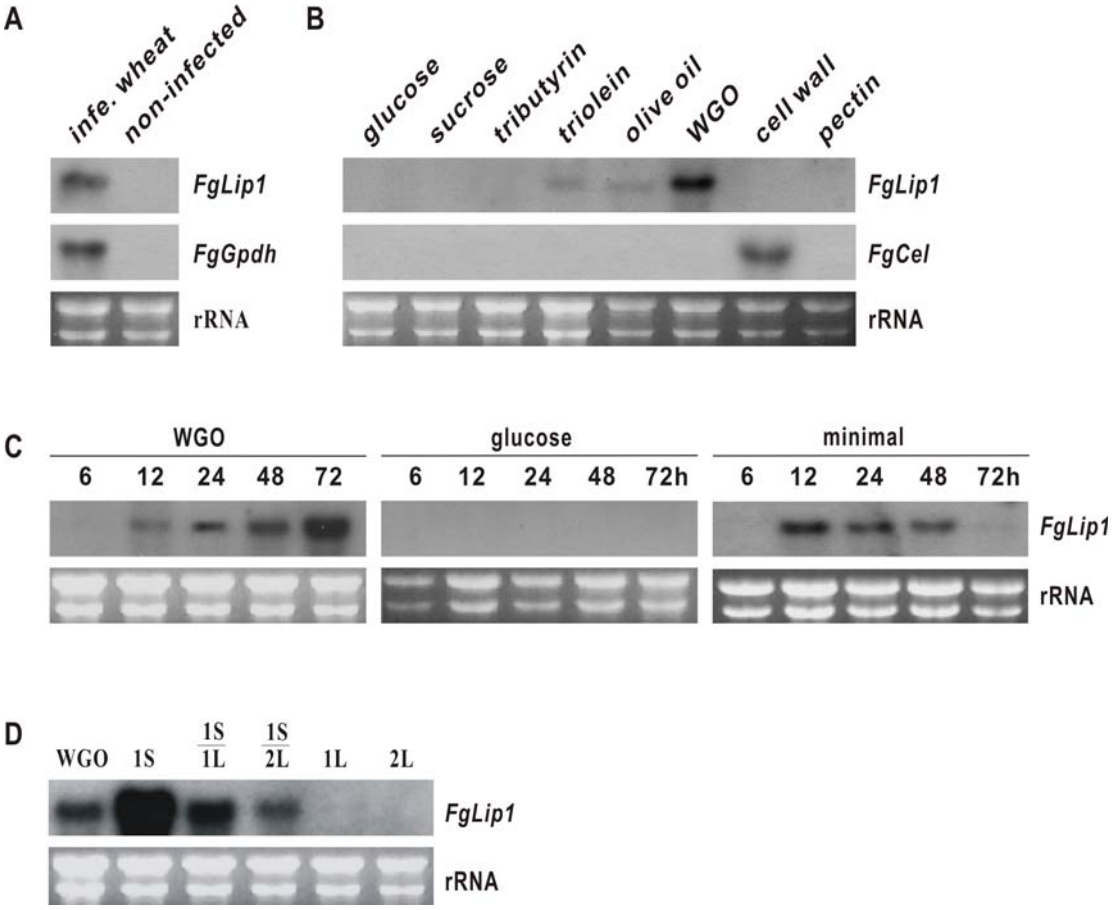

Fig. 3.7. Expression analysis of *FgLip1* by northern hybridization. **A.** Expression of *FgLip1* in infected wheat. Total RNA extracted from infected spikes 5 days after inoculation were probed with either *FgLip1* or a constitutively expressed fungal glyceraldehyde 3-phosphate dehydrogenase gene (*FgGpdh*, EAA73952). **B-D**. Expression of *FgLip1 in vitro*. Mycelium of PH-1 collected from overnight YPG culture was transferred in minimal medium supplemented with various carbon source. **B.** Expression of *FgLip1 in vitro* 48 h after culturing in minimal medium supplemented with various carbon source. **C.** Expression of *FgLip1* during fungal growth in minimal medium or minimal medium supplemented with either wheat germ oil or glucose as the sole carbon source. **D.** Specific induction and suppression of *FgLip1* expression by fatty acids after culturing for 48 h in minimal medium supplemented with 1% wheat germ oil (WGO), 1% stearic acid (1S), 1% stearic acid and 1% linoleic acid (1S/1L), 1% stearic acid and 2% linoleic acid (1S/2L), 1% linoleic acid (1L) or 2% linoleic acid (2L). Panel rRNA, the blot were stained with 0.02% methanol blue prior to hybridization showing similar amounts of total RNA was loaded.

Fatty acids have been reported to regulate gene expression in various organisms (Duplus *et al.*, 2000). The main difference between WGO and olive oil, triolein and tributyrin is that WGO contains a higher proportion of long-chain saturated fatty acids (up to 15%). Thus, it is possible that long-chain saturated fatty acids were responsible for the enhanced *FgLip1* gene expression in WGO medium. To clarify the nature of the induction of *FgLip1* by WGO, stearic acid ($C_{18:0}$), linoleic acid ($C_{18:2}$) or WGO was added into the minimal medium as the sole carbon source. After 48 h, cultures grown in stearic acid showed a markedly higher expression level compared to cultures grown with either WGO or linoleic acid. No *FgLip1* expression was evident when the fungus was grown in a linoleic acid-containing minimal medium. However, *FgLip1* expression induced by stearic acid was suppressed by adding linoleic acid to the stearic acid-containing medium (Fig. 3.7D). These results suggest that the long-chain saturated fatty acid specifically induced *FgLip1* expression and that the unsaturated fatty acid suppressed that expression.

3.3.4. Expression of *FgLip1* is different from other *F. graminearum* lipase genes

Expression of four other lipase genes from *F. graminearum* was investigated by northern analyses (Fig. 3.8). The open reading frames of these four lipase genes were used as probes against total RNA extracted from mycelia cultured under the same conditions described for *FgLip1*. Three genes from the *FgLip1* gene family showed expression in the glucose-containing medium, but this expression was absent in the *FgLip1* northern analysis (Fig. 3.7B). Of these three genes, expression of *FgLip3* (EAA71500) was induced by wheat germ oil. In the minimal media containing each of the various substrates as the sole carbon source, none of these three genes showed

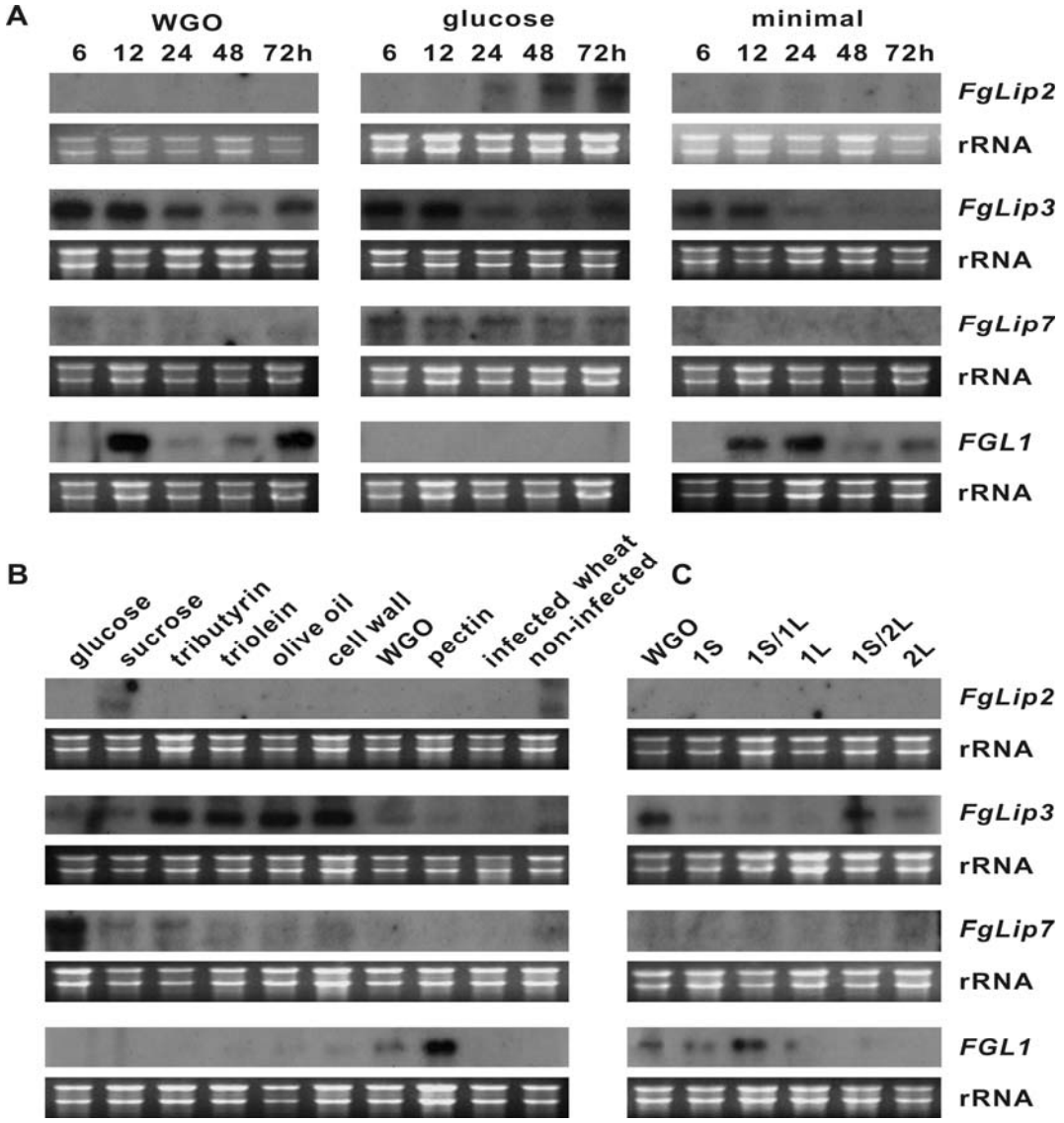

Fig. 3.8. Expression analysis of four lipase genes other than *FgLip1* from *F. graminearum*. Total RNA was extracted from infected CDC Teal spikes 5 days after inoculation with PH-1 or from mycelium of PH-1 cultured for 48 h in minimal medium supplemented with various carbon sources. The blot was probed with the open reading frame of the corresponding gene. **A.** Expression during fungal growth in minimal medium or minimal medium supplemented with either wheat germ oil or glucose as the sole carbon source. **B.** Expression *in planta* and *in vitro* with various carbon sources added into the minimal medium. **C.** Specific induction and suppression of expression by fatty acids. WGO, 1% wheat germ oil; 1S, 1% stearic acid; 1S/1L, 1% stearic acid and 1% linoleic acid; 1S/2L, 1% stearic acid and 2% linoleic acid; 1L, 1% linoleic acid; or 2L, 2% linoleic acid. Panel rRNA, the blot were stained with 0.02% methanol blue prior to hybridization showing similar amounts of total RNA was loaded.

expression patterns similar to *FgLip1* (Fig. 3.8B). The *FGL1* gene (AY292529), which has been characterized as an essential pathogenicity factor for *F. graminearum* (Voigt *et al.*, 2005), showed expression patterns similar to *FgLip1* in glucose, WGO and starvation media. However, its expression was strongly induced by pectin, a result not observed for *FgLip1*. Interestingly, no transcript was detected from infected plants for each of these four genes. The expression of *FGL1* in this experiment was inconsistent with the result reported by Voigt *et al.* (2005).

3.3.5. Development of a transformation protocol for *F. graminearum*

A protocol specific for *F. graminearum* genetic transformation, which was highly efficient for both protoplast production and transformant regeneration, was developed. For protoplast production, Novozyme 234 (Novo Nordisk, Bagsvaerd, Denmark) was used exclusively as the cell wall degrading enzyme. However, Novozyme 234 has not been commercially available since 2004 and no other equivalent products were available. Interspex Products has produced similar enzymes, but no procedure for efficient *F. graminearum* transformation has been reported to date. During this study, extensive experiments were conducted to seek the best enzyme combination and osmotic buffer.

After treatment with a mixture of D-glucanase and Driselase for one hour, release of spherical protoplasts from *F. graminearum* mycelia was detected under the light microscope (Fig. 3.9A) and after two hours most hyphal tissue was degraded and extensive protoplast production could be observed (Fig. 3.9B). Protoplasts were concentrated to 10^7/ml before mixing with the vector DNA (Fig. 3.9C). Protoplast germination was evident 12 h after transformation (Fig. 3.9D) and regenerated fungal colonies were observed on the medium surface 4-6 days after incubation (Fig. 3.9E).

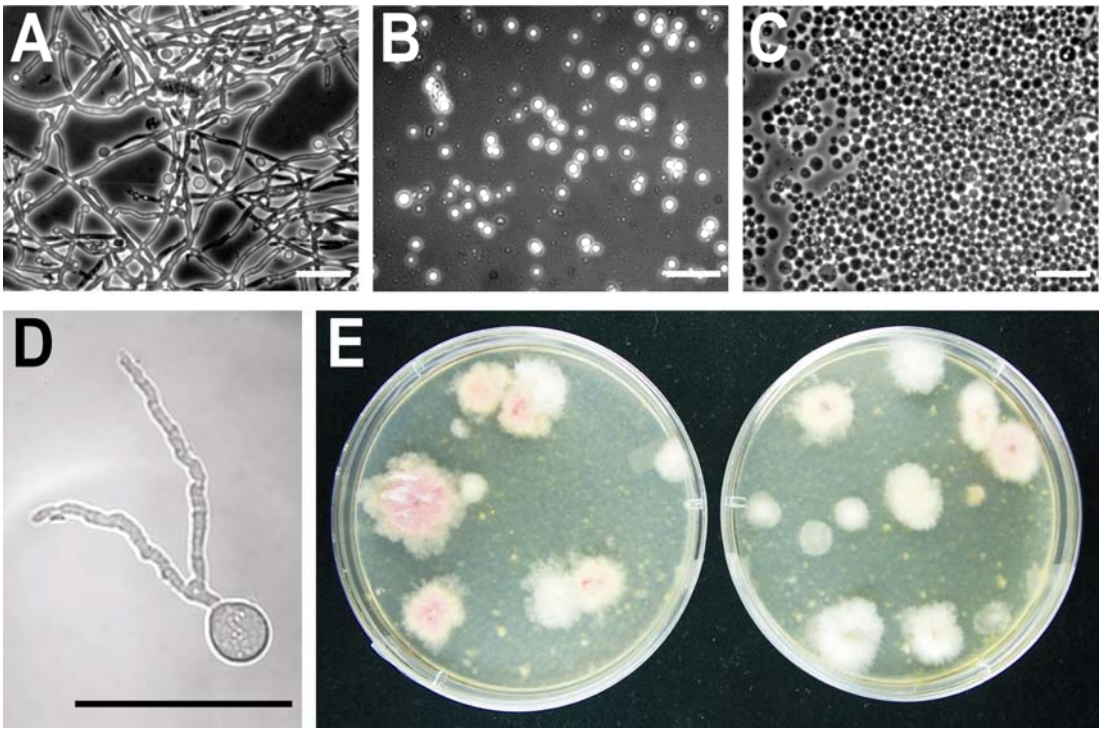

Fig. 3.9. Transformation of *F. graminearum*. **A** and **B.** Protoplast production by treating mycelia with cell wall degrading enzymes after 1 h (**A**) and after 2 h (**B**). **C.** Protoplasts were concentrated to 10^7/ml before transformation. **D.** Protoplast germination at 12 h after transformation. **E.** Colonies of transformants regenerated on the hygromycin-containing medium. Bars=10 μm.

