MOLECULAR AND GENETIC DETERMINATION OF THE ROLE OF ELSINOCHROME TOXINS PRODUCED BY *Elsinoë fawcettii* CAUSING CITRUS SCAB

By

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To my grandmother, Ma-Men Liao, who always supports my educational pursuits

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MOLECULAR AND GENETIC DETERMINATION OF THE ROLE OF ELSINOCHROME TOXINS PRODUCED BY *Elsinoë fawcettii* CAUSING CITRUS SCAB

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Citrus scab disease, caused by the fungus *Elsinoë fawcettii* (Bitancourt & Jenkins), affects all varieties of citrus, resulting in serious fruit blemishes and economic losses in Florida. My study focused on genetic determination of the role of elsinochrome phytotoxins produced by *E. fawcettii*.

Results indicated that elsinochromes function as photosensitizing compounds that exert toxicity to plant cells due to production of reactive oxygen species such as singlet oxygen and superoxides. Upon irradiation to light, elsinochromes alone rapidly killed suspension-cultured citrus and tobacco cells, induced necrotic lesions on rough lemon leaves, and provoked a steady increase of electrolyte leakage. The toxicity was drastically decreased or alleviated by the singlet oxygen quenchers, such as bixin, DABCO, or reduced glutathione. Accumulation of singlet oxygen and superoxides induced by elsinochromes after irradiation was also detected.

An *EfPKS1* gene encoding a fungal polyketide synthase (PKS) was cloned and characterized from *E. fawcettii* to confirm the roles of elsinochromes in fungal pathogenesis and lesion formation by genetic disruption and complementation strategies. In addition, accumulation of the *EfPKS1* transcript and elsinochromes by the wild-type strain were coordinately regulated

by light, carbon, nitrogen and pH. The results clearly indicate that elsinochromes play a consequential role in fungal pathogenesis.

The genes involved in the biosynthesis of fungal secondary metabolites are often clustered. I sequenced a 30-kb region beyond *EfPKS1* and identified nine putative genes; some of them encoding polypeptides are likely required for elsinochrome biosynthesis and regulation. In addition to *EfPKS1*, five genes, *RDT1* encoding a reductase, *OXR1* encoding an oxidoreductase, *TSF1*encoding a transcriptional factor, *ECT1*encoding a membrane transporter, and *PRF1* encoding a prefoldin protein subunit were identified. Other four genes (designated as *EfHP1-4*) encode hypothetical proteins that are likely not associated with biosynthetic functions. The involvement of these genes in elsinochrome production was evident by analyzing the *TSF1* gene encoding a putative pathway-specific regulator. Targeted disruption specifically occurred at the *TSF1* gene created fungal mutants that are defective in elsinochrome production. Expression of the adjacent genes in the *TSF1*-disrupted mutants was markedly down-regulated.

In addition, elsinochromes were extracted, for the first time, from affected lesions. A survey of 52 field-collected *E. fawcettii* isolates in Florida revealed that most of them were able to produce elsinochromes in cultures and/or *in planta*. A single isolate (Ef41) with distinct genetic traits from all others failed to infect rough lemon, grapefruit, and sour orange and produced no elsinochromes. Overall, my studies employing biochemical, molecular, and pathological approaches clearly demonstrated that elsinochromes play critical roles for fungal pathogenesis and lesion development.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Biology of *Elsinoë fawcettii* Causing Citrus Scab

Citrus scab (formerly called sour orange scab) is caused by the pathogenic fungus, *Elsinoë fawcettii* Bitancourt & Jenkins (Anamorph: *Sphaceloma fawcettii* Jenkins) that belongs to the kingdom Fungi, phylum Ascomycota, class Loculoascomycetes, order Myriangiales, and family Elsinoaceae (Alexopoulos et al. 1996).

The teleomorph of *Elsinoë fawcettii* is very rare and has been reported only in Brazil (Bitancourt and Jenkins 1936a; 1936b). *E. fawcettii* forms stromata that contain numerous spherical asci within the pseudothecial locules. Each ascus harbors eight filamentous ascospores (sexual spores) that are hyaline and oblong elliptical with the size of 5-6 x 10-12 µm (Holliday 1980). *Elsinoë* spp. produces two kinds of conidia (asexual spores): hyaline conidia and spindle conidia (Fig. 1-2). Hyaline conidia of *Elsinoë* spp. are one celled, elliptical, and 2-4 x 4-8 µm and are the primary source for inoculation (Whiteside et al. 1988). Conidia are produced within the acervulus which is typically a flat or saucer-shaped bed of conidiophores growing side by side and arising from a stromatic mass of hyphae (Alexopoulos et al. 1996) and capable of reproducing by formation of a new germ tube (Holliday 1980; Whiteside et al. 1988). In contrast, the colored, spindle-shaped conidia that are produced mainly on scab lesions can germinate to produce hyaline conidia. In culture, *E. fawcettii* produces raised, slow-glowing colonies that are usually beige to tan or vinaceous to black. Isolates of *E. fawcettii* grow slowly in axenic culture, forming < 10-mm colony size in 30 days. Most strains of *E. fawcettii* secrete red pigments after 10-15 day incubation in the light.

Identification of citrus scab pathogens is primarily based on their host ranges, because it is difficult to differentiate based on their morphologies. *Elsinoë fawcettii* causing common scab

was found in many citrus producing areas worldwide. Whiteside (1978; 1984) described two pathotypes from Florida and later were designated as the "Florida broad host range (FBHR)" and the "Florida narrow host range (FNHR)" (Timmer et al. 1996). The FBHR pathotype mainly attacks the leaves and fruits of lemon (*C. limon* (L.) Burm. F.), sour orange (*C. aurantium* L.), grapefruit (*C. paradisi* Macf.), and Temple/Murcott tangors (*C. sinensis* (L.) Osbeck x *C. reticulata* Blanco), and the fruit of sweet orange (*C. sinensis*). The FNHR pathotype fail to infect sour orange, Temple tangor, and sweet orange fruit (Tan et al. 1996; Timmer et al. 1996). All pathotypes of citrus scab attack rough lemon (*Citrus jambhiri* Lush).

E. australis Bitancourt & Jenkins (anamorph *S. australis* Bitancourt & Jenkins.), causing sweet orange scab differs from *E. fawcettii* in host ranges and is limited to southern South America. *E. fawcettii* rarely causes lesions on sweet orange, whereas *E. australis* attacks all sweet oranges as well as some tangerines and their hybrids (Chung and Timmer 2005). Unlike *E. fawcettii* that induces lesions on all parts of citrus, *E. australis* appears to affect only fruit. In addition, *E. australis* can be distinguished from *E. fawcettii* based on the sizes of ascospores (12-20 x 15-30 µm in *E. australis*) (Whiteside et al. 1988). Furthermore, *E. australis* does not produce spindle-shaped conidia in scab lesions that are often associated with *E. fawcettii.*

Molecular studies have been employed recently for identification and differentiation of different species and isolates of *Elsinoë* (Tan et al. 1996; Timmer et al. 1996; Hyun et al. 2001). For example: *E. fawcettii* and *E. australis* are differentiated by endonuclease restriction analysis of the amplified internal transcribed spacers (ITSs) of ribosomal DNA. *E. fawcettii* isolated from Florida and Australia could be separated by random amplified DNA polymorphism (RAPD) analysis (Tan et al. 1996).

In addition to citrus, *Elsinoë* spp. also cause diseases in a wide range of plants, such as bean scab caused by *E. phaseoli*, leaf spot of *Dracophyllum* sp. caused by *E. dracophylli*, grape anthracnose caused by *E. ampelina*, raspberry anthracnose caused by *E. veneta*, and twig disease of *Pittosporum tenuifolium* caused by *E. takoropuku* (Phillips 1994; Johnston and Beever 1994; Agrios 1997; Ridley 2005).

Symptoms, Disease Cycles and Disease Control of Citrus Scab Caused by *Elsinoë fawcettii*

Symptoms

Citrus scab affects fruit, leaves and twigs of susceptible varieties of citrus (Timmer et al. 2000) (Fig. 1-1). Symptoms start as small, pale orange or buff-colored slightly-raised pustules, which consist of a mixture of fungal and host tissues. As the pustules develop on most of the susceptible species and cultivars (e.g., lemons, Satsuma mandarins, Temples, and sour orange), those spots become warty and creaky protuberance (Fig. 1-1A, and 1-1B). In contrast, on grapefruit and sweet orange, the lesions appear to be flattened scabby sheets (Fig. 1-1C). The age of the tissues at the time of infection also affects the elevation and size of lesions. Those formed on young tissues tend to be raised and those on more mature tissues are flatter. Because of variations among cultivars and infected-tissue age, it is difficult to differentiate citrus scab from other citrus diseases on the basis of symptoms alone.

Disease Cycle

In the field, *E. fawcettii* survives under unfavorable conditions in pustules on leaves, stem lesions and fruit. Ascospores most likely play no role in the infection process. *E. fawcettii* survives in old lesions and produces conidia from acervuli on the surface of scab pustules (Fig. 1-2A). The mode of spread of conidia occurs mostly due to rain splash. Hyaline conidia die rapidly when exposed to light or dry conditions. After periods of dew, production of spindle-shaped conidia (Fig. 1-2B) on lesions are stimulated and are spread by wind. A short period of

moisture is needed for infection of *E. fawcettii* after dispersal of conidia. The minimum humidity period for production of conidia is 1-2 hr, and infection occurs within 2 to 3 hr at 24-27 °C (Timmer et al. 2000). The severity of citrus scab is markedly affected by wetness. Longer periods of humidity (up to 24 hr) greatly facilitate infection even at suboptimal temperatures. Under suitable conditions, scab lesions usually appear on the host, as early as 6 days to 7 days after infection (Fig. 1-2C and D). Leaves are susceptible immediately after they emerge from the bud, and become resistant when they are older (about 20 days after emergence). The young fruit are susceptible until 6-8 weeks after petal fall. The infection often causes fruit to be misshapen and fall prematurely (Bushong and Timmer 2000).

Disease Control

Citrus scab is a common disease in humid citrus-growing areas in many countries. Although the damage produced by scab is superficial and does not affect internal fruit quality, citrus scab reduces acceptability for the fresh-fruit market. Citrus scab is endemic in Florida where climatic conditions are highly favorable for the pathogen cycle and disease. Scab disease predominantly affects summer growth flushes as summer rain showers frequently occur in Florida and produce sufficient wetness for conidial germination and infection. The most effective strategy recommended for scab management is to remove and destroy the inoculum sources. Frequent applications of fungicides are absolutely needed to manage citrus scab if the fruit is intended for the fresh market. Several fungicides such as Topsin® (thiophanate methyl), Abound® (azoxystrobin), Gem® (trifloxystrobin), Headline®, (pyraclostrobin), ferbam, and copper fungicides are registered in Florida and can be used for control of citrus scab (Bushong and Timmer 2000). However, fungicides are often not adequate for disease control. Application of fungicides also raises concerns on resistance of the pathogens and environmental hazards (Timmer and Zitko 1997).

Secondary Metabolites of Fungi

Fungal products classified as secondary metabolites (Turner 1971; Turner and Aldridge 1983) often show significant biological activities or enigmatic properties, and they are not essential for the microorganisms to complete their lifecycles. Many of those bioactive compounds (e.g., phytotoxins and melanin) are required for development of diseases or fungal structures. Some of them may increase fungal fitness, display antimicrobial activity, and pharmaceutical activities (e.g., antibiotics and immunosuppressant) (Demain and Fang 2000). Thereby, fungal secondary metabolites have been thought to confer competitive advantages for the producing microorganisms in natural environments. In addition, fungal secondary metabolites are of medical, industrial and/or agricultural importance.

Several fungal secondary metabolites have been shown to be associated with fungal development. For example, linoleic acid-derived compounds produced by *A. nidulans* (Calvo et al. 2001), zearalenone from *Fusarium graminearum* (Wolf and Mirocha 1973), and butyrolactone I from *Aspergillus terreus* (Schimmel et al. 1998) are able to enhance sporulation. Furthermore, dark brown-melanin pigments produced by many fungi by oxidative polymerization of phenolic compounds function as fungal virulence factors due to their ability to stimulate production of conidia (e.g., *Alternaria alternata*), appressoria (e.g., *Colletotrichum lagenarium*), sclerotia (e.g., *Scherotium* spp.), and sexual bodies (e.g., *Sordaria macrospora*) (Chet and Hüttermann 1982; Kawamura et al. 1999; Takano et al. 2000; Butler et al. 2001; Engh et al. 2007). *Alternaria alternata* produces melanin to enhance the integrity of sexual and asexual spores (Kawamura et al. 1999). Melanin apparently contributes to the survival of the fungal spores by protecting against UV light damage. Cercosporin phytotoxin produced by many

pathogenic *Cercospora* spp. is not required for spore development but plays a critical role during fungal pathogenesis (Choquer et al. 2005; 2007).

Phytotoxins produced by many phytopathogenic fungi often damage plant cells or influence the course of disease development and the formation of symptoms. Cercosporin produced by *Cercospora* spp. and botrydial produced by *Botrytis cinerea* have been shown to function as virulence factors and further exacerbate disease severity (Choquer et al. 2005; van Kan 2006). Phytotoxins are classified into two classes: host-specific and non-host specific. Non-host specific toxins exhibit general phytotoxicity toward a wide range of plant species including non-host plants. However, host-specific toxins affect only plant varieties or genotypes that are the hosts of the producing microorganisms.

Elsinochromes Produced by *Elsinoë fawcettii*

Many *Elsinoë* fungal species produce light-activated, red/orange pigments, called elsinochromes. Elsinochrome pigments containing a phenolic quinine chromophore consist of at least four derivatives (A, B, C, and D) (Fig. 1-3). The bright red pigments, elsinochromes A, B, and C were originally isolated from cultures of a pecan pathogen, *Elsinoë randii* (anamorph: *Sphaceloma randii*) and their chemical structures and physical properties have been well documented (Weiss et al. 1965; Lousberg et al. 1969). Elsinochromes B and C can be quickly oxidized to A by chromium trioxide (Lousberg et al. 1969). In contrast, elsinochrome D, likely derived from elsinochrome C by forming a methylenedioxy ring (Fig. 1-3), is an orange pigment produced only by some *Elsinoë* species (Shirasugi and Misaki 1992). Elsinochromes are structural analogs to several polycyclic perylenequinones such as altertoxin I produced by *Alternaria alternata*, cercosporin produced by many *Cercospora* spp., hypericin produced by *Hypericum* spp., hypocrellin A produced by *Hypocrella bambusae*, and phleichrome produced by *Cladosporium* spp. (Yoshihara et al. 1975; Assante et al. 1977; Daub 1982a; Duran and Song

1986; Stierle and Cardellina 1989; Daub et al. 2005). All of these compounds have a common 4,9-dihydroxyperylene-3,10-quinone chromophore and only differ in attached side chains (Daub et al. 2005). Additionally, these compounds are grouped as photosensitizers based on their ability to sensitize cells to visible light, react with oxygen molecules, and produce reactive oxygen species (ROS) (Yamazaki et al. 1975; Daub 1982a). Light and oxygen are two key components for photodynamic function and toxicity of these compounds. Although elsinochromes structurally resemble many photosensitizing perylenequinones, their toxicity has never been investigated.

Photosensitizing compounds are a group of structurally diverse compounds and natural products that are able to absorb light energy and are converted to an electronically excited triplet state. The activated photosensitizers then react with oxygen molecules in two different ways to form both radical and non-radical species of activated oxygen, including superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen (Dobrowolski and Foote 1983; Girotti 1990) (Fig. 1-4). Activated oxygen species have general toxicity, as they react with biomolecules common to all cells, including lipids, proteins, and nucleic acids, and often result in cell death (reviewed by Daub et al. 2005). The toxicities of radical oxygen species have been well known. Several lines of evidence suggest that the non-radical-singlet oxygen ($^1O_2$) is also highly toxic to cells (reviewed by Daub et al. 2005).

Polyketide Biosynthesis

Polyketides are a large family of natural products that are produced by bacteria, fungi, and plants. Polyketide chain assembly is catalyzed by polyketide synthase (PKS). Three basic types of bacterial PKSs are known to date (Shen 2003). Bacterial type I PKSs are modules which contain one or more large multifunctional protein subunits similar to typical domains of fatty acid synthases [KS (ketoacyl synthase), AT (acyltransferase), KR (ketoreductase), DH

(dehydratase), ER (enoyl reductase), ACP (acyl carrier protein), and TE (thioesterase)]. Each module harbors a set of distinct, non-iterative activities responsible for the catalysis of one cycle of polyketide chain elongation. The bacterial type I polyketides which resemble branched-chain fatty acids include large carboxylic compounds (e.g., deoxyerythronolide B; erythromycin A) (Cortes et al. 1990; Donadlo et al. 1991; Hutchinson 1999; Staunton and Weissman 2001; Shen 2003). Bacterial type II PKSs are multienzyme complexes that carry a single set of iterative activities. The type II bacteria polyketides usually contain two or more aromatic rings fused into polycyclic structures, such as tetracenomycin C, actinorhodin, and doxorubicin (Malpartida and Hopwood 1984; Motamedi and Hutchinson 1987; Staunton and Weissman 2001; Shen 2003). Bacterial type III PKSs are iterative homodimeric enzymes that act iteratively and independently of ACP to synthesize aromatic polyketides, which are often monocyclic or bicyclic, such as chalcone and stilbene (Funa et al. 1999; Shen 2003).

Fungal secondary metabolites are often synthesized by defined biosynthetic pathways using various precursors such as shikimic acid, tricarboxylic acid, fatty acid, polyketides, terpenoids, or amino acids (Keller et al. 2005). Fungal polyketides contain a diverse range of structures, including a wide range of pigments, mycotoxins, and phytotoxins such as naphthopyrone, aflatoxin (AF)/sterigmatocystin (ST), fumonisin, lovastatin, compactins, melanins, and cercosporin.

Carbon skeletons of fungal polyketides are typically synthesized by the fungal iterative type I PKSs that are composed of a single multifunctional polypeptide with a set of active site domains similar to a module of bacterial type I PKSs, but they carry out biosynthetic reactions repeatedly (Shen 2003). Recently, the type III PKSs have been found in some filamentous fungi, such as *Neurospora crassa* and *Aspergillus oryzae* (Seshime et al. 2005; Funa et al. 2007).

PKSs are structurally and functionally similar to eukaryotic fatty-acid synthases (FASs), both catalyzing sequential condensations between ACP (acyl carrier protein)-linked acyl-thioesters, such as acetyl-CoA and malonyl-CoA (Fig. 1-5) via decarboxylation. However, fungal type I PKSs do not always process β-keto reduction, dehydration, and enoyl reduction that are essential for the full processing of the β-carbon in fatty acids. All fungal PKSs contain β-ketoacyl synthase (KS), acyltransferase (AT) and acyl carrier proteins (ACP) domains for assembling polyketide backbone, whereas many of them, unlike FASs, lack ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) domains (Fig. 1-5A).

Similar to FASs, fungal PKSs iteratively add a two-carbon unit to polyketide chains. Generally, but not all, biosynthesis of fungal polyketides, such as cercosporin, is initiated when acetyl and malonyl coenzyme A (CoA) are covalently linked, as thioesters, to the 4'-phosphopantotheine of an acyl carrier (ACP) domain through the acyltransferase (AT) domain. Condensation via decarboxylation then occurs with another thioester intermediate that is attached to the ketoacyl CoA synthase (KS) domain. The KS domain is involved in both chain initiation and elongation. After condensation, some of β-ketothioesters can be further modified by the action of other domains such as ketoreductase (KR), dehydratase (DH), or enoyl reductase (ER) domains if they are present. Further modifications may include cyclization, oxidation, hydration, and methylation. PKSs that are required for squalestatin biosynthesis in *Phoma* sp. and fusarin C production in *Fusarium moniliforme* and *F. venenatum*, contain a methyltransferase (MT) domain that adds methyl groups to the α-carbon of the thioester (Fig. 1-5B) (Hopwood and Sherman 1990; Bender et al. 1999; Cox et al. 2004; Song et al. 2004).

Once the polyketide chains are completed, they will be released from the enzyme complex by the function of thioesterase (TE)/cyclase (CYC) domain that also catalyzes further ring

closure. Further modification processes involving monoxygenases, dehydrogenases, esterases, O-methltransferase, reductase, and oxidase contribute to a remarkable diversity of polyketide-secondary metabolites in nature.

Gene Clusters

Biosynthetic genes that are involved in the production of fungal secondary metabolites are frequently coordinately regulated and tightly clustered in the genomes (Zhang et al. 2004). Such examples include the genes involved in the biosynthesis of penicillin by *Penicillium* spp. (Smith et al. 1990), cercosporin by *Cercospora nicotianae* (Chen et al. 2007), aflatoxin (AF)/sterigmatocystin (ST) by *Aspergillus* spp. (Yu et al. 2004), ergot alkaloids by *Claviceps pupurea* (Tudzynski et al. 1999), fumonisin by *Gibberella moniliformis* (Proctor et al. 2003), ochratoxin A by *Penicillium nordicum* (Karolewiez and Geisen 2005), aurofusarin by *Fusarium graminearum* (Malz et al. 2005), and melanin by *Alternaria alternate* (Kimura and Tsuge 1993). Regulation of the clustered genes is often governed by a pathway-specific regulator and also influenced by many global regulatory factors which usually respond to various environmental cues, such as carbon/nitrogen sources, light, and pH. The gene encoding a pathway-specific transcription factor is often embedded within the clusters. It is currently unclear why fungal genes that are responsible for secondary metabolite biosynthesis tend to be clustered. The clustering of genes in fungi may evolve vertically through meiotic or mitotic processes due to environmental selections or simply are obtained via horizontal gene transfer from prokaryotes (Rosewich and Kistler 2000; reviewed by Walton 2000). Horizontal transfer has been proposed to be a stable transfer of genetic material between microorganisms (Rosewich and Kistler 2000). Horizontal transfer via assorted processes, such as conjugation, transformation, or transduction in bacteria is very common. However, the mechanisms involved in horizontal gene transfer in

eukaryotes are largely unknown, and no direct evidence is available to explain how horizontal transfer occurs in eukaryotes including fungi.

Research Overview

The major goal of this research is to determine the function of elsinochromes produced by *Elsinoë fawcettii*. Elsinochromes are structurally similar to many photosensitizing perylenequinones, which has led to speculation that elsinochromes may function as photosenitizers and are required for fungal virulence during plant-pathogen interactions. In this study, biochemical, molecular, and genetic approaches were employed to investigate the virulence determents that are required for *Elsinoë fawcettii* to invade citrus. Particular emphasizes was placed on the elsinochromes produced by *E. fawcettii.* Protocols for extraction and quantification of elsinochromes were established and the toxicity of elsinochromes to plant cells was determined to occur through generation of reactive oxygen species, primarily singlet oxygen and superoxide (Chapter 2). Environmental stimuli affecting production of elsinochromes in culture was also investigated (Chapter 3). Molecular and genetic approaches were employed to conclusively determine the role of elsinochromes in fungal pathogenesis and lesion development by cloning and disruption of an *EfPKS1* gene, encoding a fungal type I polyketide synthase in *E. fawcettii* (Chapter 4). Chromosome walking and sequence analysis beyond the *EfPKS1* gene allowed identification of additional genes whose products might also be required for elsinochrome biosynthesis and regulation, indicating that some of the genes involved in elsinochrome biosynthesis are clustered in the genome of *E. fawcettii* (Chapter 5). Targeted disruption of a *TSF1* gene encoding a putative transcription regulator generated fungal mutants that failed to accumulate any measurable elsinochromes and displayed reduced expression of the genes including *EfPKS1* in the cluster (Chapter 5). A survey of 52 isolates of *E. fawcettii* obtained from citrus-growing areas in Florida revealed variations in fungal

pathogenicity to rough lemon, grapefruit, and sour orange and elsinochrome production among isolates. DNA polymorphism analyses identified several *E. fawcettii* variants or pathotypes that were not previously recognized in Florida (Chapter 6).

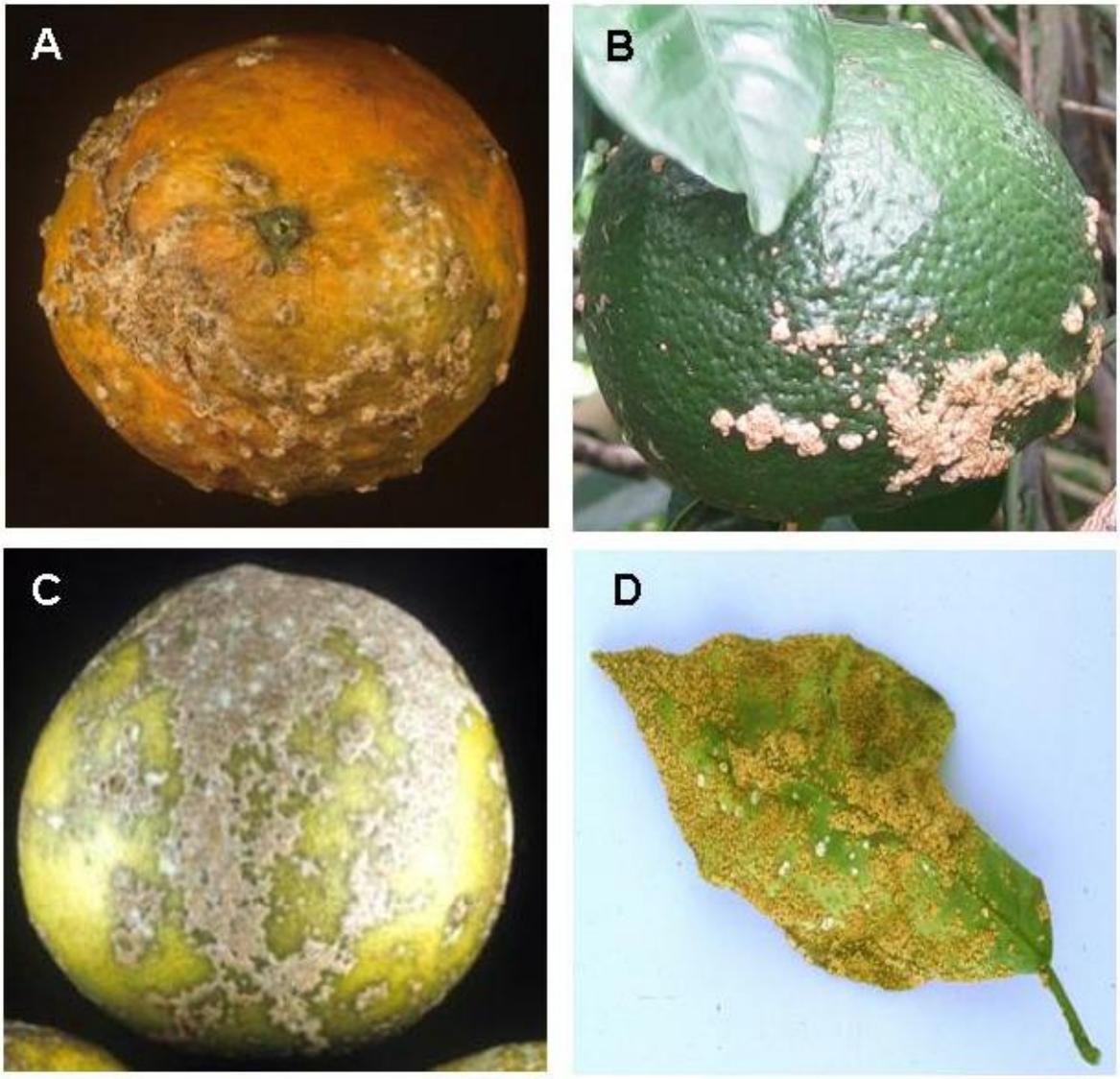


Figure 1-1. Symptoms of citrus scab caused by the pathogenic fungus, *Elsinoë fawcettii*. Warty and scabby blister-shaped growths are produced on fruit of Murcutt (A) and Temple (B). C) The infected grapefruit shows the flatter symptoms. D) The infected tangerine leaf is covered with scabby and corky pustules. (Courtesy L. W. Timmer)

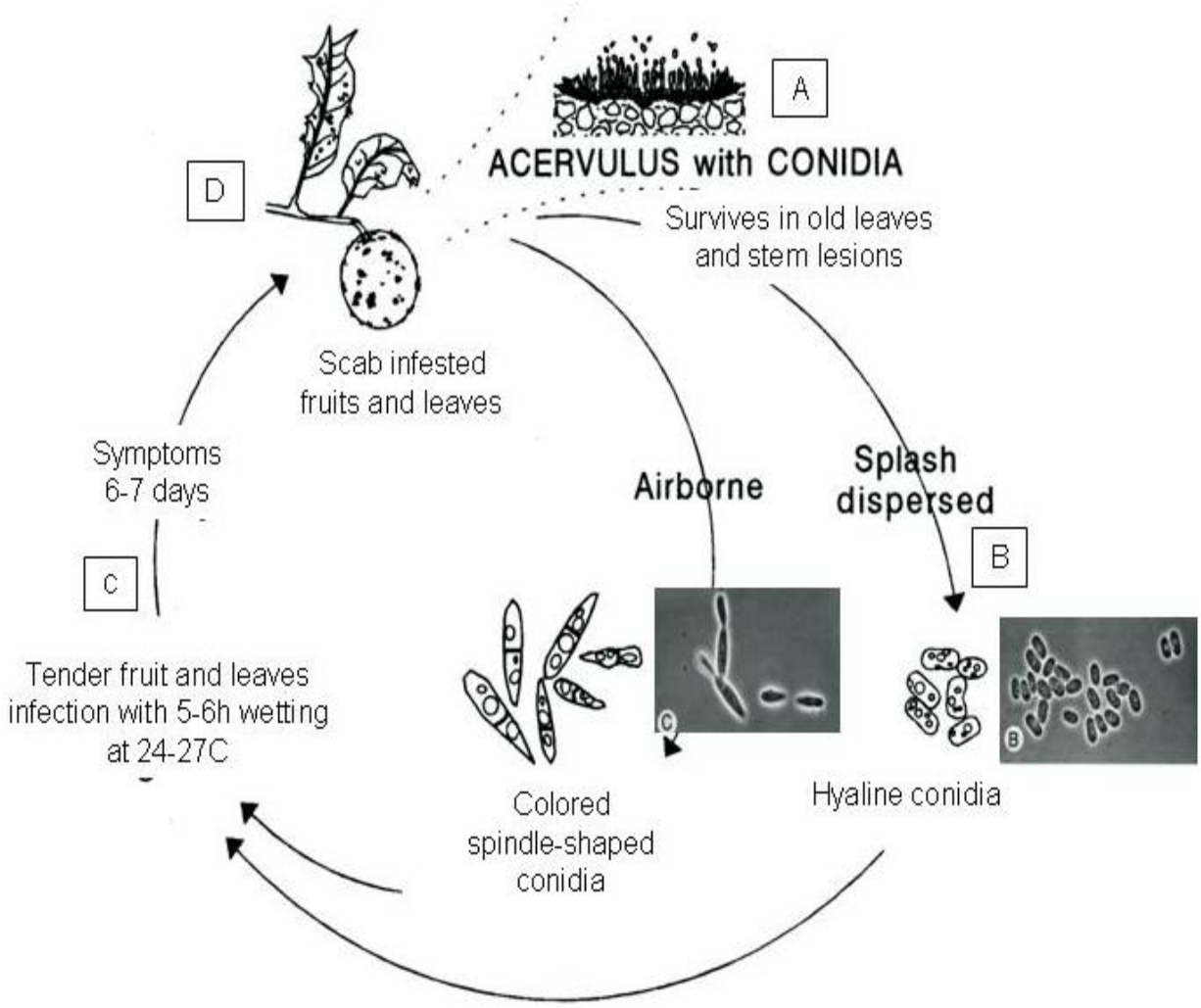
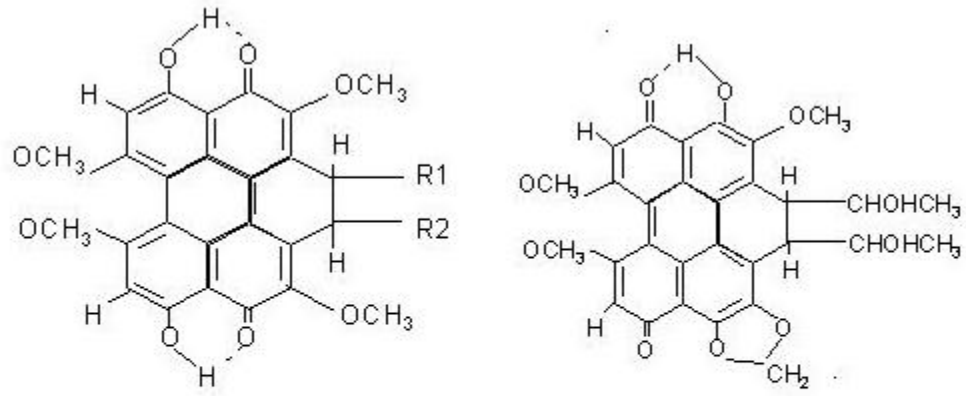


Figure 1-2. Life cycle of *Elsinoë fawcettii*, the causal agent of citrus scab (Whiteside 1988; Timmer et al. 2000).



