Determination of a transcriptional regulator-like gene involved in biosynthesis of elsinochrome phytotoxin by the citrus scab fungus, *Elsinoë fawcettii*

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Elsinochromes are nonhost-selective, light-activated, polyketide-derived toxins produced by many phytopathogenic *Elsinoë* species. We recently showed that the polyketide synthase-encoding gene *EIPKS1* is essential for elsinochrome biosynthesis in the citrus scab fungus *Elsinoë fawcettii*. Sequence analysis beyond the *EIPKS1* gene identified nine putative ORFs: four genes, designated *RDT1*, *TSF1*, *PRF1* and *ECT1*, all encode polypeptides likely to have biosynthetic or efflux functions; five additional genes, *OXR1* and *EIPHP1* to *EIPHP4*, encode hypothetical proteins of unknown function. Northern-blot analysis revealed that expression of these genes in *E. fawcettii* was not completely correlated with accumulation of elsinochromes under nitrogen limitation, alkaline pH or high concentrations of glucose. Targeted disruption of the *TSF1* gene, encoding a putative transcriptional activator, yielded fungal mutants unable to produce elsinochromes, and defective in both conidiation and expression of *RDT1*, *EIPKS1*, *PRF1* and *EIPHP1*, whereas expression of *RDT1*, *TSF1*, *PRF1* and *ECT1* was nearly abolished in *EIPKS1*-disrupted mutants. By contrast, expression of *OXR1*, *EIPHP2* and *EIPHP3* was not affected by disrupting either *EIPKS1* or *TSF1*. Taken together, the results indicate that in addition to polyketide synthase, the products of *TSF1* and other adjacent genes may also play a crucial role in elsinochrome production.

**INTRODUCTION**

*Elsinoë fawcettii* Bitancourt & Jenkins (anamorph: *Sphaceloma fawcettii* Jenkins) is the causative pathogen of citrus scab disease, causing necrotic lesions on leaves, twigs and fruit (Timmer et al., 1996). This fungal pathogen attacks a wide variety of citrus species and cultivars, including grapefruit, lemon, and many tangerines and their hybrids. Citrus scab is widely distributed in many humid citrus-growing areas of the world, reducing fruit values for the fresh-fruit market. Another *Elsinoë* species, *E. australis* Bitancourt & Jenkins, induces lesions only on the fruit of sweet orange and mandarin, but not on leaves, and is present largely in South America (Tan et al., 1996; Timmer et al., 1996). Isolates of *E. fawcettii* grow extremely slowly in culture and often form gummy colonies less than 10 mm in diameter after 4 to 5 weeks. Little is known about the molecular and genetic interactions between *Elsinoë* species and their hosts.

Many *Elsinoë* species produce red or orange pigments, collectively termed elsinochromes, in culture (Weiss et al., 1987). Elsinochromes consist of at least four interconvertible tautomers (A, B, C and D) that have a core phenolic quinone to which various side chains are attached, and they have remarkable structural similarity to many perylene-quinone phytotoxins, such as cercosporin, stemphytoxin, hypomycin, hypocrellin and phleichrome of fungal origin (Weiss et al., 1987; Daub et al., 2005). Elsinochrome A is readily converted to elsinochrome B or elsinochrome C in the presence of chromium trioxide (Lousberg et al., 1969); elsinochrome D, containing a methylenedioxy ring, is derived from elsinochrome C (Shirasugi & Misaki, 1992). The structures of elsinochromes were determined by NMR spectroscopic and X-ray crystallographic studies several decades ago (Weiss et al., 1965; Lousberg et al., 1969, 1970; Meille et al., 1989; Mebius et al., 1990). The biological function of elsinochromes as phytotoxins toward host and nonhost plant cells both in *vitro* and in *vivo* was recently...
documented to be light-dependent (Liao & Chung, 2008a). Their cellular toxicities were further shown to be mediated through production of reactive oxygen species such as superoxide and singlet oxygen, which may damage cell membranes and induce electrolyte leakage from the cells (Liao & Chung, 2008a). Further studies employing molecular and genetic approaches unambiguously confirmed a critical role for elsinochromes in lesion development and fungal pathogenesis. *E. fawcettii* mutants disrupted in the *Efpks1* gene, which encodes a fungal polyketide synthase, do not produce any detectable elsinochromes and have a drastically reduced ability to form lesions on citrus (Liao & Chung, 2008b).

