A secreted lipase encoded by \textit{LIP1} is necessary for efficient use of saturated triglyceride lipids in \textit{Fusarium graminearum}

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INTRODUCTION

During the initial association with their hosts, plant pathogens encounter epicuticular waxes and cuticle covering host epidermal cells. Epicuticular wax consists of a relatively soluble complex mixture of long-chain fatty acids, aldehydes, alkanes, primary and secondary alcohols, ketones and wax esters, whereas the cuticle is a continuous layer of lipid material that consists of insoluble polymeric material called cutin (Juniper & Jeffree, 1983). A cutin polymer matrix with embedded waxes forms an efficient barrier against desiccation. This cuticular matrix also decreases the vulnerability of plants to pathogen attacks 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 them to penetrate and infect the host tissue (Knooge, 1996; Kolattukudy, 1985; Oliver & Osbourn, 1995) and are collectively called cell-wall-degrading enzymes (CWDEs). These enzymes may contribute to pathogenesis by degrading wax, cuticle and cell walls, thus aiding tissue invasion and pathogen dissemination. Furthermore, they can act as elicitors of host defence reactions and may also play a nutritional role during certain stages of the fungal life cycle. Early studies suggested that fungal cutinases played an important role in the penetration of plant cuticle and epicuticular waxes. It is now widely accepted that fungal lipases are also involved in the process of penetration and infection of plant hosts, and that they contribute to the degradation of lipid substrates in the host plant cuticle and cuticle.

Abbreviations: CWDEs, cell-wall-degrading enzymes; FHB, fusarium head blight; WGO, wheatgerm oil.
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 (Crowhurst et al.,
1997; Stahl & Schäfer, 1992; Sweigard et al., 1992; van Kan
et al., 1997). It has been proposed that cutinolytic activities
expressed by pathogenic fungi during the infection process
could be contributed by other enzymes (Comménil et al.,
1995, 1998, 1999; Nasser Eddine et al., 2001; van Kan et al.,
1997). 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 secreted lipolytic activities
associated with the extracellular matrix has been reported
among obligately biotrophic powdery mildew (Roberts &
Mims, 1998) and rust (Deising et al., 1992), hemibiotrophic
Colletotrichum (Pain et al., 1996) and necrotrophic Botrytis
(Doss, 1999). Thus, it is likely that secreted fungal lipolytic
activities play an important role in the infection processes of
these pathogens.

Fusarium head blight (FHB) has emerged in recent years as a
major disease causing damage on wheat, barley and other
small grains in North America (McMullen et al., 1997). FHB
can reduce seed quality and yield significantly due to the
production of discoloured, shrivelled 'tombstone' kernels.
Fusarium-infected grain is also often contaminated with the
mycotoxins trichothecenes and zearalenone (McMullen
et al., 1997), making it unusable for food or feed. Fusarium
graminearum Schwabe [teleomorph Gibberella zaeae (Schw.)
Petch] is the major pathogen responsible for this disease, but
other Fusarium species, especially Fusarium culmorum, also
play a role (Gilbert & Tekauz, 2000). Pathogens of the genus
Fusarium can also cause head blight and root rot on many
other plant species. The importance of F. graminearum as
the most agriculturally significant pathogen is also high-
lighted by the recently coordinated efforts of the F.
graminearum genome project. Through the USDA/NSF
microbial genome sequencing program, a 36 Mb assembly
has been released based on ~10 x 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, specifically for those
fungi in which gene disruption is feasible through trans-
formation approaches.

Based on the unique catalytic property of fungal lipase and
its possible role in pathogenesis, we have identified and
characterized a secreted lipase gene (LIP1) from F.
graminearum strain PH-1. Expression levels of LIP1 were
examined in vitro and in planta during infection. The
function of LIP1 was further investigated by gene replace-
ment studies, indicating that LIP1 was essential for lipid
utilization rather than for fungal pathogenicity.