3.3.6. *FgLip1* was successively disrupted from the *F. graminearum* genome

To investigate the function of *FgLip1* in fungal growth and pathogenicity, targeted gene disruption was conducted by transformation. Three independent transformation experiments produced 54 hygromycin-B-resistant transformants. Because the investigation of identified gene-knockout strains was performed immediately after the transformation experiments, later-emerging transformants from the second and third transformation experiments were not included in further study. Initially, 22 transformants were screened for the absence of the native *FgLip1* sequence by PCR using primers $F_{Lip1\text{-}4}$/$R_{Lip1\text{-}4}$ (Table 3.1). The presence of only the 3.3-kb band indicated

that the hygromycin resistance gene had replaced the wild-type *FgLip1* gene in the *F. graminearum* genome (Fig. 3.10A). If both the 3.3-kb and the 2.5-kb bands were amplified, the transformants were considered to have had the hygromycin resistance gene integrated at ectopic locations. These transformants will be referred to as ectopic integration strains. Twelve putative gene replacement strains were identified and seven of them, as well as three ectopic strains, were selected for further study. Genomic DNAs isolated from the selected strains and from the wild-type PH-1 were analyzed by Southern hybridization. Digestion of genomic DNA with KpnI, which has one cutting site in the 3' homologous region and an additional cutting site in either the *hygR* or *FgLip1* coding region, resulted in a 3.2- or a 2.4-kb fragment from the replacement and the native sequences, respectively (Fig. 3.1). Southern hybridization (Fig. 3.10B) showed that only the 3.2-kb fragment was present in the seven gene replacement strains and that both 2.4 and 3.2-kb fragments were present in the three ectopic integration strains. In the wild-type strain, only the 2.4-kb fragment was present. Southern blot analysis also demonstrated the replacement of native *FgLip1* by vector DNA in all seven transformants. Null mutation of *FgLip1* in seven replacement strains was further confirmed by northern analysis (Fig. 3.10C). No *FgLip1* transcript accumulation was observed in the seven replacement strains grown for 48 h in minimal medium containing 1% WGO. In contrast, a single transcript was detected from the three ectopic integration and the wild-type PH-1 strains.

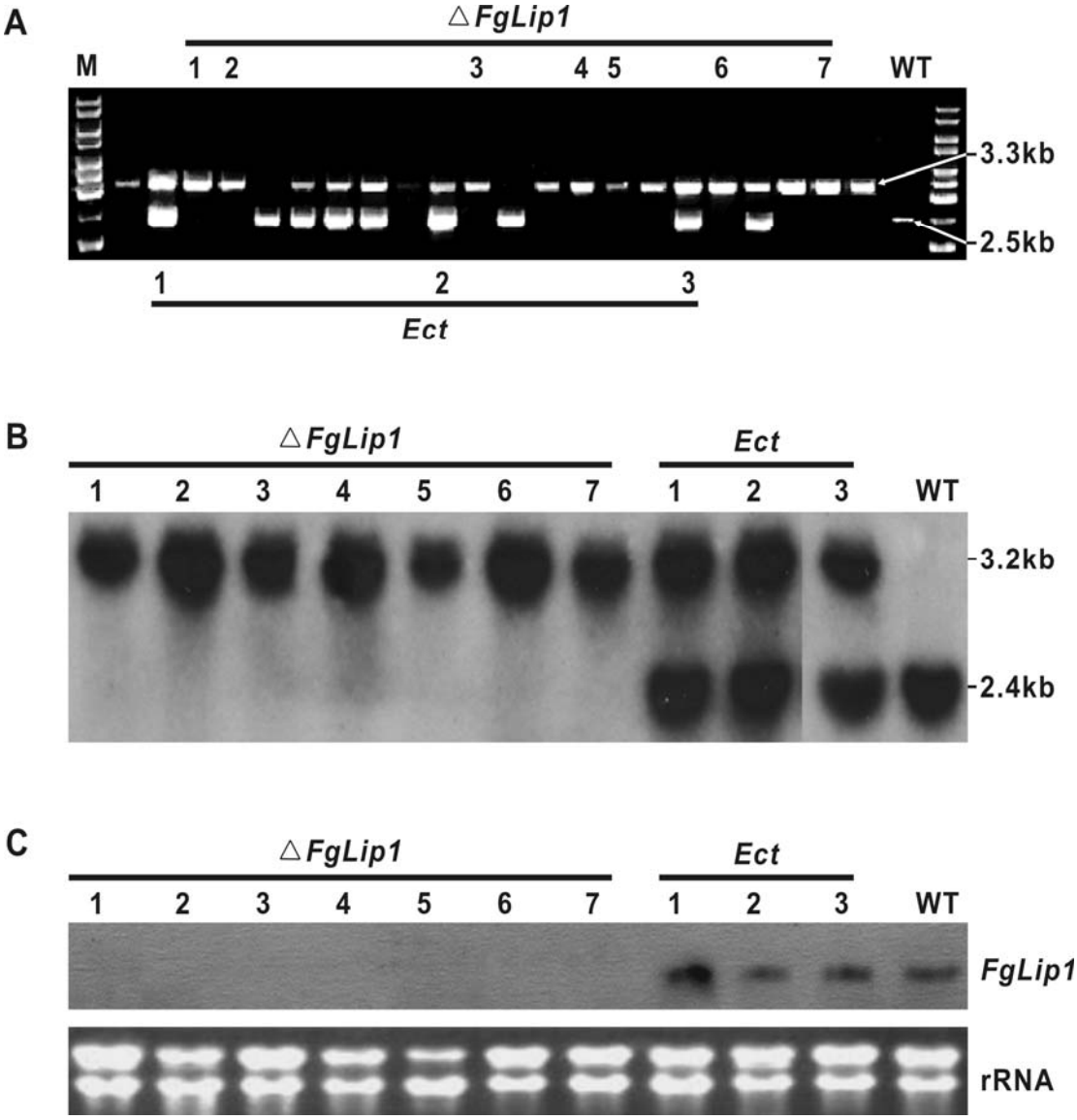

Fig. 3.10. Analysis of *FgLip1* gene disruption mutants. **A.** PCR analysis of transformants using primers $F_{Lip1\text{-}4}/R_{Lip1\text{-}4}$. The 3.3-kb and 2.5-kb bands indicate the presence of the hygromycin resistance gene and the wilt-type *FgLip1* gene, respectively. Numbers indicate the transformants selected for further study. M, GeneRuler 1 kb DNA Ladder (Fermentas, St. Leon-Rot, Germany). **B.** Southern blot of genomic DNA extracted from seven *FgLip1* replacement strains, three ectopic integration strains and wild-type strain PH-1. **C.** Northern blot of RNA extracted from seven *FgLip1* replacement strains, three ectopic integration strains and wild-type strain PH-1. Fungal strains were grown in minimal media containing 1% wheat germ oil for 48 h. Panel rRNA, the blot were stained with 0.02% methanol blue prior to hybridization showing similar amounts of total RNA was loaded.

3.3.7. *FgLip1* encodes a secreted lipase

Although secretion was predicted from the amino acid sequence analysis for 11 of the 12 lipase genes, the deduced native signal peptide of FgLip1 failed to function in recombinant *P. pastoris* (Section 3.3.3). Therefore an experiment was performed to screen the wild-type *F. graminearum* and its knock-out mutants for utilization of triolein or tristearin in agar plates containing rhodamine-B. As an additional control, the ectopic integration strains in which the native gene was intact were also used. After two days of incubation, strong fluorescence was observed around the colonies from the wild-type PH-1 and ectopic integration strains on both triolein- and tristearin-containing plates, but not from the seven null mutant strains (Figs. 3.11A and 3.11B). Rhodamine-B was not taken up by fungal mycelia since no fluorescence was detected in the mycelia of either Δ*FgLip1* mutants or PH-1 grown on rhodamine-B plates (Figs. 3.11C and 3.11D). Fluorescence was observed from oil droplets surrounding the wild-type mycelia, but was absent from the Δ*FgLip1* mutants, indicating that secreted lipolytic activity was completely lost in the Δ*FgLip1* knock-out mutants. This implied that FgLip1 is the major detectable lipase secreted under the growth conditions used in this study. The incubation time was extended by four days to determine if additional lipolytic activity could be detected in the knock-out mutants. After four days, fluorescence was also detected surrounding colonies of all seven Δ*FgLip1* mutants (Fig. 3.11E). These results established that while *FgLip1* was the major secreted lipase isoform that is induced by saturated and unsaturated lipid substrates, additional lipolytic activity appeared later during growth.

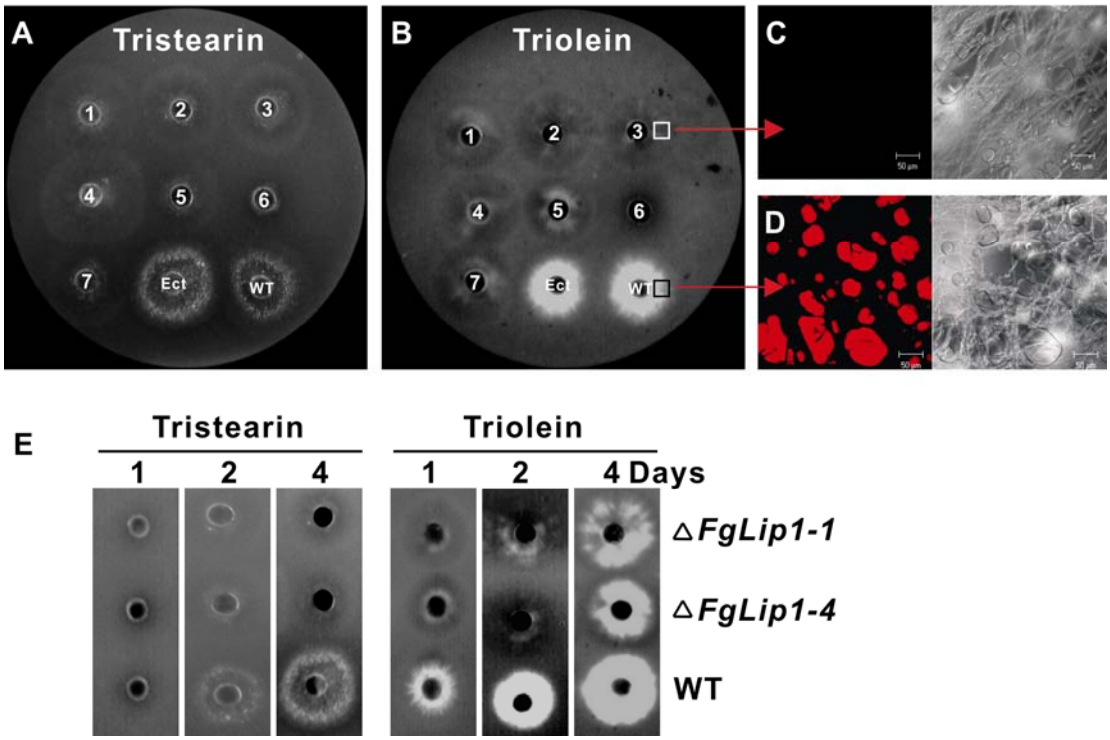

Fig. 3.11. Secreted lipolytic activity of Δ*FgLip1* strains. Wild-type PH-1 (WT) and ectopic integration (Ect) strains and Δ*FgLip1* mutants (1-7) were grown on minimal rhodamine-B agar plates with either 1% triolein or tristearin as the sole carbon source, respectively. **A** and **B.** The plates were inoculated with a mycelial plug of 5-mm diameter, incubated at 22°C in the dark for two days, and placed on a UV transilluminator (302 nm) for photography. **C** and **D.** Mycelial plugs of one Δ*FgLip1* mutant and the wild-type strain PH-1 viewed under a confocal microscope. The right column depicts the corresponding area by phase microscopy. **E.** A time course study shows fluorescence surrounding colonies of PH-1 strain and two Δ*FgLip1* mutants at one, two and four days after incubation.

3.3.8. *FgLip1* is essential for efficient utilization of triglyceride lipids

The major role of *FgLip1* on secreted lipolytic activity suggested that *FgLip1* might make a critical contribution to fungal nutrient utilization *in vitro*. Null mutants Δ*FgLip1-1* and Δ*FgLip1-4*, and wild-type PH-1 were grown on the solid minimal medium and in liquid minimal medium supplemented with either 1% triolein, tristearin or glucose as the sole carbon source. After four days of incubation, colonies of the Δ*FgLip1* strains and PH-1 grew similarly in size and morphology on solid medium when

glucose or triolein was used as the sole carbon source. In contrast, growth of the Δ*FgLip1* strains on medium containing tristearin was significantly impaired, showing very little hyphal growth (Fig. 3.12A). It was also noted that pigmentation occurred in the colonies of the wild-type PH-1 strain grown on all media, whereas pigmentation was absent from the colonies of the Δ*FgLip1* strains grown on triolein and tristearin and was present, but weak, in Δ*FgLip1* grown on glucose. While fungal biomass from liquid cultures containing glucose or triolein as the sole carbon source was similar after four days between the PH-1 strain and the Δ*FgLip1* strains, the Δ*FgLip1* strains grown in tristearin-containing medium produced significantly less biomass than the PH-1 strain (Fig. 3.12B). These results indicated that the *FgLip1* gene is needed for saturated lipid utilization *in vitro*.

3.3.9. *FgLip1* is not important for FHB pathogenesis

To determine whether *FgLip1* plays a role during pathogenesis, spikes of susceptible spring wheat cultivar CDC Teal were inoculated with spore droplets from the seven Δ*FgLip1*-disruption mutants, the wild-type and the three ectopic strains. Continuous observation over 14 days found no significant differences in symptom development between infected spikes inoculated with the wild-type, the ectopic, and all Δ*FgLip1* strains (data not shown). Similar results were obtained following a corn silk infection assay (Fig. 3.13A), in which all strains caused similar discoloration of the corn silk. Two Δ*FgLip1* mutant strains were chosen for detailed pathogenicity tests on other wheat cultivars and barley using the spore droplet and the spray inoculation methods. Symptom development caused by the Δ*FgLip1* mutants was not significantly different for both inoculation methods from that induced by the wild-type strain (Fig. 3.13B-E).

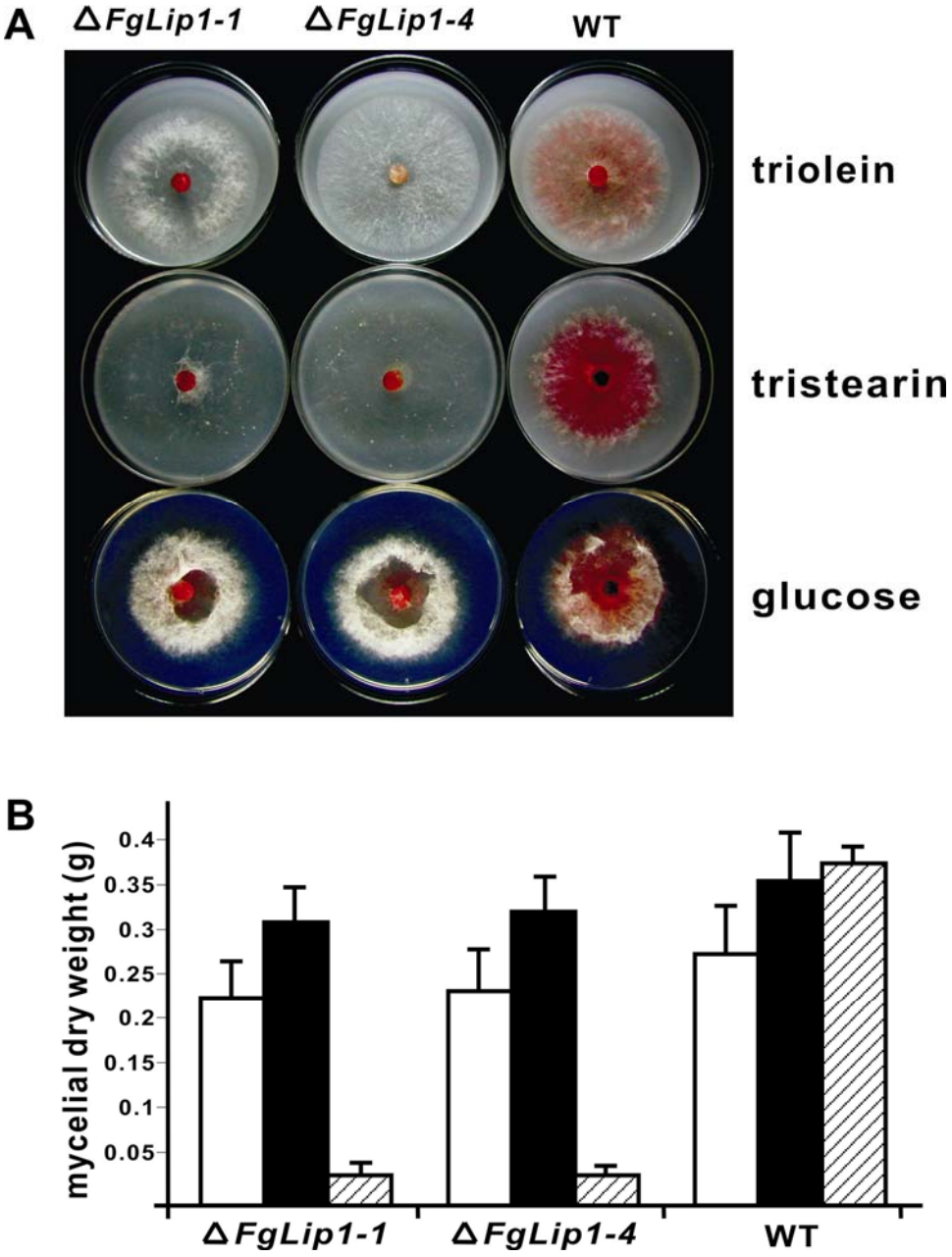

Fig. 3.12. Influence of carbon sources on the growth of wild-type and Δ*FgLip1* mutants. **A.** Wild-type PH-1 and Δ*FgLip1* mutants were grown on minimal agar plates with 1% triolein, tristearin or glucose as the sole carbon source, respectively. The plates were inoculated with a mycelial plug and incubated at 22°C in the dark for four days. **B.** Dry weight of the mycelium of the wild-type strain and Δ*FgLip1* mutants measured after four days in liquid minimal medium containing 1% glucose (blank bars), 1% triolein (black bars) or 1% tristearin (hatched bars) as the sole carbon source.