Early studies of *Elsinoë* spp. fed radioisotope-labelled substrate revealed that elsinochromes are synthesized in a polyketide pathway by condensation of acetate and malonate monomers (Chen et al., 1986; Kurobane et al., 1981). To determine if any genes adjacent to *Efpks1* might also be involved in elsinochrome production in *E. fawcettii*, we carried out a DNA sequence analysis upstream and downstream of the *Efpks1* locus and identified nine new ORFs. In the work reported here, we characterized the function of one of these genes, *Tsfl*, which encodes a polypeptide containing a Cys_His-type zinc finger and a GAL4-like Zn_Cys binuclear cluster implicated in DNA binding, and evaluated its function in relation to gene regulation and elsinochrome production using a disruption strategy in *E. fawcettii*. This study provides the molecular foundation on which to elucidate the biosynthetic and regulatory networks leading to elsinochrome production in this important citrus pathogen.

**METHODS**

**Fungal strains, media and cultural conditions.** The wild-type CAL WH-1 isolate of *Elsinoë fawcettii*, used for cloning, transformation and targeted gene disruption, was cultured from scab-affected calamondin (Citrus madurensis Lour.) fruit in Florida and has been previously characterized (Liao & Chung, 2008a). The genetically modified *Efpks1* disruptant (D4) and two complementation strains (C1 and C2) were reported in a previous study (Liao & Chung, 2008b). Fungal isolates were cultured on potato dextrose agar (PDA, Difco). Conidia were prepared using Fries medium (Fries, 1978) as described by Timmer et al. (1996). Other media used in this study were complete medium (CM), minimal medium (MM) and protoplast regeneration medium (RMM) (Jenns et al., 1989; Chung et al., 2002). The pH of media was adjusted with 0.1 M phosphate buffer as described elsewhere (You et al., 2007). For toxin production, fungal mycelium was ground with a sterile blender, spread on PDA, and incubated under continuous fluorescent light (wavelength 436 nm) at an intensity of 3.5 J m\(^{-2}\) s\(^{-1}\) for 3 days at room temperature (~25 °C). The plates were placed approximately 45 cm away from the light source. When preparation of protoplasts was desired, fungal isolates were grown in 50 ml potato dextrose broth (PDB, Difco) for 7 days, ground, mixed with fresh PDB (200 ml), and incubated for an additional 15 h. For DNA or RNA isolation, fungal DNA that was digested with restriction endonucleases and self-ligated with adaptor primers to obtain DNA fragments harboring unknown genomic regions using Titanium Advantage 2 DNA polymerase (BD Biosciences). In some cases, DNA fragments were obtained by PCR with primers from fungal DNA that was digested with restriction endonucleases and self-ligated as previously described (You et al., 2007). After being purified with a DNA-purification kit (Mo Bio Laboratories), the amplified DNA fragments were either directly sequenced or cloned into pGEM-T Easy vector (Promega) for sequence analysis at Eton Bioscience. Oligonucleotides used for PCR and sequencing were synthesized by Integrated DNA Technologies and Allele Biotechnology and Pharmaceuticals. Similarity searches using the BLASTX program (Altschul et al., 1997) were performed at the National Center for Biotechnology Information. Prediction of ORFs and exon/intron junctions was first performed using the gene-finding software at http://www.softberry.com and further confirmed by comparing genomic and cDNA sequence. Functional domains were identified using the PROSITE database available through the ExPASy Molecular Biology Server (http://us.expasy.org) (Gasteiger et al., 2003) and the Motif/ProDom and Block programs (Henikoff et al., 2000) at http://motif.genome.jp. Analysis of the promoter regions was conducted using regulatory sequence analysis tools (van Helden, 2003) at http://rsat.ulb.ac.be/rsat/. Palindrome searches were performed at http://bioweb.pasteur.fr/seqanal/interfaces/palindrome.html.