METHODS

Fungal and plant materials. F. graminearum strain PH-1 (FGSC
#9075) was obtained from the University of Kansas Medical Center
(Kansas City, KS, USA). The fungus was maintained routinely on
4 % potato dextrose agar (PDA) plates and the long-term stock was
kept at -80 °C as a spore suspension in 15 % glycerol. For inoculum
production, strain PH-1 and mutants were cultured on cornmeal
agar plates for at least 7 days before making the conidial suspension
(Groth et al., 1999). For growth assays, the wild-type strain and
mutants were cultured at 24 °C on Czapek-Dox minimal medium
(0.2 % NaNO₃, 0.05 % KCl, 0.1 % K₂HPO₄, 0.05 % MgSO₄·7H₂O and
0.001 % FeSO₄·7H₂O) supplemented with 2 % agar (for plates only)
and substrate as required. All media containing lipids as sub-
strate were emulsified for at least 30 min using a Branson 2200
ultrasonic water bath (Branson). The spring wheat (Triticum aesti-
rum) cultivar CDC Teal, highly susceptible to F. graminearum, was
used in this study for extracting total RNAs from fungus-infected
and healthy spikes. For testing the pathogenicity of transformants,
spikes of CDC Teal, spring wheat line BW799, winter wheat CDC
Clair and winter barley McDiarmid, as well as the silk of corn culti-

var Seneca 60, were used. Plants were grown in 10 cm diameter
plastic pots in a growth chamber with a 24 °C day/18 °C night
temperature and a 16 h photoperiod.

Chemicals. All restriction enzymes were purchased from New
England Biolabs. Agar, PDA, tryptone, peptone, yeast extract and
yeast nitrogen base without amino acids and without ammonium
sulfate (YNB) were purchased from Difco. β-D-Glucanase and
Driselase were purchased from Interspex Products. Other chemicals,
unless stated otherwise, were purchased from Sigma-Aldrich.

Cloning of LIP1. Three genomic sequences were amplified from F.
graminearum by PCR using Pfu polymerase (Strategene) and specific
primer pairs (Table 1). F1/R1 were used to amplify LIP1 including its
coding region and 5’- and 3’-flanking sequences. F2/R2 and F3/
R3 were used to amplify the LIP1 coding region with and without
the predicted signal peptide, respectively. The 4532 bp fragment
amplified by F1/R1 was cut with SclI/Apal and cloned into the
same restriction sites of pBluescript II KS⁺ (Stratagene). The other
two fragments were cloned into TA vector (pBluescript II KS⁺).
The resultant plasmids were named pLIP1, pLIP1ORF and
pLIP1ORF-SP and transformed into Escherichia coli strain DH5α. All
PCR products were confirmed by sequencing.

Heterologous expression in yeast and lipolytic activity
assay. To construct vectors for overexpressing LIP1 in Pichia pas-
toris, plasmid DNAs from pLP1ORF and pLP1ORF-SP were
double-digested with SflI/Apal and SflI/XbaI and the resultant frag-
ments were inserted into the same restriction sites of EasySelect
Pichia expression vectors pPICZa and pPICZaA (Invitrogen),
respectively. The difference between pPICZa and pPICZaA is that
the latter contains a native Saccharomyces cerevisiae 2-factor secre-
tion signal, which allows for efficient secretion of most proteins
from Pichia. The constructed vectors were named pPICZa-LIP1ORF
and pPICZaA-LIP1ORF-SP, respectively. Both vectors, as well as the
empty vector pPICZaA, were transformed into P. pastoris X-33 cells.
For the lipolytic activity assay, X-33 cells were grown in YPD or
BMMY plates and recombinant LIP1 expression was induced by
adding 0-5% methanol to the medium. The plates were incubated
for 48 h at 30 °C. Secreted lipolytic activities were detected using
both the emulsified tributyrin assay described by Rapp & Backhaus
(1992) and the rhodamine B assay described by Kouker & Jaeger
(1987). Rhodamine B plates were supplemented with 1 % (w/v)
substrate as required.

