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 *EfPKS1* is essential for elsinochrome biosynthesis in the citrus scab fungus *Elsinoë fawcettii*. Sequence analysis beyond the *EfPKS1* 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 *EfHP1* to *EfHP4*, 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*, *EfPKS1*, *PRF1* and *EfHP1*, whereas expression of *RDT1*, *TSF1*, *PRF1* and *ECT1* was nearly abolished in *EfPKS1*-disrupted mutants. By contrast, expression of *OXR1*, *EfHP2* and *EfHP3* was not affected by disrupting either *EfPKS1* 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.

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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

Abbreviation: CSPD, disodium 3-[4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1]decan}-4-yl]phenyl phosphate.

The GenBank/EMBL/DBJ accession numbers for the sequence data reported in this article are: *EfHP1*, EU414199; *EfHP2*, EU414200; *EfHP3*, EU414201; *RDT1*, EU401704; *TSF1*, EU401705; *OXR1*, EU401706; *EfPKS1*, EU086466; *PRF1*, EU401707; *ECT1*, EU414198; and *EfHP4*, EU414202.

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.*, 1966; 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, *TSF1*, which encodes a polypeptide containing a Cys₂His₂-type zinc finger and a GAL4-like Zn₂Cys₆ 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 F15T8 fluorescent light (wavelength 436 nm) at an intensity of 3.5 J m⁻² s⁻¹ for 5 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 isolates were grown on media with a layer of sterile cellophane (Choquer *et al.*, 2005). Screening of elsinochrome-deficient mutants was performed on thin PDA as previously described (Choquer *et al.*, 2005; Liao & Chung, 2008a).

Extraction and analysis of elsinochrome toxin. Elsinochromes were extracted from fungal cultures with 5 M KOH or acetone. Agar plugs bearing fungal mycelium were cut with a 6 mm sterilized cork borer, and soaked in 5 M KOH in the dark for at least 12 h. The A₄₈₀ of the soaking solution was measured using a model Genesys 5 spectrophotometer (Spectronic Instruments). Since elsinochromes and cercosporin have a similar structure and molecular mass (Weiss *et al.*, 1987), elsinochromes were quantified using a molar absorption coefficient of 23 300 l mol⁻¹ cm⁻¹ (Jenns *et al.*, 1989) and expressed as nmoles per plug. For TLC analysis, elsinochromes were extracted twice from dried agar medium with fungal mycelia in acetone for 16 h, condensed and separated on a TLC plate coated with 60 F254 fluorescent silica gel (5 × 20 cm, Selecto Scientific) as previously described (Liao & Chung, 2008a, b). The concentration of elsinochromes was estimated by reference to a regression line that was established using pure cercosporin (Sigma) as a standard and expressed as cercosporin equivalents.

Chromosomal walking and sequence analysis. Fungal DNA was isolated employing the DNeasy Plant Mini kit (Qiagen). A genomic library of *E. fawcettii* was constructed from DNA digested with *Dra*I, *Eco*RI, *Pvu*II and *Stu*I and ligated to adaptors using the Universal GenomeWalker kit (BD Biosciences) according to the manufacturer's instructions. Primers were designed based on the known sequences and paired with adaptor primers to obtain DNA fragments harbouring unknown genomic regions using Titanium or Advantage 2 DNA polymerase (BD Biosciences). In some cases, DNA fragments were obtained by PCR with inverse 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 *TSF1* gene in *E. fawcettii*, a 5.4 kb DNA fragment encompassing the *TSF1* ORF and flanking sequences was amplified by PCR with primers efup11 (5'-catctcgcatatcttgaccgctc-3') and efup28 (5'-cgggtctattcttagacagag-3'). The amplified DNA fragment was cloned into pGEM-T easy vector, creating pSTSF1128. A 2.1 kb fragment harbouring 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 *Xba*I, blunt-ended with DNA polymerase I (New England BioLabs), and cloned into the *Nru*I site of pSTSF1128, creating the disruption construct pTSF1128 (see Fig. 3). The *Nru*I site is approximately 1200 nt downstream from the predicted *TSF1* 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' *TSF1* fused with 3' *HYG* and a 4.2 kb fragment encompassing 3' *TSF1* linked to 5' *HYG* were amplified with, respectively, primers efup11/

hyg3 (5'-ggatgcctcgcgtcaagta-3') and efup28/hyg4 (5'-cgttgcaagaactgcctgaa-3') from pTSF1128 using the Takara Ex Tar 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 CaCl_2 and polyethylene glycol-mediated method was performed by mixing PCR fragments with 1×10^5 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* DH5 α 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, and Southern- and Northern-blot hybridizations (Sambrook & Russell, 2001). DNA probes used for Southern- and Northern-blot hybridization were labelled 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 ($\text{C}_{18}\text{H}_{19}\text{Cl}_2\text{O}_7\text{PN}_2$) chemofluorescent 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 product 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 (T_3HN) reductase involved in melanin biosynthesis (Table 1). The *OXR1* ORF is located between *EfPKS1* and *TSF1*. The predicted OXR1 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