**Targeted gene disruption and genetic complementation.** To disrupt the *Tsfl* gene in *E. fawcettii*, a 5.4 kb DNA fragment encompassing the *Tsfl* ORF and flanking sequences was amplified by PCR with primers efup11 (5′-cattgcgtatgctgaccttg-3′) and efup28 (5′-cggctattatcaggggacagag-3′). The amplified DNA fragment was cloned into pGEM-T Easy vector, creating pSTSF1128. A 2.1 kb fragment harboring the hygromycin phosphotransferase B gene (HYG) cassette under the control of the *Aspergillus nidulans* trpC gene promoter and terminator was released from pUCATPH (Lu et al., 1994) by digestion with XbaI, blunt-ended with DNA polymerase I (New England BioLabs), and cloned into the *NruI* site of pSTSF1128, creating the disruption construct pTSF1128 (see Fig. 3). The *NruI* site is approximately 1200 nt downstream from the predicted *Tsfl* start codon. A split-marker strategy was applied to promote double crossing-over recombination as previously described (Choquer et al., 2005). Briefly, a 4.4 kb DNA fragment containing truncated 5′ *Tsfl* fused with 3′ HYG and a 4.2 kb fragment encompassing 3′ *Tsfl* linked to 5′ HYG were amplified with, respectively, primers efup11/
hyg3 (5'-ggatgctccctgctgaaga-3') and efup28/hyg4 (5'-cgttgcaaga-gaagtgctgaa-3') from pTSF1128 using the Takara Ex Taq PCR system (Takara Bio). Fungal protoplasts were released from hyphae by a mixture of cell-wall-degrading enzymes as previously described (Chung et al., 2002). Fungal transformation using a CaCl₂ and polyethylene glycol-mediated method was performed by mixing PCR fragments with 1 × 10⁹ protoplasts ml⁻¹ as previously described (Chung et al., 2002). The HYG gene cannot encode a functional protein until recombination occurs between the 800 nt overlapping portions of HYG sequence in the two PCR products. Fungal transformants were recovered from RMM medium containing 200 μg hygromycin ml⁻¹ (Roche Applied Science) after 2 to 3 weeks and were screened for the loss of elsinochrome (red pigment) production on PDA.

For genetic complementation, a functional TSF1 gene and its 5' nontranslated region were amplified using primers efup11 and efup28 and the Expand High Fidelity PCR system (Roche Applied Science), and the amplified product was sequenced for confirmation. The amplified TSF1 gene cassette was co-transformed into protoplasts prepared from TSF1 null mutants with the pBARKS1 plasmid harbouring a phosphinothrin acetyltransferase gene under the control of the A. nidulans trpC promoter that is responsible for bialaphos resistance (Pall & Brunelli, 1993). Transformants were first identified from a medium supplemented with 50 μg bialaphos ml⁻¹ (Gold Biotech) and tested for production of elsinochromes.

Pathogenicity tests. Fungal pathogenicity was assessed on detached rough lemon (Citrus jambhiri Lush.) leaves inoculated with agar plugs bearing fungal mycelium as previously described (Liao & Chung, 2008b).

Manipulation of nucleic acids. Plasmid DNA was propagated in Escherichia coli DH5α and purified using the Wizard DNA purification kit (Promega). Fungal RNA was purified by Trizol reagent according to the manufacturer’s directions (Invitrogen). Single- and double-strand cDNA was prepared with a cDNA synthesis kit (BD Biosciences) and amplified by reverse transcriptase (RT)-PCR with gene-specific primers. The resulting fragments were purified and directly subjected to sequence analysis. Standard molecular techniques were used for endonuclease digestion of DNA, electrophoresis, Southern- and Northern-blot hybridizations (Sambrook & Russell, 2001). DNA probes used for Southern- and Northern-blot hybridization were prepared by PCR to randomly incorporate digoxigenin (DIG)-11-dUTP (Roche Applied Science) into DNA with gene-specific primers. Procedures used for probe labelling, hybridization, post-hybridization washing and immunological detection of the probe using the CSPD (C18H19Cl2O7PNa2) chemiluminescent substrate for alkaline phosphatase were performed following the manufacturer’s recommendations (Roche Applied Science).

**RESULTS**

**Sequence analysis and determination of ORFs**

In a prior study we showed that disruption of the *E. fawcettii* *EfPKS1* gene encoding a polyketide synthase completely blocks elsinochrome production. Sequence analysis beyond the *EfPKS1* gene was performed to determine if any of the genes adjacent to *EfPKS1* is also required for elsinochrome biosynthesis. Using a combination of chromosome walking and inverse-PCR strategies, we obtained and sequenced over 30 kb of genomic DNA after multiple rounds of PCR. Analysis of the compiled sequences identified nine putative ORFs, designated *EfHP1*, *EfHP2*, *EfHP3*, *PRF1*, *ECT1*, *TSF1*, *RDT1*, *OXR1* and *EfHP4* (Fig. 1).

