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Analysis of the promoter region of the gene *LIP1* encoding triglyceride lipase from *Fusarium graminearum*

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Abstract

Triglyceride lipases catalyze the reversible degradation of glycerol esters with long-chain fatty acids into fatty acids and glycerol. *In silico* analysis of 5′-end flanking sequence of the gene *LIP1* encoding a triglyceride lipase from the wheat head blight pathogen *Fusarium graminearum* revealed the presence of several *cis*-regulatory elements. To delineate the function of these regulatory elements, we constructed a series of deletion mutants in the *LIP1* promoter region fused to the open reading frame of a green fluorescent protein (GFP) and assayed the promoter activity. Analysis of GFP expression levels in mutants indicated that a 563-bp promoter sequence was sufficient to drive the expression of *LIP1* and regulatory elements responsible for the gene induction were located within the 563—372 bp region. To further investigate the regulatory elements, putative *cis*-acting elements spanned within the 563—372 bp region were mutated using a targeted mutagenesis approach. A CCAAT box, a CreA binding site, and a fatty acid responsive element (FARE) were identified and confirmed to be required for the basal expression of *LIP1*, glucose suppression and fatty acid induction, respectively.

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1. Introduction

Many microorganisms prefer easily metabolizable substrates over less readily metabolizable substrates as the carbon source. One of the mechanisms exploited by these microorganisms to achieve this preferential utilization 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 gene from *Fusarium solani* f. sp. *pisi* (Kämper et al. 1994), xylanase (Zeilinger et al. 1996) and cellulase (Zeilinger et al. 1998) genes from *Trichoderma reesei*, a pectinase gene from *Colletotrichum lindemuthianum* (Herbert et al. 2002), and a xylanase gene from *Hypocrea jecorina* (Rauscher et al. 2006).

Expression regulation of fungal lipases is of particular interest 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 *NhL1* from *Nectria haematococca* was induced by olive oil but repressed by glucose (Nasser-Eddine et al. 2001). Similarly, a lipase gene from *Botrytis cinerea* was induced by wax and free fatty acids extracted from grape berry cuticle but repressed by cutin hydrolysates and glucose (Reis et al. 2005). From our previous study (Feng et al. 2005), a triglyceride lipase gene (*LIP1*) was identified from *Fusarium graminearum*, the causal agent of *Fusarium* head blight of wheat and other cereals. The importance of *LIP1* in fungal utilization of saturated fatty acids was clarified through targeted gene disruption. Northern blot analysis indicated that the expression of *LIP1* was strongly induced by wheat germ oil (WGO) and saturated fatty acids, whereas repressed by unsaturated fatty acids and sugars, such as glucose. These observations implied that the expression of *LIP1* was under the control of a finely regulated promoter, which was responsible for the substrate-specific regulation of *LIP1* expression.

Since substrate specific induction and repression of *LIP1* suggested that expression control at the transcriptional level was critical for *LIP1* expression, promoter deletion and targeted mutagenesis were conducted to elucidate the *cis*-acting elements implicated in transcription and to provide an insight into the underlying mechanisms. Such a study can render information on the spatio-temporal interaction between the fungus and the wheat plant. In addition, clarifying the specific induction or repression of the *LIP1* promoter may provide the possibility of the utilization of this promoter for industrial use. Furthermore, a finely controlled promoter can be used in studies of molecular and functional characterization of essential genes, for which direct gene mutagenesis may not be possible.

2. Materials and methods

2.1. Chemicals and standard techniques

All chemicals were purchased from Fisher Scientific (Ottawa, ON, Canada) unless otherwise specified. Restriction enzymes and PCR kits, including Taq polymerase, reaction buffer and deoxynucleoside triphosphates (dNTPs), were purchased from New England Biolabs (Ipswich, MA). PCR primers (Table 1) were synthesized by Integrated DNA Technologies (Coralville, IA). Molecular techniques, if not specified, were performed according to the protocols described by Sambrook and Russell (2001). The accessibility of all websites listed in this paper was verified on the day of submission.