Gene	Probable function
<i>EfHP1</i>	Unknown
<i>EfHP2</i>	Unknown
<i>EfHP3</i>	Unknown
<i>RDT1</i>	Reductase
<i>TSF1</i>	Transcription factor containing dual Cys ₂ His ₂ zinc finger and Zn(II)Cys ₆ binuclear cluster DNA-binding motifs
<i>OXR1</i>	Unknown
<i>EfPKS1</i>	Fungal type-I polyketide synthase
<i>PRF1</i>	Prefoldin protein
<i>ECT1</i>	Membrane transporter
<i>EfHP4</i>	Unknown

Fig. 1. Physical map showing the order of genes and their putative functions identified in *E. fawcettii*. Arrows represent the orientation of transcription. The sizes (kb) of each of the genes and their intergenic regions are indicated.

Table 1. Characterization of the genes flanking *EfPKS1* in *E. fawcettii*

Gene (accession no.)	Length (bp)	No. of introns (size, bp)	Amino acids	Closest BLAST match proteins and species (accession no.)	Amino acid similarity (%)	E-value
<i>RDT1</i> (EU401704)	842	1 (47)	264	<i>Botryotinia fuckeliana</i> (EDN21223) 1,3,8-Trihydroxynaphthalene reductase, <i>Alternaria alternata</i> (BAA36503)	220/262 (83 %) 208/248 (83 %)	2×10^{-103} 4×10^{-101}
<i>TSF1</i> (EU401705)	3075	4 (49, 50, 43 and 53)	959	<i>Phaeosphaeria nodorum</i> (EAT80399) Transcription factor Cmr1, <i>Cochliobolus heterostrophus</i> (ABI81496)	643/985 (65 %) 260/357 (72 %)	0 0
<i>OXR1</i> (EU401706)	744	1 (45)	232	Hypothetical protein, <i>Pseudomonas syringae</i> (AAY36032)	65/163 (39 %)	1.1
<i>EfPKS1</i> (EU086466)	6631	1 (52)	2192	Fungal type I polyketide synthase, <i>Bipolaris oryzae</i> (BAD22832)	1675/2199 (76 %)	0
<i>PRF1</i> (EU401707)	705	1 (111)	197	<i>Phaeosphaeria nodorum</i> (EAT80391) Prefoldin subunit 3, <i>Neosartorya fischeri</i> (EAW23892)	147/178 (82 %) 151/190 (79 %)	1×10^{-67} 2×10^{-57}
<i>ECT1</i> (EU414198)	1563	1 (45)	505	<i>Phaeosphaeria nodorum</i> (EDN17791) High affinity nitrate transporter, <i>Aspergillus terreus</i> (EAU39122)	273/522 (52 %) 260/513 (50 %)	2×10^{-82} 3×10^{-69}
<i>EfHP1</i> (EU414199)	993	1 (57)	311	Hypothetical protein, <i>Chaetomium globosum</i> (EAQ88529)	71/147 (48 %)	8×10^{-06}
<i>EfHP2</i> (EU414200)	1158	0	385	Hypothetical protein, <i>Marinomonas</i> sp. (EAQ67386)	44/94 (46 %)	1.0
<i>EfHP3</i> (EU414201)	819	1 (69)	249	Hypothetical protein, <i>Coprinopsis cinerea</i> (EAU83036)	60/117 (51 %)	3×10^{-06}
<i>EfHP4</i> (EU414202)	423	1 (54)	122	Hypothetical protein, <i>Bacillus subtilis</i> (CAB13417)	39/80 (48 %)	0.26

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 *EfHP2* gene transcripts increased considerably when the fungus was incubated under alkaline conditions (Fig. 2a). Under acidic conditions (pH 4), the *RDT1* and *EfHP1* gene transcripts accumulated to slightly but not significantly higher levels compared to those under alkaline conditions. By contrast, expression of *EfHP3*, *EfHP4* and

OXR1 was not affected by pH. It appears that *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 Cys₂His₂-type zinc finger and a GAL4-like