Transcript induction of LIP1. CDC Teal wheat spikes were inocu-
lated with wild-type strain PH-1 by the spore-droplet method
following Jenczmionka et al. (2003). The samples were collected 5 days post-inoculation from healthy and infected spikes. For in vitro induction tests, PH-1 was grown at 24 °C and shaken at 130 r.p.m. in minimal medium supplemented with 1% (w/v) carbon sources as required. 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).

**Construction of gene replacement vector and fungal transformation.** To construct the gene replacement vector pALIP1, a 3–2 kb cassette containing the E. coli hygromycin B phosphotransferase gene (hyyR) that has been rendered suitable for fungal expression was cut from vector pGCI-1 (Rikkerink et al., 1994) by Sall/HindIII and inserted into the same sites of the pLIP1 backbone. As a result, hygR replaced the LIP1 coding region and was flanked by border sequences from the wild-type genomic locus. The left and right flanking sequences were 1–2 kb (Sall–Sall fragment) and 1–0 kb (HindIII–Kp I fragment), respectively (see Fig. 4a). For fungal transformation, protoplasts were prepared as described previously (Hohn & Desjardins, 1992) except that the enzymes used to degrade the fungal cell wall were 1% β-D-glucanase, 0.1% β-glucuronidase and 2% Driselase. For reliable transformation, a concentration of at least 108 protoplasts ml–1 was used. Transformation of the protoplasts with the pALIP1 plasmids was conducted according to the protocols described by Wei et al. (2004). After transformation, hygromycin B-resistant mutants were preliminarily screened by PCR amplification with primers F3/R3.

**Pathogenicity tests.** Wheat spikes were inoculated at anthesis with the F. graminearum wild-type strain PH-1 or transformants by the spore-droplet (Jenczmionka et al., 2003) or spray (Schisler et al., 2002) method. After inoculation, the spikes were misted with water and covered with a plastic bag to maintain the head in a moist environment for 48 h. Plants were then moved into a growth chamber with 85% relative humidity under a 16/8 h day/night photoperiod at a day-time temperature of 24 °C and a night-time temperature of 16 °C. Symptom development on inoculated spikes was assessed every second day from 2 to 14 days after inoculation. Detached barley spikes were inoculated by the spore-droplet method and incubated on misted filter paper. The detached corn silk infected with the spore-droplet method and incubated on misted filter paper.

**Northern and Southern hybridization.** Total RNA was extracted from 5 g fresh mycelium grown in minimal medium with different treatments. Genomic DNAs extracted from the wild-type or mutant mycelium were digested for 5 h with Kp I before being subjected to electrophoretic separation. Northern and Southern hybridizations were conducted following standard techniques (Sambrook & Russell, 2001).

**Confocal microscopy.** In the rhodamine B plate assays, oil droplets produced during hydrolysis of triglyceride lipids by secreted fungal lipases were examined under a confocal laser scanning electron microscope (LSM510; Zeiss) using excitation/emission wavelengths of 405/560–615 nm.

**RESULTS**

**Identification and analysis of the F. graminearum LIP1 sequence**

We identified a gene family of 12 members in the F. graminearum genome database that shared high sequence similarity with Candida rugosa triglyceride lipases (Longhi et al., 1992; Lotti et al., 1993). The deduced amino acid sequences suggested that they belong to the type B carboxyl-esterase/lipase family and possess a highly conserved pattern, [ED][DCL][Y][T][LIV][DNS][LIV][LIVFYW][XPQR], where C is involved in a disulfide bond (Cousin et al., 1998; http://au.expasy.org). The protein sequences also contain the serine active site signature [F][GR][GXXXX-][LIVMJ][X][LIV][XGXS][STAG] and the lipase catalytic triad, a serine, a glutamic acid and a histidine (Schrag et al., 1991). A typical signal peptide is present in the N termini of 11 out of 12 members, indicating that they are likely secreted lipases. Among the 12 members, one gene designated LIP1 (EAA67628) encodes a predicted peptide that shows the highest sequence similarity to the well-known lipase gene family from the 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 Botrytis cinerea (Comménil et al., 1999). In the present study, we focused on characterization of the LIP1 gene.