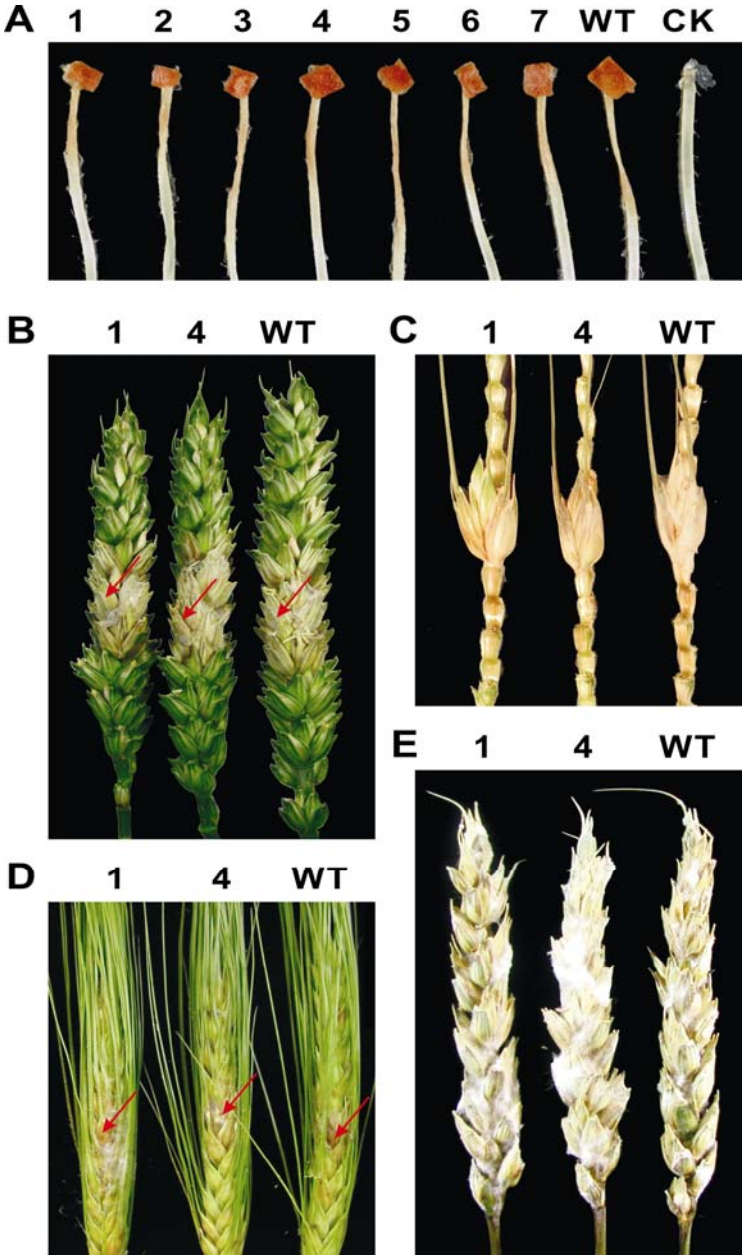

Fig. 3.13. Pathogenicity assays of *F. graminearum* Δ*FgLip1* mutants. Photographs were taken four (**A** and **D**) or five (**B**, **C** and **E**) days post-inoculation. **A.** Corn silks were inoculated at the freshly cut ends with a small block of PDA cultures of seven Δ*FgLip1* mutants (1-7), wild-type PH-1 (WT) or PDA medium only (CK). **B-D.** Spikes of spring wheat CDC Teal (**B**), winter wheat CDC Clair with the uninoculated spikelets removed to demonstrate the discoloration of the rachis (**C**) and winter barley McDiarmid (**D**) were inoculated with Δ*FgLip1-1* (1), Δ*FgLip1-4* (4), and the wild-type PH-1 (WT) by the spore droplet method. Arrow indicates the initial inoculated floret. **E.** Spikes of spring wheat Infinity were inoculated by the spray method.

Following spore droplet inoculation with the Δ*FgLip1* mutants, abundant mycelia developed from the inoculated florets and spread into the adjacent florets and spikelets (Fig. 3.13B and D). To investigate whether the disease was spread by mycelial growth on the plant surface or through the rachis, all but the inoculated spikelet were removed on the winter wheat spikes. Discoloration of the rachis in the vicinity of inoculated spikelets was evident on both Δ*FgLip1* mutant and wild-type infected spikes (Fig. 3.13C), indicating the occurrence of similar infection patterns. In the spray inoculation test, abundant mycelia grew over the entire wheat head and bleached spikelets appeared five days post-inoculation with either the Δ*FgLip1* mutant or the wild-type strain (Fig. 3.13E). The pathogenicity tests indicated that *FgLip1* was not essential for pathogenicity and virulence of *F. graminearum* on cereal hosts.

3.4. Discussion

Lipases, not traditionally considered CWDEs, are distributed widely among animals, plants, and microorganisms (Schmid and Verger, 1998). Lipases function at the interface between an insoluble substrate phase and the aqueous phase, a phenomenon known as interfacial activation (Verger, 1997), and catalyze the reversible degradation of glycerol esters with long-chain fatty acids into fatty acids and glycerol. In the present study, the *F. graminearum* FgLip1 was shown to possess lipolytic activity on lipids containing either saturated or unsaturated long-chain fatty acid.

Recently, involvement of fungal secreted lipases in infection has been demonstrated using molecular techniques. Comménil *et al.* (1995, 1998, 1999) purified and partially sequenced a 60 kDa extracellular lipase from *B. cinerea* and showed that a

specific antibody raised against this lipase suppressed lesion formation on tomato leaves. A lipase gene discovered through expressed sequence tag analysis of *Blumeria graminis* germinating conidia showed a dramatic increase in transcript abundance during conidial germination and appressorium formation (Thomas *et al.*, 2001, 2002; Yangdou Wei, unpublished data). FgLip1 shares considerable sequence identity with these *Botrytis* and *Blumeria* lipases. However, the gene disruption study showed that Δ*FgLip1* mutants and the wild-type strain caused similar patterns of FHB symptom development on susceptible hosts. Similar results were reported recently for the *Botrytis* lipase gene on its host plant bean (Reis *et al.*, 2005). In contrast, the *F. graminearum* gene *FGL1*, encoding a 35 kDa putative secreted lipase belonging to a *Rhizomucor miehei* lipase family (Schmidt-Dannert, 1999) was found to be required for the virulence of *F. graminearum* on wheat and maize (Voigt *et al.*, 2005).

Apart from their preference for triglycerides, lipases catalyze the hydrolysis and synthesis of a broad range of natural water-insoluble esters, as well as alcoholysis, acidolysis, esterification and aminolysis (Pandey *et al.*, 1999). Two *Aspergillus niger* enzymes, FAEA and FAEB, originally isolated from a commercial pectinase preparation, showed ferulic acid esterase activity in degradation of complex cell wall polysaccharides (de Vries *et al.*, 1997; 2002). These two proteins have significant sequence similarities with *F. graminearum* FGL1 and were grouped into the fungal lipase family in the database (http://bioweb.ensam.inra.fr/ESTHER/general). Although *C. rugosa* and *R. miehei* lipases belong to the same class of the α/β hydrolase fold family and share similar activation and catalytic mechanisms (Schmidt-Dannert *et al.*, 1998; Schmidt-Dannert, 1999), considerably different substrate binding sites between the two types of

lipases explain their varying substrate specificity. For instance, *C. rugosa* lipases have a tunnel-like binding site and are likely to accept substrates with long-chain fatty acids, whereas *R. miehei* lipases have a crevice-like binding site and can accept bulkier substrates (Schmidt-Dannert *et al.*, 1998; Schmidt-Dannert, 1999). Thus, information on the substrate specificity of *F. graminearum* FGL1 is necessary to determine its role in pathogenicity in contrast to the lack of that role in FgLip1. Both *F. graminearum* lipase genes *FGL1* (Voigt *et al.*, 2005; but contrary data was obtained from this research) and *FgLip1* are expressed during infection. The expression of both genes *in vitro* is induced by WGO, and suppressed by sucrose or glucose. In contrast, no *FGL1* transcripts were detected when *F. graminearum* was cultured in water without a carbon source (Voigt *et al.*, 2005), whereas this study found that expression of *FgLip1* was significantly induced when the fungus was cultured in sugar-deficient minimal medium. These results suggest that overlapping, but different regulatory mechanisms, are involved in induction of *FgLip1* and *FGL1* gene expression.

Expression of the *FgLip1* gene was induced strongly by WGO, weakly by triolein and olive oil, and not by tributyrin. Fatty acid was found to possess strong effects on the regulation of *FgLip1* expression. Long-chain saturated fatty acids such as palmitic acid ($C_{16:0}$, data not shown) and stearic acid ($C_{18:0}$) appeared to be strong inducers, whereas the long-chain unsaturated fatty acid linoleic acid ($C_{18:2}$) acted as a repressor. The association of long-chain saturated fatty acids in induction of *FgLip1* expression and long-chain unsaturated fatty acids with its repression suggests that the lipolytic products are major regulatory components controlling the transcription of *FgLip1*. In yeast, a similar regulation pattern for the *OLE1* gene, which encodes the Δ-9 desaturase, has

been reported; the mRNA level of *OLE1* was increased in response to exogenous saturated fatty acids and sharply reduced when the cells were exposed to unsaturated fatty acids (McDonough *et al.*, 1992). Although the corresponding positive and negative response elements have been characterized in the *OLE1* upstream promoter region (Choi *et al.*, 1996; McDonough *et al.*, 1992), preliminary sequence comparison analysis did not reveal the same elements present in the upstream region of the *FgLip1* gene. The transcription activation and/or repression elements required for fatty acid-mediated *FgLip1* expression remain to be determined.

The Δ*FgLip1* strains were deficient for secreted lipolytic activity on tristearin and showed delayed activity for hydrolysis of triolein. After a long incubation time, lipolytic activity particularly for triolein occurred in the Δ*FgLip1* strains, indicating that additional secreted lipases participated in exogenous lipid hydrolysis. A genome-wide survey revealed 11 additional sequences, which encode *C. rugosa* family lipases, present in the *F. graminearum* genome. Some of these putative lipases might be responsible for the enzyme activity which appeared at late stages of fungal growth under these conditions. Thus, it is reasonable to expect that the fatty acids produced by FgLip1-catalyzed lipid hydrolysis regulate expression of other lipase genes that control lipid hydrolysis in the later stages of growth.

Apart from reduced or abolished pigmentation, the colony morphology of the Δ*FgLip1* mutants was identical to the wild-type strain on minimal and complete media. However, the Δ*FgLip1* strains were unable to grow in liquid minimal medium containing tristearin as the sole carbon source. It is hypothesized that FgLip1 functions primarily when exogenous lipids containing long-chain saturated fatty acids are present,

acts as a regulator to co-ordinate expression of other lipase genes, and plays a role in fungal nutrient acquisition but not in pathogenesis.

3.5. Conclusions

1. *FgLip1* encodes a secreted triglyceride lipase.
2. FgLip1 possesses lipolytic activity against a broad range of triglyceride substrates.
3. Expression of *FgLip1* was activated *in planta* during the fungal infection process.
4. Expression of *FgLip1* is suppressed in rich media, but induced by wheat germ oil and starvation conditions.
5. Saturated fatty acid is the strongest inducer for *FgLip1* expression and this induction was proportionally suppressed by presence of unsaturated fatty acid.
6. *FgLip1* is required by the fungus for utilization of saturated triglyceride lipids.
7. *FgLip1* is not essential for the fungal pathogenicity.

4. PROMOTER STUDY OF *FgLip1* FROM *FUSARIUM GRAMINEARUM*

4.1. Introduction

During the first two years of this research project, the functions of *FgLip1* were clarified through recombinant expression of the protein in yeast and target gene disruption (Feng *et al.*, 2005). Although *FgLip1* was concluded to be non-essential for fungal pathogenicity, it was responsible for enabling the fungus to utilize saturated fatty acid as the sole carbon source. Northern analysis indicated that the expression of *FgLip1* was repressed by glucose and other simple sugars (Fig. 3.7). Furthermore, its expression was strongly induced by saturated fatty acids but repressed by unsaturated fatty acids (Fig. 3.7). Compared with other lipase genes from *F. graminearum*, the expression pattern of *FgLip1* was unique with respect to *in vitro* induction and repression (Fig. 3.7 and Fig. 3.8). *FgLip1* was found to be expressed earlier than other lipase genes when the fungus was grown in a medium containing triglycerol as the sole carbon source (Fig. 3.11). Furthermore, *FgLip1* represented a major lipase gene responsible for saturated fatty acid utilization (Fig. 3.12). These observations suggested that the expression of *FgLip1* was under the control of a fine-regulated promoter, which was responsible for substrate-specific and temporal regulation of *FgLip1* expression. Therefore, a promoter study was initiated to investigate the mechanism of *FgLip1* expression control.

Microorganisms prefer easily metabolizable substrates to less readily metabolizable substrates as the carbon source. One of the mechanisms used to achieve this is repression of the synthesis of enzymes related to the catabolism of the alternative carbon sources by a metabolic process known as catabolite or glucose repression

(Ruijter and Visser, 1997). Many fungal genes encoding cell wall degrading enzymes are subject to glucose repression, e.g., a cutinase from *Fusarium solani* f. sp. *pisi* (Kämper *et al.*, 1994), a xylanase (Zeilinger *et al.*, 1996) and a cellulase (Zeilinger *et al.*, 1998) from *Trichoderma reesei*, a pectinase from *Colletotrichum lindemuthianum* (Herbert *et al.*, 2002), and a xylanase from *Hypocrea jecorina* (Rauscher *et al.*, 2006).

Fatty acids (FAs) play important metabolic roles and can act as signaling molecules involved in regulating gene expression (Duplus *et al.*, 2000). Long chain FAs (C16 and above) (LCFAs) can be either saturated or mono- or polyunsaturated, depending upon the presence of one or more double bonds in the polycarbon chain. Most of the reported functions of FAs on gene expression have been associated with LCFAs. Saturated LCFA activation and polyunsaturated LCFA inhibition of gene expression have been reported from toll-like receptor 4 (Lee *et al.*, 2001) and toll-like receptor 2 (Lee *et al.*, 2004) genes in animals, including humans. Saturated LCFAs induced a 1.6-fold increase in transcription activity of the *S. cerevisiae OLE1* gene, whereas a large family of unsaturated LCFA repressed transcription by as much as 60-fold (Choi *et al.*, 1996). The underlying mechanism for unsaturated LCFA repression has been reported for the human *Her-2*/*neu* gene, in which oleic acid upregulated the expression of Her-2/neu *trans*-repressor (Menendez *et al.*, 2006).