Elsinochrome A: R1 = R2 = -CO-CH_3
Elsinochrome B: R1 = -CO-CH_3, R2 = -CHOH-CH_3
Elsinochrome C: R1 = R2 = -CHOH-CH3

Elsinochrome D

Figure 1-3. Structure of elsinochromes with various side-groups (R) (redrawn based on the work of Weiss et al. 1987).

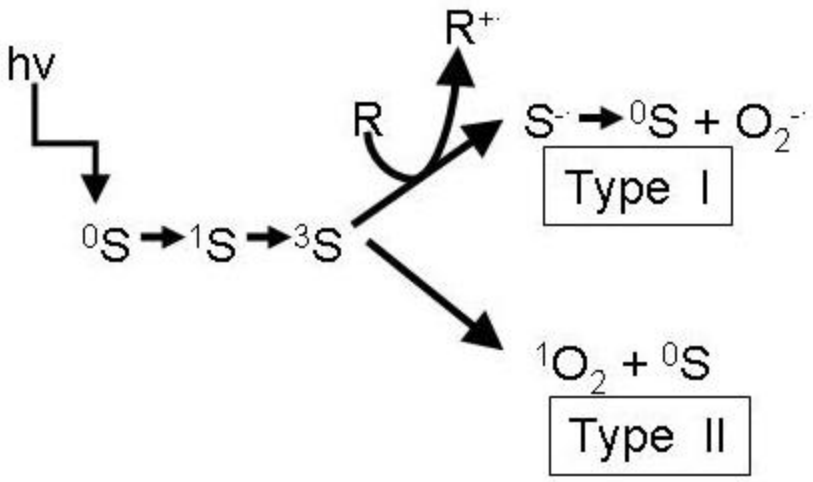


Figure 1-4. Proposed model for the formation of activated oxygen species by photosensitizers. The ground-state photosensitizers (^0S) absorb light energy, and are converted to the excited singlet (^1S) and then long-lived triplet (^3S) state. The activated sensitizers may react with oxygen molecules via electron transfers in the presence of a reducing substrate (R) to yield the radical forms of oxygen species, such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), or the hydroxyl radical ($OH^{\bullet}$) (type I reaction). Alternatively, triplet sensitizers may react directly with oxygen by an energy transfer reaction to produce non-radical singlet oxygen ($^1O_2$) (type II reaction) (Daub and Ehrenshaft 2000).

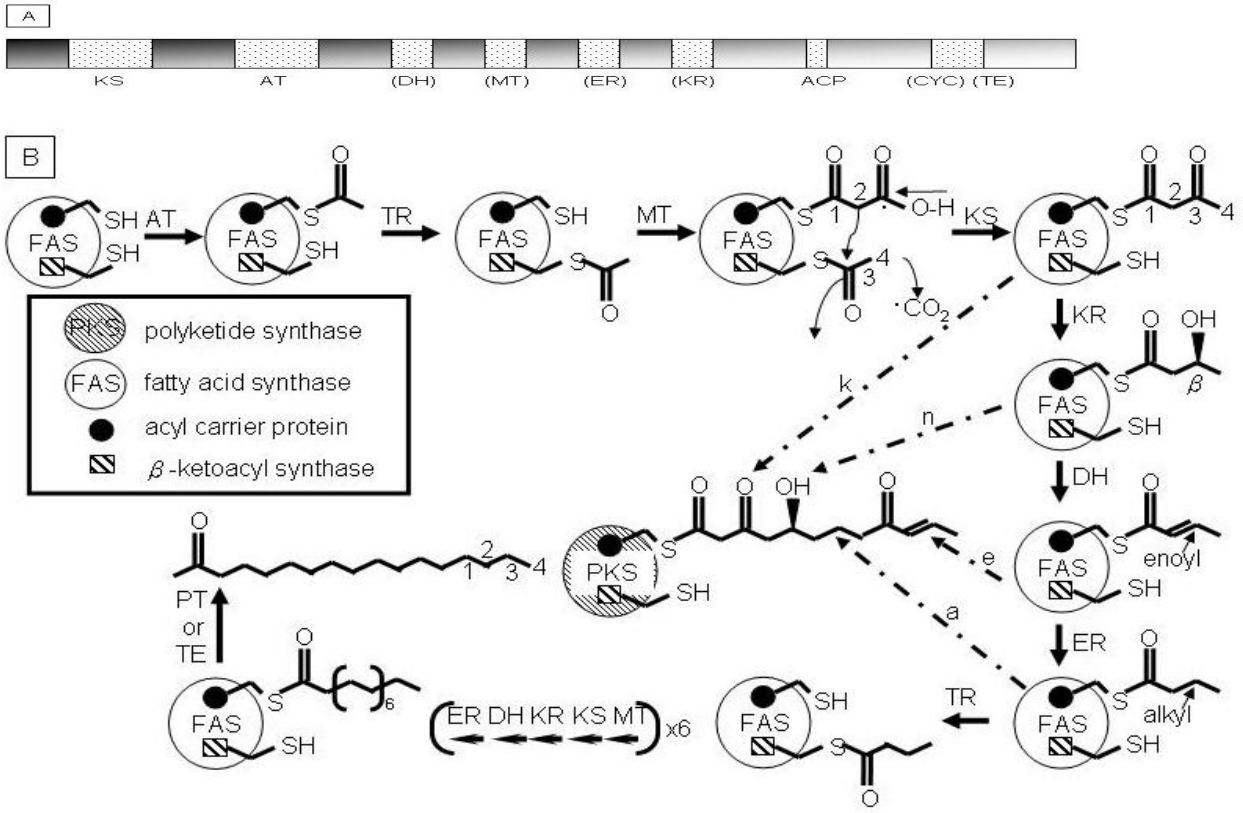


Figure 1-5. Biosynthesis of fatty acid and polyketide in fungi. A) Conserved domains found in the fungal polyketide synthase (PKS). Fungal type I PKSs produce a single, large polypeptide with multifunctional domains. Formation of the polyketide backbone requires ketoacyl CoA synthase (KS), acyltransferase (AT), and acyl carrier (ACP) domains. Other domains (such as dehydratase (DH), methyltransferase (MT), enoyl reductase (ER), ketoreductase (KR)) in brackets are not essential for all fungal PKSs. B) An overall scheme for fatty acid synthesis and polyketide biosynthesis. In fatty acid synthesis, acetyl-CoA and malonyl-CoA are converted into acetyl-ACP and malonyl-ACP, respectively, by acetyl and malonyl transacylase. Fatty acid synthase (FAS) subsequently condenses the two precursors, via a cycle of reduction, and dehydration in the keto group. Biosynthesis of polyketide in fungi is somewhat similar to fatty acid. Polyketide synthase (PKS), resembling FAS, is responsible for condensation of precursors. The major difference between FAS and PKS is that in addition to acetyl-CoA and malonyl-CoA, PKS may use different substrates as the starter and extender groups. Furthermore, PKSs produce diverse products based on the extent of reduction. The pathways, indicated by the letters (k, n, e, and a), represent various possibilities to yield keto, hydroxyl, enoyl or alkyl group into the growing polyketide chain after each condensation step. The carbon atoms of malonate and acetate contributing to polyketide chain are indicated by numerals. The carbon of malonate that is eliminated as CO_2 is indicated by asterisks. The abbreviations: KS, ketoacyl CoA synthase; AT, acyltransferase; DH, dehydrase; MT, methyltransferase; ER, enoyl reductase; KR, ketoreductase; ACP, acyl carrier protein; CYC, cyclase; TE, thioesterase; PT, palmityl transferase. (This figure is redrawn from the work of Hopwood and Sherman 1990; Bender et al. 1999).

CHAPTER 2
CELLULAR TOXICITY OF ELSINOCHROME PHYTOTOXINS PRODUCED BY *Elsinoë fawcettii*

Elsinochrome pigments (ESCs) were extracted from mycelia of cultured *Elsinoë fawcettii* by acetone and tested for cellular toxicity in this chapter. Thin-layer chromatography (TLC) analysis was used to separate and identify at least five different derivatives of ESCs. A spectrum scanning analysis of the crude extract of ESCs as well as the five distinct bands recovered from TLC plates revealed an identical absorbance spectrum, showing a major absorbance at 460 nm with two minor peaks at 530 and 570 nm. Chemical analyses revealed that the pigments extracted from *E. fawcettii* cultures contain phenolic quinines, resembling ESC phytotoxins that have been characterized in other *Elsinoë* spp. Upon irradiation with light, ESCs rapidly killed suspension-cultured citrus and tobacco cells. The toxicity was decreased by adding the singlet oxygen ($^1O_2$) quenchers, bixin (carotenoid carboxylic acid), DABCO (1, 4-diazabicyco octane), the ascorbate, or reduced glutathione. Application of ESCs onto rough lemon leaves induced necrotic lesions, whereas lesion development was inhibited by the addition of bixin, DABCO, or ascorbate, but not α-tocopherol. Incubation of rough lemon leaf discs with ESCs in the light resulted in a steady increase of electrolyte leakage. Similar with two photosensitizing compounds, hematoporphyrin and cercosporin, the accumulation of $^1O_2$ by ESCs after irradiation was indicated by successful detection of the cholesterol oxidation product, 5α-hydroperoxide. Addition of a potent quencher, β-carotene, prevented 5α-hydroperoxide production. ESCs generated superoxide ions ($O_2^{\bullet-}$), whereas accumulation of $O_2^{\bullet-}$ was blocked by addition of the superoxide dismutase, a scavenger of $O_2^{\bullet-}$, but not the $^1O_2$-quencher, DABCO. These experiments indicated that ESCs function as photosensitizing compounds that produce $^1O_2$ and $O_2^{\bullet-}$, and exert toxicity to plant cells.

Introduction

Elsinoë fawcettii Bitancourt & Jenkins is the causal agent of citrus scab. This disease is widely distributed, occurring in many citrus growing areas where rainfall conditions are conducive for infection. Conidia (asexual spores) are produced from the imperfect stage of the fungus, *Sphaceloma fawcettii* Jenkins, and serve as the primary source for inoculation in the field (Hyun et al. 2001).

A large number of *Elsinoë* species are able to produce red pigments, named elsinochromes (ESCs) (Meille et al. 1989). The structures of ESCs have been well established (Weiss et al. 1957; Weiss et al. 1965; Meille et al. 1989). ESCs contain at least four derivatives, A, B, C and D, differing in side groups. Those derivatives all have a basic 4,9-dihydroxyperylene-3,10-quinone chromophore similar to several perylenequinone compounds grouped as photosensitizers based on their ability to sensitize cells to visible light and produce reactive oxygen species (ROS) (Yamazaki et al. 1975; Daub 1982a). Light and oxygen are absolutely required for the photodynamic function and toxicity of these compounds. Photosensitizing compounds absorb light energy and convert to a stable electronically excited state (triplet state) which in turn reacts with oxygen to produce toxic reactive oxygen species (ROS) such as superoxides ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and/or singlet oxygen ($^1O_2$) (Dobrowolski and Foote 1983; Girotti 1990). In the Type I reaction, the activated triplet photosensitizers can react with oxygen molecules directly by transferring a hydrogen atom or electron from reducing substrates (such as NADPH, ascorbate, and L-cysteine), resulting in reduced oxygen species including superoxides ($O_2^{\bullet-}$) (Girotti 1990). In the Type II reaction, the activated photosensitizers react with oxygen molecules by an energy-transfer process, producing electronically reactive singlet oxygen ($^1O_2$) (Spikes 1989).

Both $^1O_2$ and $O_2^{\bullet-}$ are toxic to cells, causing the oxidation of various biological components including fatty acids, membranes, proteins/enzymes, sugars, and nucleic acids, and often resulting in cell death (reviewed by Daub et al. 2005). Many of the perylenequinone pigments produced by fungi have been shown: 1) to be toxic to mice, bacteria, and many fungi, 2) to be cytotoxic to animal tumors, 3) to be potent antiviral agents, and 4) to inactivate protein kinase C (Tamaoki and Nakano 1990; Hudson and Towers 1991; Diwu 1995; Hudson et al. 1997). Except for cercosporin produced by many members of the fungal genus *Cercospora* (Callahan et al. 1999; Choquer et al. 2005; 2007; Chen et al. 2007; Dekkers et al. 2007; Chen et al. 2007), none of the perylenequinones of fungal origin have been demonstrated to act as a crucial factor in plant diseases caused by the producing pathogen.

Many *E. fawcettii* isolates collected from Florida citrus growing areas produce red/orange pigments in culture (see details in Chapter 6). However, the identity of these pigments remains unknown. The aim of this study is first to isolate and characterize the pigments from *E. fawcettii*. This chapter describes methodology for isolation and analysis of the extracted pigments and reports their physical and chemical properties as ESC phytotoxins that have been described elsewhere by Weiss and his colleagues (1987). In addition, the extracted ESC-like pigments from one of the *E. fawcettii* isolates are demonstrated to be toxic to host and non-host plant cells. Evidence is also presented for function of ESCs as photosensitizing agents in culture and in *planta*, by producing toxic reactive oxygen species, mainly $^1O_2$ and $O_2^{\bullet-}$.

Materials and Methods

Biological Materials and Cultural Conditions

E. fawcettii Bitancourt & Jenkins (anamorph: *Sphaceloma fawcettii* Jenkins) CAL WH-1 isolate used in this study was single-conidium isolate from scab affected calamondin (*Citrus madurensis* Lour) fruit in Florida and was kindly provided by L. W. Timmer (University of

Florida, Citrus Research and Education Center, Lake Alfred, FL). The fungus was grown on a sterilized filter paper, and stored at -20 °C for long-term storage. Fungal cultures were routinely maintained on potato dextrose agar (PDA, Difco, Becton, Dickinson and Company, Sparks, MD, USA). For toxin production, fungal mycelia were minced with a sterile blender, spread on PDA and incubated under continuous fluorescent light for 5 days as previously described (Liao and Chung 2008a).

A cell suspension of tobacco (*Nicotiana tabacum* cv. Xanthi) was kindly provided by D. J. Lewandowski (Ohio State University, Columbus, OH, USA) and maintained in a Murashige and Skoog medium (Murashige and Skoog 1962) with gentle agitation under a daily regime of 16-h light and 8-h dark. Plant cells were subcultured weekly in a freshly prepared medium for toxicity assays. For protoplast isolation, 4-day-old tobacco cells were harvested and digested with cell-wall degrading enzymes (a mixture of 1.5 % cellulase and 0.15 % pectolyase) as described by Lewandowski and Dawson (1998). Sweet orange (*Citrus sinensis* (L.) Osbeck) suspension cells, kindly provided by J. W. Grosser (University of Florida, CREC, Lake Alfred, FL, USA), were maintained in a Murashige and Tucker medium (1969). Citrus protoplasts were prepared by incubating with 10 % of cell wall degrading enzyme complex (Sigma-Aldrich, St. Louis, MO) for 2 h as described by Grosser et al. (1988). Rough lemon (*Citrus jambhiri* Lush) trees were maintained in greenhouse and rapidly expanding immature leaves approx. 13-20 mm length and 4-7 mm wide, were harvested for toxicity assays.

Isolation and Analysis of the Red/Orange Pigments

The orange/red pigments secreted in culture were extracted twice from dried agar medium bearing fungal mycelia with acetone for 16 h. Organic solvent was combined and evaporated with a Model R110 of Rotavapor (Brinkmann, Buchi, Switzerland). For thin-layer chromatography (TLC) analysis, the pigments were dissolved in acetone, spotted onto TLC

plates coated with a 60 F254 fluorescent silica gel (5 x 20 cm, Selecto Scientific, Suwanee, GA, USA), and separated with a solvent system containing chloroform and ethyl acetate (1:1, v:v). The crude extracts separated by TLC were examined by a hand-held long wavelength UV lamp (UVP, San Gabriel, CA, USA). After separation, the pigments showing distinct bands were scraped from the TLC plate, dissolved in acetone, and separated from silica gel by low-speed centrifugation. The acetone was dried and the amounts of ESCs recovered were determined by weight. The pigments dissolved in acetone were examined by spectrophotometry at absorbance between 400 and 650 nm. Cercosporin was purified from cultures of *Cercospora nicotianae* in separate studies (Choquer et al. 2005; Chen et al. 2007). The extracted ESC toxins were directly mixed with plant cells for toxicity assays. Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Toxicity Assays to Plant Cells

Citrus or tobacco suspension cells or protoplasts at density 1 x 10^6 mL^{-1} were mixed with various concentrations of ESCs (dissolved in acetone) with or without singlet oxygen ($^1O_2$) quenchers, and placed on the top of 1 % agarose (in a 35 x 10 mm Petri dish). Cell cultures were illuminated with fluorescent lights at an intensity of 3.5 J m^{-2} s^{-1} at room temperature (~25 °C) for citrus cells or at 32 °C for tobacco cells. Light intensity was determined by a Dual-Display light meter (Control Company, Friendswood, TX, USA). Dark-grown cultures were wrapped in aluminum foil and incubated in the same conditions. Cells were examined over time by staining with 1 % Evan's blue (Taylor and West, 1980). Dead cells were stained blue as they cannot exclude Evan's blue, whereas live cells remained clear. Cell viability was determined with the aid of a hemocytometer using a microscope at x100 magnification. The percentage of cell viability was calculated by the number of live cells divided by the total number of cells

examined. Control cultures were untreated or cells treated with equal volumes of acetone and/or other solvents (final concentration < 1%) as appropriate.

The toxicity of ESCs on host plants was determined on detached rough lemon leaves (4-7 days after emergence). ESCs (1 mM, cercosporin equivalent) with or without antioxidants were applied onto the surface of rough lemon leaves and incubated in a moist chamber under fluorescent light (3.5 J m^{-2} s^{-1}), and monitored daily for development of necrotic lesions. Antioxidants used in this study include: bixin (carotenoid carboxylic acid), β-carotene, DABCO (1,4-diazabicyco octane), α-tocopherol (vitamin E), reduced glutathione, L-cysteine, and (+)-sodium L-ascorbate (vitamin C). Bixin, β-carotene, and α-tocopherol were dissolved in 95 % ethanol and others were dissolved in water to make a stock solution.

Detection of Singlet Oxygen and Superoxide Ions

Production of $^1O_2$ by ESCs upon irradiation was determined by their ability to oxidize cholesterol by the protocol of Daub and Hangarter (1983) with modifications. The $^1O_2$-generating photosensitizers, hematoporphyrin and cercosporin, were used as the positive controls. Photosensitizing compounds (8 mg each) in 20 mL pyridine were mixed with cholesterol (200 mg), and irradiated under a fluorescent light at intensity of 2.2 joules m^{-2} s^{-1} with gentle bubbling for 5 h. After solvent was removed, the oxidized products were suspended in 20 mL of hot methanol, passed through a filter to remove precipitation after cooling, and analyzed by TLC with a solvent system containing hexane-isopropanol (9:1 or 24:1, v:v). The oxidized products of cholesterol were visualized as bands after staining with a chromogenic reagent, N, N-dimethyl-*p*-phenylenediamine (1 %) dissolved in methanol-H_2O-glacial acetic acid (5:5:0.1, v/v/v) (Smith and Hill 1972; Smith et al. 1973). The cholesterol 5α-hydroperoxide standard was prepared by photo-oxidation with hematoporphyrin as described by Ramm and Caspi (1969).

Photochemical reduction of nitrotetrazolium blue chloride (NTB) was used to determine $O_2^{\bullet-}$ production by ESCs (Daub and Hangarter 1983). Reactions were carried out in a solution containing 5 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 10 mM methionine (as a reducing agent), 2.5 mM NBT, 2 μM riboflavin, 10 μM of ESCs or cercosporin, and superoxide dismutase (SOD, 1 mg mL^{-1}) or DABCO (1 mM). Nitrotetrazolium blue chloride was added into the buffer before the addition of photosensitizers and the reaction mixture was irradiated under a constant light at intensity of 4.7 J m^{-2} s^{-1}. Superoxide production, as shown the increase absorbance at 560 nm as a result of the reduction of NBT, was measured spectrophotometrically (Beruchamp and Fridovich 1971).

Determination of Electrolyte Leakage

Electrolyte leakage was measured by the method of Alferez et al. (2006) with some modifications. Leaf discs (0.5 cm in diameter) were cut from 4-day-old rough lemon leaves, placed in a 96-well microtiter plate, and incubated with ESCs (2 mM; dissolved in 7 % acetone) under constant fluorescent light (3.5 J m^{-2} s^{-1}) or in complete darkness at room temperature. Control leaf discs were treated with equal amounts of acetone or deionizer water as appropriate. Leaf discs (10) were randomly collected at 12-h intervals, soaked in water for an additional 1 h on a rotary shaker (60 r.p.m.), and measured for initial conductivity (IC). Leaf samples were immediately frozen in liquid nitrogen and kept at -80 °C for at least 12 h to obtain the remain conductivity of leaves. Total conductivity (TC) of leaves was determined after leaf discs were thawed at room temperature for 10-15 min. Conductivity was determined by a Model 115 Orion conductivity meter equipped with a Pentrode probe (Thermo Electron, Boston, MA, USA). Electrolyte leakage, expressed as percentage of total conductivity, was calculated by dividing initial conductivity (IC) by total conductivity (TC).

Statistical Analysis

Data were analyzed by ANOVA using SAS (PROCGLM) for PC (SAS Institute Inc., Cary, N.C.). When differences were significant ($P < 0.05$), individual treatment means were separated using Ducan's Multiple Range Test ($P = 0.05$).

Results

Isolation and Characterization of ESCs from *Elsinoë fawcettii*

Acetone extracts from cultures of an *E. fawcettii* isolate produced five distinct bands after TLC separation (Fig. 2-1). Bands 1, 2, and 3 [in order of decreasing R_f value (the ratio of the distance migrated by a substance compared with the solvent front)] appeared to be major compounds of the extracts based on band width, whereas bands 4 and 5 appeared to be minor compounds. Spectrophotometric scanning revealed that the acetone extracted pigments displayed a strong absorbance at 460 nm with two minor peaks at 530 and 570 nm (Fig. 2-2A) which resembles the ESCs previously isolated from several *Elsinoë* species (Weiss et al. 1957; 1965). Further analysis indicated that all five bands, recovered from TLC plates had similar absorption spectra, also displaying three major absorption peaks at 460, 530 and 570 nm (Fig. 2-2B, C, D, E, and F). In addition, the red/orange pigments from *E. fawcettii* had several typical characteristics of ESCs containing phenolic quinines. Similar to ESCs described by Weiss et al. (1957; 1987), the extracted red/orange pigments became bright green when dissolved in aqueous KOH or sodium carbonate (Fig. 2-3). Further, the green pigment reverted to pink when alkaline sodium dithionite was added (Fig. 2-3A). The green pigment changed to a distinct leuco compound, showing yellow/greenish fluorescence when treated with zinc dust (Fig. 2-3B). These results indicated that the red/orange pigments extracted from *E. fawcettii* were ESCs.

Toxicity of ESCs from *E. fawcettii* to Host and Non-Host Cells

The toxicity of ESCs was assayed using citrus protoplasts. As shown in Fig. 2-4A, ESCs exhibited dosage-response toxicities with respect to citrus protoplasts. At 10 μM, no viable citrus cells remained after 5 h in the light. At 5 μM, there was a decrease in the rate of cell death throughout the assay period. By contrast, untreated citrus cells or cells treated with acetone alone remained viable throughout the assay period. ESCs did not produce a toxic reaction when cells were incubated in complete darkness.

The toxic effect of ESCs was also evaluated with suspension-cultured tobacco cells and protoplasts. Similar to cercosporin produced by a tobacco pathogen, *C. nicotianae* (Daub 1982a), ESCs caused rapid death of tobacco protoplasts (Fig. 2-4B) or cultured suspension cells (Fig. 2-4C), in a dose-response manner within 1 h after irradiation with light. Untreated or acetone-treated tobacco cells in the light or cells incubated in the darkness remained viable for the duration of the experiment.

Antioxidants Reduce ESC Toxicity

The toxicity of ESCs was alleviated to various degrees by adding 400 μM bixin (Fig. 2-5A), 2 mM DABCO or 4 mM of ascorbate, or reduced glutathione (Fig. 2-5B-D). Compared with bixin, DABCO, and ascorbate, reduced glutathione had less effect on the protection against toxicity of ESCs, by showing an extended lag period. Application of antioxidants with lower concentrations had little or no effect on reduction of toxicity by ESCs (data not shown). Addition of α-tocopherol or L-cysteine (4 mM each) appeared to enhance elsinochrome phytotoxicity as the duration of incubation increased (Fig. 2-5E and F).

Similar to citrus protoplasts, the cellular toxicity of ESCs to suspension-cultured tobacco cells was alleviated by the addition of antioxidants such as bixin, DABCO, ascorbate, and α-tocopherol (Fig. 2-6A-D). In the presence of bixin or DABCO, over 40 % of tobacco cells were

viable after incubation with ESCs for 24 h in the light. Both ascorbate and α-tocopherol significantly delayed photo-induced cell death (by at least 18 h), yet neither compound was able to protect cell death beyond 24 h. Untreated tobacco cells incubated in the dark were healthy for the duration of the experiment (The inset of Fig. 2-4C).

ESCs are Toxic to Citrus Leaves

To determine if ESCs were toxic to the host, the crude extracts were applied onto detached rough lemon leaves. After incubated for 10 days, the ESC-treated spots on rough lemon leaves developed noticeable necrosis in the light (Fig. 2-7). Leaves treated with the solvent alone didn't developed necrosis (Fig. 2-7). Mixture of ESCs with bixin, DABCO, or ascorbate on the leaves prevented development of necrosis on detached rough lemon leaves (Fig. 2-7A-C). Bixin showed a light brown color when dissolved in 95 % ethanol. Application of α-tocopherol alone, however, resulted in a brownish necrotic spot and failed to alleviate the toxicity of ESCs (Fig. 2-7D). Application of superoxide dismutase (SOD) or hyperoxidase also had effects on protection against the toxicity of ESCs on rough lemon leaves (Fig. 2-8).

Production of Reactive Oxygen Species by ESCs

Production of $O_2^{\bullet-}$ by ESCs was evaluated with a superoxide scavenging assay (Beruchamp and Fridovich 1971; Daub and Hangarter 1983) based on its ability to reduce nitrotetrazolium blue chloride (NTB), and compared with the levels of $O_2^{\bullet-}$ induced by other photosensitizing compounds such as cercosporin and riboflavin known to generate $O_2^{\bullet-}$ (Oster et al. 1962; Daub and Hangarter 1983). In the absence of NTB, photosensitizers dissolved in the MOPS buffer produced lower absorbance values at 560 nm (Fig. 2-9A; Fig. A-1), representing the reaction baseline. Mixing NTB with the crude extracts of ESCs significantly elevated absorbance values because of reduction of NTB (Fig. 2-9A). However, the reactions were repressed in the presence of $O_2^{\bullet-}$ scavenging enzyme, SOD, indicating the production of superoxide. Compared with

cercosporin and riboflavin, ESCs appeared to induce high levels of $O_2^{\bullet-}$ after irradiation (Fig. 2-9B). Addition of SOD, but not DABCO, drastically reduced the accumulation of superoxides from the actions of ESCs (Fig. 2-9C).

Production of cholesterol 5α-hydroperoxide from cholesterol is one of the best and simplest ways to test for the presence of $^1O_2$ (Kulig and Smith 1973). To assess the production of $^1O_2$ *in vitro*, the extracted ESCs were mixed with cholesterol and illuminated. The resulting products were chromatographed in two different solvent systems and detected as distinct bands after staining with a chromogenic reagent, N, N-dimethyl-*p*-phenylenediamine. Reaction of cholesterol 5α-hydroperoxide with dimethyl-*p*-phenylenediamine resulted in pink pigments on the TLC plate which turned dark green 2h after staining. As with hematoporphyrin (positive control) and cercosporin photosensitizers, ESCs converted cholesterol by $^1O_2$-induced photodynamic oxidation into the 5α-hydroperoxides of cholesterol in the light (R_f 0.4 and 0.1 in Fig. 2-10A and B, respectively). No visible band was detected from the untreated cholesterol. Irradiation of cholesterol with UV light (240 nm) for 24h yielded several bands on TLC, but no cholesterol 5α-hydroperoxide was detected (Fig. 2-10C). A faint band with slightly faster migration of unknown identity was detected in products generated by cercosporin and ESCs. The cholesterol 5α-hydroperoxides and the faint bands were undetectable when β-carotene, a potent $^1O_2$ quencher, was added to the reaction mixture.

Electrolyte Leakage Induced by ESCs

When incubated with illumination for 24 h, the extracted ESCs induced an increase in electrolyte leakage of rough lemon leaf discs (Fig. 2-11A). Leaf discs treated with water or solvent alone, or leaf discs incubated in the dark showed relatively minor electrolyte leakage. Electrolyte leakage induced by ESCs in the light steadily increased over time, whereas leakage of the controls remained low (Fig. 2-11B).