2.2. Fungal material

The wild-type *F. graminearum* strain PH-1 was obtained from the University of Kansas Medical Center (Kansas City, KS). The fungus was routinely maintained in Petri dishes containing potato dextrose agar. For long-term storage, a spore suspension in 15% glycerol was stored at −80 °C.

2.3. Genetic transformation of *F. graminearum*

Polyethylene glycol (PEG)-mediated protoplast transformation was performed following the method of Feng et al. (2005) with one modification. Two vectors, *LIP1* promoter-GFP fusion vector and hygromycin resistance vector pSTU1, were used simultaneously to transform *F. graminearum* (co-transformation). Hygromycin-resistant transformants were isolated and verified by PCR using primers specific to the 5′ end of the *LIP1* promoter fragment (primers 1—5, Table 1) and the 3′-end of *GFP* coding region (primer 22, Table 1), respectively.

2.4. Database search and computational analysis

The 5′-upstream sequence of *LIP1* was obtained from the *F. graminearum*

Table 1. Oligonucleotide primers used in *LIP1* promoter study.

Primer number	Primer name	Sequence (5′—3′)	Restriction site[a]
1	F_{P1405}	CGAATTCGAATGCAATCGTAACGACT	*Eco*RI
2	F_{P865}	CGAATTCTCTAGACCGACATTTTATG	*Eco*RI
3	F_{P563}	CGAATTCGATATCTACCTATTCCCACGT	*Eco*RI
4	F_{P372}	GAATTCCCATGGACGGCGATGGCTTCCT	*Eco*RI
5	F_{P175}	GAATTCGAGCTCGTTTCCCACCTTCTCAT	*Eco*RI
6	R_{Pro}	ACTAGTCAGATCTACCATCGTGAAGTCAAAGAGTCGAT	*Spe*I
7	F′	GATTACGAATTCGAATGCAATCG	*Eco*RI
8	R′	TGGGAAACGAGCTCAATTTGAAC	*Sac*I
9	$F_{sp1\text{-}1}$	AAACTCGAGGTTCCCTCTCAAGGTCCTTT	*Xho*I
10	$R_{sp1\text{-}1}$	AAACTCGAGTGGGAGAATCACACGTGGGA	*Xho*I
11	F_{ccaat1}	AAACTCGAGGATCGATCTCGCCAATACC	*Xho*I
12	R_{ccaat1}	AAACTCGAGCCGAGGCAAGGAATGGAAA	*Xho*I
13	F_{ccaat2}	AAACTCGAGACCACACCCAAGTTAAGCG	*Xho*I
14	R_{ccaat2}	AAACTCGAGGAGATCGATCGACTGGCCG	*Xho*I
15	$F_{sp1\text{-}2}$	AAACTCGAGCAAGTTAAGCGACGGCATC	*Xho*I
16	$R_{sp1\text{-}2}$	AAACTCGAGGTATTGGCGAGATCGATCG	*Xho*I
17	F_{creA}	AAACTCGAGAGGGGAAAAGGAACTCTTT	*Xho*I
18	R_{creA}	AAACTCGAGCCCACGGAGGGATGAGATA	*Xho*I
19	F_{fare}	AAACTCGAGAAAGGAACTCTTTGGGGAT	*Xho*I
20	R_{fare}	AAACTCGAGTCCCCGCTTCCCCACGGAG	*Xho*I
21	F_{GFP}	GCCATGGTAGATCTGACTAGTAAAGG	*Nco*I
22	R_{GFP}	CGGATCCTCACACGTGGTGGTGTTGTTGTT	*Bam*HI

[a] Incorporated restriction site sequences are underlined.

whole-genome database accessible at http://www.broad.mit.edu. The whole sequence was screened for putative promoter elements using Gene Builder (http://zeus2.itb.cnr.it/~webgene/genebuilder.html). After the promoter deletion study, the first 563-bp sequence was further analyzed using the following method: a matrix of a previously reported transcription regulatory element (Ruijter and Visser 1997; Gancedo 1998; Smale and Kadonaga 2003) was obtained from TRANSFAC Transcription Factor Binding Sites Database (http://gene-regulation.com/cgi-bin/pub/databases/transfac/search.cgi). The matrix was then used to detect the putative promoter element on the *LIP1* promoter sequence with PATSER software available at the Regulatory Sequence Analysis Tools web site (http://rsat.scmbb.ulb.ac.be/rsat).