A dendrogram was constructed based on comparison of the deduced amino acid sequences of LIP1, the closest BLAST hits from other fungal species and 11 putative C. rugosa

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**Table 1. Oligonucleotide primers used in this study**

Incorporated restriction site sequences are underlined.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Restriction site</th>
</tr>
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<tbody>
<tr>
<td>F1</td>
<td>GCGGCAGCCCTCCTCAATAGATGCGTTACTTAC</td>
<td>SacI</td>
</tr>
<tr>
<td>R1</td>
<td>GCGGCCGCACTGATACGACGTTACTTAC</td>
<td>ApaI</td>
</tr>
<tr>
<td>F2</td>
<td>GCGGCCAGCCGGGCCAGTGATGCGTTACTTAC</td>
<td>SfiI</td>
</tr>
<tr>
<td>R2</td>
<td>GCGGCCCTACCAACAAAGGCACCGGCATTCGCC</td>
<td>ApaI</td>
</tr>
<tr>
<td>F3</td>
<td>GCGGCCAGGGCCGAGTCCAGCTGCCTTTCCTGC</td>
<td>SfiI</td>
</tr>
<tr>
<td>R3</td>
<td>GCTAGAACCACAAAGCACCGGCATTCGCC</td>
<td>XhoI</td>
</tr>
<tr>
<td>FCellulase-F</td>
<td>ATGGCTCCCATCAAGGTCGGCATC</td>
<td>–</td>
</tr>
<tr>
<td>FCellulase-R</td>
<td>TTACGAGAAGGCTGGTGGCA</td>
<td>–</td>
</tr>
</tbody>
</table>

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Note: The table shows the primers used in the study, their sequences, and the restriction sites incorporated into the primers.
lipase-like genes of *F. graminearum* (Fig. 1a). LIP1 clustered with and showed 61, 61 and 57% identity to the putative lipase sequences of *Neurospora crassa*, *Magnaporthe grisea* and *B. cinerea*, respectively, suggesting that they are orthologues of LIP1 in other fungal genomes. The other 11 putative lipases from *F. graminearum* showed little similarity (<37%) both to each other and to the lipase cluster containing LIP1. At the time of analysis, none of these putative genes had been investigated further.

Sequencing of the LIP1 cDNA revealed that LIP1 consisted of a 1827 bp ORF, interrupted by a 51 bp intron (beginning at nucleotide 1762). 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 & Doolittle, 1982) of LIP1 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. 1b). Although neither the presence of a lid structure nor the occurrence of interfacial activation is a strict requirement for an enzyme to be classified in this family (Verger, 1997), this architecture and mechanism of activation are common to most lipases described to date.

**LIP1 encodes a triglyceride lipase**

*P. pastoris* strain X-33 does not produce secreted lipolytic activity on culture plates. We introduced the LIP1 gene with or without the sequence encoding the signal peptide into vectors pPICZA and pPICZaA, respectively. Extracellular lipolytic activity of the transformed yeast was determined by substrate hydrolysis, which produced a clear zone around the colony on tributyrin emulsion plates. Clear zones formed only around colonies of transformants with the vector pPICZaA-Lip1ORF-SP vector (Fig. 2a), but not in yeast transformed with pPICZA-Lip1ORF or with the empty vectors. It is therefore likely that the putative LIP1 signal peptide of *F. graminearum* could not function in *P. pastoris* strain X-33.