Discussion

Elsinoë fawcettii isolates obtained in Florida citrus-growing areas produced red/orange pigments in culture similar to ESCs produced by many *Elsinoë* spp. (Weiss et al. 1957; 1987), and had a visible absorption spectra characteristic of ESCs. In addition, the orange/red pigments had other typical characteristics of ESCs, such as forming a distinct yellow/greenish fluorescence leuco compound when reacted with zinc dust in alkaline conditions (Weiss et al. 1957; 1987), sparing solubility in water, but readily soluble in several organic solvents, such as acetyl acetate and acetone. Thin-layer chromatography analysis of the acetone-extracted pigments revealed significant variations of the pigments produced in culture. The results strongly suggest that these pigments were ESC analogs containing a phenolic quinine chromophore.

Compared with studies of chemical characterization, little is known about the biological function of ESCs. The photodynamic action of ESCs leading to cellular toxicity has been predicted based on their structural similarity to many photosensitizing compounds such as cercosporin, hypericin, or hypocrellin A (Daub et al. 2005), yet this has never been demonstrated experimentally. In the present study evidence is presented for ESCs' toxicity to plant cells by functioning as photosensitizers that generate reactive oxygen species (ROS). Since citrus suspension cells aggregated to form massive clumps in culture, the toxicity assays were performed only with citrus protoplasts. ESCs extracted from *E. fawcettii* rapidly killed citrus protoplasts in a dose-dependent manner and only when cells were exposed to the light, consistent with the mode of action for other photosensitizing compounds (Yamazaki et al. 1975; Daub 1982a; Spikes 1989). The cellular toxicity of ESCs was attenuated considerably when antioxidant compounds were added into the culture, implying the involvement of reactive oxygen species, particularly $O_2^{\bullet-}$ and/or $^1O_2$. Carotenoids are efficient $^1O_2$ quenchers in the biological systems (Krinksy 1979). Bixin, a carotenoid carboxylic acid, has a lower molecular

weight than β-carotene and is more polar owing mainly to the presence of the carboxylic acid group (Daub 1982a). Both bixin and β-carotene have the same isoprenoid chain length but bixin is more soluble in aqueous solutions. Carotenoids quench $^1O_2$ via energy transfer mechanisms (Foote and Denny 1968; Foote et al. 1970) and are slowly consumed in the reaction.

DABCO (1,4-Diazabicyclo octane) also is an effective $^1O_2$ quencher. Unlike bixin, DABCO quenches $^1O_2$ by a chemical reaction, and is quickly consumed (Oannes and Wilson 1968; Daub 1982a). These contrasting properties may account for why bixin provided a more persistent protection of citrus cells than DABCO (Figs 2-5 and 2-6). In addition, the red pigmented ESCs became bright green when dissolved in aqueous DABCO, indicating a direct interaction between DABCO and ESCs. Both bixin and DABCO have been shown to decrease the toxicity of cercosporin to tobacco cells (Daub 1982a). ESCs were also toxic to tobacco, a nonhost plant of *E. fawcettii*. Similarly, addition of antioxidants also provided some protection against the toxicity of ESCs, indicating a nonhost specificity of ESCs. The antioxidants bixin, DABCO and ascorbic acid but not α-tocopherol decreased the toxicity of ESCs in culture and provided some protection on detached rough lemon leaves. However, the concentrations required for protection *in planta* were far higher than those in culture, likely owing to the poor penetration of antioxidants through the cuticle. Both ascorbic acid and the reduced form of glutathione were also effective in protecting citrus cells against ESCs. By contrast, L-cysteine and α-tocopherol had little or no effect on cellular protection against ESCs in culture and on citrus leaves. Application of α-tocopherol alone at a concentration of 500 mM on young rough lemon leaves induced necrosis. However, α-tocopherol provided some levels of protection against the toxicity of ESCs to tobacco cells. α-tocopherol, with a well-known ability to terminate radical chain oxidation by binding to cell membranes, has been often used in studies of nutritional or cell

membrane functions (Tinberg and Barber 1970; Tappel 1972). The requirement of α-tocopherol binding to membranes may contribute to its ineffectiveness in cellular protection against ESCs for citrus protoplasts and leaf tissues.

Photosensitizing compounds are able to generate reactive oxygen species upon activation by light (Yamazaki et al. 1975; Daub 1982a; Dobrowolski and Foote 1983; Spikes 1989; Girotti 1990). Results derived from this study on the toxic effect of ESCs *in vitro* and *in vivo* confirm that ESCs are damaging to citrus cells by the production of both $O_2^{\bullet-}$ and $^1O_2$. This study has demonstrated that antioxidants such as bixin, DABCO, and others capable of quenching $^1O_2$ provide substantial protection from the toxicity of ESCs in both cultured citrus and tobacco cells and on rough lemon leaves. Production of $^1O_2$ by ESCs was confirmed by successful detection of the cholesterol. 5α-hydroperoxide that is solely produced through the oxidation of cholesterol by $^1O_2$ (Kulig and Smith 1973), which is one of the best indications of $^1O_2$ production versus production of $O_2^{\bullet-}$ and other free radicals (Smith et al. 1973). Oxidation of cholesterol by $O_2^{\bullet-}$, UV or other radicals often generates multiple products, mainly the 7α- and 7β-hydroperoxides, but never produce the 5α-hydroperoxide (Smith et al. 1973). By contrast, the oxidizing reaction of $^1O_2$ with cholesterol primarily produces the 5α-hydroperoxide. Addition of β-carotene, an effective $^1O_2$ quencher, completely prevented the production of the 5α-hydroperoxide by hematoporphyrin, cercosporin and ESCs. Therefore, positive detection of the cholesterol 5α-hydroperoxide indicates that ESCs were able to generate $^1O_2$ after exposure to light. Singlet oxygen is highly reactive and has been shown to be toxic to a wide range of cells (Foote and Denny 1968; Foote et al. 1970; Daub and Ehrenshaft 2000). The results strongly implicate the involvement of $^1O_2$ in the toxicity of ESCs because $^1O_2$ quenchers reduced cultured citrus cell mortality and prevented necrosis on host leaves.

Production of $O_2^{\bullet-}$ by ESCs was demonstrated by a superoxide production assay used to determine SOD enzymatic activity with NTB as substrate (Beruchamp and Fridovich 1971; Daub and Hangarter 1983). It seemed that ESCs produced $O_2^{\bullet-}$ more efficiently than cercosporin. Reduction of NTB induced by ESCs was inhibited by adding the $O_2^{\bullet-}$ scavenging enzyme (SOD) but not the $^1O_2$ quencher, providing evidence to support that photodynamic reaction of ESCs also generates $O_2^{\bullet-}$. The detection of $O_2^{\bullet-}$ has important implication for involvement in ESC toxicity since $O_2^{\bullet-}$ also is toxic in biological systems.

To explore the potential toxic mechanisms of ESCs, electrolyte leakage of rough lemon leaf discs was measured after treatment with ESCs. Treatment with ESCs under light induced higher electrolyte leakage than water or solvent controls. The results suggested that ESCs damage cell membranes, likely by inducing lipid peroxidation as demonstrated in cercosporin-treated tobacco tissues (Daub 1982b). Unlike the rapidly (< 2 min) induced electrolyte leakage caused by cercosporin in tobacco, ESC-induced ion leakage was not observed until several hours after irradiation, indicating that the toxic activity on citrus membranes might not be direct. Alternatively, this slow response of electrolyte leakage might result from the complexity of cell membranes of citrus or the structure of its cuticle. Disruption of cell membranes followed by nutrient release is beneficial to the invading pathogen. Therefore, ESCs may play a critical role in pathogenesis during fungal penetration and colonization.

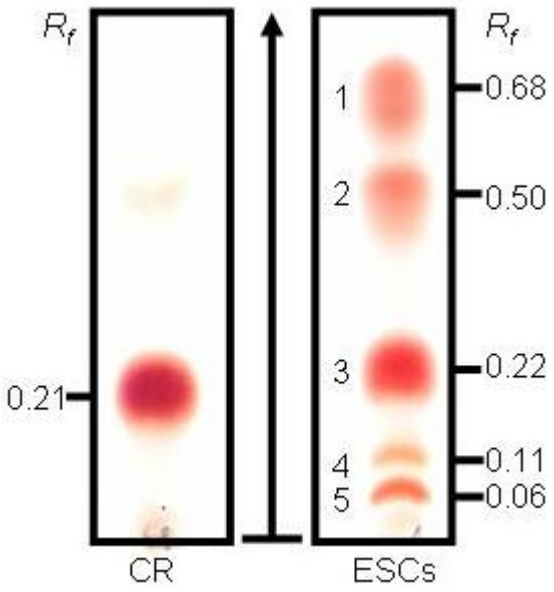


Figure 2-1. Thin-layer chromatography analysis of elsinochromes (ESCs) produced by *Elsinoë fawcettii* and compared to cercosporin (CR) extracted from a tobacco pathogen, *Cercospora nicotianae*. ESCs were extracted from agar plugs containing fungal mycelium with acetone, spotted onto a silica gel plate, and separated with chloroform and ethyl acetate, resulting in five distinct (red/orange) bands with R_f values (1-5).

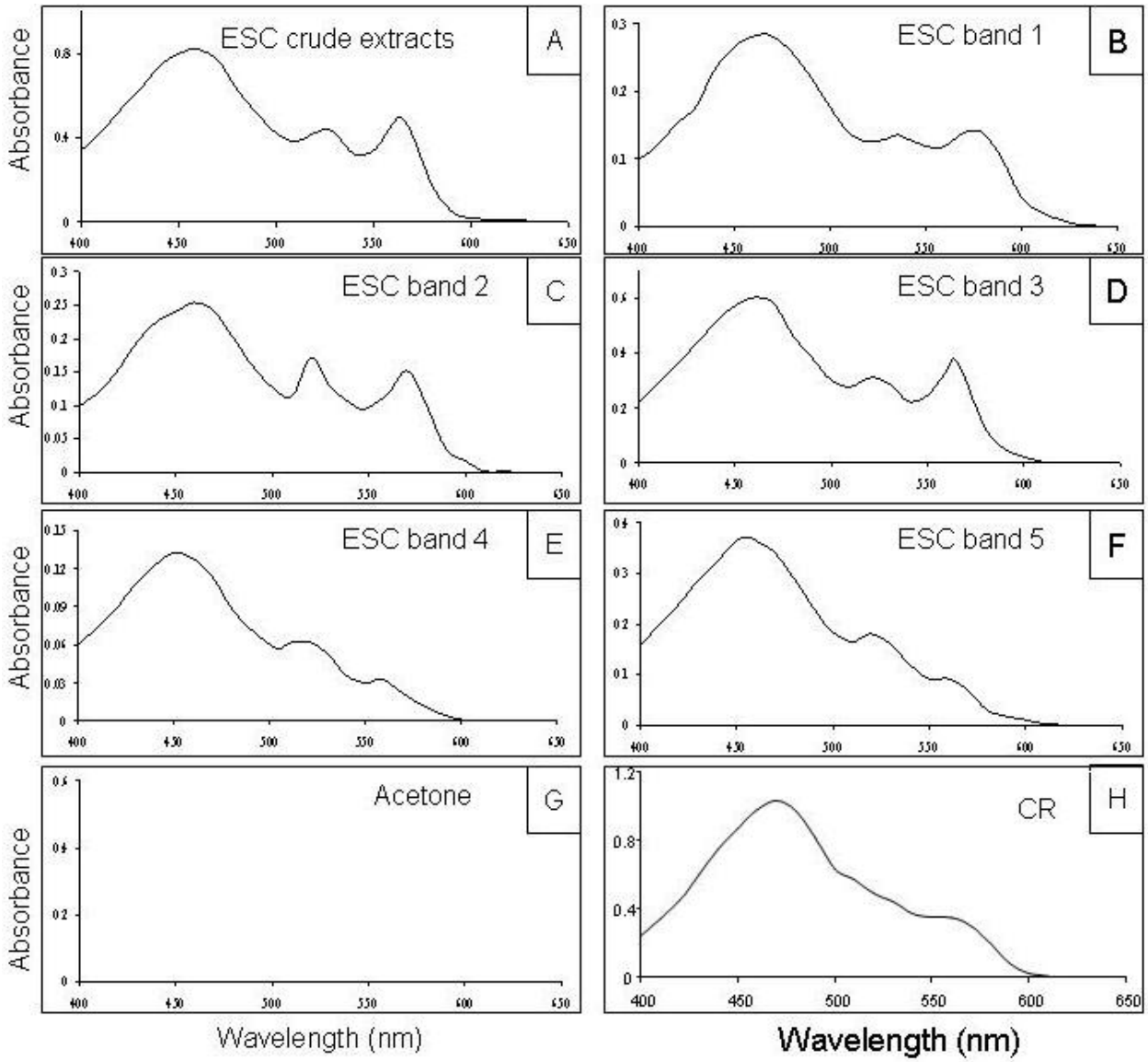


Figure 2-2. Absorption spectrum of the acetone crude extracts of ESCs (A), and individual bands, 1, 2, 3, 4 and 5, recovered from TLC plate (B, C, D, E, and F), showing three major peaks at 460, 530 and 570 nm. Solvent alone (acetone) and pure cercosporin (CR) (Sigma-Aldrich) were also used as controls (G and H).

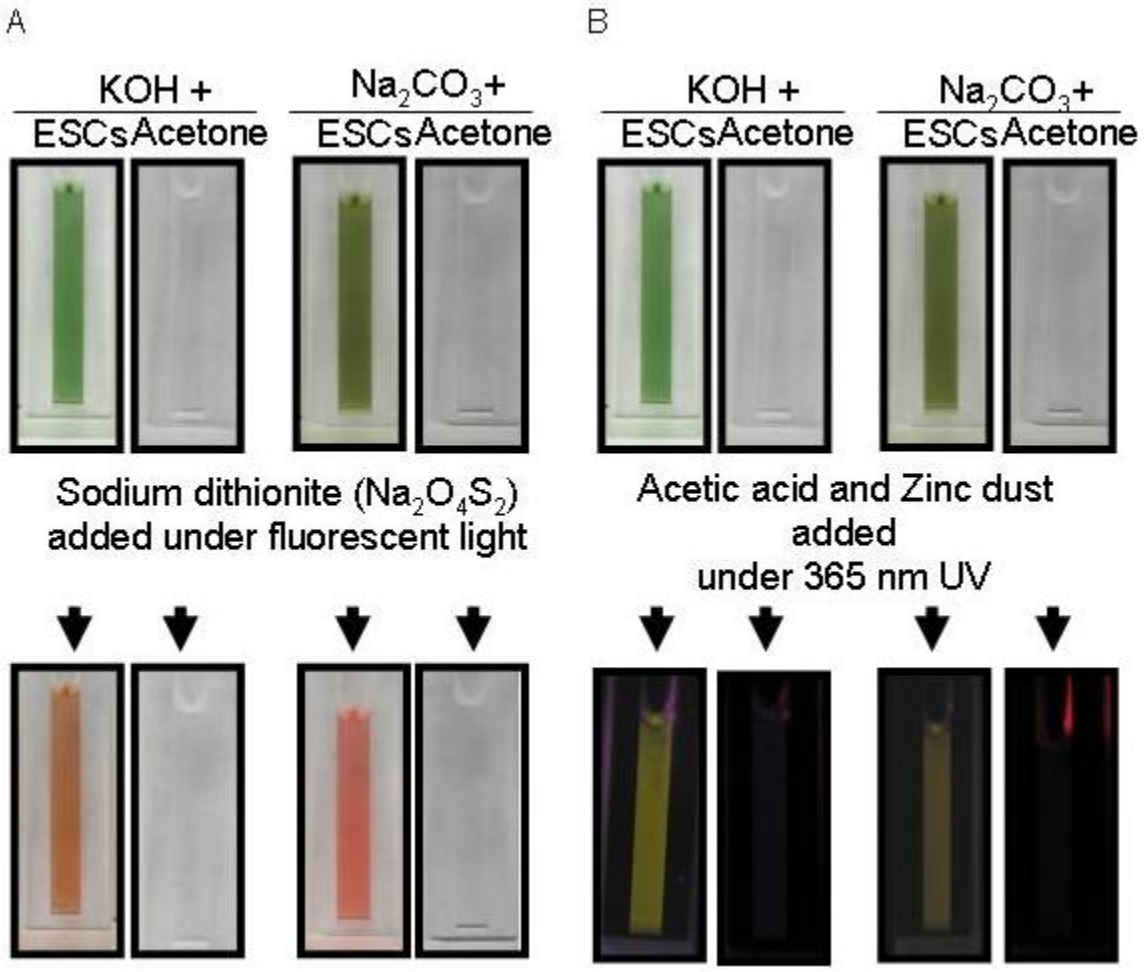


Figure 2-3. Chemical properties of phenolic quinines of the acetone extracted ESCs. The red/orange pigments appear bright green in color when dissolved in aqueous KOH or sodium carbonate. A) The green color was changed to a pink color when alkaline sodium dithionite was added. B) The green color was changed to yellow/greenish fluorescence immediately after treated with zinc dust in acidic conditions under UV light (365 nm). In all cases, acetone alone was used as a negative control.

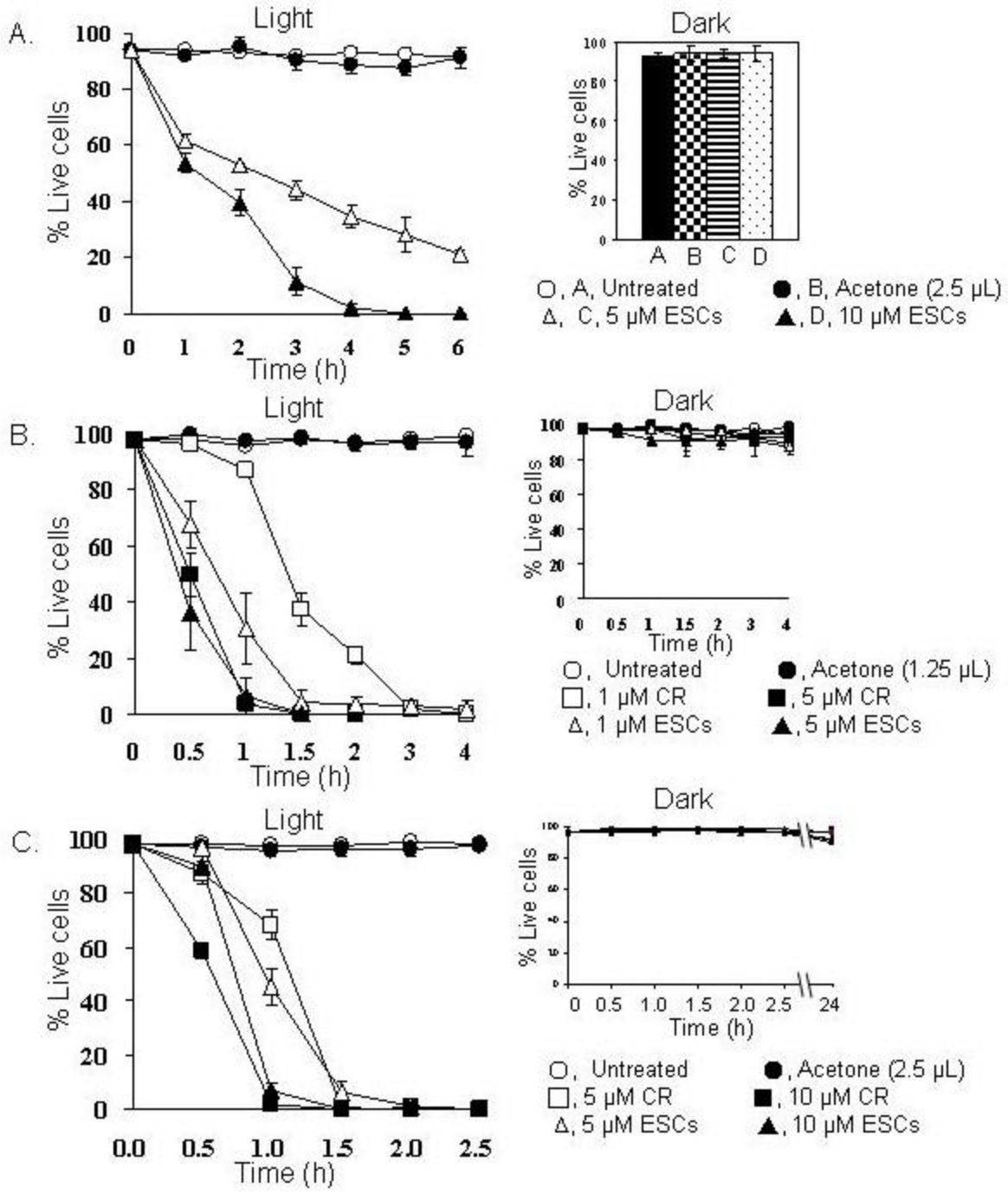


Figure 2-4. Reduction of live cells (%) of citrus protoplasts (A), tobacco protoplasts (B), or suspension-cultured tobacco cells (C) treated with elsinochrome (ESC) phytotoxins produced by *Elsinoë fawcettii* or Cercosporin (CR) toxin extracted from a tobacco pathogen, *Cercospora nicotianae*, and incubated under constant fluorescent light or in the darkness. Cell viability of citrus protoplasts in the darkness was determined only at 6 h after incubation (A). Insets indicate viability of plant cells incubated in the dark. Data represent the means of two different experiments with at least three replicates. Vertical bars represent standard deviation.

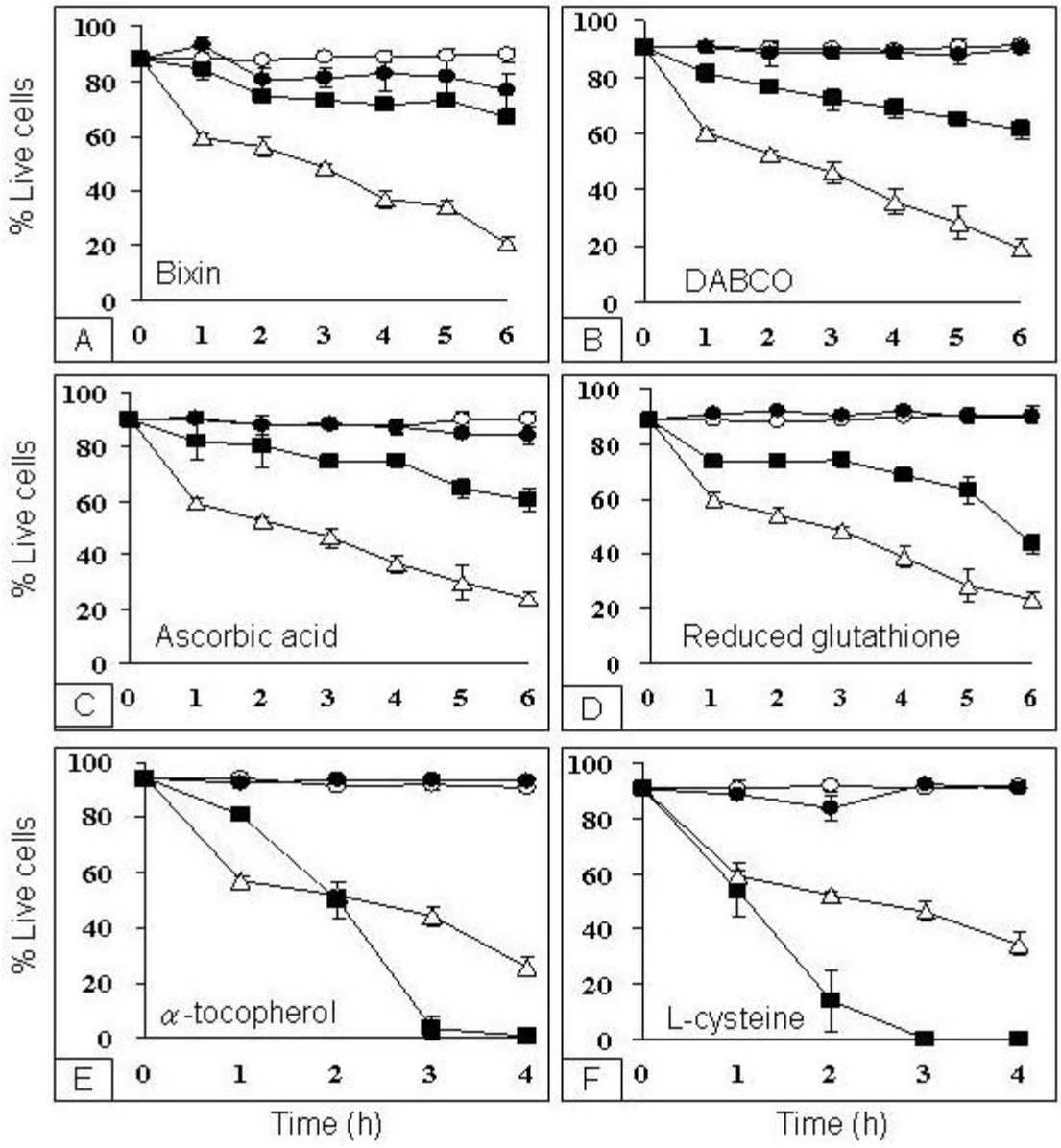


Figure 2-5. Cell viability (% live cells) of citrus protoplasts after treatment with elsinochromes (ESCs) isolated from *Elsinoë fawcettii* with and without antioxidant compounds: A) 400 µM bixin (carotenoid carboxylic acid); B) 2 mM DABCO (1,4-diazabicyco octane); C) 4 mM $_{L}$-ascorbic acid; D) 4 mM reduced glutathione; E) 4 mM α-tocopherol; and F) 4 mM $_{L}$-cysteine. Citrus protoplasts (1 x 10^6) were mixed with or without ESCs (5 µM, cercosporin equivalent) and antioxidants as indicated under constant fluorescent light. The controls were treated with acetone-H_2O or acetone-95% ethanol as appropriate. Data represent the means of two different experiments with at least three replicates. Vertical bars represent standard deviation. Open circles, control; closed circles, antioxidant; open triangles, ESCs; closed squares, ESCs + antioxidant.

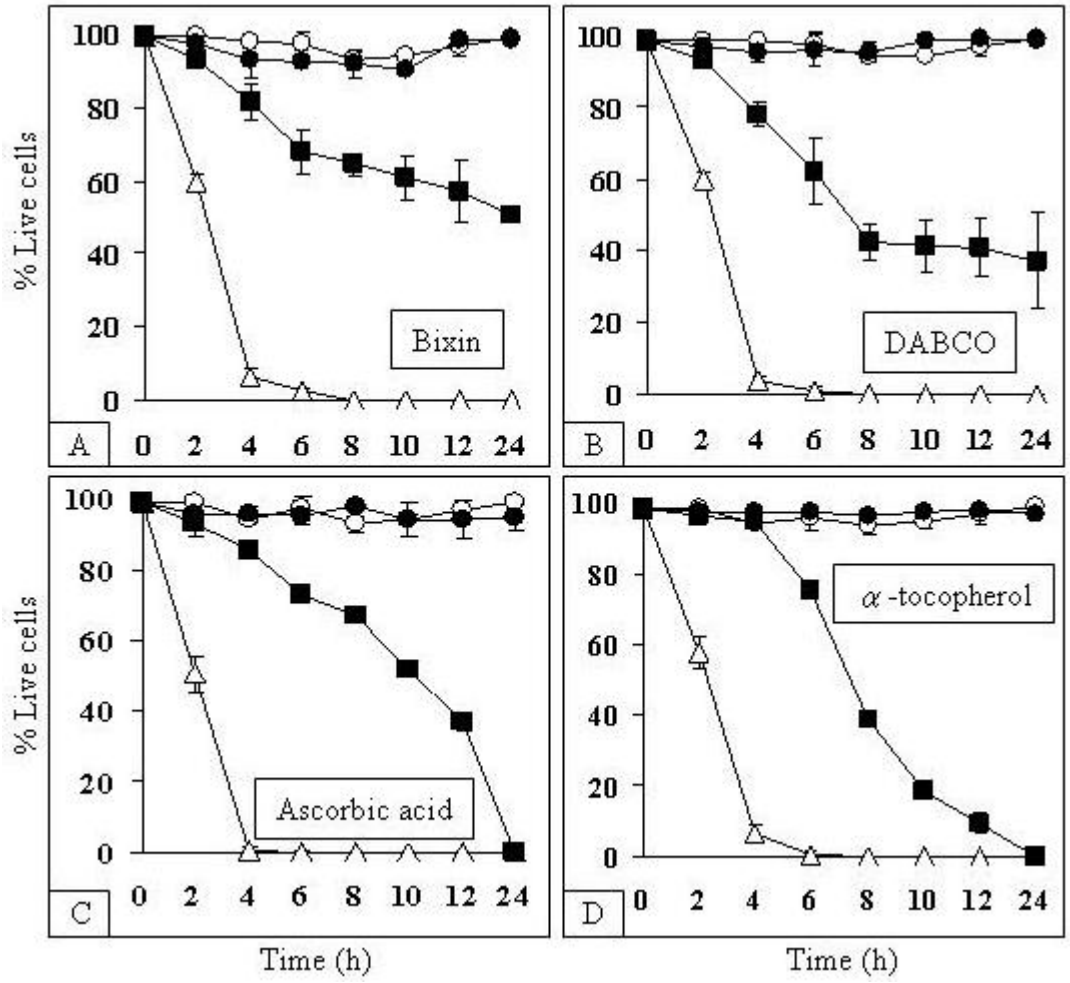


Figure 2-6. Cell viability (% live cells) in suspension-cultured tobacco cells by 1μM elsinochromes (ESCs) with antioxidants (400 μM bixin (carotenoid carboxylic acid), 2 mM DABCO (1,4-diazabicyco octane), 4 mM of $_{L}$-ascorbic acid, or α-tocopherol). Tobacco cells (1 x 10^6) were treated with ESCs in the presence or absence of antioxidants, as indicated, and incubated under constant fluorescent light. Data represent the means of two different experiments with at least three replicates. Vertical bars represent standard deviation. Open circles, control (1 μL acetone-ethanol); closed circles, antioxidant; open triangles, ESCs; closed squares, ESCs + antioxidant.