2.5. Construction of basal vectors and development of control strains

The vector pSTU1 was constructed by fusing the hygromycin resistance cassette from pGC1-1 (Rikkerink et al. 1994) to the pBluescript II KS^+ (Stratagene, La Jolla, CA) backbone. Circular vector pSTU1 carrying the hygromycin resistance gene was used in all transformations. To construct the fungal GFP expression vector, the coding region of *uidA* gene in pNOM102 (GenBank accession number: Z32701) was replaced with the coding region of *mGFP* from pCAMBIA-1302 (AF234298; Hajdukiewicz et al. 1994). The resulting vector was named pFGFP, in which *mGFP* was under the control of the *Aspergillus nidulans gpdA* gene promoter and the *A. nidulans trpC* terminator (Fig. 1). A co-transformation was conducted using pFGFP and pSTU1 to generate 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 GFP activity assays. To create a negative control, a vector designated as pP0 was constructed by removing the *A. nidulans gpdA* promoter from pFGFP and self-ligating the backbone. Both pP0 and pSTU1 vectors were used in co-transformation to produce transformants used as negative controls.

2.6. Promoter deletion

Promoter fragments of the *LIP1* gene were amplified by PCR using PH-1 genomic DNA. Each upstream primer (primers 1—5, Table 1) was tailed with the recognition sequence for *Eco*RI at the 5′ end. A common downstream primer (primer 6, Table 1),

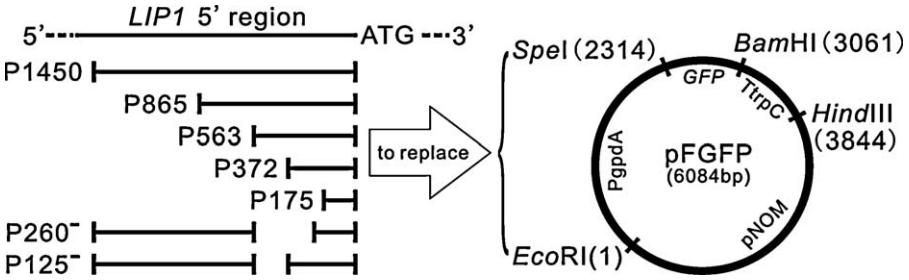

Fig. 1. Diagram of *LIP1* promoter fragments amplified by PCR and construction of promoter deletion vectors based on pFGFP. *PgpdA*, *Aspergillus nidulans gpdA* promoter; *TtrpC*, *Aspergillus nidulans trpC* terminator; *GFP*, *mGFP5** gene from pCAM1032; pNOM, pNOM102 backbone.

which contained a *Spe*I recognition sequence, was complementary to the *LIP1* sequence spanned from −20 nt to −1 nt (the first letter of the translation initiation codon was designated +1) and the sequence from +1 nt to +18 nt of the *mGFP* gene on pFGFP. The PCR products were digested with *Eco*RI/*Spe*I and introduced into the pFGFP to replace the *A. nidulans gpdA* promoter (Fig. 1). The constructed vectors were named pP1405, pP865, pP563, pP372 and pP175, according to the length of each promoter fragment. To delete the promoter region between P372 and P563, a 125-bp or a 226-bp fragment was cut off from pP1405 with *Eco*RV and *Eco*RV/*Nco*I, respectively. The backbones were blunted with the Klenow fragment and then self-ligated. The resulting vectors were designated pP125^{-} and pP260^{-}, respectively. All constructed vectors were verified by sequencing and each of them was used with pSTU1 in co-transformation.