A search for similarities in the NCBI database using BLASTX was performed to predict the functions of the translated polypeptides. The *PRF1* gene, located downstream from *EfPKS1*, encodes a putative polypeptide similar to prefoldin protein subunit 3 (Fig. 1). The *ECT1* gene encodes a protein with strong similarity to membrane transporters, with the highest hit to a nitrate transporter of *Aspergillus terreus* (Table 1). The *TSF1* gene, which is located upstream of and transcribed divergently from *EfPKS1*, encodes a putative protein showing strong similarity to many zinc finger transcriptional factors of fungi. The *RDT1* gene, located further upstream from *TSF1*, encodes a putative polypeptide similar to a wide range of reductases, with strong similarity to the 1,3,8-trihydroxynaphthalene (T₃HN) reductase involved in melanin biosynthesis (Table 1). The *OXR1* ORF is located between *EfPKS1* and *TSF1*. The predicted ORF protein of unknown function is rich in proline. Three putative ORFs (designated *EfHP1* to *EfHP3*) upstream of *RDT1* encode hypothetical proteins with unknown functions. Sequence alignment revealed that *EfHP1* and *EfHP3* share 52% similarity on both the nucleotide and amino acid level. A small ORF that defines the *EfHP4* gene located downstream of *ECT1* encodes a putative polypeptide, which is not involved in the
production of any secondary metabolites. Sequencing beyond the EfHP1 and EfHP4 genes failed to produce reliable sequences after several attempts with different primers, probably due to the presence of inverted repeat sequences (data not shown).

Expression of putative ORFs
To determine if the newly identified ORFs are expressed and coordinately regulated under conditions favourable for elsinochrome accumulation, Northern-blot hybridization of RNA from E. fawcettii was conducted to examine the expression profiles of the RDT1, TSF1, OXR1, EfPKS1, PRF1 and ECT1 genes as well as those of EfHP1 to EfHP4. All the genes except EfHP3 were upregulated when the fungus was grown on a medium without nitrogen supplement, in contrast to the barely detectable transcript levels in the presence of ammonium nitrate (Fig. 2a). Expression of EfHP3 appears to be constitutive as its transcript level remained unchanged under all conditions tested. Accumulation of the TSF1, EfPKS1, PRF1, ECT1 and OXR1 were preferentially expressed under conditions of high glucose concentration, whereas accumulation of the TSF1 gene transcript was repressed (Fig. 2a). Expression of RDT1, EfHP1, EfHP2 and EfHP4 was apparently not regulated by glucose.

Accumulation of elsinochromes
Quantitative assays revealed that large quantities of elsinochromes accumulated when the fungus was cultured in the absence of nitrogen, under alkaline conditions (pH 8.0), and in the presence of a high concentration of glucose (Fig. 2b). Addition of ammonium nitrate, reducing the glucose concentration to 2 g l\(^{-1}\), or lowering the pH to acidic conditions (pH 4.0) completely suppressed elsinochrome production.

Targeted disruption of the TSF1 gene
To determine if the genes adjacent to EfPKS1 are also involved in elsinochrome biosynthesis and regulation, we first characterized the TSF1 gene, encoding a putative transcriptional activator, in detail. The TSF1 gene contains 3075 nt and is interrupted by four introns of 49, 50, 43 and 53 bp. The predicted 959 aa product of the TSF1 gene contains a Cys\(_2\)His\(_2\)-type zinc finger and a GAL4-like transcriptional activator, in detail. The TSF1 gene contains 3075 nt and is interrupted by four introns of 49, 50, 43 and 53 bp. The predicted 959 aa product of the TSF1 gene contains a Cys\(_2\)His\(_2\)-type zinc finger and a GAL4-like
Zn$_2$Cys$_6$ binuclear cluster DNA-binding motif, and displays strong similarity to numerous conserved transcriptional regulators from the sequenced genomes of fungi. Analysis of the promoter region 1090 nt upstream from the predicted start codon of the TSF1 gene revealed the presence of three GCCARG consensus motifs, which bind the pH-responsive PACC regulator (Espeso et al., 1997), one GATA binding site that is recognized by the AREA nitrogen regulator (Marzluf, 1997), and the WC1/WC2 light-responsive regulator (Linden & Macino, 1997).