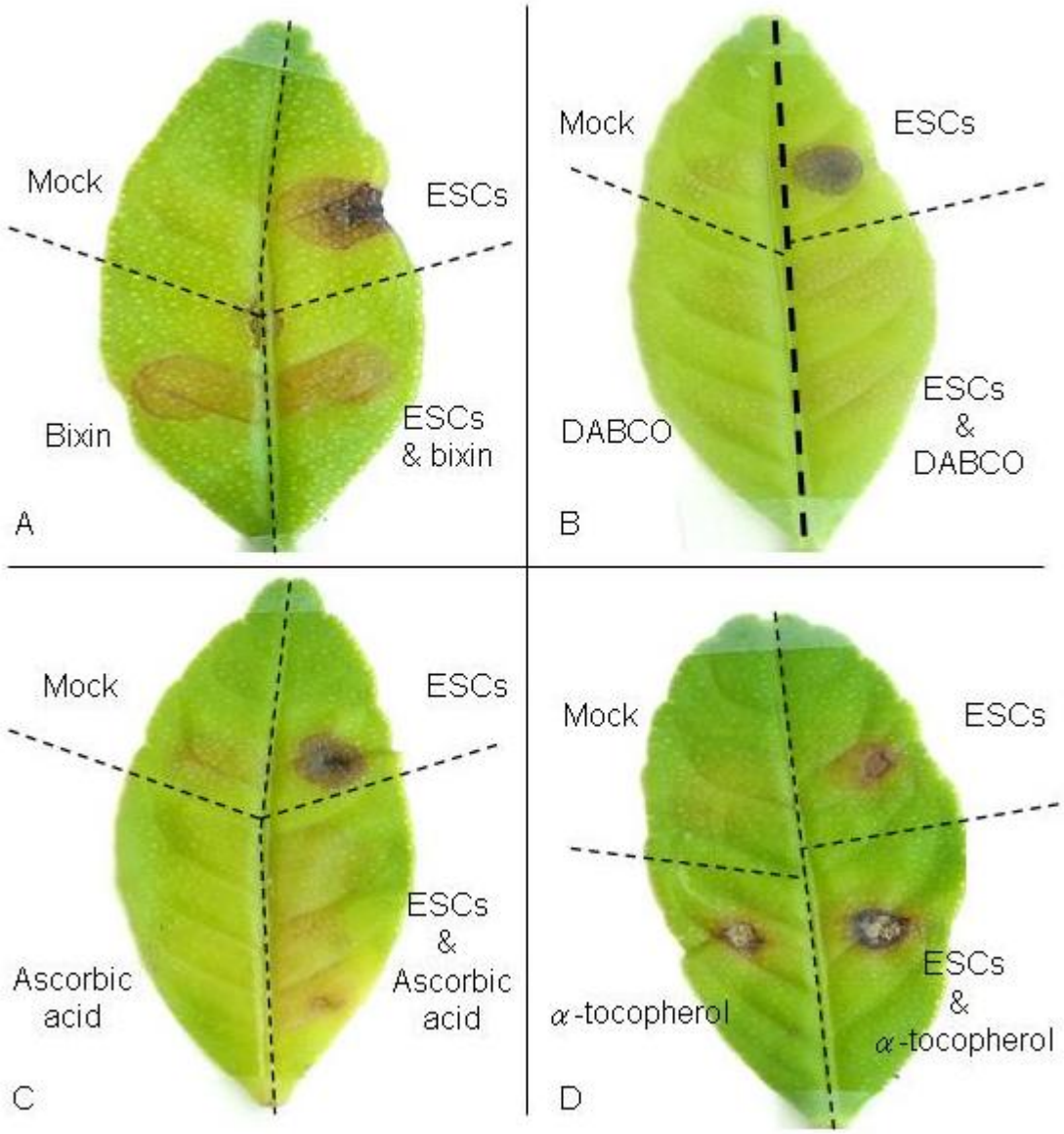


Figure 2-7. Development of necrotic lesions on detached rough lemon leaves after a 10-day application of 1 mM elsinochromes (ESCs) from *Elsinoë fawcettii*, and prevention of necrosis by co-application of antioxidants, A) 100 mM bixin (carotenoid carboxylic acid), B) 300 mM of DABCO (1,4-diazabicyco octane), C) 300 mM $_{L}$-ascorbic acid, or D) 500 mM α-tocopherol). Citrus leaves were treated with or without ESCs and antioxidants as indicated and incubated in a moist chamber under a constant fluorescent light. The mocks were treated with equal volume (3 µL) of water, 95% ethanol, and/or acetone as appropriate.

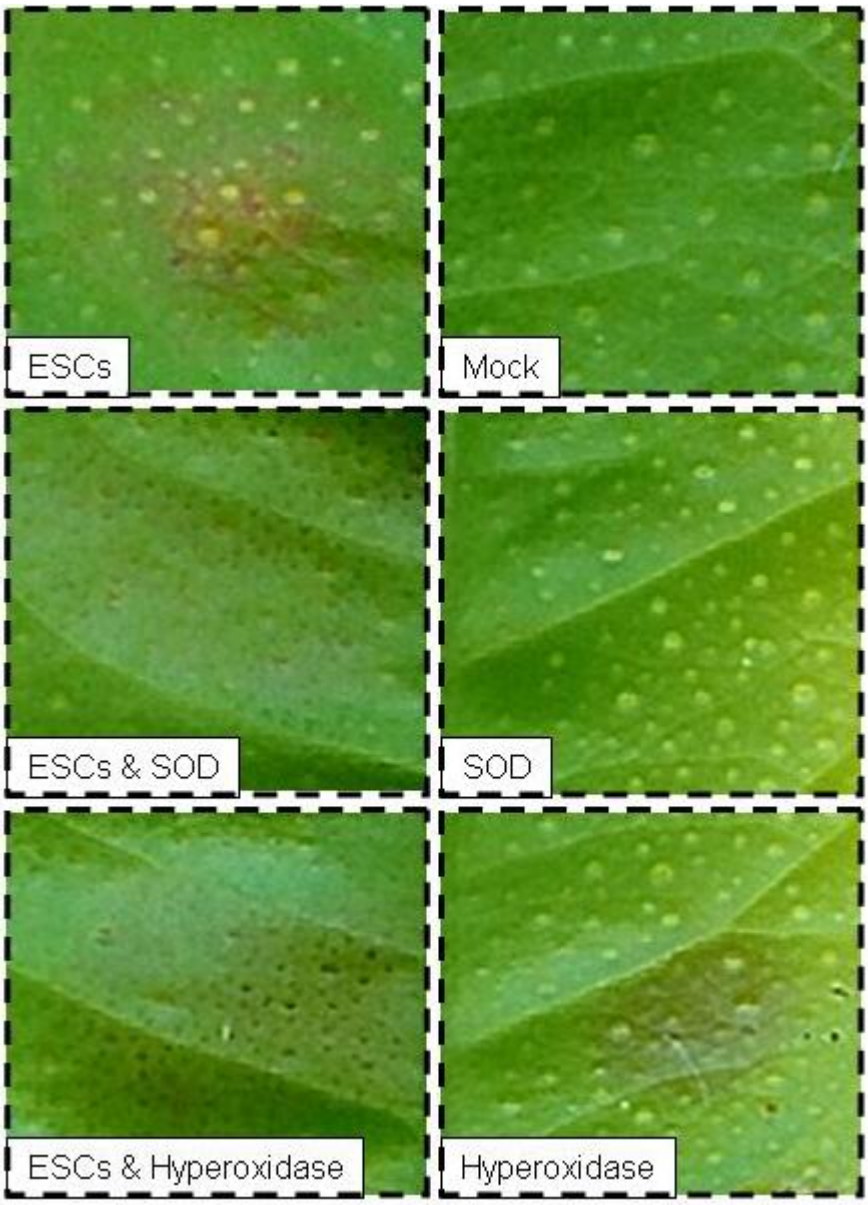


Figure 2-8. Development of necrotic lesions on detached rough lemon leaves 10 days after application of 100 μM elsinochromes (ESCs) from *Elsinoë fawcettii*. Co-application of ESCs and 100 μM superoxide dismutase (SOD) or 100 μg μL^{-1} hyperoxidase prevents lesion formation induced by ESCs alone. Citrus leaves were treated with or without ESCs and superoxide dismutase and hyperoxidase as indicated and incubated in a moist chamber under a constant fluorescent light. The mocks were treated with equal volume (3 μL) of water and/or acetone as appropriate.

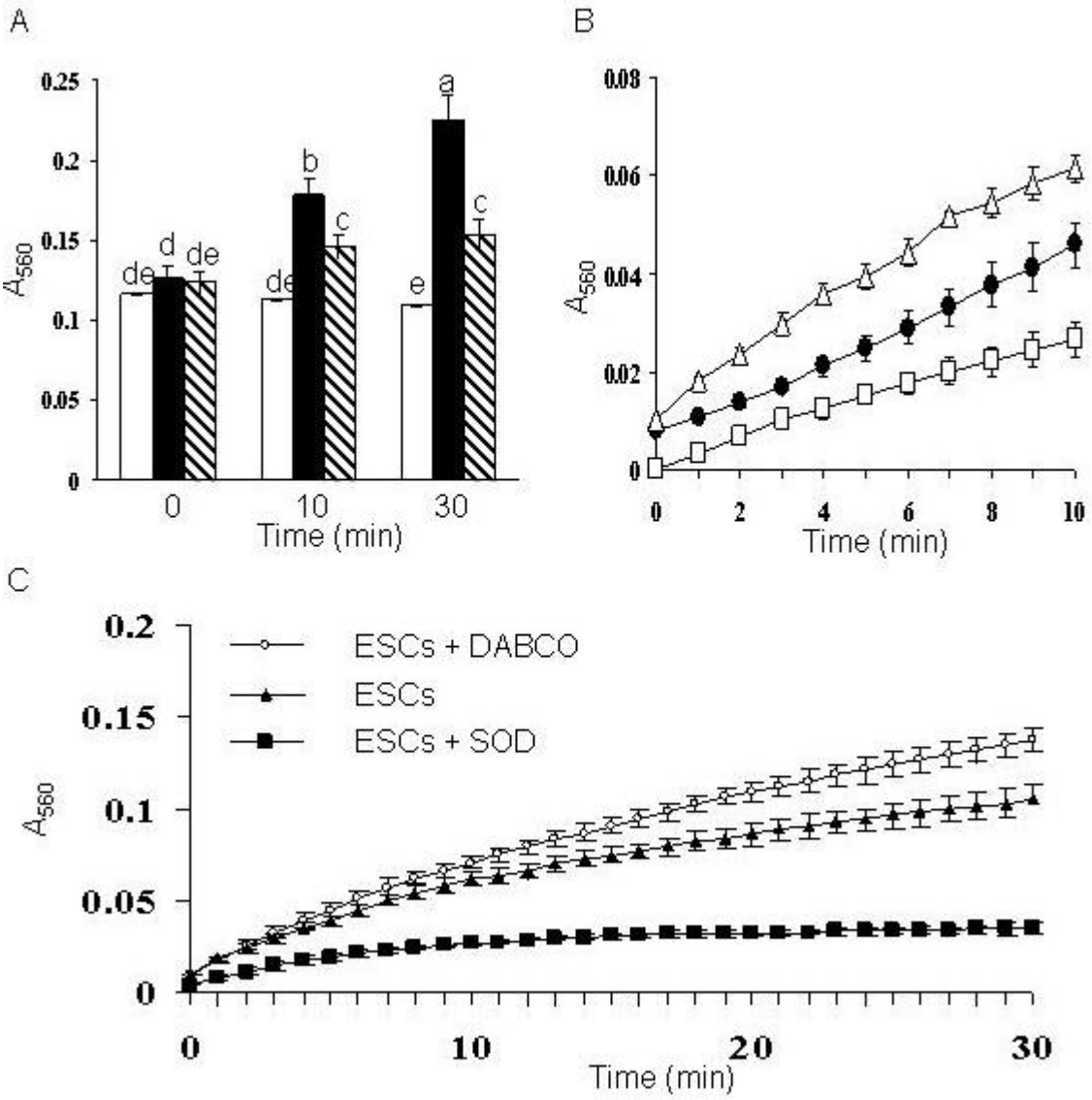


Figure 2-9. Superoxide production assays based on the reduction of nitrotetrazolium blue chloride (NBT) as substrate. A) Spectrophotometric absorbance of elsinochromes (ESCs) alone (open bars) or ESCs treated with NBT with (hatched bars) or without (closed bars) superoxide dismutase (SOD) in the 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer at 560 nm. B) Accumulation of superoxide ions ($O_2^{\bullet-}$) by photosensitizing compounds riboflavin (circles), cercosporin (squares) extracted from *Cercospora nicotianae*, and ESCs (triangles) extracted from cultures of *Elsinoë fawcettii* after irradiation with light. The respective photosensitizers were dissolved in buffer and used as the blanks. C) Suppression of ESC-induced superoxide accumulation by addition of SOD but not DABCO (1,4-diazabicyco octane). Reactions were carried out in the MOPS buffer solution containing 2.5 mM NBT, 10 mM methionine, and 10 μM of cercosporin or ESCs, or 2μM riboflavin, and/or SOD (1mg mL^{-1}) or DABCO (1 mM), and absorbance at 560 nm (A_{560}) measured. Vertical bars represent standard deviation. Means followed by the same letter are not different at judged by Duncan's multiple range test at $P < 0.0001$.

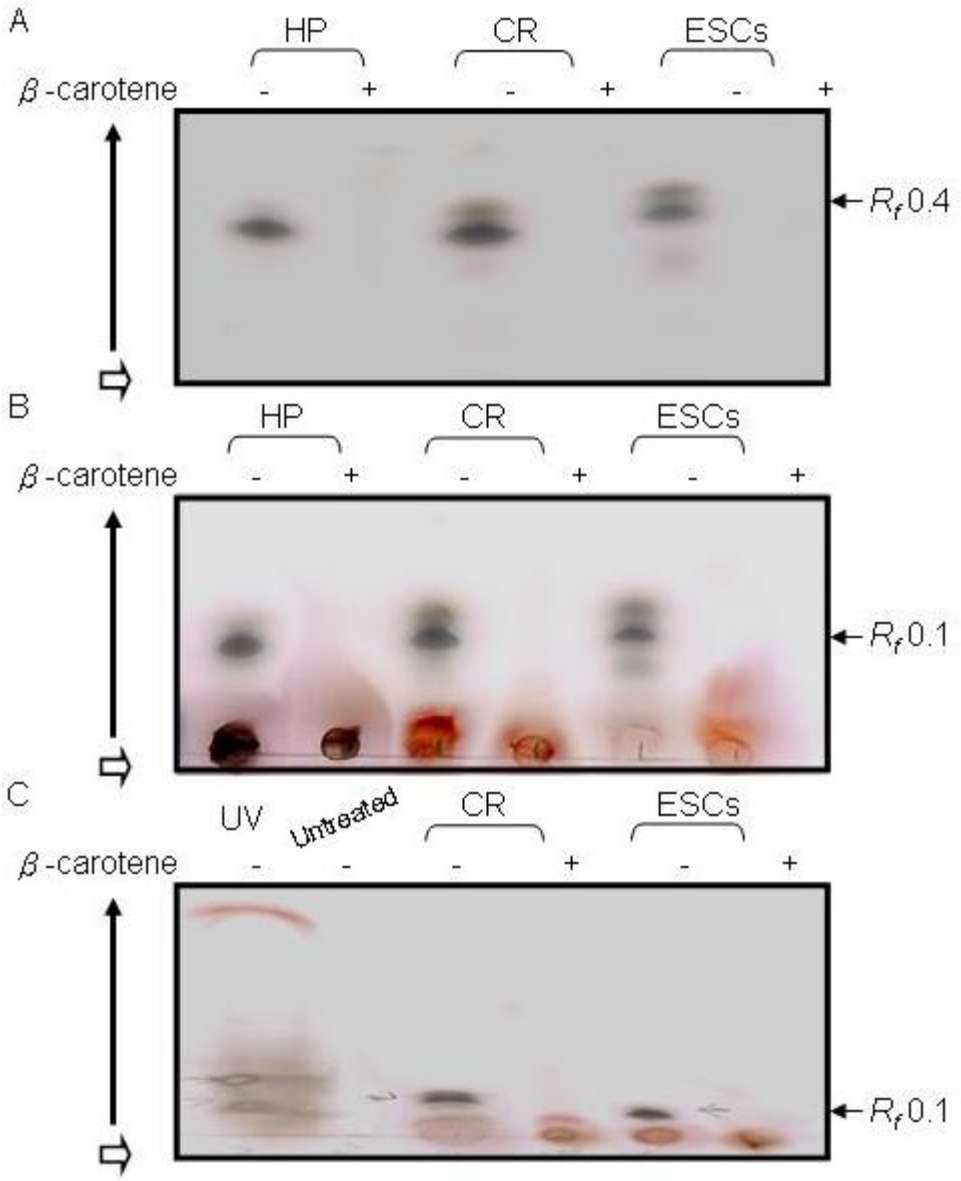


Figure 2-10. Oxidation of cholesterol by photosensitizers: hematoporphyrin (HP), cercosporin (CR), and elsinochromes (ESCs) under light. Cholesterol (200 mg) was dissolved in pyridine, mixed with photosensitizers with or without β-carotene, as indicated, and incubated for 5 h. The oxidized products were separated by thin-layer chromatography (TLC) with a hexane-isopropanol 9 : 1 (v : v) (A) or 24 : 1 (v : v) (B, C) solvent system. Cholesterol 5α-hydroperoxide (R_f 0.4 or 0.1 in solvent A or B) formed distinct bands after staining with 1 % of *N*,*N*-dimethyl-*p*-phenylenediamine. Radiation of cholesterol to UV light for 24 h resulted in multiple products, but no cholesterol 5α-hydroperoxide (C). Untreated cholesterol (in chloroform) was not visible after staining with the chromogen (C).

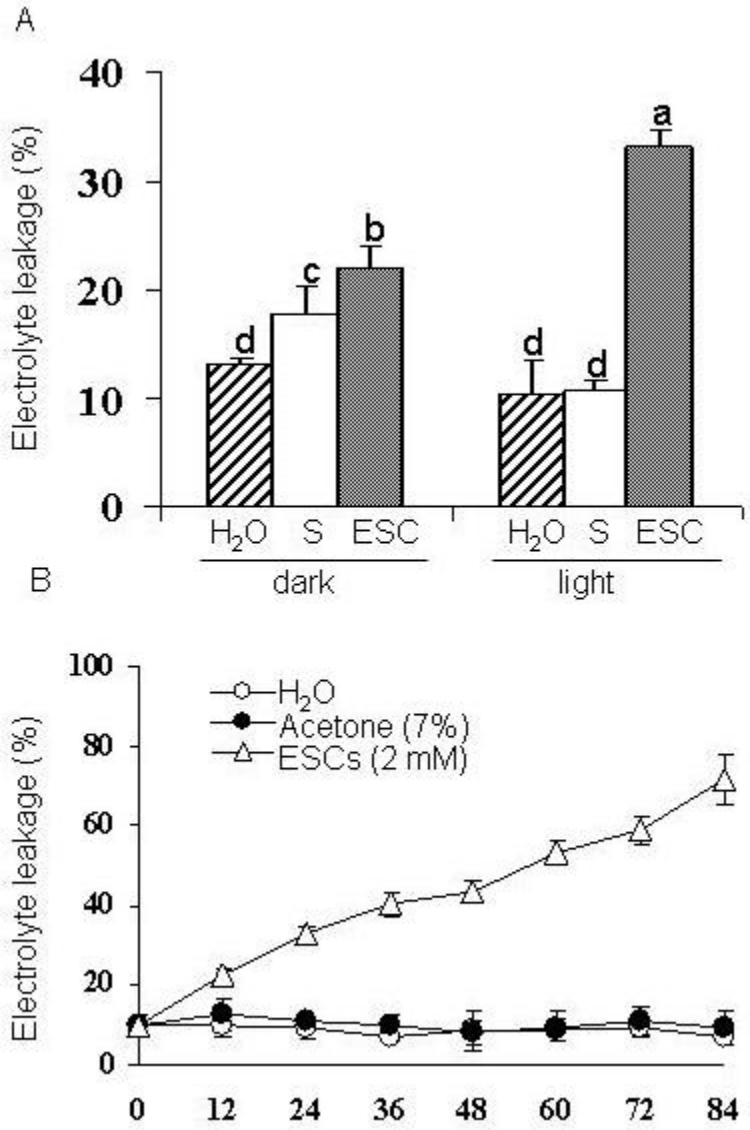


Figure 2-11. Leakage of electrolytes from illuminated rough lemon leaf discs treated with elsinochromes (ESCs). A) Light increased electrolyte leakage of citrus leaf discs treated with ESC compared with those treated with water or acetone (S) alone at 24 h. B) Increasing electrolyte leakage of rough lemon leaf discs over time after treatment with ESCs in the light. Water, open circles; acetone, closed circles; ESCs, triangles. Data represent the means of two different experiments with five replicates. Vertical bars represent standard deviation. Means followed by the same letter are not different at judged by Duncan's multiple range test at $P < 0.0001$.

CHAPTER 3
ENVIRONMENTAL FACTORS AFFECTING PRODUCTION OF ELSINOCHROMES BY *Elsinoë fawcettii*

Elsinochromes (ESCs), light-activated phytotoxins, are produced by many *Elsinoë* isolates and are required for fungal virulence. In this chapter, the effects of environmental factors in relation to ESC accumulation are investigated. The effects of environmental signals such as light, pH, medium compositions, carbon and nitrogen sources, ions, and antioxidants on fungal radial growth and ESC production by *E. fawcettii* were evaluated. Light and media compositions influenced ESC production considerably. *E. fawcettii* produced the highest titers of ESCs on PDA compared to other media tested. Production of ESCs was stimulated when the fungus was grown in a medium with ample carbon sources or under nitrogen starvation stress. An increase in ESC production correlated with an increase in the ambient pH. A reduction in ESC production was observed when antioxidant agents such as cysteine, DABCO and glutathione, were exogenously amended into PDA. Ascorbate dramatically enhanced ESC production. Addition of ions such as $CaCl_2 \cdot 2H_2O$, $Ca(NO_3)_2 \cdot 4H_2O$, $CoCl_2 \cdot 6H_2O$, or LiCl decreased ESC production, whereas other ions tested markedly enhanced ESC production. Production of ESCs was also affected by the presence of multiple colonies and the distance between two colonies on the same agar plate, indicating that nutrient competition resulting in nitrogen depletion promoted ESC production.

Introduction

Elsinoë fawcettii, the causal agent of citrus scab, produces elsinochromes (ESCs) (Liao and Chung 2008a). ESCs are phytotoxins produced by many species of *Elsinoë* (Weiss et al. 1987). ESCs have been investigated as a fungal virulence factor since ESCs produce toxic reactive oxygen species (ROS) (Liao and Chung 2008a; 2008b).

Production and accumulation of secondary metabolites by microbes are often regulated by a number of environmental and nutritional factors. Physical parameters which affect production of secondary metabolites include light intensity, temperature, and pH. Nutritional factors such as carbon source and nitrogen source also affect production of secondary metabolites. Although significant progress has been made in identifying the environmental factors for production of fungal secondary metabolites, little is known about their role in ESC production in *E. fawcettii*.

Light has been demonstrated to be required for ESC biosynthesis and toxicity (Liao and Chung 2008a; 2008b). ESCs were produced when the fungus was incubated under light and production was markedly suppressed in the dark. In addition, ESCs having structures similar to many photosensitizing compounds have been shown to produce reactive oxygen species in a light-dependent manner (Daub et al. 2005; Liao and Chung 2008b). In *Aspergillus* species, production of sterigmatocystin and aflatoxin was regulated by carbon sources (Calvo et al. 2002). Nitrogen sources have diverse effects in regulation of secondary metabolites. For example, sterigmatocystin and aflatoxin were produced in ammonium-based media (Keller et al. 1997; Morrice et al. 1998), whereas production of alternariol (AOH) and alternariol monomethyl ether (AME) by *Alternaria alternata* (Orvehed et al. 1988) was inhibited by nitrogen. Ambient pH has been reported to serve as a regulatory of production of many fungal secondary metabolites, such as aflatoxin, sterigmatocystin, and penicillin (Cotty 1988; Shah et al. 1991; Keller et al. 1997). In *Aspergillus* spp., production of fungal secondary metabolites was controlled by complex regulatory networks involved in the G-protein/c-AMP/protein kinase signaling cascades in response to environmental factors (Calvo et al. 2002).

Antioxidants, such as butylated hydroxyanisol (BHA) and prophy paraben (PP) have been shown to inhibit fungal growth and toxin production by *Fusarium verticillioides* and *F.*

proliferatum (Reynoso et al. 2002; Farnochi et al. 2004). Further, phenolic antioxidants inhibit ochratoxin A and aflatoxin B production by *Aspergillus* species (Palumbo et al. 2007; Passone et al. 2005).

Extrinsic ions are also known to affect toxin production in fungi (Marsh et al. 1975; Cuero et al. 1988; Jackson et al. 1989). For example, Zn^{2+}, Fe^{2+}, and Cu^{2+} affect production of aflatoxins in *Aspergillus flavus* and zearalenone in *Fusarium graminearum* (Cuero et al. 2003; Cuero and Ouellet 2005). Normally, metal ions influence accumulation of fungal toxins via controlling expression of the genes whose products are required for toxin biosynthesis. In this study, environmental signals were demonstrated to have multiple effects on ESC production. For example, co-culturing multiple colonies of *E. fawcettii* on the same medium tends to trigger early production of ESCs and the distance between two colonies affects the timing of ESC production.

Materials and Methods

Fungal Strains, Maintenance, and Culture Conditions

The origin of *Elsinoë fawcettii* Bitancourt & Jenkins (anamorph: *Sphaceloma fawcettii* Jenkins) isolate CAL WH-1 and the *EfPKS1* null mutants from this isolate used in this study have been previously described (Chapter 2; Liao and Chung 2008b). Fungi were grown on a sterilized filter paper, and stored at -20 °C for long-term storage.

The basal media used for fungal growth and toxin production included: potato dextrose agar (PDA, difco, Becton, Dickinson and Company, Sparks, MD), a complete medium (CM) containing 1 g Ca $(NO_3)\cdot 4H_2O$, 0.2g KH_2PO_4, 0.25g $MgSO_4\cdot 7H_2O$, 0.15g NaCl, 10g glucose, 1g each of yeast extract and casein hydrolysate, and 15g agar per liter (Jenns et al. 1989), and a minimal medium (MM) containing all components of CM but omitting yeast extract and casein hydrolysate. The pH of media was adjusted by 0.1 M phosphate buffer as described (You et al.

2007). Glucose or sodium nitrate in CM medium was substituted with equal molarity of other appropriate carbon or nitrogen sources.

To prepare fungal inoculum, the 5-day-old mycelium cultured on PDA under continuous fluorescence light at an intensity of 3.5 J m^{-2} s^{-1} was minced with a sterile blender and suspended in sterilized water. Hyphal suspension (3 µL) was placed on the surface of the test medium (5 mL) in a 60 x 15 mm Petri dish and the plates were incubated under constant light at 25 °C. The plates were wrapped with aluminum foil for the dark control and incubated under the same conditions. Fungal growth was determined by colony diameter (millimeters, mm) 3 weeks after incubation and was measured prior to ESC extraction.

ESC Purification and Quantification

For ESC purification and quantification, four 7-mm diameter agar plugs cut from mycelial cultures were extracted with 5N KOH in the dark for 16 hr, and the extracts were measured at 480 nm by a model Genesys 5 spectrophotometer (Spectromic Instruments, Rochester, NY). The ESC concentration was calculated using a molar extinction coefficient of 23,300 (Yamazaki and Ogawa 1972) and was reported as nano moles per agar plug.

Statistical Analysis

Data were analyzed by ANOVA using SAS (PROCGLM) for PC (SAS Institute Inc., Cary, N.C.). When differences were significant ($P < 0.05$), individual treatment means were separated using Ducan's Multiple Range Test ($P = 0.05$). Data are the means of two different experiments with at least three replicates.

Preparation of Chemicals

Compounds $CaCl_2 \cdot 2H_2O$, $Ca(NH_3)_2 \cdot 4H_2O$, LiCl, $MgCl_2 \cdot 6H_2O$, NaCl and KCl were purchased from Fisher Scientific (Fair Lawn, NJ.), while all other chemicals used in this study were purchased from Sigma (St. Louis, Mo.). Chemicals were dissolved in water to prepare stock

solutions as appropriate. All aqueous solutions were sterilized by filtration. All the chemical stock solutions were added to solid media. An equal volume of sterile distilled water was added as the mock control.

Results and Discussion

Fungal Growth and ESC Production in Response to Light, Media Components and pH

As assessed on PDA, *E. fawcettii* produced high amounts of ESCs under constant light (Fig. 3-1A). Accumulation of ESCs significantly decreased ($P < 0.01\%$) when the test fungus was grown under the conditions alternating in a cycle of 12-h light and 12-h dark. ESC production was almost completely suppressed when the fungus grown under darkness. *E. fawcettii* grew slightly faster under light but there was no significant difference between treatments. The results indicated that light is a critical factor for ESC production. Under constant light, production of ESCs by the *E. fawcettii* isolate was highest when the fungus was grown on PDA and significantly reduced when grown on CM or MM (Fig. 3-1B). Fungal growth was also concomitantly affected. Minimum medium (MM) has of the same ingredients in CM, except yeast extract and casein hydrolysate. MM and CM supported equivalent fungal radial growth, but the fungus produced much less ESCs on CM than on MM, indicating that yeast extract and/or casein hydrolysate had a negative effect on ESC production.

Effects of pH for fungal growth and ESC production were also tested (Fig. 3-2). A close correlation between ESC production and pH was observed, i.e., accumulation of ESCs increased as the pH of medium was elevated (Fig. 3-2). Thus, *E. fawcettii* alkaline conditions were most favorable for production of ESCs. By contrast, fungal radial growth was slightly greater under acidic pH.

Carbon Sources on Fungal Growth and ESC Production

As tested on CM, production of ESCs by *E. fawcettii* was enhanced when glucose was used as a sole carbon source, in a concentration-dependent manner (Fig. 3-3). Substitution of glucose with sucrose as the sole carbon source significantly enhanced ESC production, whereas replacement of glucose with mannitol non-significantly reduced ESC production. Apparently, the test fungus preferred higher amounts of carbon sources for radial growth and utilized the four carbon sources equally well.

Carbon sources generally have diverse effects on fungal development and production of secondary metabolites. Production of aflatoxin by *Aspergillus* spp. was greatly enhanced when fungi were cultured in the glucose-containing medium, but not the mannitol-containing medium (Calvo et al. 2002). However, *E. fawcettii* can utilize glucose or mannitol efficiently for ESC production. Given that ESC is synthesized by a polyketide pathway, likely by condensation of acetyl-CoA and malonyl-CoA (Liao and Chung 2008b), one would expect that high concentration of carbon sources enhances the acetyl-CoA pool via glycolysis, and thus, boost the ESC biosynthesis.

Effects of Inorganic and Organic Nitrogen on Fungal Growth and ESC Production

To determine if ESC production is affected by nitrogen sources, sodium nitrate in MM was substituted with ammonium chloride, ammonium nitrate, glutamine, or glycine at various concentrations as the sole nitrogen source and ESC production was measured. Ammonium chloride and ammonium nitrate suppressed ESC production but only slightly affected fungal radial growth (Fig. 3-4A). At higher concentration (4g L^{-1}) of ammonium chloride and ammonium nitrate, production of ESCs was inhibited completely. ESC production was inhibited when glutamine but not glycine was used as the sole nitrogen source even though fungal growth was slightly stimulated (Fig. 3-4B). In a prior study, expression of the *EfPKS1* gene has been

shown to be regulated by carbon/nitrogen sources and pH (Liao and Chung 2008b). In addition, several binding elements that are recognized and bound by global transcription factors for specific gene expression, such as C/EBP (cAMP-inducible genes), AreA (nitrogen or light regulatory genes), WC1/WC2 (light regulatory genes) and PacC (pH responsive genes), were found in the promoter region of the *EfPKS1* gene, suggesting that environmental signals affected ESC production via transcriptional activation of the ESC biosynthetic genes.