Fig. 2. (a) Northern-blot analyses for differential expression of the elsinochrome biosynthetic genes in wild-type *E. fawcettii*. Total RNA was purified from 7-day-old fungal mycelia grown on nonbuffered minimal medium (MM) with or without ammonium nitrate as sole nitrogen source (pH 5.3), on MM buffered to pH 4.0 or 8.0 with phosphate buffer, or on nonbuffered complete medium (CM) amended with 2 g or 60 g glucose l⁻¹ as sole carbon source (pH 5.3) under constant fluorescent light. RNA was separated and denatured in formaldehyde-containing agarose gels, blotted onto nylon membranes and hybridized to DNA probes as indicated. Sizes (kb) of the hybridizing signals are indicated. Ethidium bromide-stained rRNA shows the relative loading of the samples. (b) Quantification of elsinochromes (ESCs) produced by *E. fawcettii* grown on various conditions as described above. Elsinochromes were extracted from fungal cultures by soaking in 5 M KOH prior to RNA purification, and measured as A_{480} . The concentrations of elsinochromes were calculated using a regression line. Data are means and standard errors of two independent experiments with four replicates of each treatment.

Zn₂Cys₆ 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 predicated 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*, *EfPKS1*, *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*, *EfPKS1*, *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)PyGGGG-3') that is recognized by the CREA carbon repressor. In addition, the promoter regions of the *TSF1*, *EfPKS1* 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*, *EfPKS1*, and *ECT1* genes each have a consensus sequence, 5'-CATT(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(N₂₋₄)CGA-3' and 5'-CGG(N₃₋₁₁)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

Fig. 3. (a) Schematic illustration of the disruption of the *Tsf1* gene (indicated by shaded bars), encoding a putative transcription activator, in wild-type *E. fawcettii*, using a split-marker strategy. The 5' end of *Tsf1* joined to the *trpC* promoter of *A. nidulans* and truncated hygromycin phosphotransferase B gene (*Hy*) and the 3' end of *Tsf1* joined to the *trpC* terminator and truncated *yG* fragment were amplified from the disruption construct pTsf1128 with primers as indicated and directly transformed into protoplasts prepared from wild-type (WT). The truncated *Hy* and *yG* fragments share 0.8 kb of overlapping sequence. Dashed lines indicate regions with homologous sequences; cross symbols represent the expected occurrence of double crossing-over recombination. Sizes (kb) of the hybridizing fragments are indicated. Abbreviations for restriction enzymes: B, *Bgl*I; N, *Nru*I; P, *Pvu*II. (b, c) Southern-blot analysis of genomic DNA from WT and three *Tsf1* disrupted mutants of *E. fawcettii*, confirming successful disruption of the *Tsf1* gene. DNA was purified from 5-day-old fungal cultures grown on PDA plates under light, digested with *Pvu*II (b) or *Bgl*I/*Pvu*II (c), and hybridized to a *Tsf1* gene probe.

determined by a targeted disruption strategy to create null mutants at the *Tsf1* locus. The split-marker disruption strategy that we employed involves transforming *E. fawcettii* with two DNA amplicons that overlap by 466 nt within the coding region of the selective marker (*HYG*). The DNA 5' and 3' to the *HYG* fragments corresponds, respectively, to the target gene coding and flanking sequence (Fig. 3a). All putative transformants recovered were then screened for production of red/orange pigments (elsinochromes). Of 20 transformants tested, four failed to

accumulate the red/orange pigments and were considered to be *Tsf1* disruptants. Successful disruption of the *Tsf1* gene was confirmed by Southern-blot analysis. Genomic DNA digested with *Pvu*II and hybridized to a DNA probe corresponding to *Tsf1* coding sequence resulted in a single 4.8 kb band in the wild-type and two bands of the expected sizes of 3.9 and 3.2 kb in transformants (Fig. 3b). Hybridization of genomic DNA cleaved with *Bgl*I and *Pvu*II also yielded different banding patterns between wild-type and the disruptants as expected (Fig. 3c), indicating specific disruption at the *Tsf1* locus.

Northern-blot analysis was conducted to determine if the disrupted mutants accumulated the *Tsf1* transcript. As shown in Fig. 4(a), hybridization of total RNA identified a sole *Tsf1* transcript of 3.1 kb from wild-type, but not from three disruptants. Apparently, integration of the *HYG* cassette within the *Tsf1* ORF has completely abolished expression of the *Tsf1* gene, indicating that they are *Tsf1* null mutants. Compared to wild-type, the *Tsf1* disruptants failed to accumulate elsinochromes in axenic cultures, as assayed either spectrophotometrically after KOH extraction (Fig. 4b) or by TLC analysis (Fig. 4c). In addition to elsinochrome deficiency, the *Tsf1* disruptants were also defective in conidiation and fungal pathogenesis to rough lemon (Table 3), similar to the mutant phenotypes resulting from disruption of the *EfPKS1* gene (Liao & Chung, 2008b).