To determine the substrate specificity of LIP1, a rhodamine B staining assay was conducted for yeast strains transformed with pPICZaA-LIP1ORF-SP and the empty vector, using tributyrin, triolein, tristearin, canola oil, olive oil and wheatgerm oil (WGO) as the lipid substrates. Rhodamine B forms a fluorescent complex with free fatty acids and the hydrolysis of lipids is indicated by orange fluorescence under UV light. In spite of different intensities, fluorescence was observed from each lipid substrate assay, with tributyrin giving the strongest fluorescence. The results demonstrate that yeasts expressing LIP1 have lipolytic activity on all lipid substrates examined (Fig. 2b).

**Expression of LIP1 in planta and in vitro**

To test whether LIP1 expression occurs during the infection process, we performed Northern blot analysis using total RNA extracted from CDC Teal wheat spikes 5 days post-inoculation with *F. graminearum* wild-type strain PH-1 and from uninoculated healthy spikes. LIP1 transcripts were detected in the infected wheat spikes but not in healthy spikes (Fig. 3a). Induction of LIP1 expression was also examined in liquid minimal medium supplemented with various carbon sources. Northern blot analysis revealed that LIP1 expression was induced dramatically in medium supplemented with WGO. Lower expression of LIP1 was also detected when the fungus was grown in medium containing olive oil or triolein. In contrast, no expression was detected from fungal cultures grown in the minimal medium supplemented with glucose, sucrose, tributyrin, 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 an *F. graminearum* gene (*FgCel*; EAA73192), encoding a cellulase. Expression of *FgCel* was detected only when the fungus was grown in a medium containing cell-wall material prepared from wheat leaves. An expression time course was conducted in minimal medium supplemented with either WGO or glucose as the sole carbon source. No expression of *LIP1* was detected in fungal cultures grown in the minimal medium supplemented with glucose during the time course. Induction of *LIP1* by WGO started 12 h after culturing and the expression level increased progressively up to 72 h (Fig. 3c).

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%). We therefore suspected that long-chain saturated fatty acids were responsible for the enhanced *LIP1* gene expression in WGO culture. To clarify the nature of the induction of *LIP1* by WGO, we added stearic acid (C18:0), linoleic acid (C18:2) or WGO into the minimal medium as the sole carbon source. After 48 h, cultures grown in stearic acid showed markedly higher expression, compared with cultures grown with either WGO or linoleic acid. No *LIP1* expression was evident when the fungus was grown in a linoleic acid-containing minimal medium. Interestingly, the *LIP1* expression induced by stearic acid was suppressed by adding linoleic acid to the stearic acid-containing medium (Fig. 3d). These results suggest that long-chain saturated fatty acids specifically induce *LIP1* expression and that unsaturated fatty acids suppress that expression.

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**Fig. 2.** Plate assay for detecting LIP1 lipolytic activity. (a) *P. pastoris* cells were transformed with pPICZaA (1), pPICZaA-LIP1ORF-SP (2) or pPICZA-LIP1ORF (3); transformants were grown 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. Transformants are pPICZaA on the left and pPICZaA-LIP1ORF-SP on the right.

**Fig. 3.** Expression analysis of *LIP1* by Northern hybridization. (a) Expression of *LIP1* in infected wheat. Total RNAs extracted from infected spikes were probed with either *LIP1* or the constitutively expressed fungal glyceraldehyde-3-phosphate dehydrogenase gene (*FgGpdh*; EAA73952). (b) Expression of *LIP1* in vitro with various carbon sources added to the minimal medium. Strain PH-1 was cultured for 48 h before RNA isolation. (c) Expression of *LIP1* during fungal growth in minimal medium supplemented with either WGO or glucose as the sole carbon source; time (h) is shown above each lane. (d) Specific induction and suppression of *LIP1* expression by fatty acids. Total RNAs were isolated from *F. graminearum* cultures grown for 48 h in minimal medium supplemented with the following carbon sources: WGO, 1% wheatgerm 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; 2L, 2% linoleic acid.
WGO as the sole carbon source. (b) after culture for 48 h in liquid minimal medium containing mants. Total RNAs were extracted from the strains outlined in wild-type strain PH-1 (WT). (c) Northern analysis of transformation strains, three ectopic integration strains (Ect) and LIP1 Southern blot of genomic DNA extracted from seven native sequences, respectively. Southern hybridization probe B (0