Effects of Antioxidants on Fungal Growth and ESC Production

Since ESCs generate reactive oxygen species in aerobic conditions upon exposure to light (Liao and Chung 2008a), experiments were also performed to test if antioxidants such as ascorbate, cysteine, DABCO, and reduced glutathione influence ESC production and fungal growth. As tested on MM, addition of cysteine, DABCO, or reduced glutathione at higher concentration (cysteine at 50 mM, each of DABCO and glutathione at 10 mM) inhibited ESC production substantially (Fig. 3-5). Cysteine at 100 mM and DABCO at 50 mM completely inhibited fungal growth. By contrast, addition of ascorbate into MM promoted both fungal growth and ESC production in a dose-dependent manner (Fig. 3-5). Those antioxidants often inactivate reactive oxygen species (ROS) and have been previously shown to alleviate the cellular toxicity of ESCs (Liao and Chung 2008a). This study showed that the antioxidants might interfere with biosynthesis of ESCs. By contrast, ascorbate, which has been reported to reduce O_2 to H_2O_2 or to scavenge H_2O_2 from generating ·OH (hydroxyl radicals) *in planta* (Fry 1998), enhanced both fungal growth and ESC production in this study. However, it is unknown how the antioxidants inhibit or promote ESC production and fungal growth.

Effects of Ions on ESC Production

Addition of $CaCl_2$, $Ca(NO_3)_2{\cdot}4H_2O$, $CoCl_2$, or LiCl into PDA decreased ESC production by *E. fawcettii* (Fig. 3-6). Metal ions $CoCl_2$ at 10 mM and LiCl at 100 mM suppressed fungal

radial growth. Addition of $CuCl_2$, $FeCl_3$, KCl, $MgCl_2$, $MnCl_2$, NaCl, or $ZnCl_2$ into PDA elevated ESC production to various levels, depending on the concentration of the compound tested (Table 3-1). Addition of EGTA, the Ca^{2+} chelator, enhanced ESC production. Similar inhibitory or stimulatory effects of ions in PDA were also observed when fungus was cultured in CM (Table B-1).

Co-culturing Enhances ESC Production

As described above, production of ESCs was influenced by diverse environmental factors. The stimulatory effect of physical interactions between fungal colonies for ESC production was observed. When a single colony of *E. fawcettii* was placed in the center of PDA medium, accumulation of small amounts of ESCs was observed 10 days after inoculation and continuously increased as duration of incubation increased (Fig. 3-7A). When three or five spots (each was separated by 1-cm apart) were inoculated with the same *E. fawcettii* isolate on a PDA plate, the rate for ESC production by each inoculated colony was much faster and the magnitude of ESC accumulation was much higher as visually indicated by red pigment compared to those produced by a single colony inoculated alone (Fig. 3-7A). Interestingly, ESCs secreted by each of the colonies tended to diffuse toward one another in medium (Fig. 3-7B). Similar phenomena were also observed in a distance-response manner when a wild-type isolate was co-cultured with the *EfPKS1* null mutants, producing no ESCs (Fig. 3-7C). It is likely that competition for nutrients, particularly nitrogen source, promoted early production of ESCs.

Table 3-1. Effects of ions and EGTA on ESC production by *Elsinoë fawcettii*

Treatment	Conc. (mM)	Mean colony dai. (mm) ± SEM	ESCs (nmoles per plug), mean ± SEM
none	-	13.5 ± 0.4	14.9 ± 3.3
$CuCl_2 \cdot 2H_2O$	0.1	14.0 ± 0.3	25.12 ± 5.80
	1.0	13.5 ± 0.9	27.18 ± 2.18
	10.0	0.0 ± 0.0	nd[1]
$FeCl_3$	0.1	14.6 ± 0.4	23.3 ± 3.0
	0.2	15.1 ± 0.5	17.8 ± 4.0
	0.5	15.3 ±1.4	22.3 ± 2.9
	1.0	16.0 ± 0.8	26.0 ± 1.8
	2.0	14.3 ± 0.5	34.8 ± 2.7
	10.0	0.0 ± 0.0	nd
KCl	50.0	12.5 ± 0.8	32.8 ± 5.2
	100.0	11.9 ± 0.5	24.5 ± 7.6
$MgCl_2 \cdot 6H_2O$	50.0	13.1 ± 0.5	36.5 ± 7.5
	100.0	12.3 ± 0.3	45.7 ± 6.2
$MnCl_2 \cdot 4H_2O$	0.2	14.1 ± 0.3	14.7 ± 2.8
	1.0	12.0 ± 0.4	13.9 ± 4.3
	5.0	12.2 ± 0.6	24.9 ± 3.2
	10.0	5.1 ± 0.3	20.5 ± 4.5
	100.0	0.0 ± 0.0	nd
NaCl	50.0	12.6. ± 0.5	15.2 ± 6.9
	100.0	12.4 ± 0.2	30.4 ± 5.4
$ZnCl_2$	0.1	13.5 ± 0.4	19.9 ± 6.9
	1.0	13.8 ± 0.6	19.6 ± 3.6
	10.0	0.0 ± 0.0	nd
EGTA	1.0	13.2 ± 0.1	22.6 ± 3.4
	2.0	9.3 ± 0.9	17.6 ± 2.2
	3.0	8.8 ± 0.8	19.2 ± 1.0

[1]nd, not determined

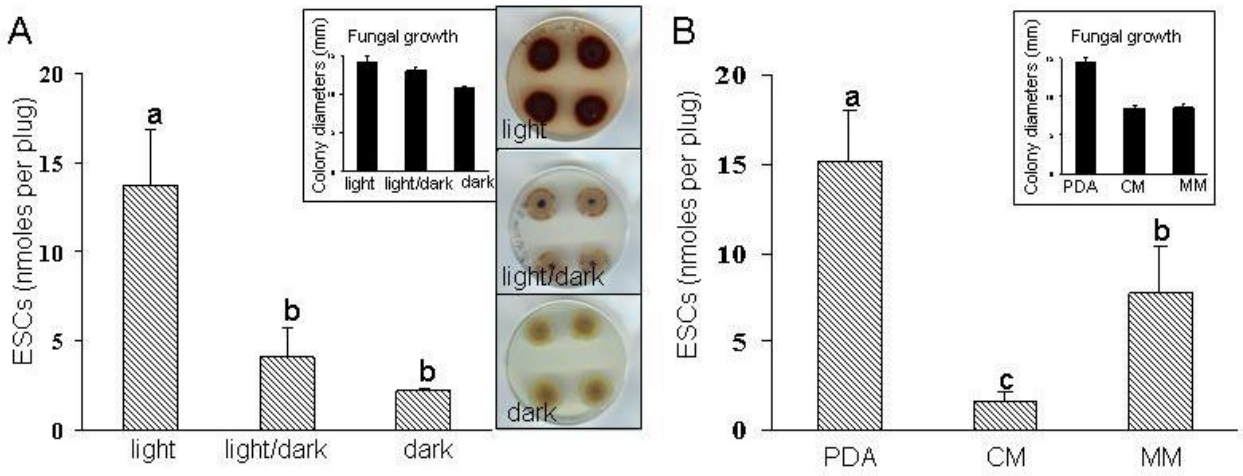


Figure 3-1. Effects of light (A) and medium compositions (B) on fungal growth and elsinochrome (ESC) production by *E. fawcettii*. Insets indicate radial growth of fungal colonies which were measured before ESC extraction. A) Fungal cultures grown on PDA were incubated at 25 °C under constant light, in complete darkness or 12-h light/dark alternation for 21 days. B) *E. fawcettii* was cultured on PDA, CM, or MM, and incubated under constant light for 21 days. ESC was extracted with KOH and measured at A_{480} nm. Vertical bars represent standard deviation. Means followed by the same letter are not different at judged by Duncan's multiple range test at $P < 0.0001$.

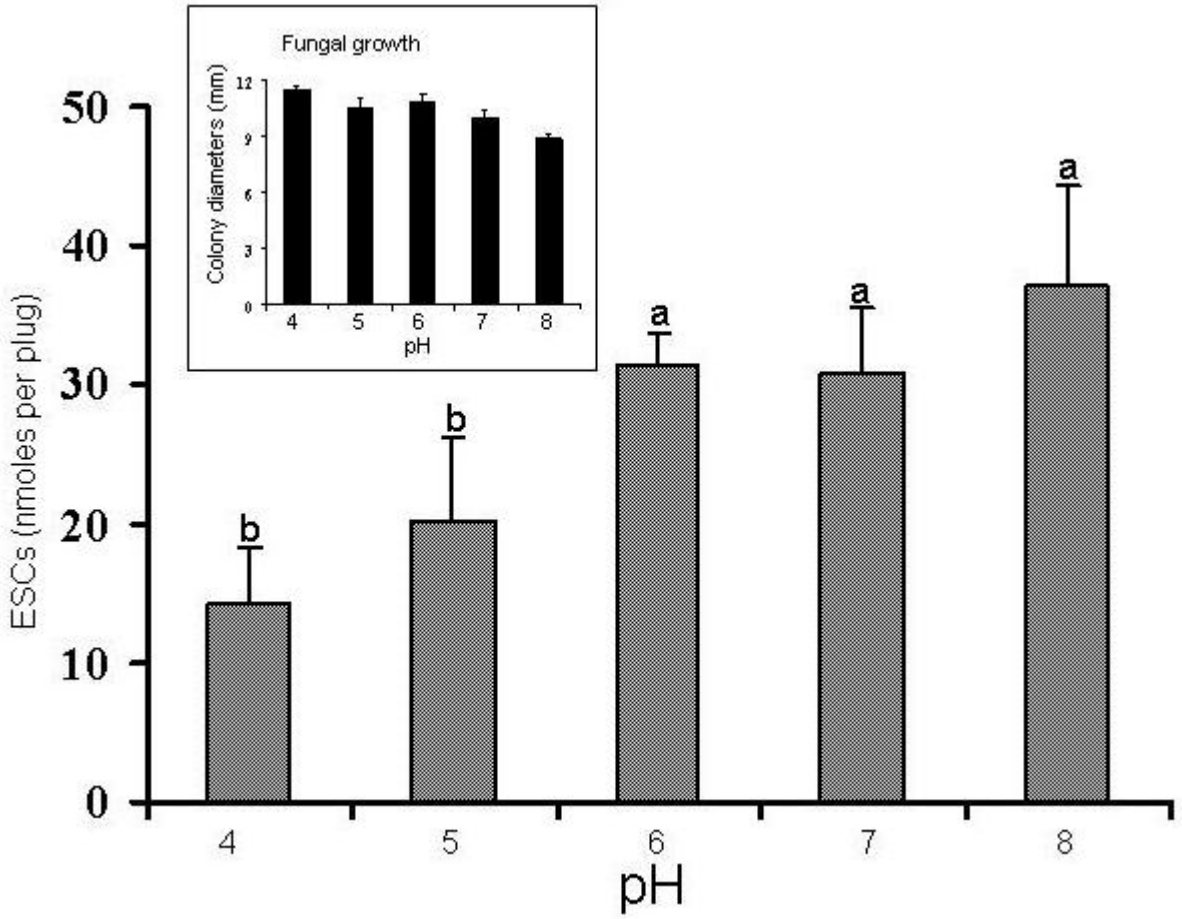


Figure 3-2. ESC accumulation and growth of *E. fawcettii* at different pH on MM. Insets indicate the diameters of fungal colonies under the appropriate conditions. pH was adjusted to 4 with sodium phosphate buffer (0.1M) buffer and 5N HCl. Vertical bars represent standard deviation. Means followed by the same letter are not different at judged by Duncan's multiple range test at $P < 0.0001$.

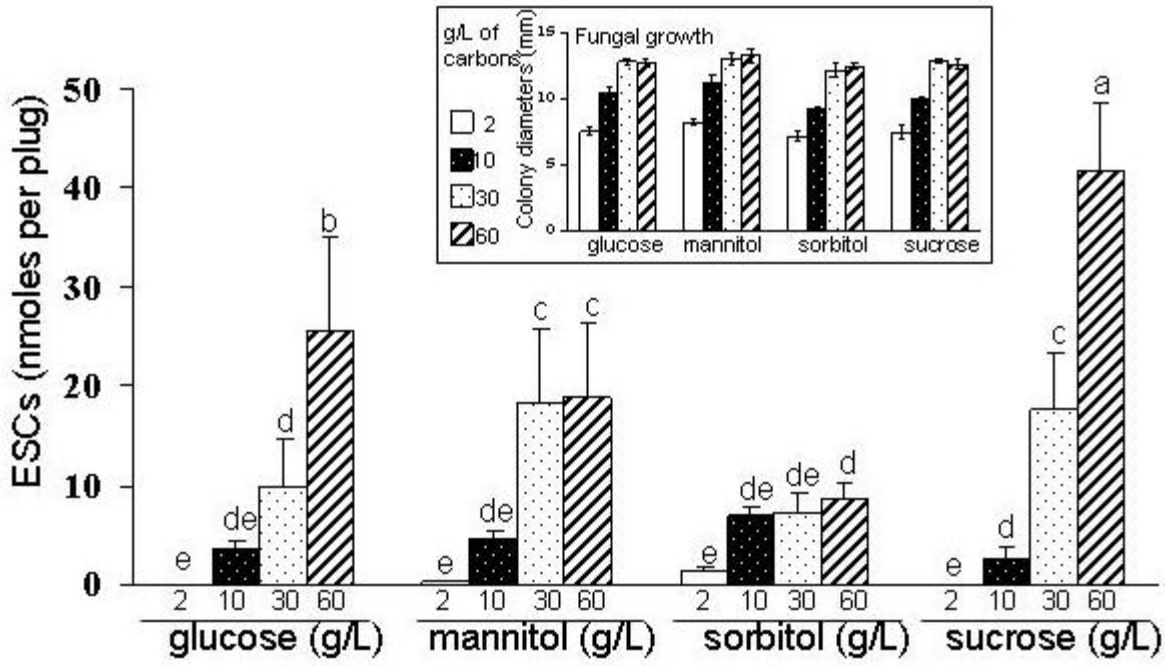


Figure 3-3. Effects of various carbon sources on fungal growth and ESC production by *E. fawcettii* assayed on CM medium. Insets indicate the diameters of fungal colonies under the appropriate conditions. Different concentration of glucose, mannitol, sorbitol, and sucrose were used to substitute glucose as the sole carbon source in the CM medium. Vertical bars represent standard deviation. Means followed by the same letter are not different at judged by Duncan's multiple range test at $P < 0.0001$.

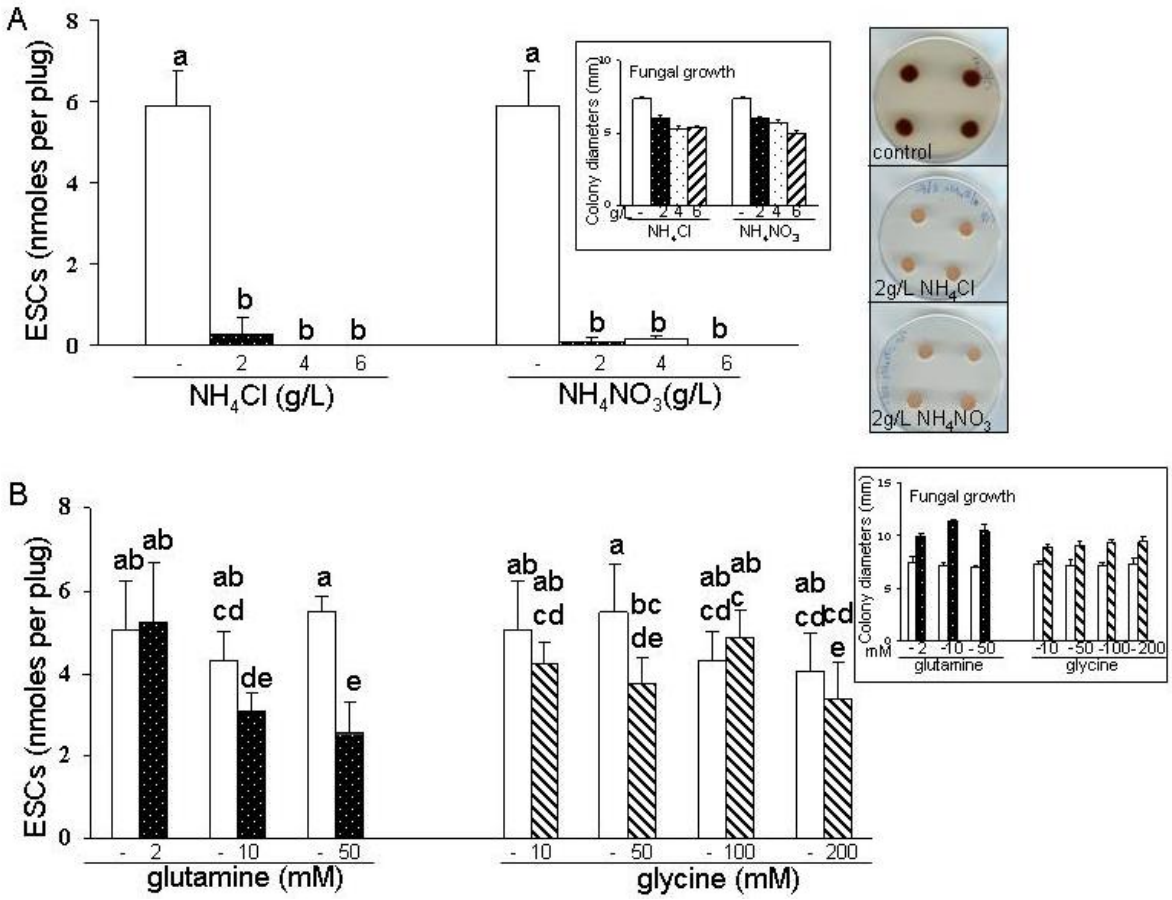


Figure 3-4. Effects of nitrogen sources on fungal growth and ESC production by *E. fawcettii* assayed in MM medium. A) Ammonium chloride and ammonium nitrate suppressed ESC production. B) ESC production was inhibited when glutamine but not glycine was used as the sole nitrogen source. Insets indicate the diameters of fungal colonies under the appropriate conditions for 21 days. Vertical bars represent standard deviation. Means followed by the same letter are not different at judged by Duncan's multiple range test at $P < 0.001$.

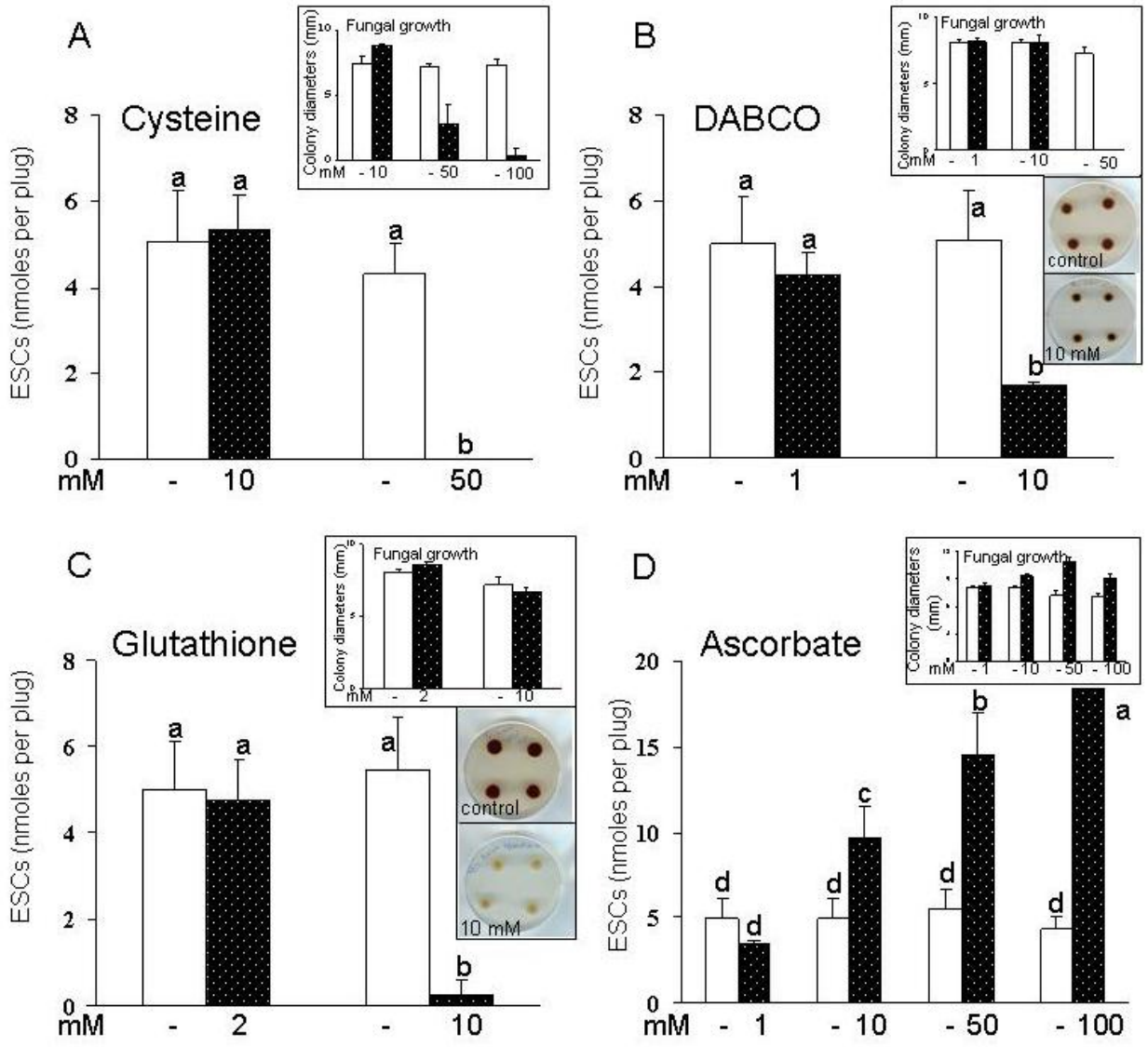


Figure 3-5. Effects of antioxidatants, A) cysteine, B) DABCO (1,4-diazabicyco octane), C) glutathione, and D) ascorbate on ESC production by *E. fawcettii* in MM medium. Insets indicate the diameters of fungal colonies under the appropriate conditions for 21 days. Vertical bars represent standard deviation. Means followed by the same letter are not different at judged by Duncan's multiple range test at $P < 0.001$.

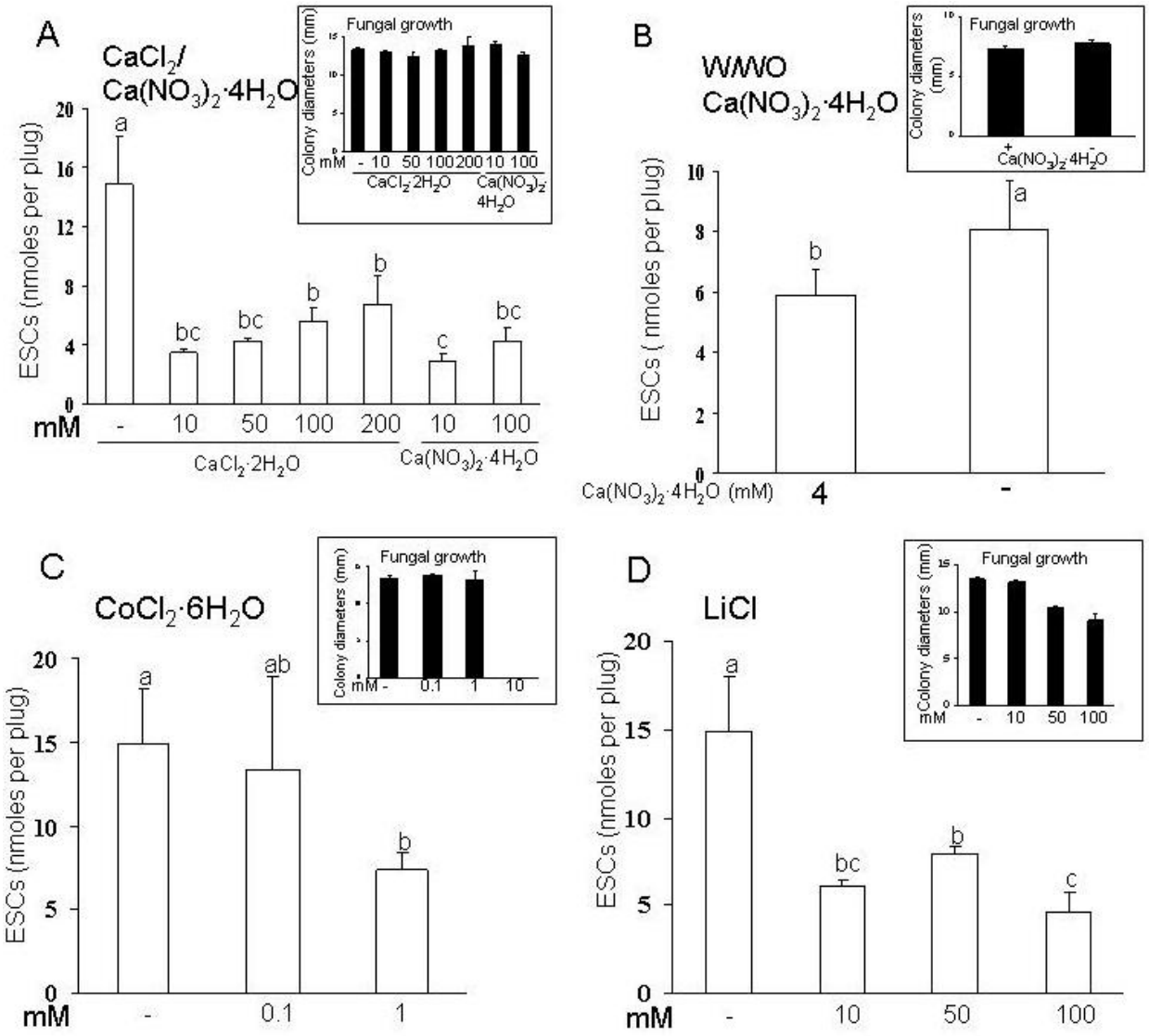


Figure 3-6. Inhibition of ESC production by Ca^{2+}, Co^{2+}, and Li^{2+} in *E. fawcettii*. Insets indicate the diameters of fungal colonies under the appropriate conditions. In A, C, and D, the fungus was grown on PDA medium either in the absence or presence of different concentration of ions. B) Fungus was grown at MM medium with (+) or without (-) the component of $Ca(NO_3)_2{\cdot}4H_2O$. Vertical bars represent standard deviation. Means followed by the same letter are not different at judged by Duncan's multiple range test at $P < 0.01$.

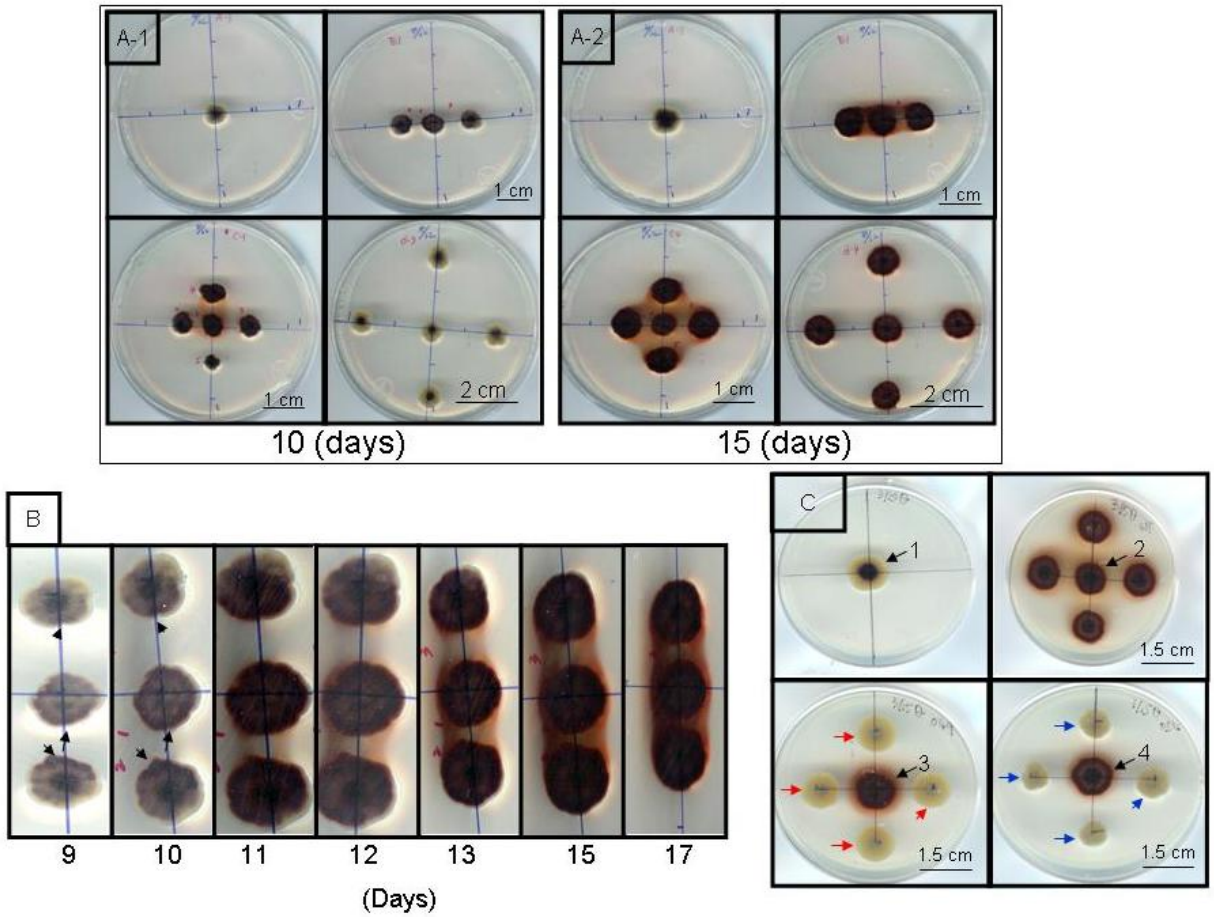


Figure 3-7. Effects of the number of fungal colonies and the distances on ESC production by *E. fawcettii*. Mycelial suspensions were place on PDA with different numbers and distances and incubated under constant light. A) Production and diffusion of ESCs were observed 10 (A-1) and 15 (A-2) days after incubation. B) Production of ESCs on PDA was promoted when three colonies of *E. fawcettii* were inoculated with 1cm apart between each and incubated for 9 to 17 days. The arrows indicate the red-pigment started to accumulate in the side of colonies that are close to another colony in 9- and 10-day-old cultures. C) The *EfPKS1* mutants [Red arrows, D3; blue arrows, D4 (Liao and Chung 2008b)] were inoculated around the wild type, showing similar stimulation on ESC production and diffusion from wild type colonies (1, 2, 3, 4) 15 days post-incubation.