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). However, Southern- and Northern-blot analyses confirmed that the two complementation strains did acquire and express the *Tsf1* gene (Fig. 5b and data not shown).

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.

Gene	TATA	ccaat or caat	AREA or WC1/WC2 gata	pH regulatory gccca(a/g)g	BRLA (a/c)(a/g) aggg(a/g)	ABAA cattc (c/t)	Palindrome	
							tcg(n ₂₋₄)cga	cgg(n ₃₋₁₁)ccg
<i>RDT1</i>	1	5	2	0	0	1	–	cgg(n ₆)ccg
<i>TSF1</i>	2	1	1	3	1	0	tcg(n ₂)cga	cgg(n ₅)ccg
<i>OXR1</i>	0	3	1	0	0	1	tcg(n _{2 and 3})cga	–
<i>EfPKS1</i>	6	12	1	3	2	1	tcg(n _{2 and 3})cga	cgg(n ₁₀)ccg
<i>PRF1</i>	0	6	2	0	1	0	tcg(n _{3 and 4})cga	cgg(n ₃)ccg
<i>ECT1</i>	1	2	1	1	0	2	–	–
<i>EfHP1</i>	1	5	0	0	0	0	–	–
<i>EfHP2</i>	3	1	2	1	0	0	tcg(n ₂)cga	–
<i>EfHP3</i>	0	4	0	0	0	0	–	–
<i>EfHP4</i>	4	1	4	0	0	0	tcg(n ₄)cga	–

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*, *TSF1*, *PRF1*, *ECT1* and *EfHP1* genes in the D4 mutant (Fig. 6). Accumulation of the *RDT1*, *TSF1*, *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 *TSF1* 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 often 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 *TSF1*, 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 *TSF1*-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, *TSF1*. For example, disruption of the *TSF1* gene does not cause marked alteration in the *ECT1* gene transcript; accumulation of the *RDT1* or *EfHP1* gene transcript is severely reduced in one *TSF1*-disruptant but not another, implying 'leaky' expression or requirement of other transcriptional regulators. The fact that the *TSF1*-disrupted mutants fail to accumulate any detectable *TSF1* transcript and elsinochromes indicates that *TSF1* has an indispensable role in elsinochrome biosynthesis. Disruption of the *TSF1* 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 *TSF1* 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 *TSF1*.

The *EfPKS1* gene, encoding a polyketide synthase, has previously been shown to be required for elsinochrome biosynthesis (Liao & Chung, 2008b). Disruption of this

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 (R_f) is indicated. Production of elsinochromes by the C2 complementation strain resulting in faint bands on TLC is not shown. (c) Quantification of elsinochromes (nmoles 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 A_{480} . 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.

Table 3. Asexual sporulation and lesion development by strains of *E. fawcettii*

Fungal isolate or treatment*	Sporulation (conidia ml ⁻¹)†	Necrotic lesions (%)‡
Agar plugs	–	0 (0/39)
Wild-type	4.5×10^6	81 (32/39)
$\Delta tsf1$ -D1	5.1×10^3	0 (0/44)
$\Delta tsf1$ -D2	2.0×10^3	0 (0/39)
$\Delta tsf1$ -D3	3.6×10^3	0 (0/39)
<i>TSF1</i> -C1	5.3×10^5	31 (12/39)
<i>TSF1</i> -C2	1.1×10^4	11 (5/44)

**TSF1* null mutants: $\Delta tsf1$ -D1, $\Delta tsf1$ -D2 and $\Delta tsf1$ -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 haemocytometer.

‡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.

gene also effectively abolished expression of the *RDT1*, *TSF1*, *PRF1* and *ECT1* genes in the cluster. Expression of a copy of the functional *EfPKS1* gene in an *EfPKS1* null mutant restored expression of *RDT1*, *TSF1*, *PRF1* and *ECT1* to wild-type levels, further implicating the requirement of these genes in elsinochrome biosynthesis.

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. 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.

consensus CATTCTY 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

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.

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 phenotypes 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.

Fig. 7. Proposed regulatory pathways leading to the production of elsinochromes and conidia, mediated by the TSF1 transcriptional factor in *E. fawcettii*. The cross indicates an inhibitory effect on elsinochrome biosynthesis, presumably by blocking expression of *TSF1* and then the elsinochrome-biosynthetic genes. A filled rectangle indicates inhibition of expression of the elsinochrome-biosynthetic genes. TSF1 is also required for conidiation. It remains uncertain whether TSF1 directly regulates expression of the genes involved in conidial formation or the elsinochrome intermediates participate directly in asexual sporulation.

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

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