locations of probe A (1-6 kb) used in Northern analysis and probe B (0-9 kb) used in Southern analysis are indicated. (b) Southern blot of genomic DNA extracted from seven LIP1-replacement strains, three ectopic integration strains (Ect) and wild-type strain PH-1 (WT). (c) Northern analysis of transformants. Total RNAs were extracted from the strains outlined in (b) after culture for 48 h in liquid minimal medium containing WGO as the sole carbon source.

Targeted gene disruption of LIP1

To investigate the potential function of LIP1 in fungal growth or pathogenicity, targeted gene disruption was conducted by PEG-mediated protoplast transformation. Three independent transformation experiments produced 54 hygromycin-B-resistant transformants. These transformants were preliminarily screened for absence of the native LIP1 sequence by PCR with primers F3/R3 (Table 1). Seven putative gene replacement strains were selected and the remaining transformants were considered ectopic integration strains. Genomic DNAs isolated from these seven strains as well as three ectopic strains and the wild-type PH-1 strain were further analysed by Southern hybridization. Genomic DNA was digested with KpnI, which has one cutting site in the 3’ homologous region and an additional cutting site in either the hygR or LIP1 coding region, resulting in a 3-2 or 2-4 kb fragment from replacement and native sequences, respectively. Southern hybridization (Fig. 4b) showed that only the 3-2 kb fragment was present in the seven gene replacement strains and both the 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 LIP1 by vector DNA in all seven transformants. Null mutation of LIP1 in the seven replacement strains was further confirmed by Northern analysis (Fig. 4c). No LIP1 transcript accumulation was observed in the seven replacement strains grown in minimal medium containing 1 % WGO for 48 h. In contrast, a single transcript was detected from the three ectopic integration strains and the wild-type PH-1 strain (Fig. 4c).

LIP1 encodes a secreted lipase

Although secretion was predicted for 11 of the 12 lipase gene products in our search, the deduced native signal peptide of LIP1 failed to function in recombinant P. pastoris. We therefore screened 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 is intact were also used. After 2 days of incubation, strong fluorescence was observed around the colonies from the wild-type strain PH-1 and ectopic integration strains on both triolein- and tristearin-containing plates, but not from the seven null-mutant strains (Fig. 5a, b). Rhodamine B was not taken up by fungal mycelium, since no fluorescence was detected in the mycelia of either ΔLIP1 mutants or PH-1 grown on rhodamine B plates (Fig. 5c, d). Fluorescence was observed from oil droplets surrounding the wild-type mycelium, but absent from that of the ΔLIP1 mutants, indicating that secreted lipolytic activities were completely abolished in the ΔLIP1 knock-out mutants. This implied that LIP1 is the major detectable lipase secreted under the growth conditions employed. We continued the incubation to see whether additional lipolytic activity could be detected in the knock-out mutants. Following an extended incubation period, fluorescence was also detected surrounding colonies of the ΔLIP1 mutants (Fig. 5e). These results establish that, while LIP1 is the major secreted lipase isoform that is induced by saturated and unsaturated lipid substrates, additional lipolytic activities appear later during growth.