Similarly, analysis of at least 528 bp of sequence upstream of the putative ATG translational start codon identified several consensus sequences in the RDT1, OXR1, EJPKS1, PRF1, ECT1 and EfHP1–4 promoters (Table 2). The consensus TATA motif was found in the promoters of all genes except OXR1, PRF1 and EfHP3. All the genes contain at least one CAAT or CCAAT consensus sequence in their promoter regions. The pH-regulatory PACC-binding sequence (5’-GCCA(A/G)G-3’) was identified only in the promoter regions of TSF1, EJPKS1, ECT1 and EfHP2. All the genes, except EfHP1 and EfHP3, have one or multiple GATA consensus sequences potentially recognized and bound by the nitrogen-regulatory AREA protein or the light-regulatory transcriptional WC1/WC2 complex. However, none of the promoter regions has the binding sequence (5’-(G/C)PyGGG-3’) that is recognized by the CREA carbon repressor. In addition, the promoter regions of the TSF1, EJPKS1 and PRF1 genes each have a consensus sequence, 5’-(A/C)(A/G)AGGG(A/G)-3’, that serves as a binding site for the conidial formation-related BRLA transcriptional activator in A. nidulans (Adams et al., 1998). The promoter regions of the RDT1, OXR1, EJPKS1, and ECT1 genes each have a consensus sequence, 5’-CATTC(C/T)-3’, that acts as a binding site for the ABAA transcriptional activator involved in conidiophore development in A. nidulans. Two palindromic sequences, 5’-TCG(N$_2$–4)CGA-3’ and 5’-CGG(N$_4$–11)CCG-3’, were also identified in the promoter regions of some but not all genes (Table 2).

The regulatory function of the TSF1 gene product in relation to the biosynthesis of elsinochromes was...
Functional complementation of a TSF1 null mutant

Functional complementation was performed to further determine the role of TSF1 in relation to biosynthesis of elsinochromes. Transformants were screened for recovery of the red-pigmented elsinochromes. Of several hundreds of transformants screened, two showed restored elsinochrome production on PDA. However, production of elsinochromes by these two complementation strains was far less than that produced by the wild-type (Fig. 4b, c). Expression of a functional TSF1 in a null mutant only partially restored conidiation and virulence (Table 3).

Gene regulation by TSF1

A general role for the TSF1 gene product in transcriptional activation was assessed by examining expression of the clustered genes in two TSF1 disruptants. As shown in Fig. 5, disruption of the TSF1 gene resulted in a considerable reduction of the levels of TSF1, RDT1, EFPKS1, PRF and EFHP1 gene transcripts, but had little or no effect on expression of ECT1, OXR1, EFHP2 and EFHP3. Acquiring and expressing a functional copy of TSF1 in a null strain restored expression of the TSF1, RDT1, EFPKS1, PRF, ECT1 and EFHP1 genes to wild-type levels (Fig. 5b).
**Table 2. Promoter analysis of the genes flanking EfPKS1 in E. fawcettii**

The numbers indicate the number of consensus sequences with 100% matches found per promoter.

<table>
<thead>
<tr>
<th>Gene</th>
<th>TATA</th>
<th>GATA or WC1/WC2</th>
<th>AREA or ccaat</th>
<th>pH regulatory</th>
<th>BRLA</th>
<th>ABAA</th>
<th>Palindrome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ccaat or</td>
<td></td>
<td></td>
<td>gcca(a/g)/g</td>
<td>aggg(a/g)</td>
<td>catt(c/t)</td>
<td></td>
</tr>
<tr>
<td>RDT1</td>
<td>1</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>tcg(n2-4)cga cgg(n3-11)ccg</td>
</tr>
<tr>
<td>TSFI</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>tcg(n2)cga cgg(n3)ccg</td>
</tr>
<tr>
<td>OXR1</td>
<td>6</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>tcg(n2)ccg cgg(n3)ccg</td>
</tr>
<tr>
<td>EfPKS1</td>
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<td>2</td>
<td>1</td>
<td>tcg(n2)ccg cgg(n3)ccg</td>
</tr>
<tr>
<td>ECT1</td>
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<td>0</td>
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<td>–</td>
</tr>
<tr>
<td>EfHP1</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>EfHP2</td>
<td>3</td>
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<td>EfHP3</td>
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<td>0</td>
<td>0</td>
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<td>1</td>
<td>–</td>
</tr>
<tr>
<td>EfHP4</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>–</td>
</tr>
</tbody>
</table>