Extensive research has been conducted on lipase gene regulation, with human lipoprotein lipases and hepatic lipases being of major interest due to the health concerns related to the encoding enzymes (Preiss-Landl *et al.*, 2002; Merkel *et al.*, 2002; Rufibach *et al.*, 2006). In bacteria, repression of lipase expression by oleic acid has been reported in *Pseudomonas aeruginosa* (Gilbert *et al.*, 1991) and *Acinetobacter calcoaceticus* (Kok

et al., 1996). Expression regulation of fungal lipases was particular interesting due to the usage of these lipases in industrial processes and the fact that filamentous fungi are amiable hosts for the production of homologous and heterologous lipases (Verdoes *et al.*, 1995; Gouka *et al.*, 1997). The expression of an extracellular lipase gene from *Nectria haematococca* was induced by olive oil but repressed by glucose (Nasser-Eddine *et al.*, 2001). Similarly, a lipase gene from *B. cinerea* was induced by wax and free fatty acids extracted from grape berry cuticle but repressed by cutin hydrolysates and glucose (Comménil *et al.*, 1999; Reis *et al.*, 2005). The expression pattern shown by *FgLip1* has, so far, not been reported for other fungal lipase genes.

The control of gene expression represents a process involving a combination of events that include decondensation of the locus, nucleosome remodeling, histone modification, binding of transcriptional activators and coactivators to enhancers and promoters, and recruitment of the basal transcription machinery to the core promoter (Smale and Kadonaga, 2003). Since induction and repression of *FgLip1* by specific substrates suggested that expression control at the transcription level was important for *FgLip1* expression, promoter deletion and linker scanning mutagenesis were conducted to reveal the *cis*-acting elements involved in transcription and to provide insight into the underlying mechanisms.

Such a study can provide information on the spatio-temporal interaction between the fungus and the wheat plant. As well, clarifying the specific induction or repression of the *FgLip1* promoter provides the possibility for utilization of this promoter in industry. Finally, a finely controlled promoter can be used in studies of functional characterization of essential genes, for which direct gene mutagenesis may not be possible.

4.2. Materials and methods

4.2.1. Fungal material

The same *F. graminearum* strain (PH-1) and procedures for maintenance and culture as described in Section 3.2.1 were used in this study. Transformation was performed following the protocol described in Section 3.2.8 with two modifications. Firstly, two vectors, an *FgLip1* promoter-GFP vector and a hygromycin resistance vector pSTU1, were used simultaneously for each transformation (co-transformation), and secondly, in the promoter deletion study, plasmid DNA of the *FgLip1* promoter-GFP vector was linearlized with HindIII or EcoRI before transformation. In other transformations, unlinearized plasmid DNA was used.

4.2.2. Database search and computational analysis

The *FgLip1* 5' upstream sequence was obtained from the *F. graminearum* whole genome database accessible at http://www.broad.mit.edu. The whole sequence was analyzed for putative promoter elements with the computer analysis tool GeneBuilder (Milanesi *et al.*, 1999; http://www.itba.mi.cnr.it/tradat). After the promoter deletion study, the first 600-bp sequence was further analyzed. A consensus sequence of a previously reported transcription regulatory element was obtained as a matrix from TRANSFAC Transcription Factor Binding Sites Database (Wingender *et al.*, 2000; http://www.gene-regulation.com). The matrix was then used to detect the putative promoter element for the *FgLip1* promoter sequence with PATSER software available at the Regulatory Sequence Analysis Tools web site (van Helden, 2003; http://rsat.scmbb.ulb.ac.be/rsat).

4.2.3. Construction of basal vectors and making of control strains

The fungal transformation vector pGC1-1 containing the *E. coli* hygromycin B phosphotransferase gene (*hygR*) was used to construct the gene knockout vector pΔFgLip1 (Section 3.2.9). However, for unknown reasons, *F. graminearum* transformation using intact pGC1-1 failed to produce stable hygromycin resistant transformants; instead, many tiny colonies emerged on the surface of the selection medium but lost hygromycin resistance after being transferred to fresh medium. A new vector containing the hygromycin resistant gene, named pSTU1, was constructed by fusing the hygromycin resistance cassette (HindIII/SalI) from pGC1-1 to the pBluescript backbone (Fig. 4.1A). Circular pSTU1 DNA was used in all transformations as the hygromycin resistance vector.

The binary vector pCAMBIA-1302 (AF234298) harbors an *mgfp5* His6* gene under the control of a CaMV 35S promoter (Hajdukiewicz *et al.*, 1994). To construct a fungal GFP expression vector, the *mgfp5* His6* open reading frame was amplified by PCR from pCAMBIA-1302 using two primers tailed with an NcoI and a BamHI site, respectively (Table 4.1). The expression vector pNOM102 (Z32701) contains a *uidA* gene under the control of the *Aspergillus nidulans gpdA* gene promoter and the *A. nidulans trpC* terminator. The open reading frame of the β-glucuronidase gene was released by NcoI/BamHI double digestion and replaced with the same restriction fragment of the *mgfp5* His6* PCR product. The resultant vector was named pFGFP (Fig. 4.1B).

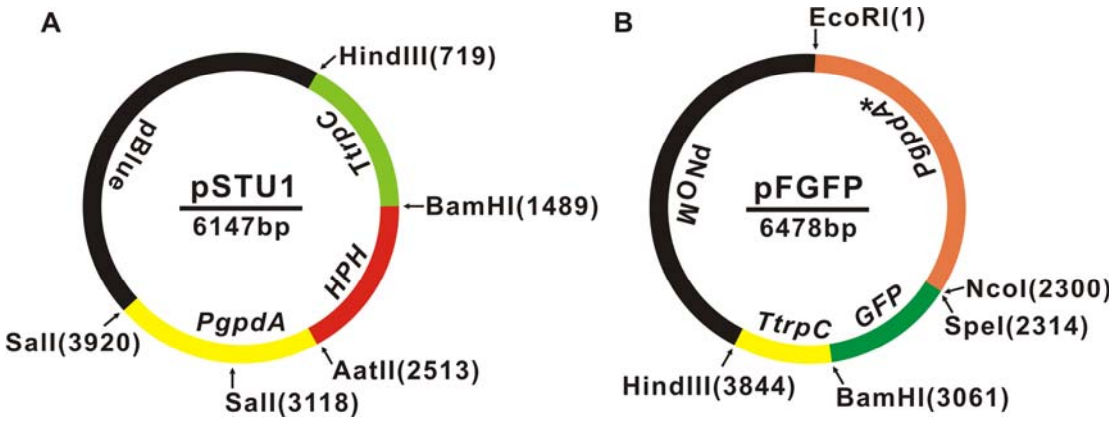

Fig. 4.1. Maps of fungal transformation vectors pSTU1 (**A**) and pFGFP (**B**). *PgpdA*, *Glomerella cingulata gpdA* promoter; *HPH*, *Escherichia coli* hygromycin phosphotransferase (hygromycin resistance) gene; *TtrpC*, *Aspergillus nidulans trpC* terminator; pBlue, pBluescript II KS^+ backbone; *PgpdA**, *A. nidulans gpdA* promoter; *GFP*, *mGFP5** gene from pCAM1032; pNOM, pNOM102 backbone. Important restriction sites are indicated.

A co-transformation was conducted using pFGFP and pSTU1 to create transformants containing the *GFP* gene under the control of the *A. nidulans gpdA* promoter. One transformant, confirmed to possess strong GFP signals under various culture conditions, was selected as the positive control in the GFP activity assay. To create a negative control, vector pP0 was constructed by removing the *A. nidulans gpdA* promoter from pFGFP with EcoRI/NcoI (Fig 4.1B), blunting the backbone with the Klenow fragment of DNA polymerase I from *E. coli* (MBI Fermentas), and self-ligating the blunt-end backbone. pP0 and pSTU1 were used in co-transformation and two transformants, confirmed by PCR using the primer pair F_{GFP}/R_{GFP} (Fig. 4.7), were selected as the negative controls.

Table 4.1. Oligonucleotide primers used in *FgLip1* promoter study

Primers	Sequence (5'→ 3')	Restriction site*
F_{P2060}	CGAATTCGAGATGATGACAAGCAATGC	EcoRI
F_{P1405}	CGAATTCGAATGCAATCGTAACGACT	EcoRI
F_{P865}	CGAATTCTCTAGACCGACATTTTATG	EcoRI
F_{P563}	CGAATTCGATATCTACCTATTCCCACGT	EcoRI
F_{P372}	GAATTCCCATGGACGGCGATGGCTTCCT	EcoRI
F_{P175}	GAATTCGAGCTCGTTTCCCACCTTCTCAT	EcoRI
F_{P70}	CGAATTCCACTTGGCTCATTCTTTGTC	EcoRI
R_{Pro}	ACTAGTCAGATCTACCATCGTGAAGTCAAAGAGTCGAT	SpeI
F_{LSM}	GATTACGAATTCGAATGCAATCG	EcoRI
R_{LSM}	TGGGAAACGAGCTCAATTTGAAC	XhoI
F_{LSM1}	AAACTCGAGGTTCCCTCTCAAGGTCCTTT	XhoI
R_{LSM1}	AAACTCGAGTGGGAGAATCACACGTGGGA	XhoI
F_{LSM2}	AAACTCGAGGATCGATCTCGCCAATACC	XhoI
R_{LSM2}	AAACTCGAGCCGAGGCAAGGAATGGAAA	XhoI
F_{LSM3}	AAACTCGAGACCACACCCAAGTTAAGCG	XhoI
R_{LSM3}	AAACTCGAGGAGATCGATCGACTGGCCG	XhoI
F_{LSM4}	AAACTCGAGCAAGTTAAGCGACGGCATC	XhoI
R_{LSM4}	AAACTCGAGGTATTGGCGAGATCGATCG	XhoI
F_{LSM5}	AAACTCGAGAGGGGAAAAGGAACTCTTT	XhoI
R_{LSM5}	AAACTCGAGCCCACGGAGGGATGAGATA	XhoI
F_{LSM6}	AAACTCGAGAAAGGAACTCTTTGGGGAT	XhoI
R_{LSM6}	AAACTCGAGTCCCCGCTTCCCCACGGAG	XhoI
F_{LSM7}	AAACTCGAGCCAGTCGATCGATCTCGCCA	XhoI
R_{LSM7}	AAACTCGAGCAAGGAATGGAAAGGACCTT	XhoI
F_{GFP}	GCCATGGTAGATCTGACTAGTAAAGG	NcoI
R_{GFP}	CGGATCCTCACACGTGGTGGTGTTGTTGTT	BamHI

* Incorporated restriction site sequences are underlined.

4.2.4. Promoter deletion

Seven fragments differing in the length of the 5' flanking region of *FgLip1* were amplified by PCR with *F. graminearum* genomic DNA as the template (Fig 4.2). The longest fragment was 2,060 bp in length and extended from the stop codon of the gene (EAA67627) upstream of *FgLip1* to the ATG translation start codon of *FgLip1*. Each upstream primer (F_{P2060} to F_{P70}, Table 4.1) was tailed with the recognition sequence (GAATTC) for EcoRI at the 5' end. A common downstream primer R_{Pro} (Table 4.1) was complementary to the sequence from -20 nt to -1 nt (the first letter of the translation initiation codon was designated +1) of *FgLip1* and the sequence from +1 nt to +18 nt of the *GFP* gene on pFGFP. The PCR products were subcloned into the TA (pBluescript II KS^+) vector and sequenced (Plant Biotechnology Institute, Saskatoon, Canada).

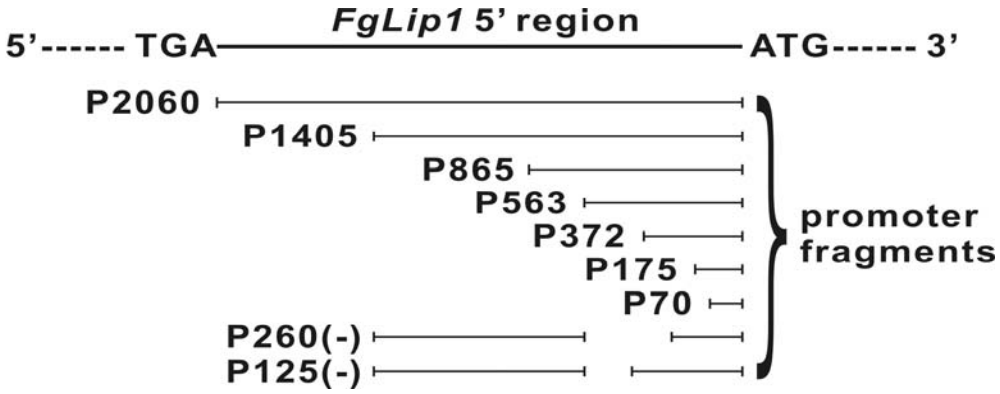

Fig. 4.2. Diagram of *FgLip1* promoter fragment amplified by PCR for the promoter deletion study. Fragments are labeled by the number of base pairs.

The digits in the names of the plasmids listed below describe the promoter length relative to the ATG start codon of *FgLip1*. Each promoter fragment was excised from the corresponding TA-promoter vector by EcoRI/SpeI digestion. The SpeI site at the 3' end, which is originally present at position +13 in the *GFP* coding sequence, was used

for the excision. Each fragment was introduced into the EcoRI/SpeI site of pFGFP. After digestion, the pFGFP backbone contained the *GFP* coding sequence fused with a *TtrpC* but without any promoter (Fig. 4.1B). The expression of *GFP* was controlled by the heterologous promoter fragments introduced from *FgLip1*. The constructed vectors were named pP1405 (Fig. 4.3), pP865, pP563, pP372 and pP175 (pP2060 and pP70 were not obtained). To delete the promoter region between P372 and P563, a 125-bp or a 226-bp fragment was cut off from pP1405 with EcoRV and EcoRV/NcoI, respectively. The backbones were end-blunted with the Klenow fragment and then self-ligated. The resulting vectors were named pP125(-) and pP260(-), respectively. All constructed vectors were verified by sequencing and each of them was used with pSTU1 in co-transformation.

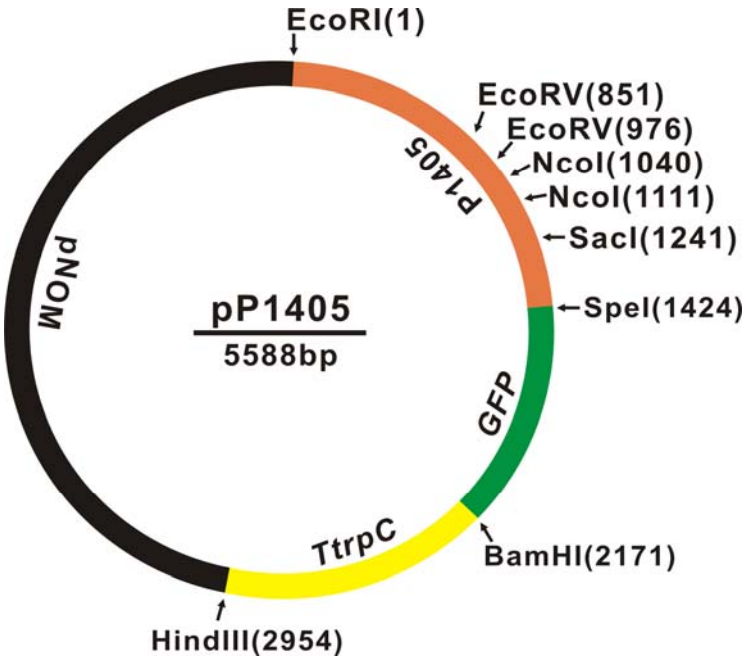

Fig. 4.3. Map of promoter deletion vector pP1405. *FgLip1* promoter fragment P1405 was inserted into the backbone of pFGFP which consists of *mGFP5** gene from pCAM1032 (*GFP*), *A. nidulans trpC* terminator (*TrpC*), and pNOM102 backbone (pNOM). Important restriction sites are indicated.