2.7. Mutagenesis of putative *cis*-elements

For targeted mutagenesis, the *Eco*RI—*Sac*I fragment from pP1405 contained all putative *cis*-elements included in this study, was manipulated in order to generate constructs mutated in the putative *cis*-elements. For each mutation, two PCR fragments were amplified using two primer pairs, e.g., F′/$R_{sp1\text{-}1}$ and $F_{sp1\text{-}1}$/R′ (Table 1). Primers F′ and R′ were specific to pP1405 sequences and contained *Eco*RI and *Sac*I restriction sites, respectively. Primers 9—20 (Table 1) were tailed with the *Xho*I site and specific to pP1405 sequences immediately up- (reverse primers) or down-stream (forward primers) of the targeted mutation site. For each mutation, the two PCR products were digested with *Xho*I, and ligated using T4 DNA ligase. Thereafter, another PCR was conducted using the primer pair F′/R′ with the ligation solution as the template. The purified PCR product was digested with *Eco*RI/*Sac*I and inserted into the backbone of pP1405. Using this approach, six to ten base pairs at the target locations were replaced by an *Xho*I sequence. Six vectors were constructed with the target putative *cis*-element mutated. To construct a vector for double mutagenesis on both Sp1 and CreA sites, a 125-bp fragment was cleaved from the vector of sp1-1 by *Eco*RV and inserted into the same restriction site of the vector of creA backbone. All vectors were confirmed by sequencing.

2.8. GFP assay

Seven-day old mycelia of PH-1 or the transformants on PDA plates were transferred into YPG medium (Feng et al. 2005) containing 50 μg mL^{-1} hygromycin in 1.5-mL Eppendorf tubes and shaken overnight at 30 °C overnight. The freshly grown mycelium was collected by centrifugation and washed four times with sterile distilled water before being transferred into tubes containing 0.5 mL Czapek-Dox minimal medium, minimal medium with 1% wheat germ oil (WGO), or minimal medium with 1% glucose. After shaking at 30 °C for 24 h, the mycelium was mounted on a glass slide and GFP signal was examined under a LSM510 confocal laser scanning microscope (Zeiss) using excitation/emission wavelengths of 488/515 nm. For GFP signal quantification, ImageJ software (http://rsbweb.nih.gov/ij/) was used. High-resolution images (1600 × 1200 pixels) of individual hyphae were separated into red, blue and green channels and the green channel images were converted to binary. The background mean gray values were determined by measuring five 100 × 100 pixel areas adjacent to each hypha. The hypha was selected by the freehand selection tool and the mean gray value was measured. The GFP signal intensity was calculated by subtracting the averaged mean gray values of the background from the mean gray value of the hypha. If no hypha could be visually detected from the binary image, a signal intensity of aero was given to the corresponding sample. For each promoter fragment, five samples

from at least three transformants were assayed. For the wild-type and the two control mutants (P0 and GFP), only one strain was assayed.

3. Results

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

Five fragments differing in their lengths at the 5′ flanking region of *LIP1* were amplified by PCR to generate *LIP1* promoter-*GFP* vectors (Fig. 1). Co-transformations using the resulting construct and pSTU1 produced over 160 hygromycin-resistant transformants. All of them were screened using PCR to verify the presence of the intact *LIP1* promoter-*GFP* sequence. For each *LIP1* promoter-*GFP* construct, at least three transformants containing the intact *LIP1* promoter-*GFP* sequence were identified and subjected to GFP activity assay.

When the transformants were grown in WGO-containing medium or medium without any carbon source, those with transformed vectors pP1405, pP865, and pP563 showed GFP activity whereas those with pP372, pP175 and pP0 did not (Fig. 2; if the mean intensity was less than 1.0, the mutant was considered as no activity). This indicated that *LIP1* promoter fragment as small as P563 were sufficient to control expression of *GFP* under WGO and starvation condition. No GFP signal was detected from transformants grown in glucose-containing medium (Fig. 2), suggesting the presence of repressors in all the promoter fragments.