**Gene expression in an EfPKS1 null mutant**

Accumulation of the gene transcripts was also examined in an EfPKS1 null mutant (D4) that is defective in elsinochrome production (Liao & Chung, 2008b). Northern-hybridization analysis revealed a marked reduction of transcripts of the RDT1, TSFI, PRF1, ECT1 and EfHP1 genes in the D4 mutant (Fig. 6). Accumulation of the RDT1, TSFI, PRF1 and ECT1 gene transcripts was nearly restored to wild-type levels in strains (C1 and C2) expressing a functional copy of EfPKS1. In contrast, expression of the OXR1, EfHP2, and EfHP3 gene transcripts was apparently not affected by disruption of the EfPKS1 gene (Fig. 6). A regulatory network deciphering environmental cues, signal transduction, transcriptional activation and feedback suppression of the genes required for elsinochrome biosynthesis and conidial formation via TSFI is proposed (Fig. 7).

**DISCUSSION**

We previously showed that deletion of the E. fawcettii EfPKS1 gene encoding a polyketide synthase leads to loss of both elsinochrome production and full fungal virulence, thus establishing an essential role for elsinochromes in fungal pathogenesis (Liao & Chung, 2008b). The genes involved in the biosynthesis of polyketide compounds are closely linked in fungal genomes (Hoffmeister & Keller, 2007), and this also appears to be the case for some of the genes governing biosynthesis of elsinochromes in E. fawcettii. In the present study, we sequenced the regions flanking EfPKS1 to obtain a 30 kb contig in which we detected nine additional ORFs. These new genes include RDT1, encoding a putative reductase, and TSFI, encoding a putative transcriptional activator; both are likely to be involved in elsinochrome biosynthesis. The ECT1 gene, encoding a probable membrane transporter, might function in toxin secretion. The role of the PRF1 gene, encoding a putative prefoldin protein, in relation to elsinochrome production remains unknown. Similarly, the remaining five ORFs (OXR1 and EfHP1 to EfHP4) encode polypeptides with low similarity to hypothetical proteins of unknown function in the database. Whether they are involved in elsinochrome production will require further investigation.

Our analysis of gene expression patterns in EfPKS1- or TSFI-disrupted mutants suggests that at least some of the newly identified genes are needed for elsinochrome production. As was evident from Northern-blot analysis, all the putative ORFs identified in the cluster are expressed. However, accumulation of gene transcripts does not fully correspond to all the conditions conducive to elsinochrome production. Expression of all ORFs, apart from EfHP3, and accumulation of elsinochromes did occur under limited nitrogen, but such coordinate gene expression and elsinochrome accumulation was not observed in response to increased pH or glucose concentration. Northern-blot analysis showed that not all of the genes are coordinately controlled by the putative transcriptional regulator, TSFI. For example, disruption of the TSFI gene does not cause marked alteration in the ECT1 gene transcript; accumulation of the RDT1 or EfHP1 gene transcript is severely reduced in one TSFI-disruptant but not another, implying ‘leaky’ expression or requirement of other transcriptional regulators. The fact that the TSFI-disrupted mutants fail to accumulate any detectable TSFI transcript and elsinochromes indicates that TSFI has an indispensable role in elsinochrome biosynthesis. Disruption of the TSFI gene also resulted in considerably reduced expression of the RDT1, EfPKS1 and PRF1 genes but had little or no effect on ECT1 and OXR1. It is yet not clear whether the OXR1 and EfHP1–4 genes flanking EfPKS1 and TSFI are also involved in elsinochrome biosynthesis, even though expression of some of them was regulated by nitrogen limitation and altered by disruption of EfPKS1 or TSFI.