CHAPTER 4
GENETIC DISSECTION DEFINES THE ROLES OF ELSINOCHROME PHYTOTOXIN FOR FUNGAL PATHOGENESIS AND CONIDIATION OF THE CITRUS PATHOGEN *Elsinoë fawcettii*

Elsinochrome pigments produced by many phytopathogenic *Elsinoë* species are non-host selective toxins which react with oxygen molecules after light activation to produce highly toxic reactive oxygen species. This chapter describes the cloning, expression, and functional characterization of the polyketide synthase-encoding gene, *EfPKS1*, which is required for the production of elsinochromes (ESCs) and fungal pathogenesis. Target disruption of *EfPKS1* in *E. fawcettii* completely abrogated ESC production, drastically reduced conidiation, and significantly decreased lesion formation on rough lemon leaves. All mutant phenotypes were restored to the wild type in fungal strains expressing a functional copy of *EfPKS1*. Accumulation of the *EfPKS1* transcript and ESCs by a wild-type strain appears to be coordinately regulated by light, nutrients, and pH. The results clearly indicate that the product of *EfPKS1* is involved in the biosynthesis of ESCs via a fungal polyketide pathway, and that ESCs play an important role in fungal pathogenesis.

Introduction

Many phytopathogenic fungi produce perylenequinone pigments, which are light-activated and nonhost-selective phytotoxins (Daub et al. 2005). For example, the compounds, alteichin, altertoxin, alterlosin, and stemphyltoxin are produced by *Alternaria* spp. (Stack et al. 1986; Davis and Stack 1991) and *Stemphylium botryosum* (Davis and Stack 1991); cercosporin and isocercosporin are produced by many *Cercospora* spp. (Daub et al. 2005); ESCs are produced by many *Elsinoë* and *Sphaceloma* spp. (Weiss et al. 1987; Liao and Chung 2008a); hypomycin A is produced by *Hypomyces* spp. (Liu et al. 2001); hypocrellin is produced by *Hypocrella bambusae* (Weiss et al. 1987); Shiraia bambusicola (Wu et al. 1989), and phleichrome, calphostin C,

cladochrome, and ent-isophleichrome are produced by *Cladosporium* spp. (Weiss et al. 1987; Arnone et al. 1988). Among all phytotoxins identified, perylenequinone toxins are unique because they contain a chromophore of phenolic quinine that absorbs light energy (photosensitizers) and produces reactive oxygen species (ROS) such as the hydroxyl radical ($OH^{\bullet}$), superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) (Daub and Ehrenshaft 2000; Daub et al. 2005). Although the biological functions of the light-activated perylenequinone toxins for the producing fungi remain largely unknown, their production by a wide range of plant pathogens suggests an important role for these toxins in fungal pathogenesis (Daub and Ehrenshaft, 2000). Perylenequinone toxins were originally investigated because of their possible pharmaceutical application (Hudson and Towers 1991) or because of their potential as food contaminants (Stack et al. 1986). In contrast, the role of perylenequinone toxins in fungal pathogenesis has been investigated very little compared to many other host-specific phytotoxins. Only the role of cercosporin in Cercospora diseases has been demonstrated genetically (Upchurch et al. 1991; Callahan et al. 1999; Shim and Dunkle 2003; Choquer et al. 2005; Choquer et al. 2007; Dekkers et al. 2007).

ESCs are red/orange pigments produced by a number of phytopathogenic *Elsinoë* species. ESCs comprise of at least four derivatives (A, B, C, and D) with a common perylenequinone backbone but differing in side groups (Weiss et al. 1987). The bright red pigments, elsinochrome A, B, and C were originally isolated from cultures of a pecan pathogen, *Elsinoë randii* (anamorph: *Sphaceloma randii*). Elsinochrome D is likely derived from elsinochrome C by formation of a methylenedioxy ring. *Elsinoë fawcettii* infects lemons, grapefruit, and some tangerines and their hybrids, producing exterior blemishes (citrus scab) on the fruit, which is a serious problem for the fresh-fruit market worldwide. In the previous studies, elsinochrome

pigments were extracted from a field isolate of *E. fawcettii* and the production of $^1O_2$ and $O_2^{\bullet-}$ by ESCs upon exposure to light was demonstrated (Chapter 2; Liao and Chung 2008a). Crude extracts containing a mixture of five elsinochrome derivatives from *E. fawcettii* cultures were shown to be highly toxic to citrus and tobacco cells in suspension culture, and induced electrolyte leakage of host leaves. The toxicity of ESCs was reduced considerably when $^1O_2$ quenchers such as bixin, ascorbate, and reduced glutathione were present. Furthermore, ESCs induced necrosis on rough lemon leaves when exposed to visible light, and the development of necrosis was reduced by co-applying $^1O_2$ quenchers (Liao and Chung 2008a). Thus ESCs function as photosensitizing compounds that are toxic to plant cells by generating $^1O_2$ and $O_2^{\bullet-}$.

Discovery of ESCs' toxicity to the plants led to studies of the role of ESCs in fungal pathogenesis and the molecular mechanisms leading to its biosynthesis. ESCs were implicated as the polyketide-derived based on their structural similarity with several perylenequinones. However, no direct evidence of this structural of ESCs has been established. This chapter describes the cloning, coordinate expression, and functional characterization of the polyketide synthase-encoding gene, *EfPKS1*, for the production of ESCs. This was demonstrated by creating and analyzing loss-of-function *EfPKS1* mutants of *E. fawcettii*. The results indicate that ESCs are required for fungal pathogenesis.

Materials and Methods

Fungal Isolate and Growth Conditions

The wild type CAL WH-1 isolate that was single-spore isolated from scab affected calamondin (*Citrus madurensis* Lour) fruit has been previously characterized (Liao and Chung 2008a; Chapter 2 and 3) and was used as the recipient host for transformation and targeted gene disruption. This isolate was routinely maintained on potato dextrose agar (PDA, Difco, Sparks, MD). For toxin production, fungal mycelia were mincing with a sterile blender, spread on media

and incubated under continuous fluorescent light for 5 or 7 days at room temperature (~25 °C). For generation of protoplasts, fungal isolates were grown in 50 mL of potato dextrose broth (PDB, Difco) for 7 days, minced, and mixed with fresh PDB (200 mL), and incubated for additional 15 h. Fungal cultures used for DNA or RNA isolation were grown on media with a layer of sterile cellophane as previously described (Choquer et al. 2005). Complete medium (CM), minimal medium (MM), and protoplast regeneration medium (RMM) used in this study have been described in Chapter 3 and/or elsewhere (Jenns et al. 1989; Chung et al. 2002). The pH of media was adjusted by 0.1 M phosphate buffer as previously described (You et al. 2007).

Extraction and Analysis of ESC Toxins

Isolation of fungal toxins from culture and TLC analysis are described in chapter 2. Screening of ESC-deficient mutants was conducted on thin PDA as previously described (Choquer et al. 2005; Chen et al. 2007).

Molecular Cloning and Analysis of the *EfPKS1* Gene

Two degenerate oligonucleotides LCKS1 (5'-GTNCCNGTNCCRTGCATYTC-3') and LCKS2 (5'-GAYCCNMGNTTYTTYAAYATG-3') that are complementary to the conserved β-keto synthase (KS) domain of fungal type-I polyketide synthase genes (Bingle et al. 1999) were synthesized and used for amplification from *E. fawcettii* genomic DNA using Taq DNA polymerase (GenScript, Piscataway, NJ). The amplified DNA fragment (~700 bp) was cloned into pGEM-T easy vector (Promega, Madison, WI) for sequencing analysis from both directions at Eton Bioscience (San Diego, CA). Sequences were blasted against the databases at the National Center for Biotechnology Information (NCBI) using the BLAST network service (Altschul et al. 1997) to determine the similarity of the amplified fragment. The full-length *EfPKS1* gene was obtained by PCR using a chromosome walking strategy as described previously (Chen et al. 2005; You et al. 2007) and by PCR with two inverse primers (Table C-1;

Fig. C-1) (Choquer et al. 2005). A chromosome library of *E. fawcettii* was prepared from genomic DNA cleaved with four different enzymes (*Eco*RV, *Pvu*II, *Sma*I and *Stu*I), and ligated to the adaptors from the Universal Genome Walker kit following the manufacturer's instructions (BD Biosciences, San Jose, CA). To walk upstream and downstream into unknown genomic regions, primer were synthesized to complement the known regions and used for multiple rounds of PCR amplification with adaptor primers supplied with the kit. For PCR with inverse primers, fungal DNA was digested with restriction endonucleases, self-ligated, and used as a template for amplification. Oligonucleotide primers used for PCR amplification and sequence analysis were synthesized by Integrated DNA Technologies (Coralville, IA), Allele Biotechnology and Pharmaceuticals (San Diego, CA), respectively. Open reading frame (ORF) and exon/intron positions were predicted using the Softberry gene finding software and confirmed by comparisons of genomic and cDNA sequences. Functional domains were predicted according to the PROSITE database using ExPASy (Gasteiger et al. 2003) or Motif/ProDom and Block programs (Henikoff et al. 2000). Analysis of the promoter region was conducted using regulatory sequence analysis tools (van Helden 2003).

Targeted Gene Disruption

To disrupt the *EfPKS1* gene in *E. fawcettii*, a 5.8-kb DNA fragment encompassing the *EfPKS1* ORF was obtained by PCR with primers efup3 (5'-CAATTACGCGAATGGGTCACAGAGC-3') and efdown11 (5'-CGTCAAGGACATCAGCGAGTC-3'). The amplified DNA fragment was purified with a DNA purification kit (Mo Bio Laboratories, Carlsbad, CA), and cloned into pGEM-T easy vector to create pSPKS0311. A 1.3-kb *Eco*RV-*Kpn*I DNA fragment, corresponding to the conserved acyl transferase (AT) domain of EfPKS1, was removed and replaced with an end-filled 2.1-kb fragment harboring the hygromycin phosphotransferase B gene (*HYG*) cassette under the control

of the *Aspergillus nidulans trp*C gene promoter from pUCATPH (Lu et al., 1994) to yield the disruption construct, pPKS0311 (Fig. 4-4A). A split marker strategy was used to enhance the efficiency of double crossing-over recombination as previously described (Choquer et al. 2005). A 4.6-kb DNA fragment containing truncated 5' *EfPKS1* fused with 3' *HYG* and a 3.3-kb fragment encompassing 3' *EfPKS1* joined with 5' HYG were amplified, respectively, with primers efup3/hyg1 (5'-AGGAGGGCGTGGATATGTCCTGCGGG-3') and efdown11/hyg2 (5'-CCGACAGTCCCGGCTCCGGATCGG-3') from pPKS0311 using the Takara Ex Tar PCR system (Takara Bio USA, Madison, WI). Fungal protoplasts were prepared by the method of Chung et al. (2002) except that hyphae were incubated with cell wall degrading enzyme cocktails for 6 h instead of 2 h. The resulting DNA fragments were mixed and transformed into protoplasts (1×10^5) of wild type using $CaCl_2$ and polyethylene glycol as previously described (Chung et al. 2002). The two hybrid fragments share 400-bp of overlapping sequence within the *HYG* gene. The *HYG* gene is not functional unless recombination occurs between the two truncated HYG DNA fragments. Fungal transformants appearing on RMM medium supplementing with 200 μg mL^{-1} hygromycin (Roche Applied Science, Indianapolis, IN) after 2 to 3 weeks were selected and tested for lack of ESC production (red/orange pigments) on thin PDA.

Genetic Complementation

For genetic complementation, a DNA fragment (8.4 kb) including the entire *EfPKS1* ORF and its endogenous promoter was amplified from genomic DNA with primers efup3 and efdown 18 (5'-CTTTCGTCGTCGGCCCAAC-3') by an Expand High Fidelity PCR system (Roche Applied Science) and co-transformed with plasmid pBarKS1 carrying a phosphinothrin acetyltransferase gene responsible for bialaphos resistance under control of the *A. nidulans trp*C promoter (Pall and Brunelli 1993) into an *EfPKS1* disruptant (D4). Transformants were selected

against 100 μg mL^{-1} of DL-phosphinothricin (chlorimuron ethyl; Chem Service, West Chester, PA) and tested for restoration of ESC production.

Miscellaneous Methods of Processing Nucleic Acids

Fungal DNA was isolated with a DNeasy Plant kit (Qiagen, Valencia, CA). Standard procedures were used for endonuclease digestion of DNA, electrophoresis, and Southern-and Northern-blot hybridizations. Plasmid DNA was purified using a Wizard DNA purification kit (Promega) from transformed *Escherichia coli* DH5α bacterial cells. Fungal RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA). Double stranded cDNA of *EfPKS1* was synthesized with a cDNA synthesis kit (BD Biosciences) following the manufacturer's instructions and amplified with gene-specific primers. The amplified fragments were purified and directly subjected to sequence analysis. DNA probes used for hybridization were labeled with digoxigenin (DIG)-11-dUTP (Roche Applied Science) by PCR with gene-specific primers. The manufacturer's recommendations were followed for probe labeling, hybridization, post-hybridization washing and immunological detection of the probe using a CSPD chemofluorescent substrate for alkaline phosphatase (Roche Applied Science).

Preparation of Fungal Inoculum and Pathogenicity Assays

Assays for fungal pathogenicity were conducted on detached rough lemon (*Citrus jambhiri* Lush) leaves inoculated with conidial suspension or agar plugs covered with fungal hyphae. Rough lemon is susceptible to all *Elsinoë* pathotypes of citrus (Timmer et al. 1996). Conidia were prepared as described by Timmer and colleagues (1996) with modifications. Briefly, fungal hyphae were minced in Fries medium (Fries 1978), placed on Petri dish (15 x 90 mm), and incubated for 2 to 3 days in the dark to trigger conidial formation. Rough lemon seedlings were maintained in a greenhouse and expanding immature leaves of approximately 13-20 mm length and 4-7 mm width were collected for pathogenicity assays. A conidial suspension (2 μL, 1 x 10^5

mL^{-1}) was carefully placed on the leaves and the inoculated leaves were incubated in a moist chamber under constant fluorescence light for lesion formation. Pathogenicity also was evaluated using agar plugs. Briefly, PDA agar plugs (4 mm in diameter) were cut from mycelial mats cultured on PDA for 7 days and placed on the underside of rough lemon leaves (~12 days after emergence). The inoculated leaves were incubated in a mist chamber and examined for lesion formation daily.

Results

Cloning and Characterization of *EfPKS1* Gene

A 0.7-kb DNA fragment was amplified from genomic DNA of *E. fawcettii* with degenerate primers designed to anneal to the conserved β-keto-synthase (KS) domains of many type-I fungal polyketide synthases. Sequence analysis revealed that the amplified fragment has strong similarity to the KS of PKSs. The gene was named *EfPKS1* (*E. fawcettii* polyketide synthase gene 1). Subsequently, the entire *EfPKS1* ORF sequences as well as its 5' and 3' nontranslated regions were obtained by multiple rounds of PCR from chromosome walking library or with two inverse primers from restriction enzyme-digested and self-ligated DNA pools. As a result, over 8 kb of genomic sequences were obtained, assembled, and deposited within EMBL/GenBank Data Libraries under accession number EU086466.

Computer prediction and comparison between cDNA and genomic DNA sequences revealed that *EfPKS1* contains two exons interrupted by a 52-bp intron near the 5' end of *EfPKS1* (data not shown). The intron has characteristic splicing (5'-/gt---ag/-3') and internal lariat (cta/gat/c) consensus sequences often found in genes of filamentous fungi. Conceptual translation revealed that *EfPKS1* encodes a polypeptide containing 2192 amino acids that displays considerable similarity and identity to numerous type-I PKSs of fungi, particularly those involved in pigment formation and biosynthesis of secondary metabolites (Fig. 4-1). Similar to

many fungal type-I PKSs, the translated product of *EfPKS1* has a β-keto-synthase (KS) domain, an acyltransferase (AT) domain, two acyl carrier protein (ACP) domains, and a thioesterase/claisen cyclase (TE/CYC) domain (Fig. 4-1A). Phylogenetic relationships of EfPKS1 to other fungal polyketide synthases, inferred from the conserved KS or AT domain, revealed that EfPKS1 is highly similar to the fungal non-reducing PKSs, including those involved in the biosynthesis of melanin, cercosporin, bikaverin, sirodesmin, aflatoxin, and other pigments (Fig. 4-1B and C).

Promoter Analysis of *EfPKS1* Gene

To gain a better understanding in the regulation of *EfPKS1* gene expression, I analyzed 1.1-kb sequences upstream of the putative ATG translational start codon of *EfPKS1* and identified several putative binding sites for diverse transcriptional regulators (Fig. 4-2). A TATA box-like sequence (TATATC) on the sense strand was identified 264 bp upstream from the ATG codon. The promoter region of *EfPKS1* has multiple GATA consensus motifs, potential binding sites for the nitrogen-induced AreA (Marzluf 1997) and the light-regulated WC1/WC2 (Linden et al. 1997) transcriptional activators. The *EfPKS1* promoter contains four ambient pH-regulated PacC-binding consensus motifs (GCCARG; Espeso et al. 1997) and multiple cAMP-inducible C/EBP-binding motifs (CCAAT or CAAT; Rangan et al. 1996). In addition, *EfPKS1* promoter contains three MRAGGGR and two CATTCY consensus motifs that have been shown to serve as binding sites for the conidial formation-related BrlA and AbaA transcriptional activators in *A. nidulans* (Adams et al. 1998). Analysis of the complementary strand of the *EfPKS1* promoter also identified multiple AreA, WC complex, PacC, C/EBP, and BrlA binding motifs (Fig. 4-2).

Expression and Regulation of *EfPKS1* Gene and ESC Production

To determine the factors affecting ESC production and expression of the *EfPKS1* gene, fungal cultures were grown on media with different carbon, nitrogen sources, pH values, or

incubated in the light or complete darkness. ESCs were extracted and analyzed by spectrophotometry and total RNA was extracted and analyzed by Northern-blot hybridization. A time-course analysis of *EfPKS1* RNA levels and ESC production revealed that the *EfPKS1* gene transcript accumulated to a detectable level by day 3 and was elevated by days 4 and 5 (Fig. 4-3A). Similarly, ESCs were detected at low concentration at days 3 and 4, yet accumulated rapidly to high levels at day 5. Accumulation of *EfPKS1* transcript and ESCs was much higher when fungal cultures were incubated under continuous light compared to those grown in the dark (Fig. 4-3B). The *EfPKS1* gene was preferentially expressed and ESCs accumulated to high levels when *E. fawcettii* was cultured in glucose-rich medium under illumination (Fig. 4-3C). By contrast, ammonium nitrate deprivation stimulated the accumulation of both the *EfPKS1* transcript and ESCs (Fig. 4-3D). Both expression of *EfPKS1* and production of ESCs were favored when the fungus was grown under alkaline conditions (Fig. 4-3E).

EfPKS1 is Required for ESC Production

The function of *EfPKS1* in relation to ESC production was determined by targeted gene disruption in *E. fawcettii*. A disruption plasmid (pPKS0311) carrying the hygromycin phosphotransferase B gene (*HYG*) cassette flanked by truncated *EfPKS1* sequences on each side was constructed. Two separate DNA fragments overlapping within the *HYG* gene (Fig. 4-4A) were amplified and directly transformed into the wild type *E. fawcettii* for targeted gene disruption. In total, 600 transformants were recovered from media containing hygromycin. Among them, approximately 100 transformants failed to accumulate the red/orange pigments on PDA after 30-days of incubation under constant light and were considered putative *EfPKS1* mutants. Southern-blot analysis of genomic DNA isolated from 13 putative *EfPKS1* mutants revealed that all transformants tested were missing the expected 3.5-kb hybridizing band of the wild-type locus, when fungal DNA was cleaved with restriction endonuclease *Cla*I, and

hybridized to probe I that recognizes sequences in the 5' end of *EfPKS1* (Fig. 4-4B and data not shown). All 13 transformants displayed a 5.1-kb hybridizing band as a result of *HYG* insertion within *EfPKS1*. Four mutants had a single 5.1-kb hybridizing band (Fig. 4-4B), whereas the other nine mutants displayed additional hybridizing signals larger or smaller than 5.1 kb (data not shown), likely resulting from ectopic insertions. When fungal genomic DNA was cleaved with *Xba*I and hybridized to probe II that recognizes the 3' end sequences of *EfPKS1*, an expected 7.9-kb band was detected in wild type (Fig. 4-4C). By contrast, all 13 transformants had a single 4.6-kb hybridizing band resulting from the *HYG* insertion at *EfPKS1* locus (Fig. 4-4C and data not shown). The hybridizing profiles obtained from Southern-blot analysis with two different probes indicated that *EfPKS1* of each transformant was replaced with the *HYG* gene cassette via homologous integration.

Successful disruption of *EfPKS1* was confirmed by Northern-blot analysis (Fig. 4-5A). The DNA probe (probe 3, Fig.4-4A) hybridized to total RNA of wild type displays a 6.6-kb hybridizing signal. However, the probe failed to detect the *EfPKS1* transcript in three randomly selected mutants, indicting that they are *EfPKS1* null mutants. Quantitative assays of ESC production by the *EfPKS1* null mutants after KOH extraction revealed that the null mutants did not accumulate measurable ESCs compared to wild type (Fig. 4-5B). No ESCs were detected from the acetone extracts of four null mutants on TLC, indicating the disruption of *EfPKS1* completely obliterated ESC production (Fig. 4-5C).

Genetic complementation experiments revealed that the ESC-deficient phenotype of a null mutant (D4) was reverted by acquiring and expressing a functional *EfPKS1* gene with its endogenous promoter (Fig. 4-5C), confirming the requirement for *EfPKS1* in ESC production in *E. fawcettii*.

Disruption of *EfPKS1* Reduces Conidiation

The *EfPKS1* null mutants displayed normal radial growth comparable to wild type, yet produced far fewer conidia relative to wild type (Table 4-1). Genetic complementation by introducing a functional copy of *EfPKS1* gene into protoplasts of D4 null mutant, resulted in two transformants with restored conidial production (Table 4-1), indicating a close link between *EfPKS1* function and conidiation.

The *EfPKS1* Gene is Required for Full Virulence

Since the *EfPKS1* null mutants were severely defective in conidial production, fungal pathogenicity was first evaluated on detached rough lemon leaves inoculated with agar plugs cut from fungal mycelium (without conidia). As shown in Fig. 4-6, inoculation of wild type and two genetically reverted strains (C1 and C2) resulted in pink to light brown scab lesions with round pustules around the edges, whereas the *EfPKS1* null mutants did not incite any visible lesions. Quantitative assays indicated that over 65% of the sites inoculated with agar plugs from the cultures of wild type or the complementation strains developed necrotic lesions (Table 4-1). By contrast, none of the sites inoculated with *EfPKS1* null mutants or agar plugs alone produced visible necrosis.

Pathogenicity assays also were performed on detached leaves inoculated with conidial suspension. In this assay, only the D4 null mutant was evaluated for pathogenicity because the other null mutants produced very few conidia. When assayed on young leaves (approximately 3 days after emergence), D4 mutant induced necrotic lesions at rates and magnitudes indistinguishable from those induced by wild type and the C2 strain genetically expressing a functional *EfPKS1* (Fig. 4-7A). Wild type and the C2 strain produced characteristic scab lesions when inoculated onto older leaves (~7 days after emergence), whereas the D4 null mutant with similar amounts of conidia (1×10^5 mL^{-1}) incited fewer and smaller lesions presumably due to

lack of the production of ESCs (Fig. 4-7B). Nevertheless, disruption of *EfPKS1* yielded mutants that were completely devoid of ESC production and defective in conidiation and scab lesion formation, indicating that ESCs are a determinant of fungal virulence in *E. fawcettii*.

Discussion

ESCs, produced by many phytopathogenic fungi of the *Elsinoë* genus, consist of at least four derivatives differing mainly in their side groups. In the present study, the fungal *EfPKS1* gene was demonstrated to be required for ESC biosynthesis, and thereby ESCs were conclusively shown to play a role in fungal virulence. The results also indicate that ESCs are synthesized via a fungal polyketide pathway.

As with many type-I fungal polyketide synthases (Kroken et al. 2003; Choquer et al. 2005; Keller et al. 2005), the *EfPKS1* product contains five functional domains, including a KS, an AT, a TE/CYC, and two ACP domains, which are involved in catalyzing iterative condensations of acetates and malonates, and chain elongation and cyclization of polyketomethylenes (Watanabe and Ebizuka 2004). EfPKS1 also has a conserved cysteine residue in the KS domain and conserved serine residues in the AT, ACP, and TE/CYC domains, similar to other fungal PKSs belonging to the non-reducing group (Kroken et al. 2003). Integration of a hygromycin-resistance gene cassette specifically at the *EfPKS1* locus resulted in fungal mutants that were completely devoid of ESC production, severely defective in sporulation, and delayed in lesion development on older rough lemon leaves. The mutant phenotypes were fully complemented in a strain that genetically acquired and functionally expressed *EfPKS1*, confirming that mutation of *EfPKS1* is responsible for the observed deficiencies and that ESCs are important virulence factors. Pathogenicity assays on detached rough lemon leaves revealed that *E. fawcettii* incites more severe necrotic lesions when inoculated with conidial inoculation compared to mycelium cut from agar plugs. Although the *EfPKS1* null mutants were also defective in conidial formation,

reduction of fungal virulence was mainly attributed to the impairment of ESC production rather than conidial formation and germination. When pathogenicity was evaluated with conidial suspension, the D4 null mutant induced scab lesions on young leaves (3 days after emergence), comparable to those induced by the wild type. Hence, the *EfPKS1* null mutant was apparently not deficient in conidial germination. However, the D4 null mutant induced fewer lesions on older leaves (7 days after emergence) strongly indicating that production of ESCs in essential for full virulence of *E. fawcettii*.

Expression of the *EfPKS1* gene was correlated with accumulation of ESCs. The *EfPKS1* transcript and ESCs accumulated to high levels when the fungus was grown in the light, in the presence of higher amounts of glucose, at alkaline pH, or in the absence of a nitrogen source. Differential expression of *EfPKS1* in response to various environmental conditions was further supported by analyzing the promoter region for putative DNA binding sites for global transcriptional activators. For example, two GATA consensus sites, presumably involved in the binding of the AreA/Nit2 nitrogen regulatory proteins (Marzluf 1997) and/or the WC1/WC2 light responsive transcriptional regulators (Linden et al. 1997) were identified. The promoter region of *EfPKS1* has three consensus GCCARG motifs that are likely involved in the binding of the pH-responsive transcriptional regulator, PacC (Espeso et al. 1997). The *EfPKS1* promoter also has multiple CCAAT or CAAT consensus motifs involved in binding cAMP-activated proteins such as CCAAT/enhancer-binding protein (C/EBP) (Ramji and Foka 2002).

In addition to complete abrogation of ESC production, *EfPKS1* null mutants also were severely defective in conidial production. The ESC-producing phenotype and conidiation were fully restored by expressing a dominant-activated allele of an *E. fawcettii* EfPKS1 protein in a null mutant. These findings indicate a tight connection between ESC biosynthesis and

conidiation. It has long been known that production of natural compounds by microorganisms is often linked with cell development and/or differentiation (reviewed in Adams and Yu 1998; Calvo et al. 2002; Yu and Keller 2005; Brodhagen and Keller 2006). It appears that both conidiation and production of secondary metabolites in fungi respond to common environmental cues, subsequently trigger signaling transduction pathways, and regulate gene expression inside the cells. It is well known that environmental factors such as light, the types of carbon and nitrogen sources, and ambient pH via a PacC-mediated pathway regulate both developmental differentiation and biosynthesis of secondary metabolites in various fungi (Calvo et al. 2002). The genetic mechanisms linking both processes are multifaceted and beginning to be unveiled through molecular studies in several fungal species. The involvement of a G-protein/cAMP/protein kinase A-mediated pathway in both asexual sporulation and mycotoxin production in *Aspergillus nidulans* has provided significant insight into the close regulatory interactions between these two phenotypes in other fungi. In addition, the mitogen-activated protein (MAP) kinase and cyclin-dependent kinase pathways have also been shown to regulate both conidiation and production of cercosporin in *C. zeae-maydis* (Shim and Dunkle 2003) and fumonisins in *F. verticillioides* (Shim and Woloshuk 2001), respectively. Other cellular regulators such as oxylipins and other lipid derivatives, polyamines, the CCAAT-binding protein complex, and the proteins containing Trp-Asp (WD) repeats have also been shown to coordinate regulation of both secondary metabolite production and sporulation in *Aspergillus* spp. (Calvo et al. 2002; Tsitsigiannis and Keller 2006).

Coordinate regulation for conidiation and production of ESCs by *E. fawcettii* may mediate similar regulatory pathways, as described in *Aspergillus* species. Such speculation in supported by analysis of the *EfPKS1* promoter region, which identified multiple binding sequences for

transcriptional activators, including GATA factors of nitrogen and light regulation, PacC of pH regulation, and the C/EBP-binding protein induced by cAMP. Furthermore, our results also revealed that production of ESCs via a defined polyketide pathway is highly affected at the transcriptional level by light, the levels of glucose and nitrogen sources, and pH values. In addition to the binding motifs for global regulatory proteins, the *EfPKS1* promoter contains two distinct MRAGGGR motifs found in the promoter region of the gene encoding a conidiation-specific Bristle (BrlA) transcriptional activator of *A. nidulans* (Adams et al. 1998). Furthermore, the *EfPKS1* promoter has two CATTCY motifs (Y is a pyrimidine) that serve as the binding sites for the AbaA transcriptional activator (Andrianopoulos and Timberlake 1994). AbaA, regulated via BrlA, has been shown to be required for the final stages of conidiphore development in *A. nidulans* (Andrianopoulos and Timberlake 1994). The presence of conserved BrlA and AbaA binding elements in the promoter of *EfPKS1* strongly suggests that the *EfPKS1* product might be directly involved in conidial formation. Whether or not conidiation of *E. fawcettii* is regulated by light, pH, carbon/nitrogen sources, or cAMP remains to be determined.

Table 4-1. Conidial production in axenic cultures and pathogenicity assays on detached rough lemon leaves by inoculating with agar plugs cut from cultures of *Elsinoë fawcettii* wild type (WT), *EfPKS1* null mutants (D1, D2, D3, and D4), and strains (C1 and C2) expressing a functional copy of *EfPKS1*.

Fungal strains or treatment	Conidial formation (conidia per mL)[1]	Lesion development[2] Leaf spots showing lesions/total spots inoculated (%)
Wild type	$3.4 \pm 1.7 \times 10^6$	21/29 (72%)
D1	$5.0 \pm 7.0 \times 10^2$	0/21 (0%)
D2	$4.1 \pm 4.1 \times 10^3$	0/21 (0%)
D3	$1.1 \pm 0.6 \times 10^3$	0/21 (0%)
D4	$6.2 \pm 7.0 \times 10^4$	0/28 (0%)
C1	$3.9 \pm 1.9 \times 10^6$	11/17 (65%)
C2	$2.4 \pm 1.4 \times 10^6$	14/14 (100%)
Agar plugs	-	0/50 (0%)

[1]Conidia were produced in Fries medium in the dark and were determined with the aid of a hemacytometer by microscopy. Data are means and standard errors (± SEM) of ten different experiments with four replicates of each isolate.