As fragment P563 exhibited promoter activity but P372 did not, it was hypothesized that important *cis*-acting elements were located within the region from P563 to P372. To confirm this hypothesis, a 125-bp sequence, part of the region 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. 2).

3.2. Sequence analysis of the first 563-bp of LIP1 promoter revealed putative promoter elements

The 563-bp 5′-upstream region of *LIP1* was analyzed by computational tools (Fig. 3). 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 Gene Builder 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. The DPE is required for the binding of purified TFIID to a subset of TATA-less promoters. But this binding has also been noticed 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 to *S. cerevisiae* negative acting regulatory protein Mig1, which plays a critical 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. *In silico* analysis of fungal genomes has shown that this motif is present in one or more copies within 1 kb of the predicted start codon of a large number of fungal genes predicted to encode proteins involved in fatty acid metabolism (Hynes et al. 2006). This motif is referred to as the fatty acid response element (FARE) hereafter in this study. Using the PATSER software, three putative CCAAT boxes and two putative Sp1 binding sites were identified.

3.3. Targeted mutagenesis identified *cis*-acting elements responsible for *LIP1* regulation

Since the promoter region P563—P372 was shown to contain *cis*-acting elements responsible for WGO and starvation induction of *LIP1* expression, target mutagenesis was conducted to mutate the putative elements identified from *in silico* analyses within this region. This approach allows targeted replacement of the *cis*-element with a linker without changing other sequences. Six putative elements were mutated (Fig. 3), including two Sp1 binding sites (sp1-1 and sp1-2), two CCAAT boxes (ccaat-1 and ccaat-2), one 10-bp overlapping sequence shared by the two CreA (creA), and one FARE. Six vectors harboring the mutated elements and one vector containing mutations (sp1-1/creA) at both sp1-1 and creA were constructed.