The EfPKS1 gene, encoding a polyketide synthase, has previously been shown to be required for elsinochrome biosynthesis (Liao & Chung, 2008b). Disruption of this
Table 3. Asexual sporulation and lesion development by strains of E. fawcettii

<table>
<thead>
<tr>
<th>Fungal isolate or treatment*</th>
<th>Sporulation (conidia ml⁻¹)†</th>
<th>Necrotic lesions (%)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar plugs</td>
<td>–</td>
<td>0 (0/39)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>4.5 x 10⁶</td>
<td>81 (32/39)</td>
</tr>
<tr>
<td>Δtsf1-D1</td>
<td>5.1 x 10³</td>
<td>0 (0/44)</td>
</tr>
<tr>
<td>Δtsf1-D2</td>
<td>2.0 x 10³</td>
<td>0 (0/39)</td>
</tr>
<tr>
<td>Δtsf1-D3</td>
<td>3.6 x 10³</td>
<td>0 (0/39)</td>
</tr>
<tr>
<td>TSF1-C1</td>
<td>5.3 x 10⁵</td>
<td>31 (12/39)</td>
</tr>
<tr>
<td>TSF1-C2</td>
<td>1.1 x 10⁶</td>
<td>11 (5/44)</td>
</tr>
</tbody>
</table>

*TSF1 null mutants: Δtsf1-D1, Δtsf1-D2 and Δtsf1-D3. Complementation strains: TSF1-C1 and TSF1-C2.
†Conidia were produced in Fries medium in the dark and were determined by microscopy with the aid of a haemacytometer.
‡Pathogenicity assays were performed on detached rough lemon leaves. A 4 mm agar plug cut from mycelial mats was placed onto detached rough lemon leaves. Inoculated leaves were incubated in a moist chamber under fluorescent light for 25 days. Lesion formation is indicated as the number of inoculated leaf spots developing scab lesions relative to the total number of spots inoculated.

In a prior study, we observed that disruption of the EFPKS1 gene gave rise to mutants displaying a remarkable reduction in conidiation and deficient in elsinochrome production (Liao & Chung, 2008b). Similar to the EFPKS1-disrupted mutants, TSF1 null mutants are also severely defective in conidial production, further suggesting a close linkage between elsinochrome production and asexual sporulation in E. fawcettii. The connections between production of secondary metabolites and cell development and/or differentiation have also been investigated in other filamentous fungi (Adams & Yu, 1998; Calvo et al., 2002; Yu & Keller, 2005; Brodhagen & Keller, 2006). In aspergilli, both asexual sporulation and production of sterigmatocystin and aflatoxin mycotoxins are negatively regulated by a G-protein signalling pathway (Hicks et al., 1997). It is unknown whether expression of the TSF1 gene is regulated by a G-protein pathway. Since loss of elsinochrome production by disrupting TSF1 greatly reduced conidial formation, it appears that the proteins or intermediates involved in elsinochrome production might directly participate in asexual sporulation. In addition, examination of upstream sequences revealed the presence of consensus MRAGGGR motifs in the promoter regions of TSF1, which are presumably involved in binding the conidiation-specific Bristle (BRLA)-like transcriptional activator in A. nidulans (Adams et al., 1998). The