4.2.5. Linker scanning mutagenesis

All vectors for linker scanning mutagenesis were constructed based on pP1405 (Fig. 4.3). The methodology can be illustrated using the first constructed vector as an example (Fig. 4.4). Two PCR fragments were amplified using primer pairs F_{LSM}/R_{LSM1} and F_{LSM1}/R_{LSM} (Table 4.1). The two PCR products were purified from gels, digested with XhoI, and ligated with T4 DNA ligase. Then another PCR was conducted using the primer pair F_{LSM}/R_{LSM} against the ligation solution. The purified PCR product was digested with EcoRI/SacI and inserted into the backbone of pP1405 digested with the same enzymes (Fig. 4.3). By this approach, six base pairs at the target location were replaced by an XhoI sequence. Six vectors (pLSM1 to pLSM6) were constructed with the target putative *cis*-element mutated (Fig. 4.5). To construct a double-mutation vector containing mutations at both the LSM1 and the LSM5 sites, a 125-bp fragment was cut from pLSM1 by EcoRV and inserted into the same restriction site of the pLSM5 backbone (Fig. 4.5). The resultant vector was named pLSM7. All vectors were sequenced (Plant Biotechnology Institute, Saskatoon, Canada).

4.2.6. Confirmation of co-transformation

All hygromycin resistant transformants were examined by PCR to confirm the integration of the *FgLip1* promoter-*GFP* using primer pairs F_{GFP}/R_{GFP} for the promoter deletion study or F_{P563}/R_{GFP} for the linker scanning study (Table 4.1). The presence of the expected band indicated that the intact *GFP* gene had been inserted into the *F. graminearum* genome. Those transformants were subjected to analysis for GFP activity.

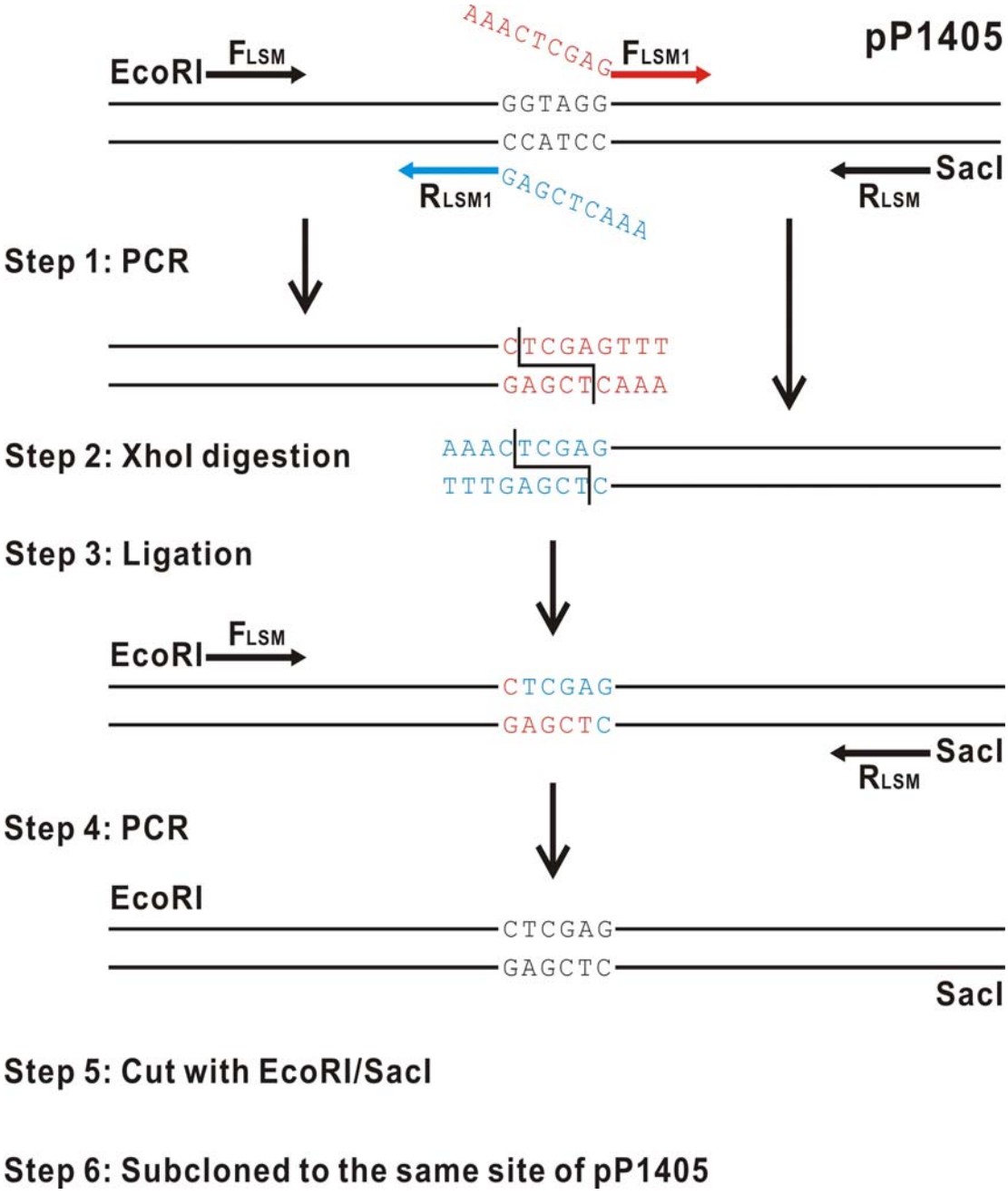

Fig. 4.4. Diagram outlining the method for linker scanning mutagenesis. The principle is to replace the target sequence with an XhoI site, while the other sequences remain unchanged.

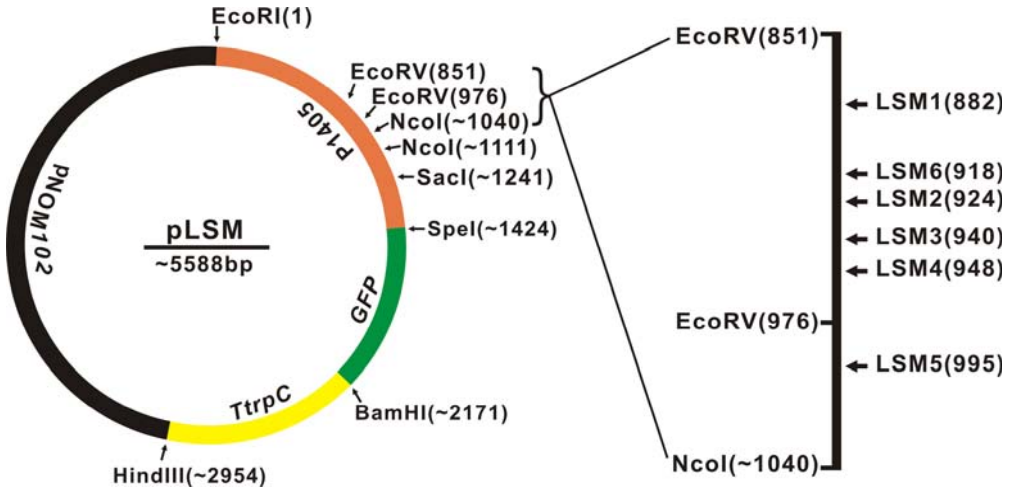

Fig. 4.5. Construction of linker scanning mutagenesis vectors. All vectors were based on pP1405. Promoter fragments after mutation were inserted into the EcoRI/NcoI site of pP1405. Important restriction sites and the location of mutation (LSM1-LSM6) are indicated. *TtrpC*, *Aspergillus nidulans trpC* terminator; *GFP*, *mGFP5** gene from pCAM1032; pNOM103, pNOM102 backbone.

4.2.7. GFP assay

Seven-day old mycelium of the wild type stain PH-1 or the transformants on the PDA plate were transferred into YPG medium (Section 3.2.10) containing 50 μg/ml hygromycin in 1.5-ml Eppendorf tubes. After being shaken at 30°C overnight, the fresh-grown mycelium was centrifuged down and washed four times with sterilized distilled water. Water was removed from the pellets on a piece of sterilized filter paper. White, cottony mycelium was transferred into tubes containing 0.5 ml Czapek-Dox minimal medium (Section 3.2.1), minimal medium with 1% wheat germ oil, or minimal medium with 1% glucose. After shaking at 30°C for 24 h, the mycelium was mounted on a glass slide and GFP fluorescence was examined under a confocal laser scanning electron microscope (LSM510; Zeiss) using excitation/emission wavelengths of 488/515 nm.

4.3. Results

4.3.1. Sequence analysis of the first 600-bp of *FgLip1* promoter revealed putative promoter elements

To identify potential *cis*-acting signals, the 2,060-bp (data not shown) and 600-bp promoter sequences were analyzed with computer tools GeneBuilder and TRANFACT. The result of the 600-bp analysis, presented in Fig. 4.6, is explained below. Degenerate nucleotides are designated according to the IUPAC code (Nomenclature Committee of the International Union of Biochemistry, 1986; Appendix I).

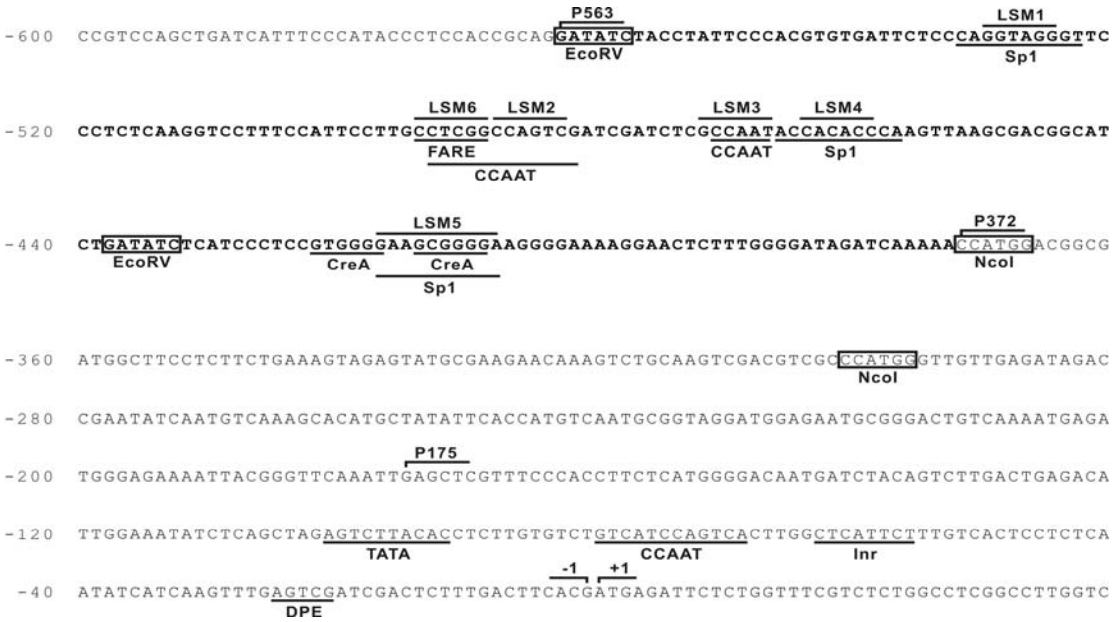

Fig. 4.6. Sequence analysis of the first 600 base pairs of the *FgLip1* promoter. Promoter sequence between P563 and P372 is bolded. Putative *cis*-acting elements are underlined and labeled with their designations. Locations of mutagenesis are indicated by a line on top. Important restriction sites are boxed.

No canonical TATA box consensus sequence TATAAA (Smale and Kadonaga, 2003) could be detected from the region immediately upstream of the start codon ATG. However, analysis using GeneBuilder identified a putative TATA sequence, AGTCTTACAC, from -101 to -92. Sequence CTCATTCT at -63 to -56 conforms to the

consensus Initiator (Inr) sequence CTCANTCT, which encompasses the transcription start site (Smale *et al.*, 1990). A downstream promoter element (DPE) that conforms to the consensus sequence RGWYV (Butler and Kadonaga, 2002) was found at -25 to -21. DPE is required for binding of purified TFIID to a subset of TATA-less promoters but is also found in TATA-containing promoters (Kutach and Kadonaga, 2000).

Two putative CreA binding sites with the consensus sequence SYGGGG (Kulmburg *et al.*, 1993) or SYGGRG (Cubero and Scazzocchio, 1994) were present within the 600-bp region. CreA is the negative regulator mediating carbon catabolism repression in *A. nidulans* and is homologous with the Mig1 negative acting regulatory protein found in *S. cerevisiae* which plays an important role in controlling the repression of a number of genes involved in the utilization of carbon sources (Gancedo, 1998). Another sequence, CCTCGG, was present at -494 to -489. This sequence has been reported to be responsible for fatty acid induction of *A. nidulans* genes (Hynes *et al.*, 2006) and is named the fatty acid responsive element (FARE) in this study.

Consensus sequences of other transcription regulatory elements were obtained as a matrix from the TRANSFAC Transcription Factor Binding Sites Database. The matrixes were used to detect putative promoter elements from the 600-bp *FgLip1* promoter sequence using PATSER software. Three putative CCAAT boxes and three putative Sp1 binding sites were identified.

4.3.2. Promoter deletion study indicated that the promoter region P563-P372 contains important *cis*-acting elements

To identify the promoter region controlling *FgLip1* induction and repression, *FgLip1* promoter-*GFP* constructs harboring sequential 5' deletions of *FgLip1* promoter regions were introduced into the genome of *F. graminearum*. Co-transformations using

pSTU1 and the vector containing *FgLip1* promoter-*GFP* cassette produced more than 60 hygromycin-resistant transformants. Forty eight of them were examined with PCR to verify the presence of the intact *GFP* gene, which would indicate the integration of both vectors. The primers used were F_{GFP}/R_{GFP} specific to the *GFP* open reading frame. Co-transformation efficiency was very high because 34 out of 48 transformants contained the intact *GFP* gene (Fig. 4.7). For each *FgLip1* promoter-*GFP* construct, more than two transformants containing the intact *GFP* gene were identified. However, this result does not give information as to whether the intact *FgLip1* promoter sequence was present in the genome of the transformant. Thus, as a complementary approach, all transformants containing intact *GFP* were subjected to GFP activity assay.

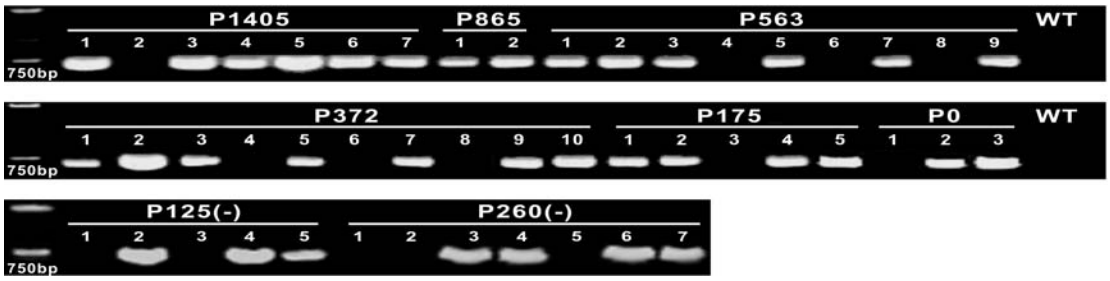

Fig. 4.7. PCR confirmation of promoter deletion transformants. The primers used were F_{GFP}/R_{GFP}. The 750-bp band indicates the presence of intact *GFP* gene in the genome of the transformant. The marker is GeneRuler 1 kb DNA ladder (Fermentas).