[2]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 florescent light for 25 days. The frequency of lesion formation is indicated as the number of inoculated spots developing scab lesions relative to the total number of spots inoculated.

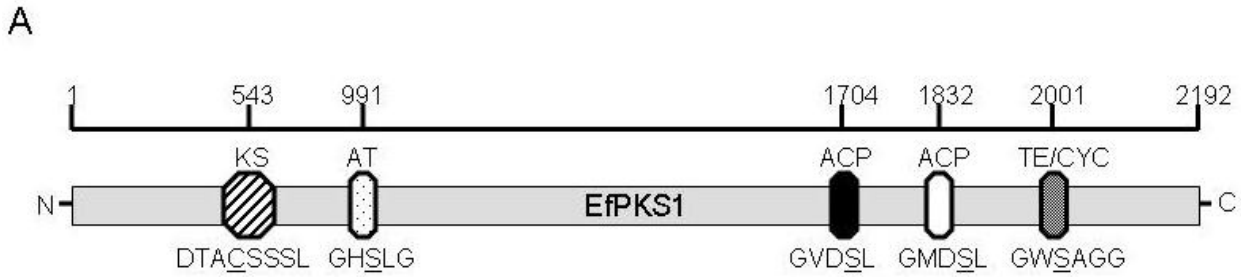


Figure 4-1. Putative polyketide synthase, EfPKS1, in *Elsinoë fawcettii*. A) the putative polyketide synthase, EfPKS1 containing 2192 amino acids in *Elsinoë fawcettii*, showing a keto synthase (KS) domain with acyl binding cysteine, an acyltransferase (AT) domain containing pantotheine binding serine, a thioesterase/claisen cyclase motif (TE/CYC), and two acyl carrier protein (ACP) domains containing phosphopantotheine binding serine. Conserved amino acids for each of the domains are also indicated under the map, and the active amino acids are underlined. EfPKS1 lacks dehydratase (DH), enoyl reductase (ER), ketoacyl reductase (KR), and methyltransferase (ME) domains. B) and C) Phylogenetic relationships of EfPKS1, based on the conserved amino acids in the KS (B) or AT (C) domain, to other fungal PKSs (accession number) including: *Bipolaris oryzae* BoPKS (BAD22832); *Cochliobolus heterostrophus* ChPKS18 (AAR90272); *Botryotinia fuckeliana* BfPKS13 (AAR90249); *Xylaria* sp. XyPKS (AAM93545); *Nodulisporium* sp. NoPKS (AAD38786); *Ophiostoma piceae* OpPKS (ABD47522); *Glarea lozoyensis* GlPKS (AAN59953); *Colletotrichum lagenarium* ClPKS1(BAA18956); *Sordaria macrospore* SmPKS (CAM35471); *Aspergillus clavatus* AcPKSP (XP_001276035); *Ceratocystis resinifera* CrPKS1 (AAO60166); *B. fuckeliana* BfPKS12 (AAR90248); *A. fumigatus* AfPKSP (AAC39471); *Chaetomium globosum* CgPKS (XP_001219763); *Emericella nidulans* wA (1905375A); *Nectria haematococca* NhPKSN (AAS48892); *Gibberella zeae* GzPKS12 (AAU10633); *A. nidulans* AnSTCA (XP_681094); *C. heterostrophus* ChPKS19 (AAR90273); *Cercospora nicotianae* CnCTB1 (AAT69682); *G. moniliformis* GmPKS3 (AAR92210); *G. moniliformis* GmPKS4 (AAR92211); *G. fujikuroi* GfPKS4 (CAB92399); *Mycosphaerella pini* MpPKS (AAZ95017); *A. flavus* AfPKSA (AAS89999); *A. oryzae* AoPKS (BAE71314); *A. parasiticus* ApPKSL1 (Q12053); *Leptosphaeria maculans* LmPKS1 (AAS92537); *C. heterostrophus* ChPKS1 (AAB08104); *G. moniliformis* GmPKS11 (AAR92218); *C. heterostrophus* ChPKS25 (AAR90279); and *Penicillium patulum* Pm6MSAS (P22367). The phylogram was created with the program PHYLP (Saitou and Nei 1987).

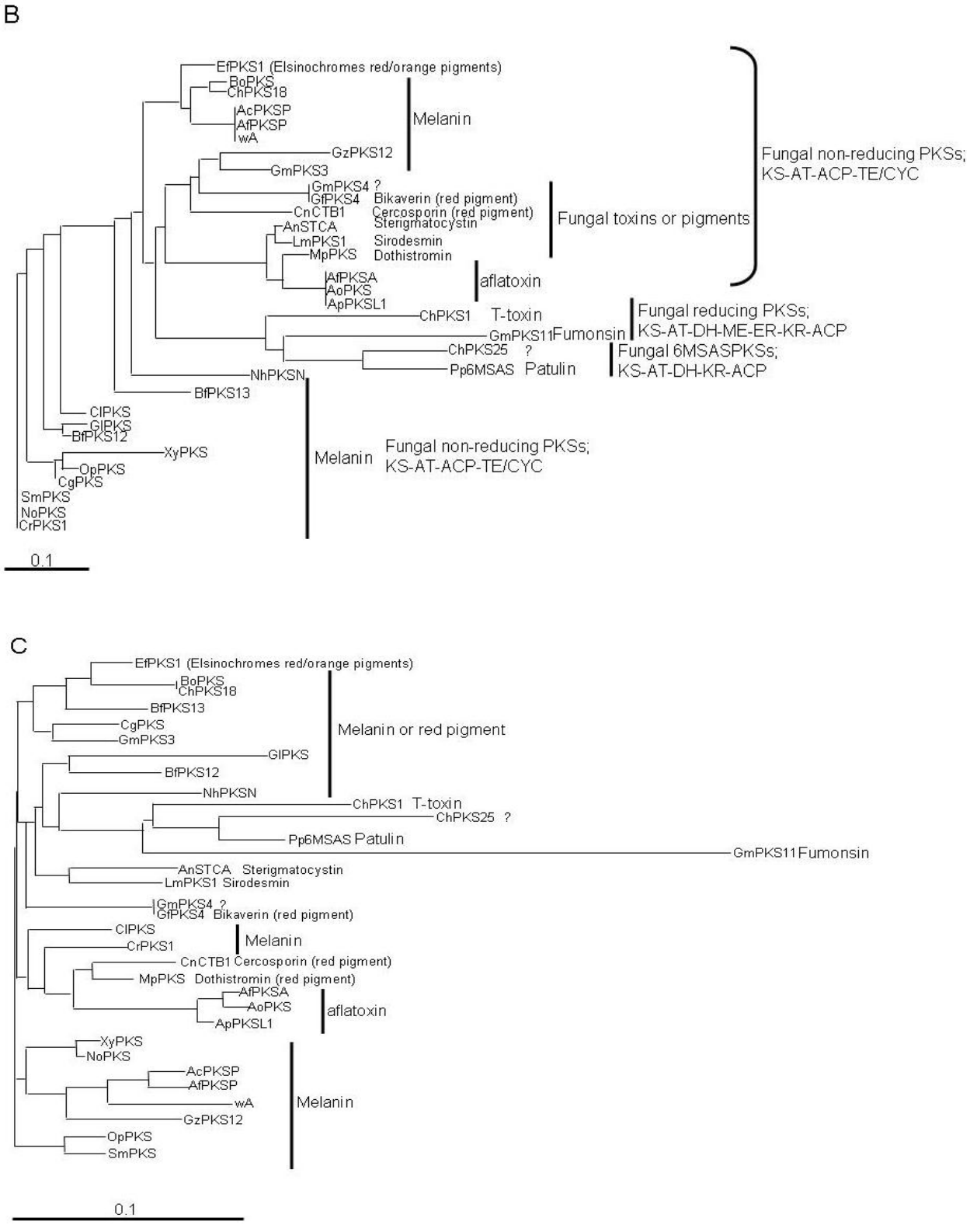


Figure 4-1. (Continued)

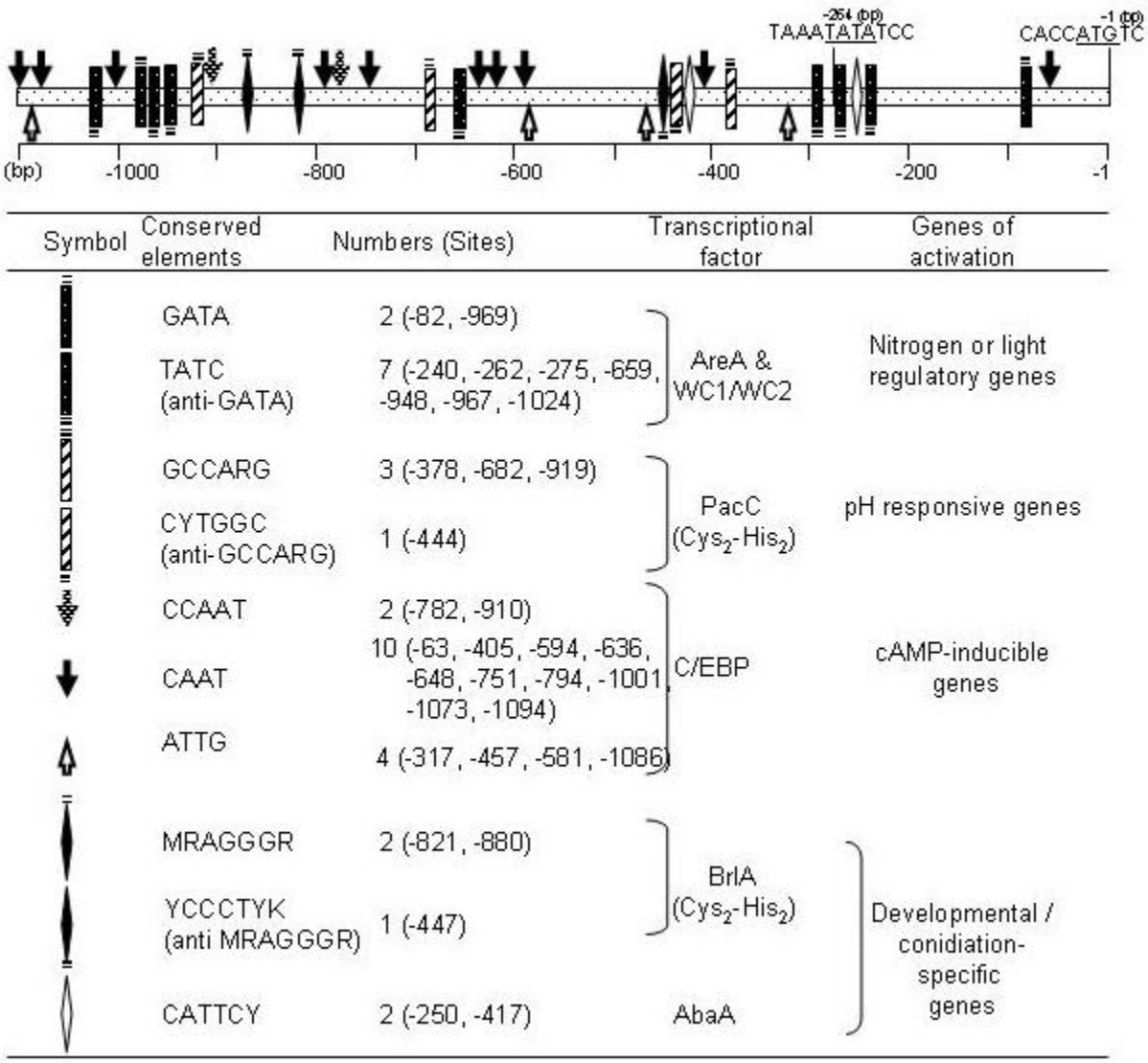


Figure 4-2. Promoter analysis of 1.1 kb sequences upstream of the putative ATG translational start codon of *EfPKS1* in both directions, identifying a number of putative binding sites for global transcriptional regulators such as AreA (nitrogen regulatory protein), the WC1/WC2 complexes (light regulatory proteins), PacC (ambient pH regulatory protein), and C/EBP (cAMP activated protein). The *EfPKS1* promoter also has conserved sequences for recognition and binding of the conidial formation-associated BrlA and AbaA transcriptional activators in *A. nidulans*. Definition of mixed bases: R = A or G; M = A or C; Y = C or T.

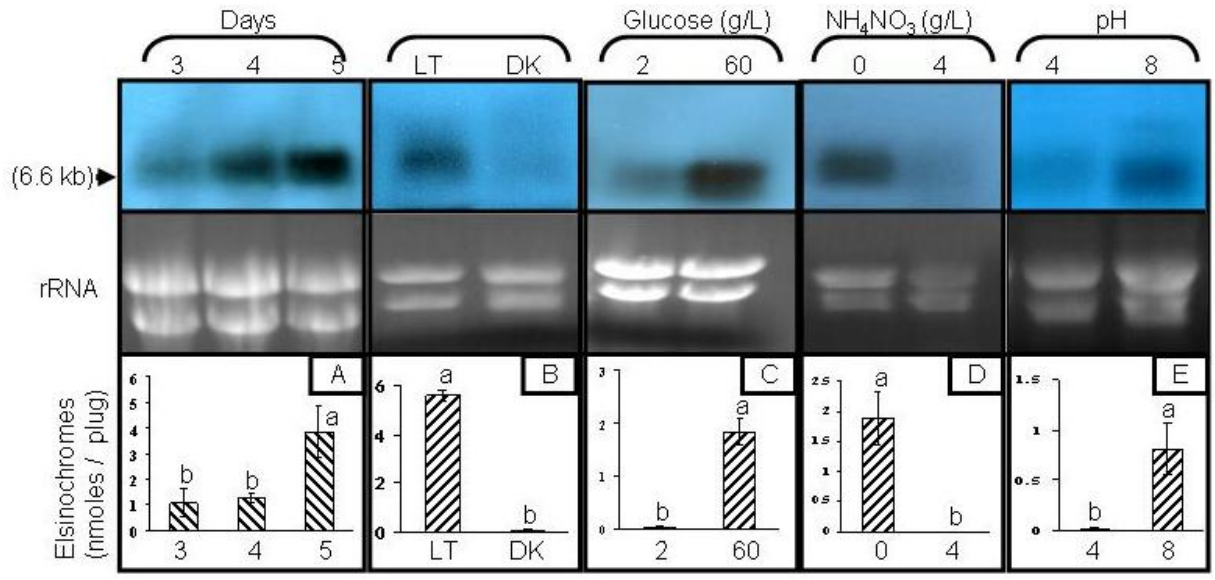


Figure 4-3. Differential expression of the *EfPKS1* gene encoding a fungal polyketide synthase and accumulation of ESC phytotoxins in a wild-type isolate *Elsinoë fawcettii*. Fungal isolate was grown on PDA (A and B), on complete medium containing different amounts of glucose (C), or on minimal medium containing different concentrations of ammonium nitrate (D) or with different pH values (E) under continuous light (LT) or darkness (DK). Unless indicated, fungal RNA was isolated from 7-day-old cultures, electrophoresed in an agarose gel containing formaldehyde, transferred onto a nylon membrane, and hybridized to an *EfPKS1* gene-specific probe. Ribosomal RNA (rRNA) stained with ethidium bromide is shown for relative loading of the samples. ESCs were extracted with 5N KOH and measured at A_{480}. The amounts of ESCs were determined by reference to a regression line was established using ethyl acetate-purified ESCs. ESC data represent the means of two different experiments with four replicates of each treatment. Vertical bars represent standard deviation. Means followed by the same letter are not different at judged by Duncan's multiple range test at $P < 0.0001$.

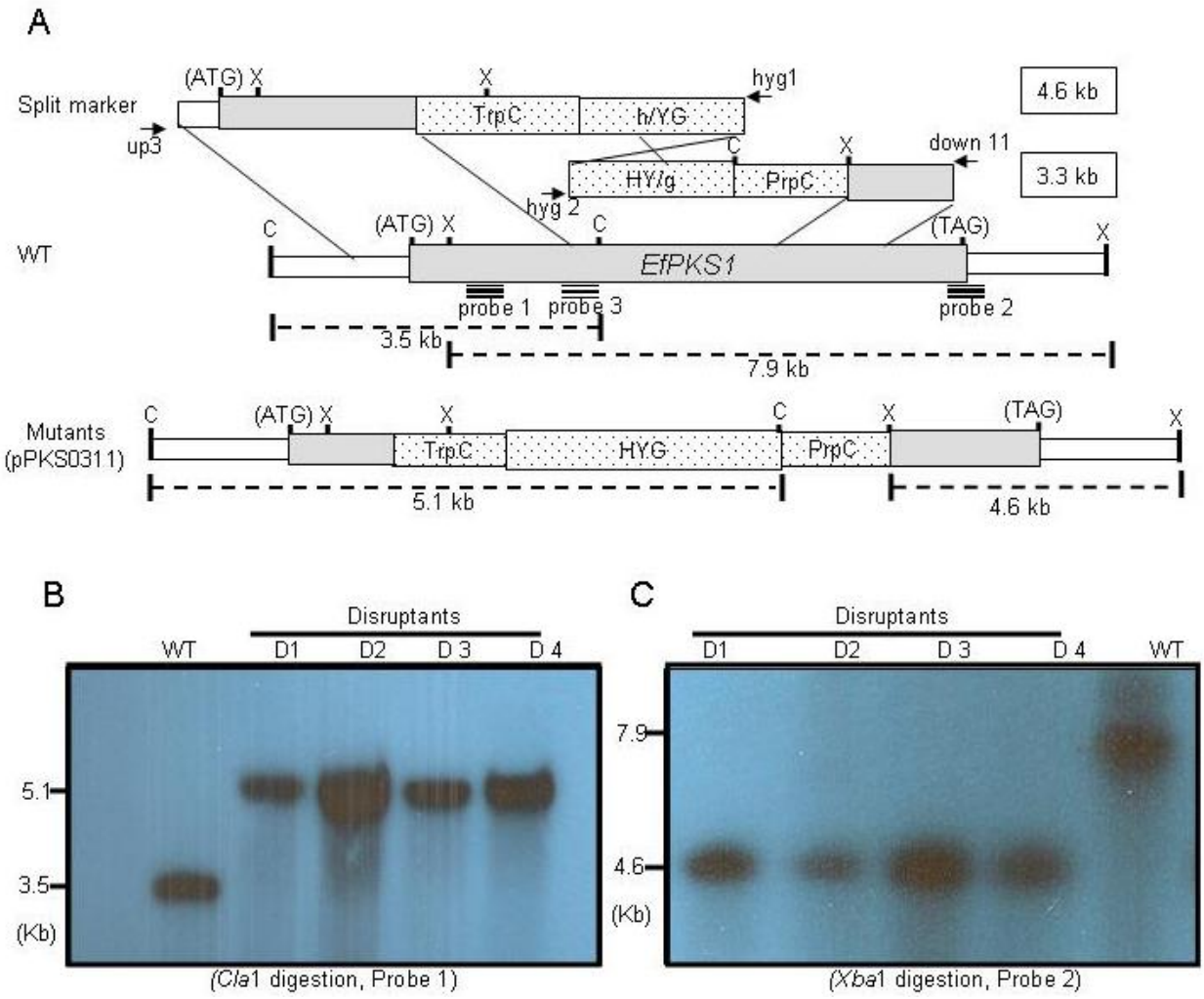


Figure 4-4. Strategy of *EfPKS1* targeted disruption in *Elsinoë fawcettii*. A) Schematic depicting a split-marker strategy for *EfPKS1* (indicated by shaded) targeted disruption in *Elsinoë fawcettii*. A 4.6-kb DNA fragment containing the 5' end of EfPKS1 fused with the *Trp*C terminator of *A. nidulans* and truncated hygromycin phosphotransferase B gene (*h/YG*) and a 3.3-kb fragment containing the 3' end of *EfPKS1* joined with the *Trp*C promoter and truncated *HY/g* were amplified from the disruption construct pPKS0311 with the primers as indicated. The *h/YG* and *HY/g* fragment share 400 bp of overlapping sequence. The split marker fragments were directly transformed into protoplasts of the *E. fawcettii* wild-type strain for targeted gene disruption via double crossing-over recombination. The relative locations of DNA probes used for Southern and Northern-blot analyses are also indicated. Abbreviations for restriction endonuclease site: C, *Cla*I; X, *Xba*I. B) Southern-blot analysis of genomic DNA from wild type (WT) and four putative *EfPKS1* disruptants (D1, D2, D3, and D4). Fungal DNA was cleaved with endonuclease *Cla*I, electrophoresed, blotted to a nylon membrane, and hybridized with the Probe 1. C) Southern-blot analysis of genomic DNA digested with *Xba*I and hybridized with the Probe 2. Sizes of hybridization bands are indicated in kilobase pairs (kb). Banding patterns confirm disruption of *EfPKS1* gene.

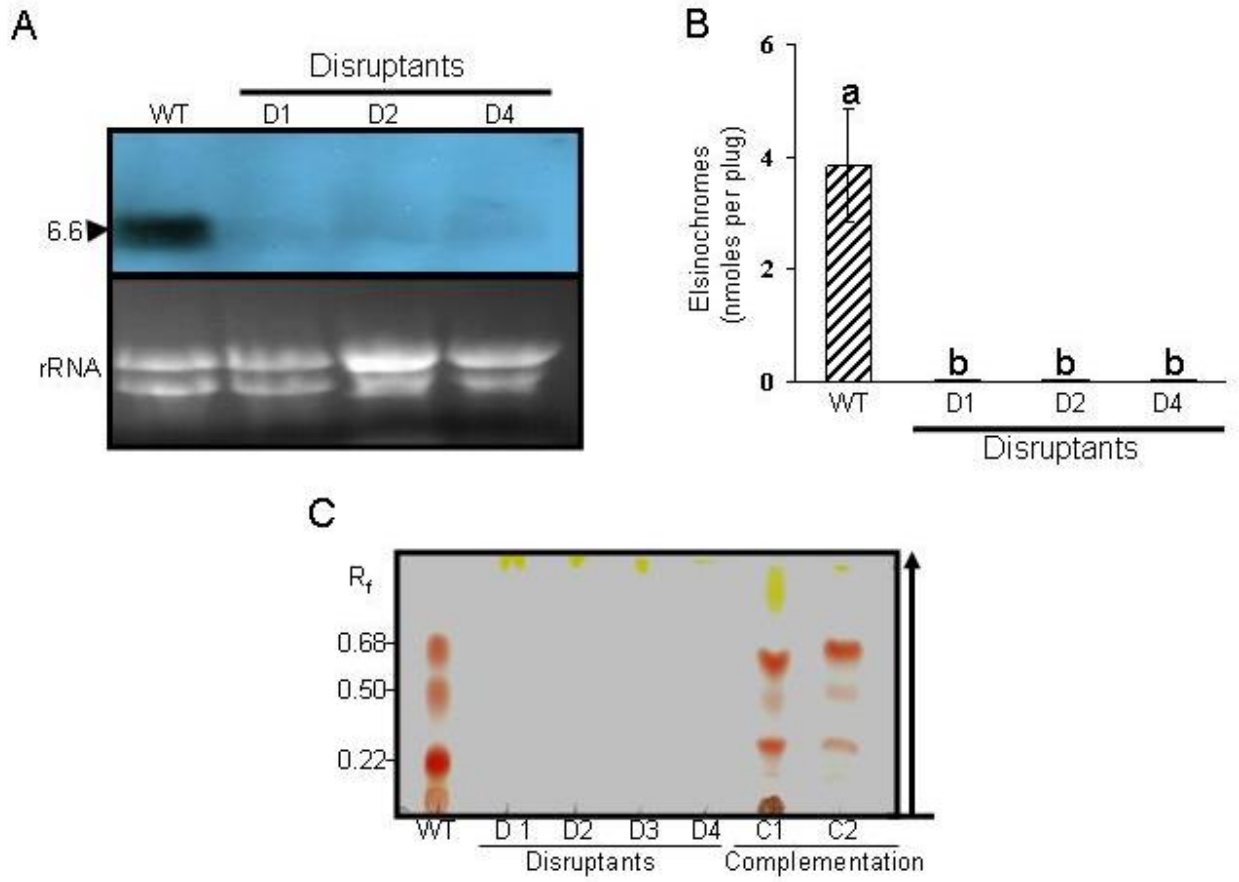


Figure 4-5. *EfPKS1* gene expression and ESC production in *EfPKS1* disruptants. A) Northern-blot analysis of fungal RNA purified from wild type (WT) and three putative *EfPKS1* disruptants (D1, D2, and D4) of *Elsinoë fawcettii.* RNA was denatured in a formaldehyde-containing gel, blotted to a nylon membrane, and hybridized to a DNA probe (Probe 3 in Fig. 4-4A). Gel stained with ethidium bromide indicates relative loading of RNA samples. Sizes of hybridization bands are indicated in kilobase pairs. B) Quantitative analysis of elsinochromes (ESCs) produced by wild type and *EfPKS1* disruptants. Fungal isolates were grown on PDA under continuous light for 5 days. ESC pigments were extracted with 5N KOH from agar plugs covered with fungal hyphae and measured at A_{480}. The concentrations of ESCs were calculated by referring to a regression line. Data represent the means of two different experiments with four replicates of each treatment. Vertical bars represent standard deviation. Means followed by the same letter are not different at judged by Duncan's multiple range test at $P < 0.0001$. C) TLC analysis of ESCs extracted with acetone from the cultures of wild type, four *EfPKS1* disruptants, and two strains (C1 and C2) expressing a functional *EfPKS1* gene. ESCs were separated on a TLC plate coated with a 60 F254 fluorescent silica gel and developed with chloroform and ethyl acetate (1:1 by volume).

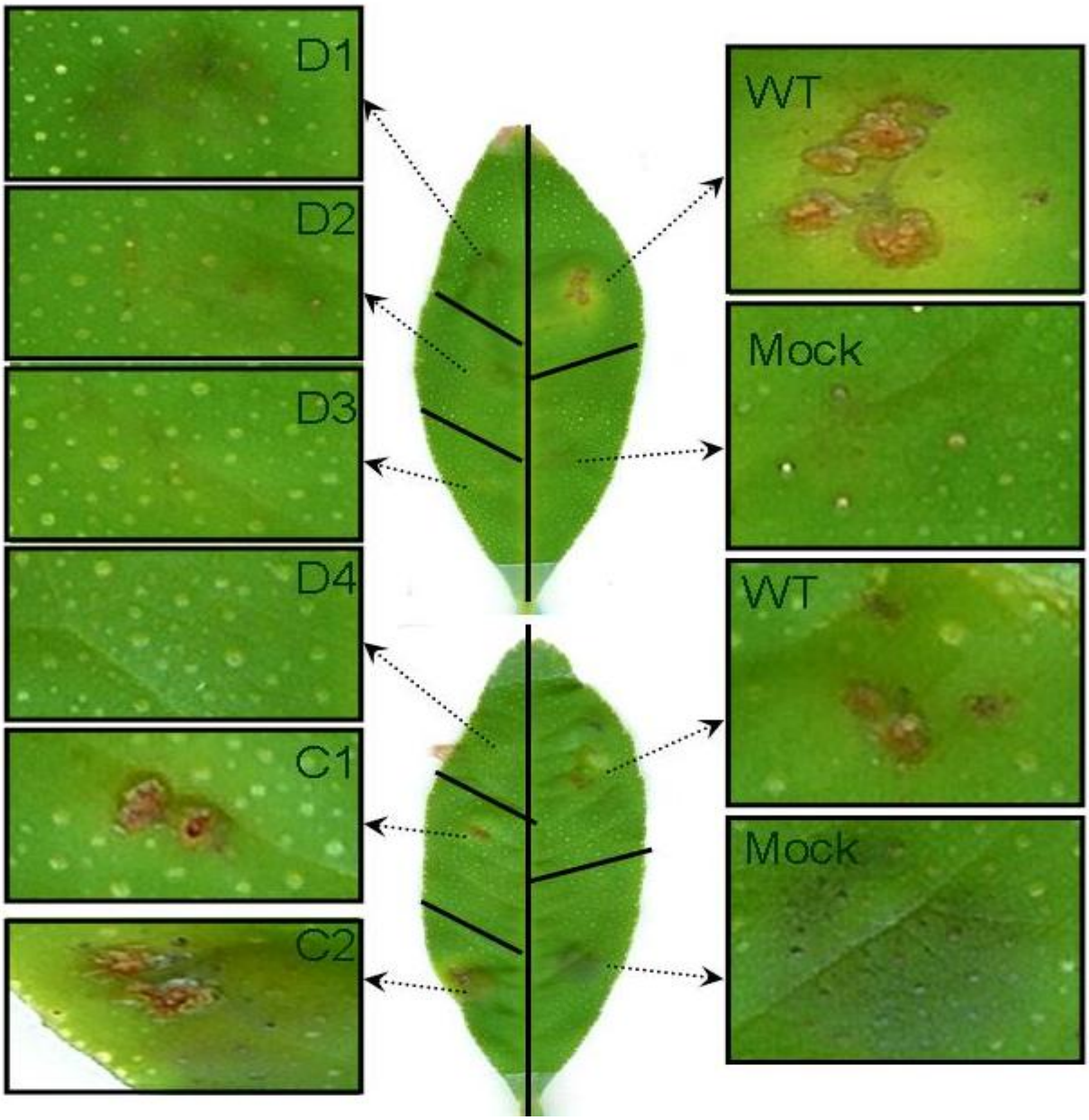


Figure 4-6. Pathogenicity assays on detached rough lemon leaves inoculated with agar plugs (4-mm in diameter) of wild type, four *EfPKS1* disruptants, and two strains (C1 and C2) expressing a functional *EfPKS1* gene of *Elsinoë fawcettii.* Fungal isolates were grown on PDA for 7 days and agar plugs covered with mycelial mats were cut and placed onto detached rough lemon leaves (14 days after emergence) in which fungal mycelium directly contacts the leaf surface. Inoculated leaves (> 20 leaves) were incubated for lesion development in a moist chamber under constant fluorescent light. Photos were taken 25 days post inoculation. The inoculated areas were cropped and enlarged for better illustration of necrotic lesions. The mocks were treated with agar plugs without fungal mycelium.