Fig. 4. (a) Northern-blot hybridization of total RNA purified from wild-type (WT), three TSF1-disrupted mutants (D1, D2 and D3), and two complementation strains (C1 and C2) of E. fawcettii. RNA was purified from 7-day-old mycelia grown on PDA under light, electrophoresed in a formaldehyde-containing gel, transferred to a nylon membrane, and hybridized with a TSF1 gene probe. Ethidium bromide-stained rRNA shows the relative loading of the samples. Sizes (kb) of the hybridizing signals are indicated. (b) TLC analysis of elsinochromes purified with acetone from cultures of wild-type, three TSF1 disruptants, and a C1 strain expressing a functional TSF1 gene. Elsinochromes were separated on a TLC plate coated with a fluorescent silica gel and developed with chloroform/ethyl acetate (1:1, v/v). The distance each substance migrated relative to the solvent front (Rf) is indicated. Production of elsinochromes by the C2 complementation strain resulting in faint bands on TLC is not shown. (c) Quantification of elsinochromes (nmol per agar plug) produced by E. fawcettii. Elsinochromes were extracted from fungal cultures by soaking in 5 M KOH prior to RNA isolation and measured as A480. The concentrations of elsinochromes were determined by referring to a regression line. Data represent the means and standard errors of two experiments with four replicates of each treatment.
A consensus CATTCY motif (Y=pyrimidine) that is recognized by the ABAA transcriptional activator (Andrianopoulos & Timberlake, 1994), is also found in the RDT1, OXR1, EfPKS1 and ECT1 gene promoters (Table 2). ABAA is activated by BRLA and is involved in conidiphore development of A. nidulans (Andrianopoulos & Timberlake, 1994). To provide a regulatory framework for better understanding the elsinochrome biosynthesis and conidial formation in E. fawcettii, a hypothetical model describing intertwined regulatory controls via TSF1 is proposed (Fig. 7). In this model, environmental cues activate signalling transduction cascades mediated by cAMP, G-protein or MAP kinase, which in turn activates transcriptional regulators such as AREA, WC1/WC2 complex, PACC, BRLA and ABAA. These transcriptional regulators may directly or indirectly trigger expression of TSF1 and the other genes, which eventually lead to elsinochrome production and conidial formation. It appears that accumulation of the EfPKS1, TSF1, RDT1 and PRF1 gene transcripts is controlled by TSF1. By contrast, expression of ECT1, OXR1, EfHP1, EfHP2 and EfHP3 is probably regulated by different transcriptional activators because disruption of TSF1 has little effect on their expression patterns. We also observed that disruption of EfPKS1 leads to coordinate inhibition of expression of the RDT1, TSF1, PRF1 and ECT1 genes, suggesting the presence of a feedback inhibition mechanism, in which disruption of one of the biosynthetic genes in the pathway abolishes elsinochrome production and in turn abrogates expression of the other biosynthetic genes.

Disruption of the EfPKS1 or TSF1 gene in E. fawcettii yielded mutants with pleiotropic phenotypes. All mutant phenotypes resulting from inactivation of the EfPKS1 gene were restored to the wild-type by expressing a functional copy of the EfPKS1 gene cassette (Liao & Chung, 2008b). It is surprising that expressing a functional TSF1 gene cassette under control of its own promoter (including 1 kb of upstream sequence) failed to fully restore conidiation, elsinochrome production or fungal pathogenesis to the TSF1 null mutants. It is very unlikely that failure to completely reverse the mutated phenotype is due to an extraneous mutation in the transformed TSF1 gene. Sequence analysis of the TSF1 cassette revealed no nucleotide substitution, deletion or insertion (data not shown). Moreover, Northern-blot analysis showed expression of the TSF1 gene in the recombinant transformants. This failure of full complementation may reveal a specific requirement for TSF1 gene regulation within the context of the elsinochrome biosynthetic gene cluster.

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**Fig. 5.** (a) RNA gel blots showing expression of the elsinochrome biosynthetic genes in wild-type (WT) and TSF1-disrupted mutants (D2 and D3) of E. fawcettii. Fungal RNA was purified from 7-day-old cultures grown on PDA under light, electrophoresed, blotted to membranes, and hybridized to appropriate DNA probes as indicated. Ethidium bromide-stained rRNA indicates the loading of the samples. Sizes (kb) of hybridizing signals are indicated. (b) RNA gel analysis confirms restoration of expression of the EfPKS1 gene encoding a polyketide synthase in a TSF1 null mutant that had acquired and expressed a functional TSF1 gene cassette. Fungal RNA purified from wild-type (WT) and two TSF1 complementation strains (C1 and C2) was denatured and hybridized to an EfPKS1 probe.

**Fig. 6.** RNA gel blots indicating transcriptional suppression of expression of the elsinochrome biosynthetic genes in E. fawcettii. Total RNA was purified from 7-day-old cultures of wild-type (WT), the EfPKS1-disrupted mutant (D4), and two EfPKS1 complementation strains (C1 and C2), and hybridized to gene-specific probes as appropriate. Ethidium bromide-stained rRNA indicates the loading of the samples. Sizes (kb) of the hybridizing signals are indicated.

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Alternatively, ectopic insertion of the TSF1 cassette may have affected the functions of nearby genes.

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr Daryl Henderson for initial editing. This research was supported by the Florida Agricultural Experiment Station and grants from the Florida Citrus Production Research Advisory Council (FCPRAC) #012-04P and #033-03P.

REFERENCES


Edited by: S. D. Harris