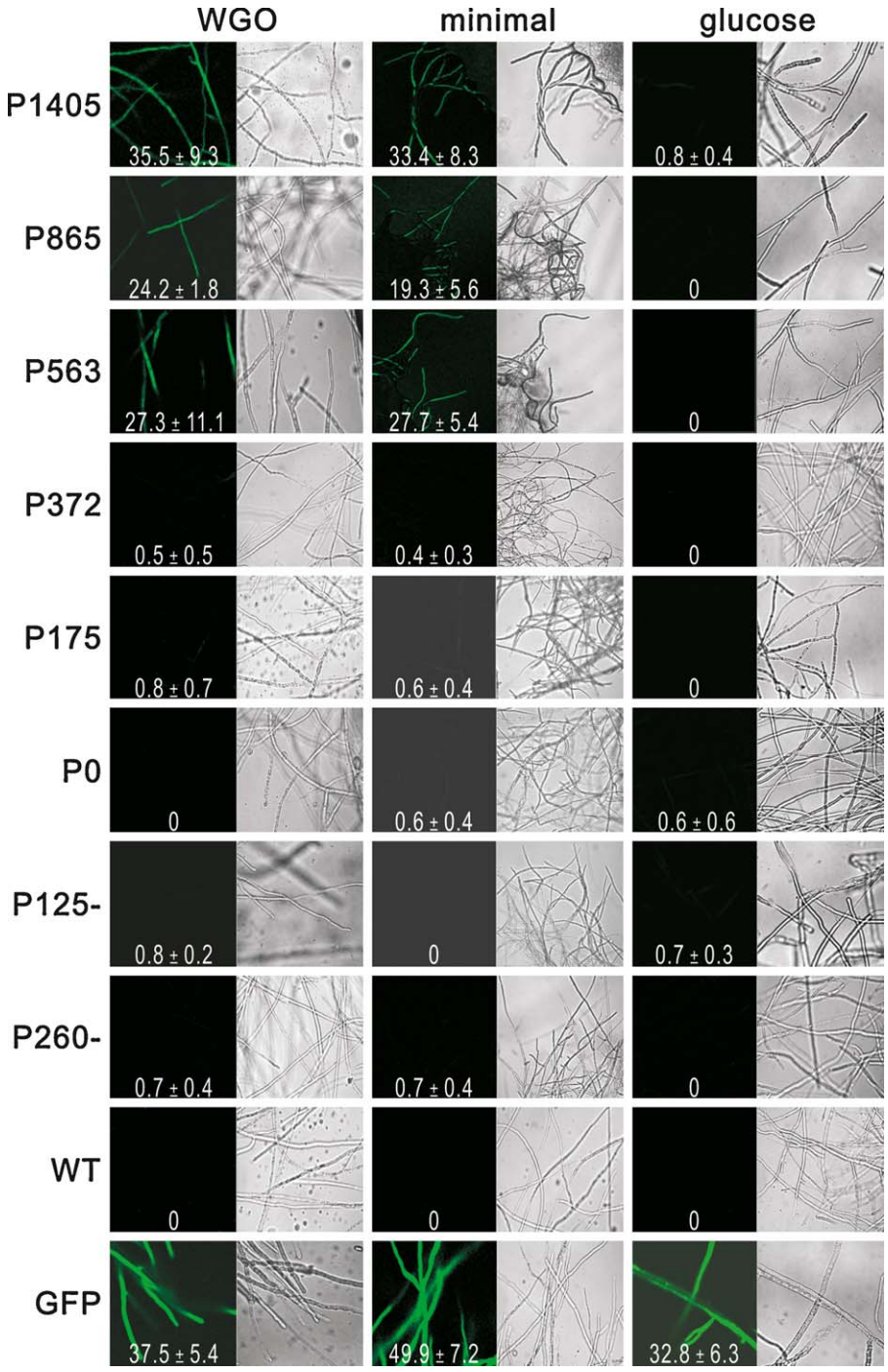

Fig. 2. GFP assay of promoter deletion transformants grown for 24 h in minimal medium, minimal medium supplemented with 1% wheat germ oil (WGO) or 1% glucose. The means of signal intensity of five hyphae with standard deviations from one (P0, WT and GFP) or at least three (others) transformants of each mutant are indicated in the panels.

After transformation, the confirmed transformants were scanned for GFP activity (Fig. 4). Promoter mutations at the putative Sp1 binding sites (sp1-1 and sp1-2) or at one of the two putative CCAAT boxes (ccaat-2) did not affect the specific induction/repression of *LIP1* expression. In contrast, the mutation at another CCAAT box (ccaat-1) prevented promoter function under all culture conditions, indicating the essential role of this CCAAT box in gene expression. Mutation creA, which is a part of the two putative CreA binding sites, produced GFP activity under all culture con-

Fig. 3. Sequence analysis of the first 563 base pairs of the *LIP1* promoter. Promoter sequence between P563 and P372 is bolded. Putative *cis*-acting elements are underlined and labeled with their designations. Locations of mutagenesis are presented as italic font and labeled on top. Important restriction sites are boxed.

ditions, including the glucose containing medium, indicating that this element is required for glucose repression. The double mutation creA/spe1-1 displayed the same effect as creA. More significantly, mutation at the FARE site 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.

4. Discussion

In this study, a promoter sequence responsible for *LIP1* 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 FARE, and one element embracing 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.

In silico analysis using Gene Builder software identified a putative TATA box on the *LIP1* promoter. However, the sequence of this TATA box does not conform to the consensus sequence TATAAA. Of the 12 lipase members from *F. graminearum* (Feng et al. 2005), a perfect TATAAA consensus sequence was found only in one gene within 200 bp upstream from the translation start codon. This raised questions regarding the *LIP1* TATA box identified by Gene Builder. However, an initiator (Inr) with a perfect match to the consensus sequence and a downstream promoter element (DPE) were present 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). In TATA-less promoters, the DPE can serve as a TATA box by functioning in coordination with the Inr as the binding site of TFIID (Kutach and Kadonaga 2000). Therefore, it is likely that the *LIP1* promoter is TATA-less, in which case the initiation of transcription is under the control of Inr and DPE.