When the transformants were grown in WGO-containing medium or medium without any carbon source, those with the constructed vectors pP1405, pP865, and pP563 showed GFP activity, whereas those with pP372, pP175 and pP0 did not (Figs. 4.8 and 4.9). This indicated that an *FgLip1* promoter sequence as small as P563 is sufficient to control expression of *GFP* under WGO and starvation condition. No GFP activity was observed from any transformant grown in glucose-containing medium, (Fig. 4.10), probably because repressors were present in all the promoter fragments.

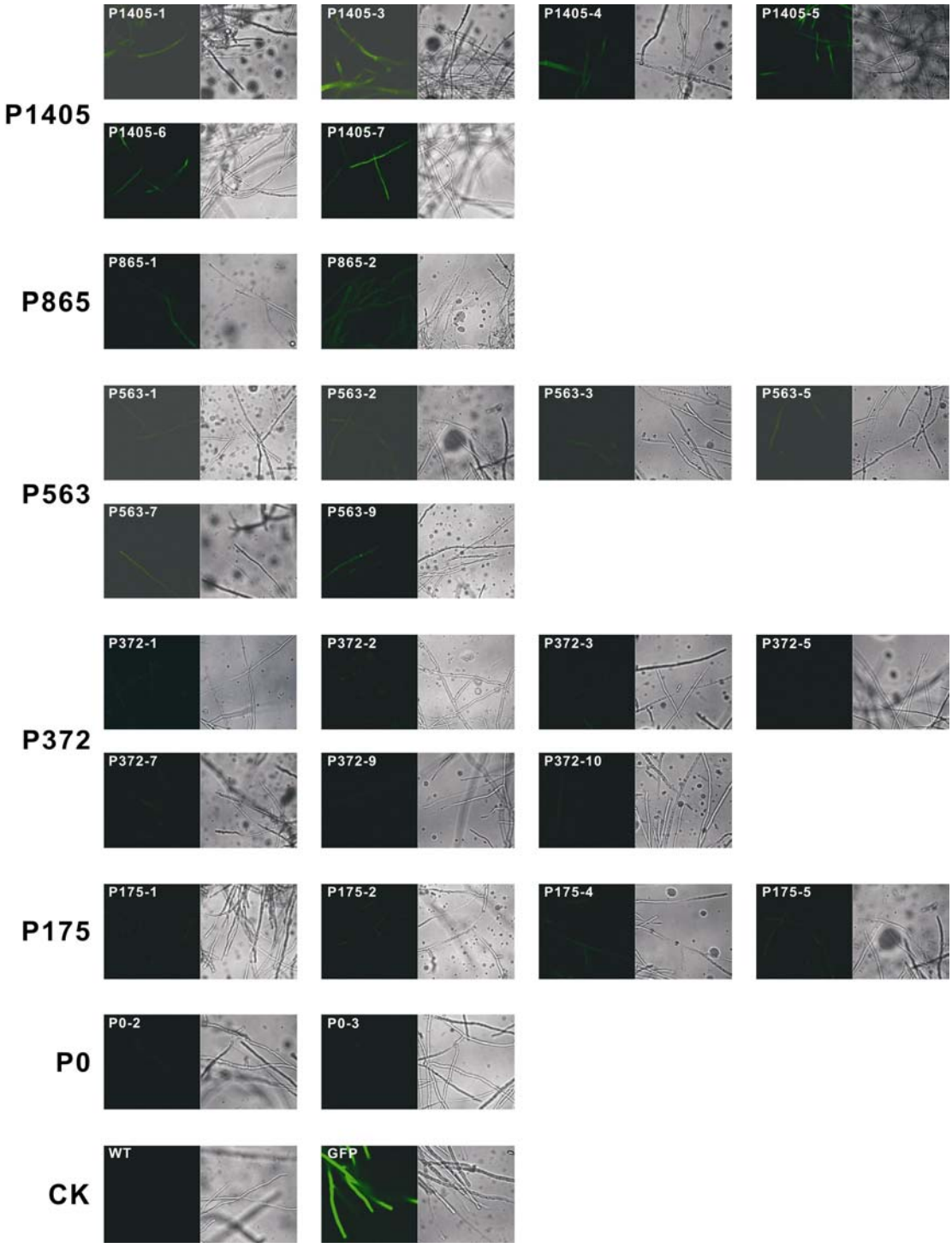

Fig. 4.8. GFP assay of promoter deletion transformants grown for 24 h in minimal medium supplemented with 1% wheat germ oil.

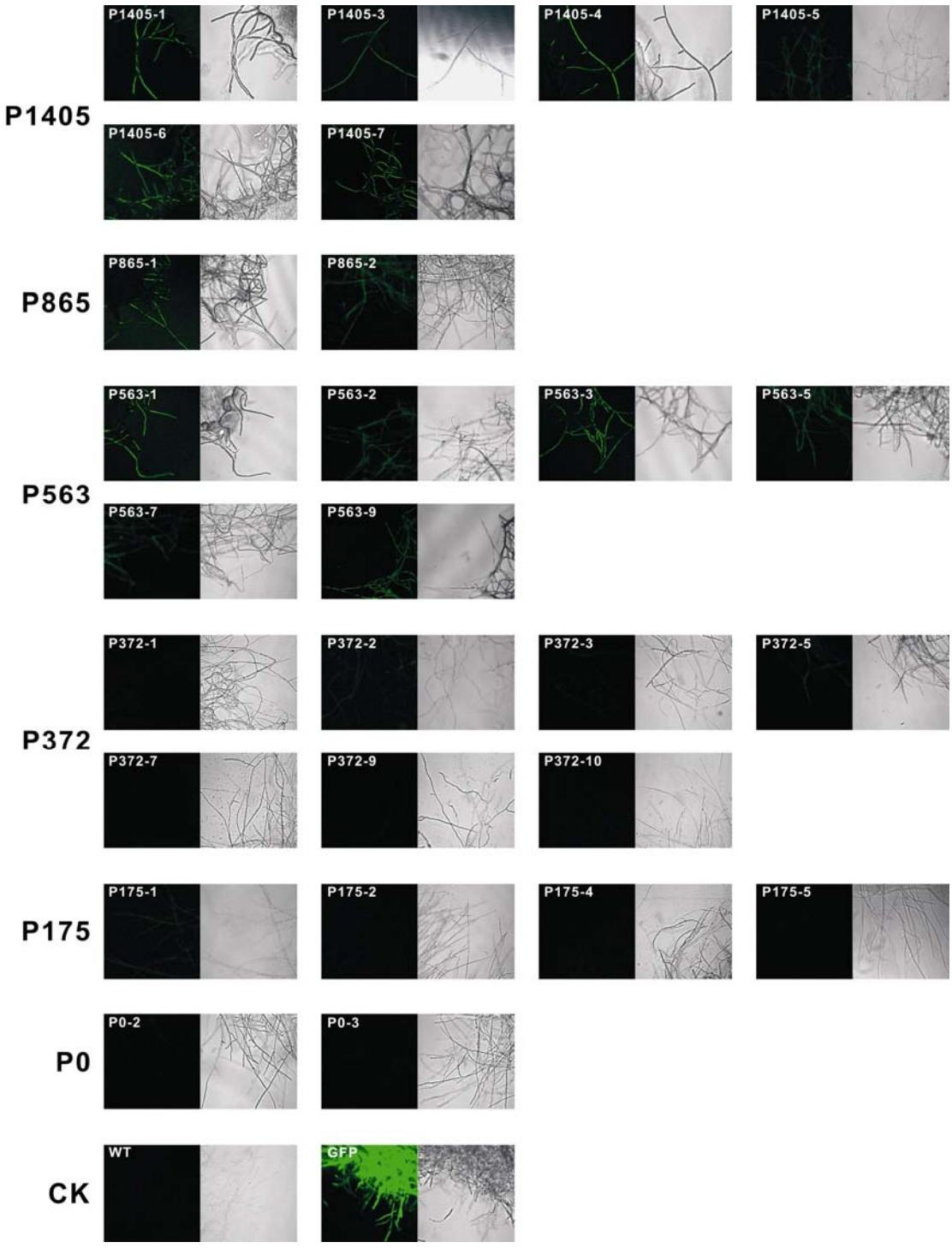

Fig. 4.9. GFP assay of promoter deletion transformants grown for 24 h in minimal medium.

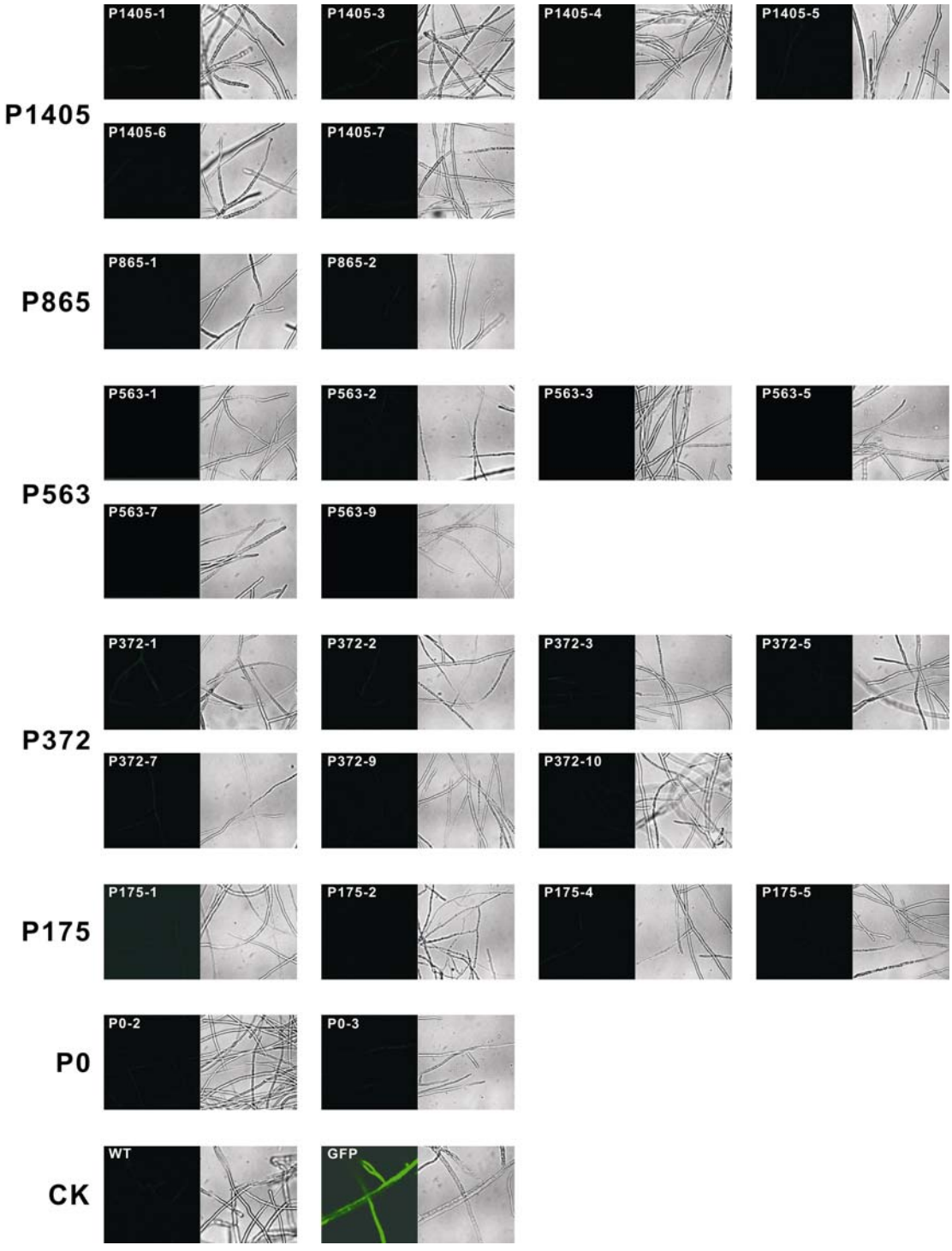

Fig. 4.10. GFP activity of promoter deletion transformants grown for 24 h in minimal medium supplemented with 1% glucose.

The 563-bp-controlled WGO induction and glucose repression was further investigated by culturing two selected pP563-transformants in medium containing both WGO and glucose. GFP activity was observed in mycelium grown in this medium (Fig. 4.11), indicating that in the 563-bp promoter, *cis*-elements controlling WGO induction are stronger or more abundant than those controlling glucose repression. Alternatively, this may suggest that the mechanisms of induction and repression are independent.

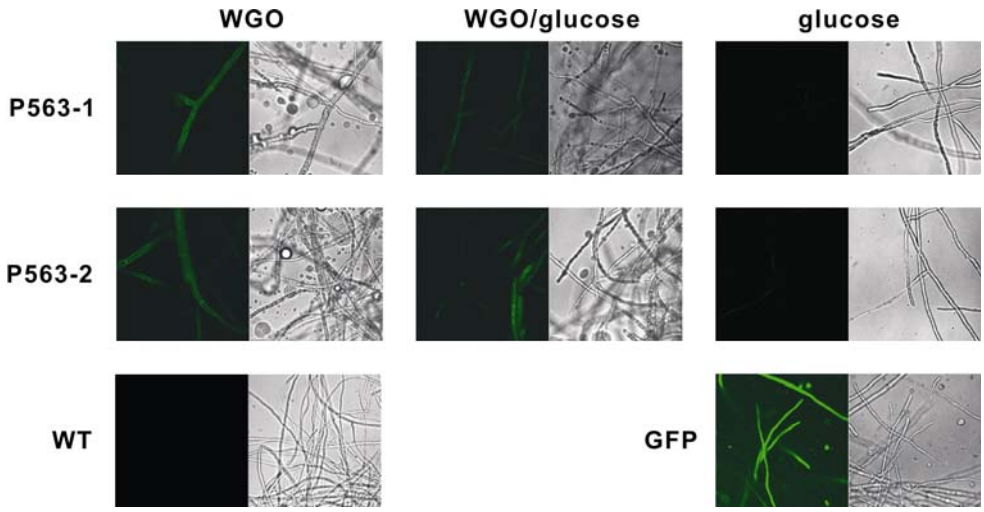

Fig. 4.11. GFP activity assay of two pP563 transformants grown for 24 h in minimal medium supplemented with 1% wheat germ oil and 1% glucose separately and in combination.

Because the promoter fragment P563 showed promoter activity but P372 did not, it is hypothesized that important *cis*-acting elements are located within the region from P563 to P372. To confirm this, a 125-bp sequence, part of P563-P372, and a 260-bp sequence stretching over this region were deleted from pP1405, producing pP125(-) and pP260(-), respectively. No GFP activity was observed in transformants containing pP125(-) or pP260(-), confirming that regulatory elements were present within the P563-P372 region (Fig. 4.12).

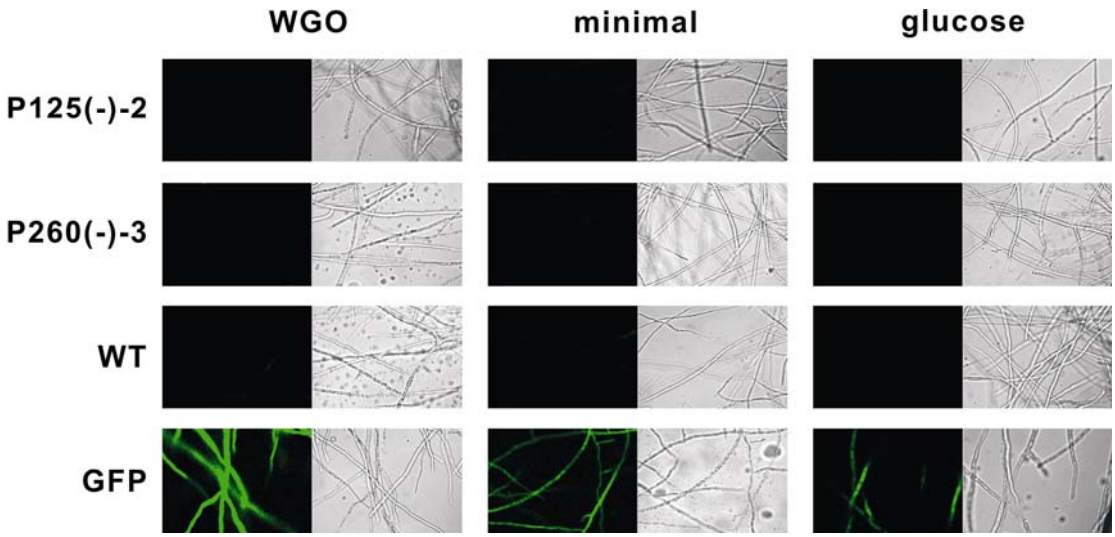

Fig. 4.12. GFP assay of pP125(-) and pP260(-) transformants grown for 24 h in minimal medium or minimal medium supplemented with 1% wheat germ oil or 1% glucose. Only one transformant from each promoter deletion is shown.