LIP1 is essential for efficient utilization of triglyceride lipids

The significance of LIP1 on secreted lipolytic activities suggested that LIP1 might make a critical contribution to fungal nutrient utilization in vitro. We grew null mutants ΔFgLIP1-1 and ΔFgLIP1-4 and wild-type PH-1 on solid minimal medium and in liquid minimal medium supplemented with 1 % triolein, tristearin or sucrose as the sole carbon source. After 4 days of incubation, colonies of the ΔLIP1 strains and PH-1 grew with similar size and morphology on plates when sucrose or triolein was used as the sole carbon source. In contrast, growth of the ΔLIP1

![Diagram of pSLIP1 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.](image-url)
strains on plates containing tristearin was significantly impaired, showing very little hyphal growth (Fig. 6a). We also noted that pigmentation occurred in colonies of the wild-type strain PH-1 on all cultures, whereas pigmentation was absent from colonies of the ΔLIP1 strains grown on triolein and tristearin and was present but weak in ΔLIP1 strains grown on sucrose. After 4 days, while fungal biomass from liquid cultures containing sucrose or triolein as the sole carbon source was similar between the PH-1 strain and the ΔLIP1 strains, the ΔLIP1 strains grown in tristearin-containing medium produced significantly less biomass than PH-1 (Fig. 6b). These results indicate that the LIP1 gene is needed for saturated lipid utilization in vitro.

F. graminearum pathogenicity does not depend on production of functional LIP1

Having determined that LIP1 was required for utilization of saturated lipids as a carbon source in vitro, we examined whether it plays a role during pathogenesis. We first inoculated spikes of spring wheat cultivar CDC Teal with spore droplets from the seven ΔLIP1-disruption mutants, the wild-type and the three ectopic strains. Based on continuous observations over 14 days, no significant differences in symptom development were observed between infected spikes inoculated with the wild-type, ectopic and all ΔLIP1 strains. Similar results were also obtained following a corn silk infection assay (Fig. 7a), in which all strains caused similar discolouration of the corn silk. We further chose two of the ΔLIP1 mutants for detailed pathogenicity tests on other wheat cultivars and barley by spore-droplet and spray inoculation methods. Under these different inoculation conditions, symptom development caused by ΔLIP1 mutants was not significantly different from that induced by the wild-type strain in wheat and barley spikes (Fig. 7b–e). Following spore-droplet inoculation from ΔLIP1 mutants, abundant mycelium developed from inoculated florets and spread into adjacent florets and spikelets (Fig. 7b, d). To investigate whether the disease was spread by mycelial growth on the plant surface or through the rachis, we removed all but the inoculated spikelet from winter wheat spikes. Decolourized rachides in the vicinity of inoculated spikelets were evident on both ΔLIP1 mutant- and wild-type-infected spikes (Fig. 7c), indicating the occurrence of similar infection patterns. In the spray assay, heavy mycelia grew over entire wheat heads and
bleached spikelets appeared 5 days post-inoculation with either the ΔLIP1 mutant or the wild-type strain (Fig. 7e). Taken together, our data indicate that LIP1 is not essential for pathogenicity and virulence of F. graminearum on cereal hosts.

**DISCUSSION**

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3), not traditionally considered CWDEs, are distributed widely among animals, plants and micro-organisms (Jaeger *et al.*, 1994). Lipases function at the interface between an insoluble substrate phase and the aqueous phase, a phenomenon known as interfacial activation (Verger, 1997), and catalyse the reversible degradation of glycerol esters with long-chain fatty acids into fatty acids and glycerol. Although microbial lipases have been widely used in industrial applications such as the production of detergents, perfumes, foods, leather and other synthetic organic materials (Jaeger & Reetz, 1998), the role of secreted lipases in fungal physiology or pathogenesis remains largely unknown. In the present study, we showed that the F. graminearum LIP1 possesses lipolytic activity on lipids with both saturated and unsaturated long-chain fatty acids.

