A secreted lipase encoded by *LIP1* is necessary for efficient use of saturated triglyceride lipids in *Fusarium graminearum*

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A triglyceride lipase gene *LIP1* was identified in the genome of *Fusarium graminearum* strain PH-1. The predicted protein encoded by *LIP1* contains 591 amino acid residues with a putative N-terminal signal peptide and shows 57 and 40–44 % identity to a *Botrytis cinerea* lipase and five *Candida rugosa* lipases, respectively. Yeast cells overexpressing *LIP1* showed lipolytic activity against a broad range of triglyceride substrates. Northern blot analyses revealed that expression of *LIP1* was activated *in planta* during the fungal infection process. *LIP1* expression was strongly induced in minimal medium supplemented with wheatgerm oil, but only weakly induced by olive oil and triolein. In contrast, supplementation with other carbon sources, including glucose, sucrose, apple pectin and wheat cell-wall material, did not induce *LIP1* expression. Saturated fatty acids were the strongest inducers for *LIP1* expression and this induction was suppressed proportionally by the presence of the unsaturated fatty acid. To determine the potential function of *LIP1*, gene replacement was conducted on strain PH-1. When compared with wild-type PH-1, Δ *LIP1* 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 *LIP1* encodes a secreted lipase for exogenous lipid hydrolysis. Moreover, the Δ *LIP1* mutants exhibited growth deficiency on both liquid and solid minimal media supplemented with the saturated triglyceride tristearin as the sole carbon source, suggesting that *LIP1* is required for utilization of this substance. Despite these differences, no variation in disease symptoms between the Δ *LIP1* mutants and the wild-type strain was observed on susceptible cereal hosts.

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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 (Knogge, 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

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 unsuitable for food or feed. *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (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 highlighted 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 $\sim 10 \times$ 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 transformation 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 replacement 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% NaNO_3 , 0.05% KCl , 0.1% K_2HPO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) supplemented with 2% agar (for plates only) and substrate as required. All media containing lipids as substrate were emulsified for at least 30 min using a Branson 2200 ultrasonic water bath (Bransonic). The spring wheat (*Triticum aestivum*) 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 cultivar 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 Interspeex 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 (Stratagene) 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 *SacII*/*ApaI* 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 DH5 α . All PCR products were confirmed by sequencing.

Heterologous expression in yeast and lipolytic activity assay. To construct vectors for overexpressing *LIP1* in *Pichia pastoris*, plasmid DNAs from pLIP1ORF and pLIP1ORF-SP were double-digested with *SfiI*/*ApaI* and *SfiI*/*XbaI* and the resultant fragments were inserted into the same restriction sites of EasySelect *Pichia* expression vectors pPICZA and pPICZ α A (Invitrogen), respectively. The difference between pPICZA and pPICZ α A is that the latter contains a native *Saccharomyces cerevisiae* α -factor secretion signal, which allows for efficient secretion of most proteins from *Pichia*. The constructed vectors were named pPICZA-LIP1ORF and pPICZ α A-LIP1ORF-SP, respectively. Both vectors, as well as the empty vector pPICZ α A, 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 inoculated with wild-type strain PH-1 by the spore-droplet method

Table 1. Oligonucleotide primers used in this study

Incorporated restriction site sequences are underlined.

Primer	Sequence (5'–3')	Restriction site
F1	G <u>CCGCGGCCCT</u> CTCAATAGATGCGTTACTTAC	<i>Sac</i> II
R1	C <u>GGGCCCCG</u> GATACATGAACCTCAGTCTGAGCC	<i>Ap</i> aI
F2	C <u>GGCCCAGCCG</u> GCCACGATGAGATTCTCTGGTTTCGTC	<i>Sfi</i> I
R2	C <u>GGGCCCTACC</u> ACCAAAGCACCGGCATTTCGCC	<i>Ap</i> aI
F3	C <u>GGCCCAGCCG</u> GCCGAGTCCAGTGCCTTTTCCTGC	<i>Sfi</i> I
R3	<u>GTCTAGAACC</u> ACCAAAGCACCGGCATTTCGC	<i>Xba</i> I
F _{GPDH} -F	ATGGCTCCCATCAAGGTCGGCATC	–
F _{GPDH} -R	TTACTTGGAGGCATCGACCTTGGC	–
F _{Cellulase} -F	ATGACGGCCTACAAGCTTTCC	–
F _{Cellulase} -R	TTACAGGAAAGAGGGGTTGGCG	–

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 pΔLIP1, a 3·2 kb cassette containing the *E. coli* hygromycin B phosphotransferase gene (*hygR*) that has been rendered suitable for fungal expression was cut from vector pGC1-1 (Rikkerink *et al.*, 1994) by *Sall*/*Hind*III 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 (*Sac*II–*Sall* fragment) and 1·0 kb (*Hind*III–*Kpn*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 pΔLIP1 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 infection assay was conducted as described by Seong *et al.* (2005) with the exception that small blocks of PDA cultures were used as the inoculum. All tests were conducted at least twice and, for each cultivar/strain combination, more than 50 spikes or corn silks were inoculated.