4.3.3. Linker scanning mutagenesis identified *cis*-acting elements responsible for *FgLip1* regulation

Since the promoter region P563-P372 was shown to contain *cis*-acting elements responsible for WGO and starvation induction of *FgLip1* expression, linker scanning mutagenesis (LSM) was conducted to mutate the putative elements identified from computer analyses within this region. LSM allows targeted replacement of the *cis*-element with a linker without changes in other sequences. Six putative elements were mutated (Fig. 4.6), including two Sp1 binding sites (LSM1 and LSM4), two CCAAT boxes (LSM2 and LSM3), one 10-bp overlapping sequence shared by two CreA and one Sp1 (LSM5), and one fatty acid responsive element (FARE, LSM6). Six vectors were constructed containing the corresponding mutated elements. Another vector containing mutations (LSM7) at both LSM1 and LSM5 was also constructed.

Fragments upstream and downstream of each target mutated site were amplified by PCR (Fig. 4.13A). Purified PCR products for each mutation were ligated and another

PCR was conducted to amplify the ligated fragment using the ligation aliquot as the template (Fig. 4.13B). To confirm that the XhoI restriction site correctly replaced the target sequence, the PCR products were digested with XhoI and examined on an agarose gel (Fig. 4.13C). After hygromycin resistant transformants were obtained, integration of the promoter-*GFP* cassette was confirmed by PCR using primer pair F_{P563}/R_{GFP} (Table 4.1). Amplification of a 1.3-kb band indicated that intact *GFP* fused to a site-mutated *FgLip1* promoter at least 563 bp in length was present in the genome of the corresponding transformant. Among 52 hygromycin resistant transformants, 20 were confirmed to possess the mutated promoter-*GFP* cassette (Fig. 4.14).

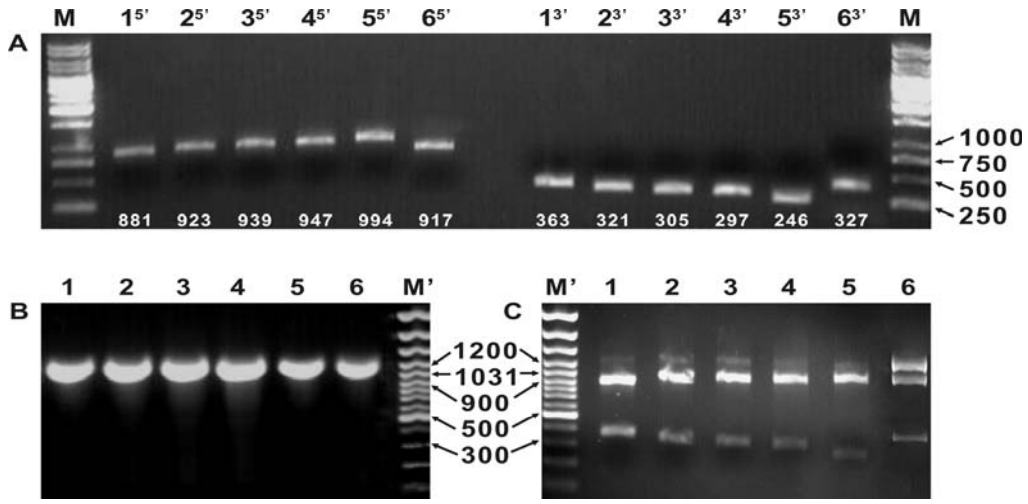

Fig. 4.13. Linking scanning mutagenesis of promoter fragments. **A.** PCR amplification of fragments from pP1405. For each mutation, two fragments were amplified. $1^{5'}$-$6^{5'}$, fragments upstream of the mutation site; $1^{3'}$-$7^{3'}$, fragments downstream of the mutation site. M, GeneRuler 1 kb DNA ladder (Fermentas). **B.** PCR amplification of ligated fragments after ligation of upstream and downstream fragments. **C.** XhoI digestion of PCR products. The presence of two bands with correct sizes indicates replacement of the target sequence by the XhoI site. M', GeneRuler 100 bp DNA ladder plus (Fermentas).

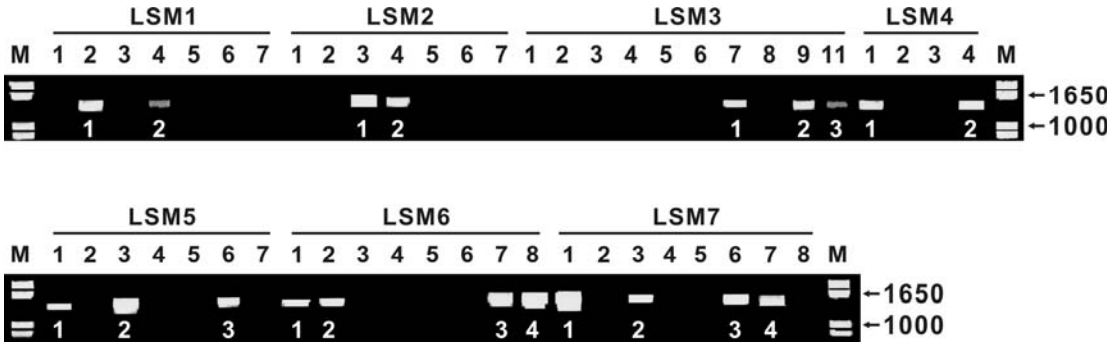

Fig. 4.14. PCR confirmation of transformants from linker scanning mutagenesis. The primers used were F_{P563}/R_{GFP}. The 1.3-kp band indicates the presence of intact P563-*GFP* in the genome of the transformant. M, 1 kb plus DNA ladder (Invitrogen).

The confirmed mutants were grown in minimal medium or in minimal medium supplemented with wheat germ oil or glucose and assayed for GFP activity. The result from one transformant for each mutation is presented in Figure 4.15. Promoter mutations at the putative Sp1 binding sites (LSM1 and LSM4) or at one of the two putative CCAAT boxes (LSM3) did not affect the specific induction/repression of *FgLip1* expression. In contrast, a mutation at another CCAAT box (LSM2) prevented promoter function under all culture conditions, indicating the essential role of this CCAAT box in gene expression. Mutation LSM5, which is a part of a Sp1 and two CreA binding sites, produced GFP activity under all culture conditions including the glucose containing medium, indicating that this element is responsible for glucose repression. A double mutation (LSM7) involving both LSM1 and LSM5 showed the same effect as LSM5. More significantly, mutation at the FARE site (LSM6) prevented the promoter function in WGO medium but retained promoter function under starvation conditions, indicating that this element was responsible for WGO and fatty acid induction.

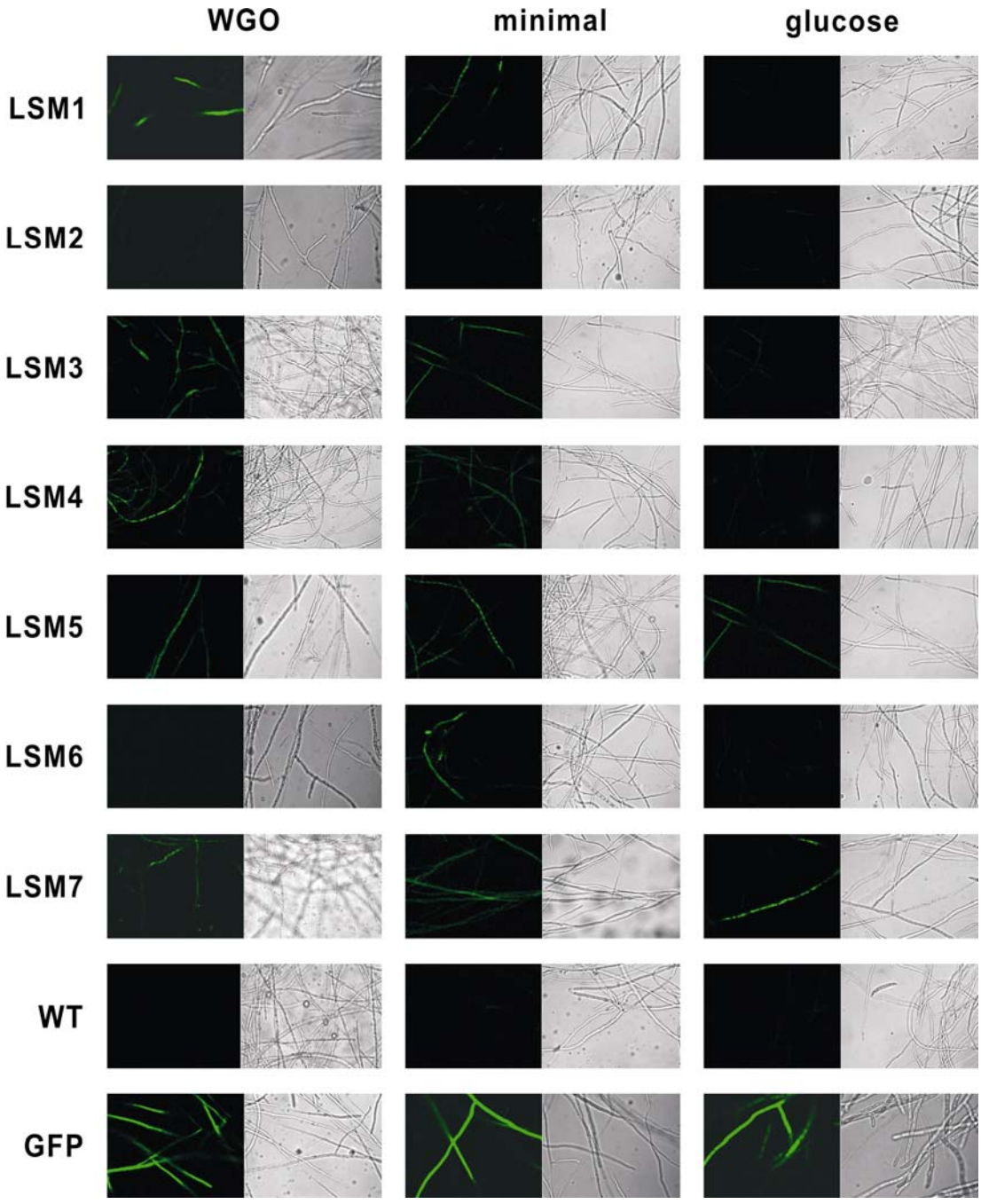

Fig. 4.15. GFP assay of linker scanning mutated transformants. Transformants were grown for 24 h in minimal medium or minimal medium supplemented with 1% wheat germ oil or 1% glucose. Only one transformant from each mutation is shown.

4.4. Discussion

In the present study, a promoter sequence responsible for *FgLip1* regulation was demonstrated to be located within the region of 563-bp upstream of the coding region. *Cis*-acting elements, including one CCAAT box, one fatty acid responsive element (FARE), and one element embracing one Sp1 and two CreA binding sites, were identified within the promoter region between P563 and P372. These *cis*-acting elements were essential for basal transcription, fatty acid induction and glucose repression, respectively.

The GeneBuilder software identified a putative TATA box on the *FgLip1* promoter. However, the sequence of this TATA box does not conform to the consensus sequence TATAAA. Of the 12 lipase members from *F. graminearum*, a perfect TATAAA consensus sequence was found only in EAA71242 within 200 bp upstream from the translation start codon. A sequence, containing one mismatch with the consensus sequence and which has been proposed to be present in more than one third of promoters investigated in *Drosophila* and humans (Smale and Kadonaga, 2003), was found within the 100-bp promoter of all the other *F. graminearum* lipase members except *FgLip1* and EAA78149. This raised questions regarding the *FgLip1* TATA box identified by GeneBuilder. However, an initiator (Inr) with a perfect match to the consensus sequence and a downstream promoter element (DPE) were found within the first 50 bp of the 5' sequence. Although some promoters contain both DPE and TATA motifs, the DPE is most commonly present in TATA-less promoters (Kutach and Kadonaga, 2000). Both TATA and Inr elements can independently direct accurate transcription initiation *in vitro*, and can also function in concert in a synergistic manner

(Martinez *et al.*, 1994). In TATA-less promoters, the DPE can be used as a TATA box by functioning in coordination with the Inr as the binding site of TFIID (Kutach and Kadonaga, 2000). It is likely that *FgLip1* is a TATA-less promoter, in which the initiation of transcription is under the control of Inr and DPE.

The promoter deletion analysis showed that an approximately 563-bp 5' upstream region can control expression of green fluorescent protein (GFP) in a pattern that closely mimics endogenous *FgLip1* expression. This indicated that this region contained sufficient *cis*-acting elements to direct wheat germ oil- and starvation-specific regulation of *FgLip1*. The absence of activity from a promoter sequence smaller than 372 bp indicated that *cis*-elements were located within the region between P563 and P372. Location of *cis*-elements distant from the transcription start site seems the rule rather than the exception among lipase or lipase-related genes. In the *F. solani* f. sp. *pisi* cutinase gene promoter, the sequence between -360 and -255 was essential for gene regulation by plant cutin monomers and glucose (Bajar *et al.*, 1991). A promoter deletion analysis of the *OLE1* gene from yeast identified a 111-bp region, approximately 580 bp upstream of the start codon, which was responsible for transcription activation and unsaturated fatty acid repression. Deletion of an 88-bp sequence within that region resulted in complete loss of transcription activity and unsaturated fatty acid regulation (Choi *et al.*, 1996). In the human lipoprotein lipase gene, promoter sequential deletion indicated that the negative and positive *cis*-acting regulatory elements were located within -724 to -565, and -368 to -35, respectively (Previato *et al.*, 1991). A lipid inducible enhancer, resembling the σ54 element responsible for nitrogen regulation in

other prokaryotic genes, was located between -129 and -113 bp of the promoter of a *Pseudomonas alcaligenes* lipase gene (Cox *et al.*, 2001).

The sequence CCAAT has been found between 50 and 200 bases from the start point of transcription in the 5' region of approximately 30% of eukaryotic genes and can be present in either orientation (Bucher, 1990). In *S. cerevisiae*, the CCAAT element functions in the control of a large number of genes involved in oxidative phosphorylation (Zitomer and Lowry, 1992). Multiple CCAAT-like sequences flanked by GC-rich motifs are also conserved features of mammalian and plant *BiP* promoters and have been shown to be important for basal and enhanced expression of the gene (Lee, 2001; Buzeli *et al.*, 2002). In *A. nidulans*, A CCAAT sequence is present in the promoter region of the acetamidase gene and has been shown to be required for high-level expression of *amdS* under all conditions tested (Littlejohn and Hynes, 1992). Among the various DNA binding proteins that interact with the CCAAT sequence, only NF-Y requires all nucleotides (Mantovani, 1998). CCAAT sequences with alternative nucleotides, which can be bound by other proteins, have been reported from cellular and viral promoters (Mantovani, 1998). In this study, an imperfect CCAAT box located ~400 bp upstream of *FgLip1* was identified and demonstrated to be essential for gene expression under all conditions tested. The sequence of this CCAAT box (CGGCCAGTCGA) conforms to the consensus CCAAT sequence (Mantovani, 1998) with one mismatch. A survey of putative CCAAT boxes against promoter regions of all the twelve lipase members was conducted and in six of them, the identified CCAAT boxes were comprised of the CCAGT sequence. It is likely that in *F. graminearum*, CCAGT sequence is common and functions as a CCAAT box.