Recently, involvement of fungal secreted lipases in infection has been shown 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 similar lipase gene discovered through expressed sequence tag analyses of Blumeria graminis germinating conidia showed a dramatic increase in transcript abundance during conidium germination and appressorium formation (Thomas et al., 2001, 2002; Y. Wei, unpublished results). In the F. graminearum genome database, we identified the gene LIP1, which shares high sequence similarity with these Botrytis and Blumeria lipases. However, our gene disruption study showed that ΔLIP1 mutants and the wild-type strain caused similar patterns of FHB symptom development on susceptible hosts. Similar results were recently reported for the corresponding 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), is required for virulence of F. graminearum on wheat and maize (Nasser Eddine et al., 2001; Voigt et al., 2005).

Apart from their preference for triglycerides, lipases catalyse 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 fumigase esterase activity in degradation of complex cell-wall polysaccharides (de Vries et al., 1997, 2002). Remarkably, these two proteins have significant sequence similarity to 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, 1999; Schmidt-Dannert et al., 1998), 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, 1999; Schmidt-Dannert et al., 1998). Thus, it will be necessary to have information on the substrate specificity of F. graminearum FGL1 to determine its role in pathogenicity in contrast to the lack of such a role for LIP1. Both F. graminearum lipase genes FGL1 and LIP1 are expressed during infection. The expression of both genes is induced in vitro by WGO and suppressed by sucrose or glucose. In contrast, no FGL1 transcripts were detected in fungal culture incubated in water (Voigt et al., 2005), whereas expression of LIP1 was significantly induced when the fungus was cultured in sugar-deficient minimal medium (data not shown). These results suggested that overlapping, but distinct regulatory mechanisms are involved in induction of LIP1 and FGL1 gene expression.

Expression of the LIP1 gene was induced strongly by WGO, weakly by triolein and olive oil, but not by tributyrin. Surprisingly, we found that the hydrolytic products had strong and different effects on regulation of LIP1 expression. Long-chain saturated fatty acids such as palmitic acid (C16:0; data not shown) and stearic acid (C18:0) appeared to be strong inducers, whereas long-chain unsaturated fatty acids such as linoleic acid (C18:2) acted as repressors. The association of long-chain saturated fatty acids with induction of LIP1 expression and long-chain unsaturated fatty acids with repression suggests that the lipolytic products are major regulatory components controlling the transcription of LIP1. In S. cerevisiae, a similar pattern of regulation of the OLE1 gene, which encodes the Δ-9 desaturase, has been reported; the level of OLE1 gene transcription was increased in response to exogenous saturated fatty acids, whereas exposure to unsaturated fatty acids sharply reduced transcription (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 did not reveal the same elements present in the upstream region of the LIP1 gene. The transcription activation and/or repression elements required for fatty-acid-mediated LIP1 expression remain to be determined.

The ΔLIP1 strains were deficient in secreted lipolytic activity on tristearin and showed delayed activity for hydrolysis of triolein. After a long incubation period, lipolytic activities, particularly for triolein, also appeared in the ΔLIP1 strains, indicating that additional secreted lipases participate in exogenous lipid hydrolysis. A genome-wide survey revealed 11 additional sequences encoding C. rugosa-family lipases present in the F. graminearum genome. Some of these putative lipases might be responsible for the enzyme activity that appeared at late stages of fungal growth under these conditions. In the ΔLIP1 strains, besides their lack of activity on saturated tristearin, hydrolytic activities on triolein were also delayed significantly. Thus, it is reasonable to expect that the product fatty acids produced by LIP1-catalysed lipid hydrolysis regulate expression of other lipase genes that control lipid hydrolysis in the later stages of growth.

Apart from reduced or abolished pigmentation, colony morphology of ΔLIP1 mutants was identical to the wild-type on minimal and complete media. However, the ΔLIP1 strains were unable to grow in liquid minimal medium containing tristearin as the sole carbon source. We propose that LIP1 functions primarily when exogenous lipids with long-chain saturated fatty acids are present, acts as a regulator to coordinate other lipase gene expression and plays a role in fungal nutrient acquisition but not in pathogenesis.

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