The promoter deletion analysis revealed that an approximately 563-bp sequence of 5′- upstream region can control expression of GFP in a pattern that closely mimics endogenous *LIP1* expression. This indicated that this region contained sufficient *cis*-acting elements to direct WGO- and starvation-specific regulation of *LIP1*. The absence of activity from a promoter sequence smaller than 372 bp pointed out 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).

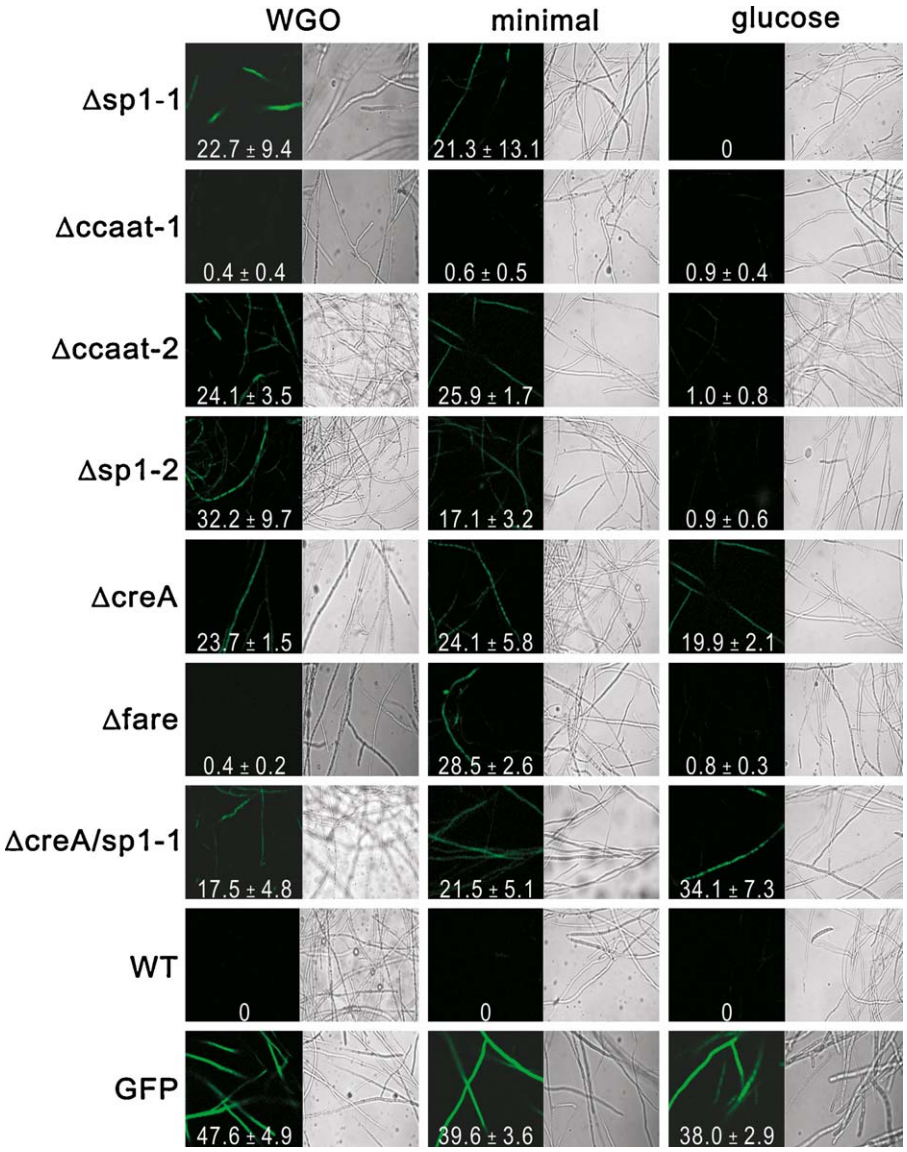

Fig. 4. GFP assay of targeted-mutagenesis transformants. Transformants were grown for 24 h in minimal medium or minimal medium supplemented with 1% wheat germ oil (WGO) or 1% glucose. The means of signal intensity of five hyphae with standard deviations from one (WT and GFP) or at least three (others) transformants of each mutant are indicated in the panels.