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 *Kpn*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 carboxylesterase/lipase family and possess a highly conserved pattern, [ED]DCL[YT][LIV][DNS][LIV][LIVFYW]X[PQR], 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-[LIV]X[LIV]XGXS[STAG]G 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*

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 pPICZαA, 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 pPICZαA-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 pPICZαA-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

Fig. 1. Analysis of the deduced amino acid sequence of the *LIP1* gene. (a) Dendrogram of lipases from *F. graminearum* and other fungi. GenBank accession numbers are indicated following the gene names. CrLIP1 and CrLIP5, Lipase 1 and 5 from *C. rugosa*, respectively; GcLIP2, lipase 2 from *G. candidum*; FgLIP1–FgLIP12, putative lipases from *F. graminearum*; NcLIP, MgLIP and BcLIP, putative lipases from *N. crassa*, *M. grisea* and *B. cinerea*, respectively. Dendrogram was constructed using TREEVIEW (Page, 1996), based on a CLUSTAL W alignment (<http://www.ebi.ac.uk/clustalw>). (b) Hydropathy plot of LIP1. Bars above the plot indicate the predicted signal peptide and lid region.

Fig. 2. Plate assay for detecting LIP1 lipolytic activity. (a) *P. pastoris* cells were transformed with pPICZ α A (1), pPICZ α A-LIP1ORF-SP (2) or pPICZ α A-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 pPICZ α A on the left and pPICZ α A-LIP1ORF-SP on the right.

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).

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.

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 ($C_{18:0}$), linoleic acid ($C_{18:2}$) 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.

Fig. 4. Analysis of *LIP1* gene-disruption mutants. (a) Diagram of p Δ LIP1 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. (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 Δ *LIP1* 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 Δ *LIP1* mutants, indicating that secreted lipolytic activities were completely abolished in the Δ *LIP1* 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 Δ *LIP1* 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 Δ *FgLIP1-1* and Δ *FgLIP1-4* 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 Δ *LIP1* 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 Δ *LIP1*

Fig. 5. Secreted lipolytic activity of $\Delta LIP1$ strains. (a–d) Wild-type strain PH-1 (WT), ectopic integration (Ect) strains and $\Delta LIP1$ mutants (1–7) were grown on minimal rhodamine B agar plates with either 1 % tristearin or 1 % triolein as the sole carbon source. (a, b) Plates were inoculated with a mycelial plug of 5 mm diameter, incubated at 22 °C in the dark for 2 days and placed on a UV transilluminator (302 nm) for photography. (c, d) Mycelial plugs of a $\Delta LIP1$ mutant and wild-type strain PH-1 viewed under a confocal microscope. The right column depicts the corresponding area by phase microscopy. (e) Time-course study showing fluorescence surrounding colonies of strain PH-1 and two $\Delta LIP1$ mutants at 1, 2 and 4 days after incubation.

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 $\Delta LIP1$ strains grown on triolein and tristearin and was present but weak in $\Delta LIP1$ 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 $\Delta LIP1$ strains, the $\Delta LIP1$ 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 $\Delta LIP1$ -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 $\Delta LIP1$ 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 $\Delta LIP1$ 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 $\Delta LIP1$ 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 $\Delta LIP1$ 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 $\Delta LIP1$ 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

Fig. 6. Influence of carbon sources on the growth of the wild-type strain and $\Delta LIP1$ mutants. (a) Wild-type PH-1 and $\Delta LIP1$ mutants were grown on minimal agar plates with 1% triolein, tristearin or sucrose as the sole carbon source. The plates were inoculated with a mycelial plug of 5 mm diameter and incubated at 24 °C in the dark for 4 days. (b) Dry mycelium weight was measured after 4 days in liquid minimal medium containing 1% sucrose (open bars), 1% triolein (shaded bars) or 1% tristearin (hatched bars) as the sole carbon source.

bleached spikelets appeared 5 days post-inoculation with either the $\Delta LIP1$ 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

Fig. 7. Pathogenicity assays of *F. graminearum* $\Delta LIP1$ mutants. Photographs were taken 4 (a, d) or 5 (b, c, e) days post-inoculation. (a) Corn silks inoculated at the freshly cut ends with a small block of PDA cultures of seven $\Delta LIP1$ 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 (c) and winter barley McDiarmid (d) inoculated with $\Delta FgLIP1-1$ (1), $\Delta FgLIP1-4$ (4) or the wild-type PH-1 (WT) by the spore-droplet method. Arrows indicate the initial inoculated floret. (e) Spikes of spring wheat line BW799 inoculated by the spray method.

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 Δ *LIP1* 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 ferulic acid 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.enscm.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 ($C_{16:0}$; data not shown) and stearic acid ($C_{18:0}$) appeared to be strong inducers, whereas long-chain unsaturated fatty acids such as linoleic acid ($C_{18:2}$) acted as repressors. The association of long-chain saturated fatty acids with induction of *LIP1* expression and long-chain unsaturated fatty acids with its 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 Δ *LIP1* 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 Δ *LIP1* 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 Δ *LIP1* 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 Δ *LIP1* mutants was identical to the wild-type on minimal and complete media. However, the Δ *LIP1* 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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