Sp1 is a DNA binding protein found in a wide variety of viral and cellular genes and can regulate gene expression in both a positive and a negative direction (Gidoni *et al.*, 1984; Kadonaga *et al.*, 1987, 1988). It is the founding member of a family of zinc finger transcription factors, which includes at least four Sp transcription factors (Philipsen and Suske, 1999). These transcription factors play important roles in a wide variety of physiological processes including cell cycle regulation, hormonal activation, apoptosis and angiogenesis (Gidoni *et al.*, 1984; Kadonaga *et al.*, 1987, 1988). Among the Sp transcription factors, Sp1 binds GC box motifs in promoters. Sp1 induces the expression of the triacylglycerol hydrolase gene during liver development in humans (Douglas *et al.*, 2001). Sp1 has also been implicated, as a negative regulatory factor, in the glucose response of the acetyl CoA carboxylase and leptin genes in adipocytes (Daniel and Kim, 1996; Fukuda and Iritani, 1999). Expression repression of the human macrophage lipase gene by interferon-γ has been proposed to be mediated by Sp1 and the related member Sp3. Interferon-γ decreased the efficiency of binding between the Sp factor and the binding sites (Hughes *et al.*, 2002). The mechanism of Sp1 regulation of gene expression has been reported for the TATA-less promoter of zebra fish *HuC* gene (Pugh and Tjian, 1991). Sp1 played an important role in assembling the TBP and TBP-associated factors (TAFs) with RNA polymerase II. Sp1-controlled gene expression has been studied almost exclusively for human and animal genes. Only two fungal genes, the cutinase genes from *Colletotrichum* species (Kolattukudy, 1987) and *F. solani* f. sp. *pisi* (Bajar *et al.*, 1991), have been proposed to be affected by Sp1. In this study, deletion of two Sp1 binding sites did not affect the expression pattern of the *GFP* gene, indicating that the putative Sp1 binding sites were not important for *FgLip1* expression.

In many organisms, glucose represses genes whose products are used to metabolize other carbon sources. In yeast a zinc-finger-containing protein, Mig1, binds GC-boxes (GCGGGG) in the promoters of several genes (Bu and Schmidt, 1998) in the presence of glucose and inhibits the transcription of genes required for the utilization of alternative sugars (Lutfiyya and Johnston, 1996). This inhibition, also known as carbon catabolite repression, is mediated by the CreA protein in *A. nidulans*, which is a homolog of the yeast Mig1 (Dowzer and Kelly, 1991). CreA binds to promoters and prevents transcription of several *A. nidulans* genes in the presence of glucose (Ruijter and Visser, 1997). A carnitine acetyltransferase gene *facC* was found to be induced by acetate and repressed by glucose. The repression was regulated by two CreA binding sites located at the promoter region from -220 to -19 (Stemple *et al.*, 1998). Glucose repression controlled by a CreA-independent mechanism was also reported for an *A. nidulans* extracellular lipase gene (Kawasaki *et al.*, 1995). The gene encoding the CreA protein, *CreA*, is present also in other fungi including *Trichoderma* species (Ilmen *et al.*, 1996), *Sclerotinia sclerotiorum* (Vautard *et al.*, 1999), and *Cochliobolus carbonum* (Tonukari *et al.*, 2003). In the present study, promoter mutation at two CreA binding sites (LSM5) eliminated glucose repression of *FgLip1* expression. BLASTP of the protein sequence of CreA (AAR02858, Dowzer and Kelly, 1991) against *F. graminearum* genome produced a single orthologue (EAA77764), which shares 53% identity with CreA. This finding strengthened the possibility that the CreA protein binds to the identified *cis*-elements and is responsible for glucose repression of *FgLip1*.

The fatty acid responsive element (FARE) has recently been identified as a promoter element responsible for fatty acid induction of *A. nidulans* genes. Utilization of

fatty acid in fungi depends on peroxisomal β-oxidation to produce acetyl-CoA. The genes for metabolism of acetyl-CoA by the glyoxalate bypass and gluconeogenesis in *A. nidulans* are controlled by the proteins FarA and FarB acting at a six-bp element present in the 5' upstream regions (Hynes *et al.*, 2006). The identical core six-bp binding site for each protein has been identified in other genes encoding glyoxalate bypass, β-oxidation, and peroxisomal functions. Comparative analyses of the genomes of *Aspergillus* species indicate a conservation of this sequence in the upstream region of orthologous genes enriched for lipid metabolism and peroxisomal functions (Hynes *et al.*, 2006). In the present study, the same six-bp element was identified from the *FgLip1* 5' upstream region. Deletion of this element removed the promoter function responsible for wheat germ oil induction but retained its function for starvation induction. Single orthologues are present in the *F. graminearum* genome and share 62% (EAA68832) and 39% (EAA77790) identities with FarA and FarB, respectively. This indicated that the fatty acid responsive element (FARE) was responsible for saturated fatty acid induction. The *cis*-elements responsible for starvation induction remain to be investigated further.

Research in the past few decades has indicated that transcription factors play a critical role in many biological events by virtue of their regulation of gene expression. It is well established that specific combinations of transcription factors exert unique effects on individual gene promoters, allowing spatio-temporal specificity of gene expression, using only a small number of transcription factors under widely varying physiological and pathological conditions (Lee and Young, 2000). Based on the results obtained from the present study, a model can be proposed to explain the expression pattern of *FgLip1*. Presumably, the gene is expressed constitutively at a very low level, hardly detectable by

northern analysis. When the fungus encounters an appropriate substrate such as a saturated fatty acid or a lipid consisting of saturated fatty acids, this basal activity provides hydrolytic products which in turn lead to transduction of signals inside the cell to activate transcription of the gene. This has been reported for other inducible fungal genes (Gonzalez-Candelas and Kolattukudy, 1992). However, when glucose was present, the CreA protein bound to the *cis*-acting element continuously inhibited gene expression. If both glucose and saturated fatty acid were present, low expression resulted from the combination of positive and negative regulation. The effects of positive and negative regulation are concurrent and functionally independent. Expression under all conditions requires the identified CCAAT box, which appears to be essential for the basal transcription mechanism of *FgLip1* gene.

4.5. Conclusions

1. A 563-bp sequence in the *FgLip1* 5' upstream region contains elements necessary for gene expression.
2. Promoter elements responsible for saturated fatty acid induction and glucose repression of gene expression are located within the promoter region between P563 and P372.
3. Within the promoter region P563-P372:
    a. a CCAAT box is required for *FgLip1* expression;
    b. an element overlapping two CreA and one Sp1 binding sites is responsible for glucose repression of *FgLip1* expression; and
    c. a fatty acid responsive element (FARE) is responsible for saturated fatty acid induction of *FgLip1* expression.

5. GENERAL DISCUSSION

5.1. Expression of *FgLip1*

One key observation from the northern study was that *FgLip1* was induced under starvation conditions, but not in the presence of cell wall material or pectin (Fig. 3.7C). Both cell wall material and pectin resulted in poor fungal growth, indicating that they are not sufficient carbon source to support *F. graminearum* growth. However, there was no *FgLip1* expression under such a carbon deficient condition. It is possible that there were signals released from cell wall material and pectin, which either served as down-regulatory factors to repress gene expression or interacted with the up-regulatory elements responsible for starvation activation. The mechanisms controlling starvation activation and cell wall or pectin repression of *FgLip1* expression were not determined in the present study and need further investigation.

Expression of *FgLip1 in planta* suggests its involvement in fungal pathogenesis. There are two possible mechanisms to explain the activation of *FgLip1 in planta*. For other plant pathogenic fungi, both starvation and *in planta* conditions can produce similar fungal gene expression profiles (Pieterse *et al.*, 1994; van den Ackerveken *et al.*, 1994; Talbot *et al.*, 1997; Trail *et al.*, 2003). Therefore, expression of *FgLip1* during infection may be regulated by the same mechanism as in the starvation conditions. The other possible mechanism is that encounter of the fungus with suitable inducers during fungal development on or in the plant is responsible for the gene activation. Hydrolysis of plant surface wax by lipase and induction of lipase expression by wax or wax hydrolysis products have been observed in other plant pathogenic fungi (Comménil *et al.*,

1999; Nasser-Eddine *et al.*, 2001; Yangdou Wei, unpublished data). If *FgLip1* possessed the same function, it should be expressed immediately after the fungus contacted the plant surface but before penetration. If this is true, *FgLip1* would contribute to the adhesion of fungal spores on the plant surface and the utilization of external nutrients during this time period. Furthermore, *FgLip1* may be induced after infection when the fungus comes into contact with the developing embryo. The prolific long-chain fatty acids in the embryo (Wang and Johnson, 2001) can be suitable inducers for *FgLip1* expression.

5.2. Function of *FgLip1*

The pathogenicity tests indicated that *FgLip1* was not important for FHB pathogenesis (Fig. 3.13). This result was not unexpected because there are 12 genes that belong to the same lipase gene family present in the *F. graminearum* genome. Therefore, loss-of-function of *FgLip1* could be complemented by other gene members. However, the gene replacement strains completely lost lipolytic activity during the early growth stage (Fig. 3.11), suggesting that, of the members of this lipase gene family, *FgLip1* is more sensitive to the external inducers and is the major gene that contributes to the utilization of both saturated and unsaturated fatty acids. Voigt *et al.* (2005) reported that knockout of another lipase gene, *FGL1*, dramatically reduced fungal lipolytic activity within 12 hours of growth in a medium with wheat germ oil as the sole carbon source. This result and that from the present study suggested that both *FgLip1* and *FGL1* are essential for fungal lipolytic activity, at least in young cultures. Since *FGL1* and *FgLip1* are present in Δ*FgLip1* and Δ*fgl1*, respectively, the reasons why *FGL1* did not function

in Δ*FgLip1*, and *FgLip1* did not function in Δ*fgl1*, in the growth assays are unclear. One possibility is that these two genes function synergistically; disruption of one gene will affect the function of the other. It would be interesting to compare the lipolytic activities of these two mutant strains under identical conditions or to create a double-mutation strain to investigate the loss of function with regard to the lipolytic activity and lipid utilization.

The present study did not investigate the possible contribution of *FgLip1* on the pre-infection process. Artificial inoculation under greenhouse conditions bypassed the steps of spore attachment and of spore utilization of external nutrients while the environment was temporarily unsuitable for germination. However, studies of an *FgLip1* orthologue from the wheat powdery mildew fungus *Blumeria graminis* f. sp. *tritici* indicated that the lipase gene plays an important role in spore attachment (Yangdou Wei, unpublished data). Utilization of glycerol, which can be released by lipase degradation of triglyceride lipids by plant pathogenic fungi during infection, has been reported (Wei *et al.*, 2004).

Fatty acids are used in the formation of new membranes during the rapid growth of the developing embryo and are assembled into triacylglycerols that are specifically synthesized during embryo development (Wurtele and Nikolau, 1992). The wheat embryo contains 8-14% oil, which consists of triglycerides containing long chain fatty acids (LCFA) including up to 15% saturated LCFAs (Wang and Johnson, 2001). During the infection process, the fungus may obtain its lipid or fatty acid nutrients by making use of *FgLip1* function.

5.3. Lipases versus cutinases

The role of cutinases in the penetration process of phytopathogenic fungi is one of the most controversial topics in plant pathology. Different cutinases have been associated with fungal spore adhesion to the host cuticle and have been implicated in the initiation of the infection process (Nicholson *et al.*, 1988; Pascholati *et al.*, 1992, 1993; Sugui *et al.*, 1998). By using serine esterase inhibitors, such as ebelactones and other organophosphorus pesticides, infection by several plant pathogenic fungi was prevented (Maiti and Kolattukudy, 1979; Köller *et al.*, 1982, 1995; Chun *et al.*, 1995; Francis *et al.*, 1996; Davies *et al.*, 2000) and was believed to be the result of cutinase inhibition. However, this was not confirmed by the production and phenotypic analysis of cutinase knockout mutants, for example in *Fusarium solani* (Stahl and Schäfer, 1992; Stahl *et al.*, 1994; Crowhurst *et al.*, 1997), *M. grisea* (Sweigard *et al.*, 1992) and *B. cinerea* (van Kan *et al.*, 1997), although disruption of a cutinase gene in the oilseed rape pathogen *Pyrenopeziza brassicae* resulted in complete loss of penetration and pathogenicity (Li *et al.*, 2003).

One possible reason for the inconsistent results regarding cutinase function is that using serine hydrolase inhibitors may not only inhibit cutinases, but also lipases. The gene knockout study would only disrupt the targeted gene with other genes including lipases still remaining functional in the mutant. This suggests two points that need to be considered when investigating the function of fungal extracellular enzymes. Firstly, these enzymes are required for fungal pathogenesis, as indicated from the inhibitor studies and the disruption of the *Cochliobolus carbonum SNF1* gene whose wild-type can release the catabolite repression of multiple cell wall degrading enzymes (Tonukari

et al., 2000). Secondly, results from inhibitor studies generally do not reveal the function of a particular enzyme, as exemplified in the cutinase studies, function of lipases may also be inhibited where serine hydrolase inhibitors were used.

5.4. Possible utilization of *FgLip1* and its promoter in industries

Lipases from microbes have been used extensively in biotechnology and industry (Pandey *et al.*, 1999). Fungi are preferred lipase sources because their eukaryotic post-translational protein processing machinery provides ideal facilities for protein glycosylation, proteolytic cleavage or formation of multiple disulfide bonds (Nevalainen *et al.*, 2005). Furthermore, fungal enzymes are usually excreted extracellularly, facilitating extraction from culture media. FgLip1 exhibits all the features needed to be a biological agent for various uses. The vigorous growth of *F. graminearum* in media with lipids as the sole carbon source and the specific induction of *FgLip1* by saturated fatty acids make this fungus a good candidate for use in the oil and fermentation industries. It could also be used for the treatment of lipid wastes from refineries and restaurants, as well as biodegradable plastics. Furthermore, the understanding of promoter function provided by this study now gives further opportunities for use of FgLip1 through the ability to control enzyme activity by adding inducers or inhibitors, for example, saturated or unsaturated fatty acids.

5.5. Future studies

The research conducted in this study addressed many questions regarding the function and mechanism of lipase gene expression. However, additional studies are

needed. Firstly, the function of *FgLip1* and other lipase genes in that gene family needs further investigation. To do this, gene expression during infection should be monitored over time to provide information on the spatio-temporal expression of the lipase genes *in planta*. Disruption of all lipase members within the gene family, performed by RNA-mediated gene silencing (RNA interference) (Nakayashiki *et al.*, 2005), would provide direct evidence for the importance of lipase during pathogenesis. Secondly, FgLip1 activity needs to be further characterized with the aim of expanding its potential for industrial utilization. This characterization would include study of its function using a broader range of substrates and identify the ideal conditions to maximize lipolytic activity, including temperature and pH value. Thirdly, more direct evidence is needed to confirm the results of the *FgLip1* promoter study, including conduct of gel mobility assays (Molloy, 2000) to verify the *cis*-acting elements and other experiments to investigate the *trans*-acting factors.

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7. APPENDICES

Appendix I. Summary of single-letter code of degenerate nucleotides. Table is adapted from Nomenclature Committee of the International Union of Biochemistry (1986).

Symbol	Meaning	Origin of designation
G	G	**G**uanine
A	A	**A**denine
T	T	**T**hymine
C	C	**C**ytosine
R	G or A	pu**R**ine
Y	T or C	p**Y**rimidine
M	A or C	a**M**ino
K	G or T	**K**eto
S	G or C	**S**trong interaction (3 H bonds)
W	A or T	**W**eak interaction (2 H bonds)
H	A or C or T	not-G, **H** follows G in the alphabet
B	G or T or C	not-A, **B** follows A
V	G or C or A	not-T (not-U), **V** follows U
D	G or A or T	not-C, **D** follows C
N	G or A or T or C	a**N**y

Appendix II. Map of homologous promoter vector pGC1-1. PRO, *Glomerella cingulata gpdA* promoter; *HPH*, *E. coli* hygromycin phosphotransferase (hygromycin resistance) gene; TERM, *Aspergillus nidulans trpC* terminator; pGEM7, phagemid pGEM7 (-) backbone. Adapted from Rikkerink *et al.* (1994).