In the human lipoprotein lipase gene, sequential deletion into the promoter region indicated that the negative and positive *cis*-acting elements were located within −724 to −565, and −368 to −35, respectively (Previato et al. 1991). A lipid inducible enhancer, resembling the σ54 element required 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 controls 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 among mammalian and plant *BiP* promoters and have been reported to be important for basal and enhanced expression of the gene (Buzeli et al. 2002). In *A. nidulans*, a CCAAT sequence is present in the promoter region of the acetamidase gene and is required for high-level expression of *amdS* under all conditions tested (Littlejohn and Hynes 1992). 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 approximately 400 bp upstream of *LIP1* was identified and demonstrated to be essential for gene expression under all conditions tested. The sequence of the 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 12 lipase members present in *F. graminearum* revealed that in six of them the identified CCAAT boxes were comprised of the CCAGT sequence. It is likely that in *F. graminearum*, the 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 positive and negative directions (Gidoni et al. 1984; Kadonaga et al. 1987, 1988). However, 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 essential for *LIP1* expression.

In many organisms, glucose represses genes whose products are used to metabolise other carbon sources. The yeast zinc-finger-containing protein Mig1 binds GC-boxes (GCGGGG) of the promoters of several genes in the presence of glucose (Bu and Schmidt 1998) and inhibits the transcription of genes required for the utilization of alternative sugars (Lutfiyya and Johnston 1996). This inhibition is mediated by the Mig1 homolog CreA protein in *A. nidulans* (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 reported to be induced by acetate and repressed by glucose. The repression was regulated by two CreA binding sites located at the promoter region ranging from −220 to −19 (Stemple et al. 1998). The CreA protein coding gene is conserved in other fungal species, including *Trichoderma* spp. (Ilmen et al. 1996), *Sclerotinia sclerotiorum* (Vautard et al. 1999), and *Cochliobolus carbonum* (Tonukari et al. 2003). In this study, promoter mutation at the two CreA binding sites (creA) eliminated glucose repression of *LIP1* expression. The analysis of the protein sequence of CreA (AAR02858, Dowzer and Kelly 1991) using BLASTP algorithm against the *F. graminearum* genome produced a single orthologue (EAA77764), which shared 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 *LIP1*.

FARE has been identified as a promoter element required for fatty acid induction of *A. nidulans* genes. Utilization of fatty acid in fungi relies on peroxisomal β-oxidation to produce acetyl-CoA. The genes for metabolism of the resulting acetyl-CoA by the glyoxalate bypass and gluconeogenesis in *A. nidulans* are regulated by FarA and FarB proteins 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 this study, the same six-bp element was identified from the *LIP1* 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 implied that FARE was responsible for saturated fatty acid induction. Further research is needed to characterize the *cis*-elements responsible for starvation induction.

Hitherto research 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 this study, a model can be proposed to explain the expression pattern of *LIP1*. 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, can transduce signals inside the cell to switch on the transcription of gene. This has also been reported for other inducible fungal genes (Gonzalez-Candelas and Kolattukudy 1992). However, when glucose, but not saturated fatty acid is present, the CreA protein bound to the *cis*-acting

element continuously inhibits gene expression. If both glucose and saturated fatty acid are present, low expression results from the combination of positive and negative regulation. The effects of positive and negative regulation are concurrent and functionally independent. Nevertheless, expression under all conditions requires the presence of the identified CCAAT box, which appears to be essential for the basal transcription mechanism of *LIP1* gene.

Microbial lipases have been exploited 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 secreted extracellularly, facilitating extraction from culture media. The LIP1 protein 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 *LIP1* 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 brings about further opportunities for use of LIP1 through the ability to control enzyme activity by adding inducers or inhibitors, for example saturated or unsaturated fatty acids.

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