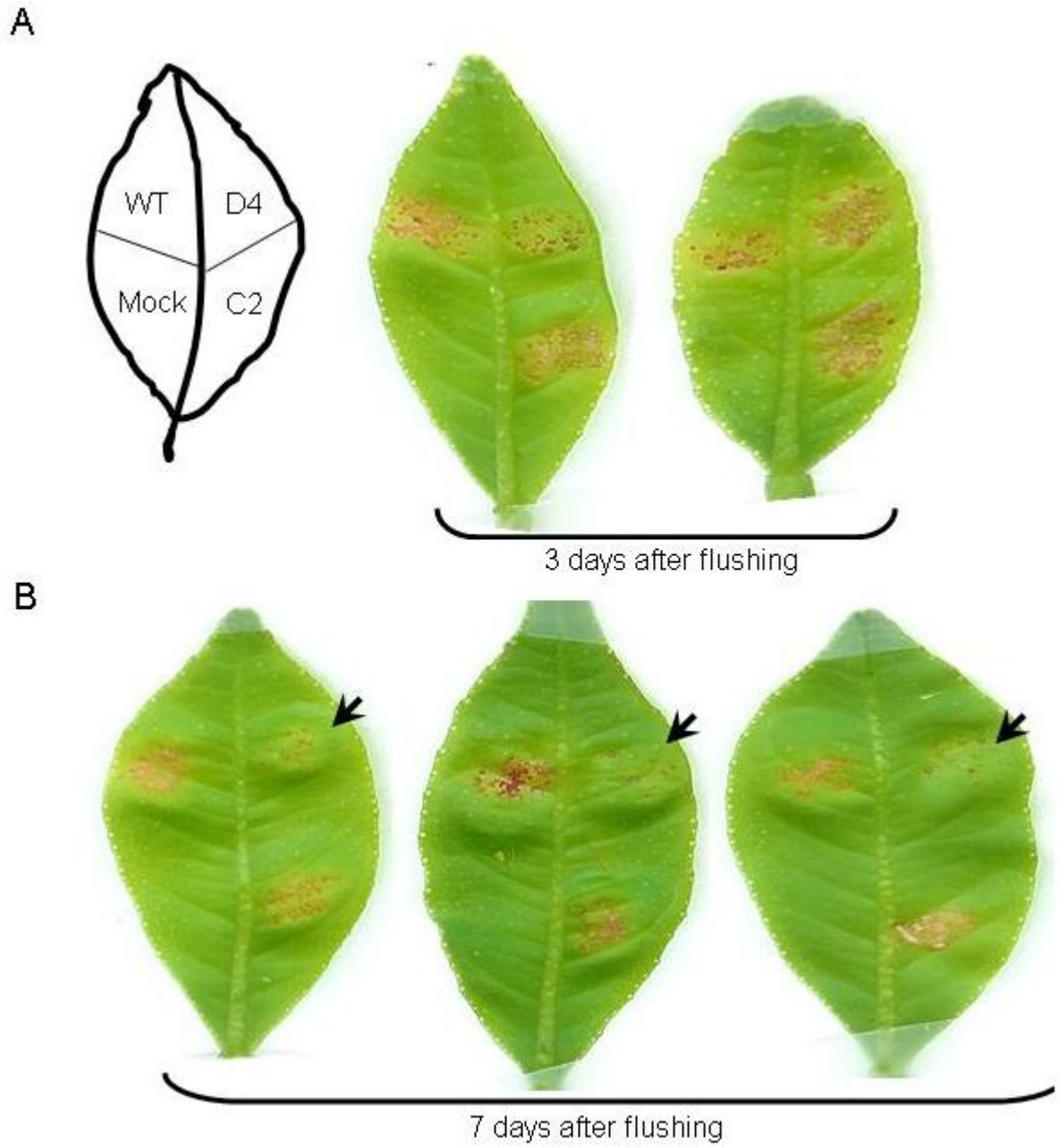


Figure 4-7. Pathogenicity assays using conidial suspensions of wild type, the *EfPKS1* disruptant (D4), and the complementation strain (C2) of *Elsinoë fawcettii* on detached rough lemon leaves 3 days (A) or 7 days (B) after emergence. Fungal conidia (1×10^5 mL^{-1}) were harvested and applied by applying 2 µL of suspension onto rough lemon leaves. Inoculated leaves (> 20 leaves) were incubated in a moist chamber. Formation of lesions was recorded 14 days post inoculation. The mocks were inoculated with water only. Reduction in lesion numbers and sizes by inoculation of *EfPKS1* disruptant (D4) are indicated by arrows. Only some of the representative replicates of the inoculated leaves are shown.

CHAPTER 5
CHARACTERIZATION OF A GENE CLUSTER REQUIRED FOR THE BIOSYNTHESIS OF ELSINOCHROMES BY *Elsinoë fawcettii*

In this chapter, a mini-gene cluster required for elsinochrome (ESC) biosynthesis is described. In the previous chapter, a polyketide synthase-encoding gene, *EfPKS1* (*Elsinoë fawcettii* polyketide synthase gene 1), was demonstrated to be required for ESC biosynthesis. Chromosome walking and inverse PCR were utilized to extend the sequences adjacent to *EfPKS1* fragment and identify additional genes that might be involved in ESC production. In addition to *EfPKS1*, nine putative open reading frames were identified in a span of 30 kb and were designated as the ESC biosynthetic gene cluster. The ESC cluster includes genes encoding a polyketide synthase (EfPKS1), a reductase (RDT1), an oxidoreductase (*OXR1*), a transcription factor (TSF1), a membrane transporter (ECT1) and a prefoldin protein subunit (PRF1) that are likely required for ESC biosynthesis, regulation, and translocation. Four other putative genes (*EfHP1*, *EfHP2*, *EfHP3*, and *EfHP4*) apparently do not encode polypeptides with obvious association with biosynthetic functions.

To determine if newly identified genes were required for ESC accumulation, the *TSF1* gene was deleted. The *TSF1* gene encodes the dual Cys_2His_2 zinc finger and $Zn(II)_2Cys_6$ binuclear cluster DNA-binding motifs and likely functions as a transcriptional regulator for ESC production. The *TSF1* deletion mutants of *E. fawcettii* failed to produce ESCs. Expression of the *RDT1*, *EfPKS1*, *PRF1*, and *EfHP1* but not the *ECT1, OXR1*, *EfHP2*, *EfHP3*, and *EfHP4* genes was markedly down-regulated in the *TSF* or *EfPKS1* null mutants. The results indicate that the *RDT1*, *TSF1*, *PRF1*, *ECT1, EfPKS1,* and *EfHP1* genes are required for ESC biosynthesis and accumulation.

Introduction

Elsinoë fawcettii, the causal agent of scab disease of citrus, produces elsinochromes (ESCs), causing cell death, necrotic lesions, and electrolytic leakage via production of reactive oxygen species (Liao and Chung 2008a). An *EfPKS1* (*Elsinoë fawcettii* polyketide synthase gene 1) was cloned and shown to be required for ESC production, and that ESCs are virulence factors during citrus pathogenesis (Liao and Chung 2008b).

Biosynthesis of secondary metabolites in fungi has long been known to respond to environmental cues, including the carbon/nitrogen source, light and pH, presumably mediated through the global regulatory factors such as CreA responding to carbon suppression; AreA responding to nitrogen signaling; PacC responding to ambient pH (Dowzer and Kelly 1989; Kudla et al. 1990; Tilburn et al. 1995). The global regulatory networks have been proposed to be regulated by a signaling pathway, involving the G protein/c-AMP/protein kinase-mediated signaling (Calvo et al 2002; Yu and Keller 2005). In Chapter three, accumulation of ESCs in culture was demonstrated to be affected by carbon and nitrogen sources, pH, and additional environmental factors. Thus, it seems likely that production of ESCs is also regulated by similar signaling pathways.

Genes for biosynthesis of secondary metabolites in filamentous fungi are often clustered and coordinately regulated by a pathway-specific transcriptional regulator (Yu and Keller 2005). A number of polyketide-derived fungal secondary metabolites, such as fumonisin, aflatoxin, ochratoxin A, aurofusarin, and cercosporin, are synthesized by clustered genes within the genomes of fungi (Proctor et al. 2003; Yu et al. 2004; Karolewiez and Geisen 2005; Malz et al. 2005; Chen et al. 2007). In this chapter, a mini gene cluster comprised of ten genes (*RDT1*, *TSF1*, *OXR1, EfPKS1*, *PRF1*, *ECT1* and *EfHP1-4*) is described in relation to ESC production in *Elsinoë fawcettii*. To determine if the genes are coordinately regulated and are involved in ESC

production, a *TSF1* gene was cloned and characterized as embedded in the cluster and encoding for a putative transcription activator containing dual Cys_2His_2 zinc finger and $Zn(II)_2Cys_6$ binuclear cluster DNA-binding domains.

Materials and Methods

Fungal Strains, Their Maintenance and Extraction/Analysis of ESC Toxin

The wild-type CAL WH-1 isolate of *Elsinoë fawcettii* Bitancourt & Jenkins (anamorph: *Sphaceloma fawcettii* Jenkins) was previously characterized (Liao and Chung 2008b). All procedures used for fungal culturing, toxin extraction, and screening for ESC-deficiency mutants were previously described (Chapter 2, 3, and 4).

Chromosomal Walking and Sequence Analysis

Fungal DNA was isolated employing a DNeasy Plant Mini kit (Qiagen, Valencia, CA). A genomic library of *E. fawcettii* was constructed from DNA digested with *Dra*I, *Eco*RI, *Pvu*I and *Stu*I, and ligated to adaptors using the Universal GenomeWalker kit (BD Biosciences, Palo Alto, CA) according to the manufacture's instructions. Primers were designed based the known sequences and paired with adaptor primers to obtain DNA fragments harboring unknown genomic regions using a 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 (Choquer et al. 2005). After purified with a DNA purification kit (Mo Bio Laboratories, Inc., Carlsbad, CA), the amplified DNA fragments were either directly sequenced or first cloned into pGEM-T easy vector (Promega) for sequence analysis at Eton Bioscience, Inc. (San Diego, CA). Oligonucleotides used for PCR and sequencing were synthesized by Integrated DNA Technologies (Coralville, IA) and Allele Biotechnology and Pharmaceuticals, Inc., (San Diego, CA). Similarity searches using a BlastX program (Altschul et al., 1997) were performed at the

National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Prediction of open reading frames (ORFs) and exon/intron junctions were first performed using the gene-finding software at http://www.softberry.com and were further confirmed by comparing genomic and cDNA sequence. Functional domains were identified using the PROSITE database in the ExPASy Molecular Biology Server (http://us.expasy.org) (Gasteiger et al. 2003) and 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 5.34-kb DNA fragment encompassing the whole *TSF1* ORF and flanking sequences was amplified by PCR with primers efup 11 (5'-CATCTCGCATATCTGGACCCGTC-3') and efup28 (5'-CGGGCTATTCTTAGAGCAGAG-3'). The amplified DNA fragment was purified with a DNA purification kit and cloned into pGEM-T easy vector to become pSTSF1128. Plasmid pSTSF1128 was digested with *Nru*I, blunted, and to accommodate an end-filled 2.1-kb fragment harboring the hygromycin phosphotransferase B gene (*HYG*) cassette under the control of the *Aspergillus nidulans trp*C gene promoter and terminator from pUCATPH (Lu et al. 1994) to create the disruption construct, pTSF1128 (Fig. 5-3). A split marker strategy was applied to facilitate double crossing-over recombination as previously described (Choquer et al. 2005). Briefly, a 4.4-kb DNA fragment containing truncated 5' *TSF1* fused with 5' *HYG* and a 4.2-kb fragment encompassing 3' *TSF1* linked to 3' *HYG* were amplified, respectively, with primers efup11/hyg3 (5'-GGATGCCTCCGCTCGAAGTA-3') and efup28/hyg4 (5'-CGTTGCAAGAACTGCCTGAA-3') from pTSF1128 using the Takara Ex Tar PCR system (Takara Bio USA, Madison, WI). Fungal

protoplasts were released from fungal 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 protoplasts (1×10^5) as previously described (Chung et al. 2002). The two hybrid fragments share 800 bp of overlapping sequence within the *HYG* gene. The *HYG* gene cassette is not functional until recombination takes place between the two truncated *HYG* DNA fragments. Fungal transformants were recovered from RMM medium containing 200 μg mL^{-1} hygromycin (Roche Applied Science, Indianapolis, IN) after 2 to 3 weeks and were screened for the loss of ESC production (red/orange pigments) on thin PDA.

For genetic complementation, a functional *TSF1* gene and its 5' non-translated region was amplified from genomic DNA with primers efup11 and efup28 by an Expand High Fidelity PCR system (Roche Applied Science) and confirmed by sequencing. The amplified *TSF1* gene cassette was co-transformed with plasmid pBARKS1 plasmid harboring a phosphinothrin acetyltransferase gene under control of the *A. nidulans trp*C promoter that is responsible for bialaphos resistance (Pall and Brunelli, 1993). Transformants were first identified from a medium supplemented with 50 μg/mL bialaphos (Gold Biotech, St. Louis, MO) and tested for production of the red/orange pigments (ESCs) on thin PDA plate as previously described (Liao and Chung 2008a; 2008b).

Molecular Techniques

Unless otherwise specified, standard molecular techniques were used for endonuclease digestion of DNA, electrophoresis, and Southern- and Northern-blot hybridizations. Plasmid DNA was propagated in *Escherichia coli* DH5α bacterial cells and purified using a Wizard DNA purification kit (Promega). Fungal RNA was purified by Trizol reagent per manufacture's directions (Invitrogen, Carlsbad, CA). 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. DNA probes used for Southern- and Northern-blot hybridization were labeled by PCR to randomly incorporate digoxigenin (DIG)-11-dUTP (Roche Applied Science) into DNA with gene-specific primers. Procedures used for probe labeling, hybridization, post-hybridization washing and immunological detection of the probe using a CSPD ($C_{18}H_{19}Cl_2O_7PNa_2$) chemofluorescent substrate for alkaline phosphatase were performed following the manufacturer's recommendations (Roche Applied Science).

Conidia Production and Pathogenicity Assays

Conidia were prepared as described in the Chapter 4. Fungal pathogenicity was evaluated on detached rough lemon (*Citrus jambhiri* Lush) leaves inoculated with agar plugs as previously described (Liao and Chung, 2008b). The inoculated leaves were incubated in a moist chamber under constant fluorescence light at an intensity of 3.5 joules m^{-2} s^{-1} and lesion formation was monitored weekly. Rough lemon trees were maintained in greenhouse and leaves of 7-10 days after flushing, with size approximately length 2.2-3.2 cm and width 1-1.5 cm, were harvested for pathogenicity assays.

Nucleotide Sequences

Sequence data reported in this article can be retrieved from the EMBL/GenBank Data Libraries under Accession nos. *EfHP1* (EU414199), *EfHP2* (EU414200), *EfHP3* (EU414201), *RDT1* (EU401704), *TSF1* (EU401705), *OXR1* (EU401706), *EfPKS1* (EU086466), *PRF1* (EU401707), *ECT1* (EU414198), and *EfHP4* (EU414202).

Results

Sequence Analysis and Determination of Open Reading Frames (ORFs)

A polyketide synthase gene (*EfPKS1*) required for elsinochromes (ESCs) biosynthesis has been determined as described in Chapter 4. The *EfPKS1* gene disrupted mutants, D1, D2, D3, and D4, were completely blocked in ESC production. Sequence analysis beyond the *EfPKS1* gene was performed to determine if any of the genes adjacent to *EfPKS1* are also required for ESC biosynthesis. Using a combination of chromosome walking and inverse PCR cloning strategies, over 30-kb of adjacent sequences were obtained after multiple rounds of polymerase chain reaction (PCR). In each round, new primers complementary to the end of assembled sequences were designed, synthesized and used to amplify overlapping DNA fragments from the genome of *E. fawcettii*. The presence of exons/introns and the identification of putative ORFs were first performed using computer software and were further confirmed by comparison of genomic and cDNA sequences. Analysis of the compiled sequences led to identify nine putative ORFs, in addition to the *EfPKS1* gene (Table 5-1; Fig. 5-1).

A similarity search in the NCBI database using BLASTX was performed to predict the function of the translated polypeptide. A *PRF1* gene, located downstream from *EfPKS1*, contains a 111-bp intron and encodes a putative polypeptide similar to prefoldin subunit 3. The putative *ECT1* gene product, excluding a 45-bp intron, displays strong similarity to numerous hypothetical proteins and membrane transporters, with the highest hit to nitrate transporter of *Aspergillus terreus* (E value = 3e-69; Table 5-1). A *TSF1* gene that is located upstream and transcribed divergently from *EfPKS1* encodes a putative protein, showing strong similarity to many zinc finger transcriptional factors of fungi. A *RDT1* gene, located further upstream from *TSF1*, has a 47-bp intron and encodes a putative polypeptide similar to a wide range of reductases, with strong similarity to 1,3,8-trihydroxy naphthalene (T_3HN) reductase involved in

melanin biosynthesis (Table 5-1). An *OXR1* gene contains a 45-bp intron and is located between the *EfPKS1* and *TSF1* genes. The OXR1 protein, rich in proline, has low similarity to malate:quinone oxidoreductases of bacteria (Table 5-1). Three putative ORFs (designated as *EfHP1-3*) upstream of the *RDT1* gene encode hypothetical proteins with unknown functions. A small segment, consisting of 44 amino acids, in the translated product of *EfHP*2, however, displays low similarity to putative cation/multidrug efflux pump of *Marinomonas* sp. (Table 5-1). The *EfHP1* and *EfHP3* genes have introns of 57 and 69 bp, respectively, whereas *EfHP2* is an intronless gene. A small *EfHP4* ORF, interrupted by 54-bp introns, is located downstream of *ECT1* and encodes a putative polypeptide, showing low similarity to isoleucyl t-RNA synthase which did not appear to involve in metabolic functions. Sequencing beyond the *EfHP1* or *EfHP4* gene failed to produce reliable sequences after several attempts with different primers (data not shown).

Expression of Putative ORFs and ESC Accumulation

In order to determine if the genes identified within the cluster were expressed and coordinately regulated under the conditions favorable for ESC accumulation, Northern-blot hybridization of RNA from *E. fawcettii* was conducted to examine the expression profiles of the *RDT1, TSF1,OXR1, EfPKS1*, *PRF1*, *ECT1* genes and four *EfHP* genes (Fig. 5-2A). All genes except for *EfHP*3 were favorably expressed when the fungus was under nitrogen starvation and their transcripts were nearly undetectable in the presence of ammonium nitrate. Expression of the *EfHP*3 gene appeared to be constitutive as its transcript remained unchanged in all conditions tested. Noticeably, accumulation of the *TSF1, EfPKS1*, *PRF1*, *ECT1,* and *EfHP2* gene transcripts increased considerably when the fungus was incubated in alkaline conditions. Under acidic conditions (pH 4), the *RDT1* and *EfHP1* gene transcripts accumulated slightly but not significantly higher compared to those in alkaline conditions. By contrast, expression of the

EfHP3 or *OXR1* gene was not affected by pH, as their transcripts were detected at similar levels both in acidic and alkaline conditions. Apparently, the *EfPKS1*, *PRF1* and *ECT1*genes were preferably expressed in the conditions with higher amounts of glucose, whereas accumulation of the *TSF1* and *RDT1* gene transcripts were repressed. Expression of the *EfHP3* gene was not regulated by glucose as its transcript accumulated equally well in both conditions (Fig. 5-2A). Quantification assays for the accumulation of elsinochromes (ESCs) revealed that large quantity of ESCs was exclusively detected when the fungus was cultured in the absence of nitrogen, in alkaline conditions (pH 8.0), or in the presence of higher amounts of glucose (Fig. 5-2B). Addition of ammonium nitrate, reducing the glucose concentration to 2 g L^{-1}, or changing the medium pH to acidic conditions (pH 4.0) completely suppressed ESC production.

Promoter Analysis for Identifying DNA Binding Motifs

The promoters of ten putative genes were analyzed to identify potential binding sequences for common regulatory elements such as the nitrogen regulatory protein (AreA), the carbon regulatory protein (CREA), the pH regulatory protein (PacC), and the light responsive proteins (WC1/WC2 complex). Analysis sequences upstream of the putative ATG translational start codon identified several consensus sequences in the *RDT1, TSF1, OXR1, EfPKS1*, *PRF1, ECT1*, and *EfHP1-4* promoters (Table 5-2). The consensus TATA motif was found in the promoters of all genes but *TSF1*, *OXR1*, *PRF1*, and *EfHP3*. All genes, but *EfHP4* 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*, *OXR1*, *EfPKS1*, *PRF1, ECT1*and *EfHP2-4*. All genes, except for *EfHP3*, have one or multiple GATA consensus sequence that is potentially recognized and bound by the nitrogen regulatory AreA protein or the light regulatory transcriptional WC1/WC2 complex (Kudla et al. 1990; Marzluf 1997; Linden et al. 1997). However, none of the promoter regions has the binding sequence (5'-

(G/C)PyGGGG-3') that is recognized by the CreA carbon repressor. The promoter regions of the *RDT1*, *EfPKS1*, and *ECT1* genes 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, however, have a consensus sequence, 5'-CATTC(C/T)-3' that acts a binding site for the AbaA transcriptional activator involved in conidiophore development in *A. nidulans* (Andrianopoulos and Timberlake 1994). Two palindrome sequences, 5'-TCG(N_{2-4})CGA-3' and 5'-CGG(N_{3-11})CCG-3', were also identified in the promoter regions of some but not all genes (Table 5-2).

Targeted Disruption of the *TSF1* Gene and Genetic Complementation

To determine if the genes that are clustered with *TSF1* are also involved in ESC biosynthesis and regulation, I characterized the *TSF1* gene encoding a putative transcriptional activator. The *TSF1* gene contains 3075 nucleotides, interrupted by four introns of 49, 50, 43, and 53 bp in lengths. The translated 959 amino acids of the *TSF1* gene displays strong similarity to numerous conserved proteins from the sequenced genomes of fungi and contains dual Cys_2His_2 type zinc finger (CTYCGHSFTRDEHLERHILTH and CFTCHMSFARRDLLQGHYTVH) and GAL4-like Zn_2Cys_6 binuclear cluster DNA-binding (CSNCAKTKTKCDKKFPCSRCASRNLRC) domain signatures. The regulatory function of the *TSF1* gene product in relation to the biosynthesis of ESCs was determined genetically, by creating and analyzing targeted disruptants that were specifically mutated at the *TSF1* locus. To increase the efficiency of recovery of transformants with targeted gene disruption, two split DNA fragments overlapping 800 bp within the *HYG* cassette were amplified separately from the disruption construct pTSF1128 (Fig. 5-3A) and directly transformed into wild-type of *E. fawcettii*. Since the *HYG* cassette was truncated in either fragment, only transformants with the

restored *HYG* function via homologous recombination would survive and form colonies on the regeneration medium supplemented with hygromycin. All transformants recovered were then screened for production of red/orange pigments (ESCs) on a thin PDA under constant fluorescent light. Of 20 transformants tested, three failed to accumulate the red/orange pigments and were considered *TSF1* disruptants. Successful disruption of the *TSF1* gene was confirmed by Southern blot analysis. Hybridization of genomic DNA digested with *Pvu*II endonuclease and hybridized to a *TSF1* gene probe resulted in a 4.8-kb hybridizing band in the wild type and two hybridizing bands with expected sizes of 3.9 and 3.2 kb, as a result of integration of the *HYG* cassette, in the putative disruptants (Fig. 5-3B). Hybridization of genomic DNA cleaved with *Bgl*I and *Pvu*II also yielded different banding patterns between the wild type and the putative disruptants as expected (Fig. 5-3C), indicating a specific disruption at the *TSF1* locus.

Northern-blot analysis was conducted to determine if the putative mutants accumulated the *TSF1* transcript. As shown in Fig. 5-4A, hybridization of total RNA identified a sole *TSF1* transcript of 3.1-kb in size 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 they were *TSF1* null mutants. Functional complementation was performed to further determine the role of *TSF1* in relation to the biosynthesis of ESCs. Transformants were screened for recovery of the red-pigmented fungal strains that acquired and expressed a functional *TSF1* gene cassette. Two fungal strains (CA and CB) accumulated low amounts of the red/orange pigments were selected for further characterization. Northern-blot analysis revealed that the *TSF1 t*ranscript was detected in both CA and CB strains, but not in their progenitor T3 strain (Fig. 5-4A). Phenotypic analyses indicated that the *TSF1* disruptants failed to accumulate ESCs in axenic cultures compared to wild type, as either assayed spectrophotometry after KOH

extraction (Fig. 5-4B) or by TLC analysis (Fig. 5-4C). Compared to wild type, the CA and CB complementation strains produced much less ESCs on PDA (Fig. 5-4B). TLC analysis of the pigments extracted from the CA strain indicated that the band with R_f 0.68 was the most abundant pigment (band 1), whereas the extracts from wild type showed three derivatives of pigments (R_f 0.68, R_f 0.50, and R_f 0.22) with band 3 as the major compound (Fig. 5-4C). Spectrophotometric scanning indicated that the acetone extracted pigments from wild type, CA, and CB strains, displayed a strong absorbance at 460 nm with two minor peaks at 530 and 570 nm, whereas all Δtsf1-disrupted mutants (T1, T2, and T3) had no absorbance peaks.

Gene Regulation by TSF1 and Feedback Inhibition

The central role of the *TSF1* gene product as a transcriptional activator was assessed for expression of the clustered genes in two *TSF1* disruptants (Fig. 5-5). As shown in Fig. 5-5, disruption of the *TSF1* gene caused a decreased accumulation of the *RDT1*, *EfPKS1*, *PRF1*, and *EfHP1* gene transcripts, but did not cause a drastic reduction for expression of the *OXR1*, *ECT1, EfHP2*, and *EfHP3* genes. Expression of the *EfPKS1* gene was restored in the CA and CB complementation strains. Accumulation of the gene transcripts was also examined in an *EfPKS1* null mutant (D4) that is defective in ESC production. Northern hybridization analysis revealed a marked reduction of transcripts of the *RDT1*, *TSF1*, *PRF1*, *ECT1*, and *EfHP1* genes in the D4 mutant (Fig. 5-6). Accumulation of the *RDT1*, *TSF1*, *PRF1*, *ECT1*, and *EfHP1* gene transcripts was nearly restored in strains (C1 and C2) expressing a functional copy of the *EfPKS1* gene. In contrast, expression of the *OXR1*, *EfHP2*, and *EfHP3* gene transcripts was apparently not influenced by disruption of the *EfPKS1* gene, as evidenced by the fact that their transcripts accumulated to the levels comparable to those of wild type (Fig. 5-6).

Discussion

Elsinochrome (ESC) phytotoxins produced by many *Elsinoë* species contain a perylenequinone backbone and is likely synthesized via a defined polyketide pathway. I have previously demonstrated that deletion of the *EfPKS1* gene encoding a fungal polyketide synthase in an isolate of *E. fawcettii* leads to complete inability in the production of ESC which demonstrates that ESC plays an essential role in fungal virulence (Liao and Chung 2008b). The genes involved in the biosynthesis of fungal polyketide compounds are often closely linked in the genome. The case for the biosynthesis of ESCs by *E. fawcettii*, is no exception. In this chapter, the regions flanking *EfPKS1* were sequenced to obtain a 30-kb continuous DNA stretch. Nine additional putative ORFs were identified, some of which encode polypeptides likely involved in biosynthesis, regulation, and accumulation of ESCs.

EfPKS1 functions in the assembly of the polyketide backbone of ESCs in an analogous fashion to fatty acid and polyketide biosynthesis (Fig. 5-7). The putative biosynthetic pathway begins with the condensation of acetyl-CoA (starter) and malonyl-CoA (extender) units. EfPKS1 contains several conserved domains including a keto synthase (KS), an acyltransferase (AT), a thioesterase (TE) and two acyl carrier protein (ACP) domains that may act cooperatively to form the polyketomethylene backbone of ESCs. The malonyl-CoA subunit is assumed to attach to the ACP domains of EfPKS1 to form a phosphopantotheine (PPT) complex (Watanabe and Ebizuka 2004) that accepts a unit from acetyl-CoA via the function of the AT domain. The KS domain of EfPKS1 catalyzes condensation of the malonyl- and acetyl-CoAs by decarboxylation. After each cycle of condensation, the malonyl keto group is reduced. The putative polyketide synthase encoded by EfPKS1 iteratively catalyses the synthesis by incorporating two carbons in each cycle into a linear polyketide chain which is then released and cyclized through a TE domain in the *EfPKS1* (Birch 1967). Additional modification steps are present to produce a heptaketide

backbone of ESCs. ESCs have a bilateral symmetrical structure (Fig. 5-7). Thus, formation of the mature ESCs is likely mediated by dimerization.

In addition to the *EfPKS1* gene, the *RDT1* gene encoding a putative reductase, the *TSF1* gene encoding a putative transcriptional activator, and the *OXR1* gene encoding a putative oxidoreductase are likely involved in modification of ESC backbone (Fig. 5-7). The *ECT1* gene encoding a probable membrane transporter is likely functioning in toxin secretion outside the fungal cells to avoid toxicity (Fig. 5-7). In addition, there is no methyltransferase-coding gene in the ESC cluster even though ESC has four methyl groups at position C3, C7, C8, and C12 (Weiss et al. 1965; Lousberg et al. 1969). It appears that the identified ESC gene cluster does not contain all the genes responsible for ESC biosynthesis. The role of the *PRF1* gene encoding a putative prefoldin protein in relation to ESC production remains unknown. Similarly, the remaining four ORFs (*EfHP1-4*) encode hypothetical proteins with unknown functions. Whether or not they are involved in the ESC production will require further investigation by a deletion strategy for the corresponding genes.

All the putative ORFs identified in the cluster were expressed as judged by Northern-blot analysis. Apparently, ESCs were favorably produced when the fungus was cultured in lower nitrogen, higher pH, and higher carbon supply. However, accumulation of the gene transcripts was not fully congruent with the conditions conductive for ESC production. Expression of all ORFs other than *EfHP3* and accumulation ESCs were regulated under nitrogen starvation, but obviously lacked strong correlation between gene expression and ESC accumulation in response to pH or carbon source. Analysis of the promoter regions of the ESC clustering genes for possible DNA binding sites of global transcriptional regulators revealed that all ten genes contain nitrogen or light regulatory binding elements (GATA) in their promoter regions that are

recognized by AreA nitrogen regulator (Marzluf 1997; Wilson and Arst 1998), and the WC1/WC2 light-responsive regulator (Linden et al. 1997). The GCCARG consensus motif involved in the binding of the pH-responsive PacC regulator (Espeso et al. 1997) was found in the 5'-untranslated regions of the *TSF1*, *EfPKS1*, *PRF1*, *ECT1* and *OXR1* genes but not *RDT1* promoter regions. Expression of *TSF1*, *EfPKS1*, *PRF1*, and *ECT1* but not *RDT1* and *OXR1* genes increased at alkaline pH. Interestingly, PacC has been reported to regulate fungal virulence genes required for phytopathogens (Rollins and Dickman 2001; Caracuel et al. 2003; You et al. 2007). Thus, I predict that PacC may also function as a fungal virulence factor in *E. fawcettii*, likely via regulation of the genes involved in ESC biosynthesis.

Many of the Zn_2Cys_6 harboring transcriptional activators have been shown to recognize and bind the palindrome DNA sequence with inverted repeats of CGG or TCG elements separated by a spacer with various nucleotides, such as [5'-CGG(N_3-N_{11})CCG-3'] or [5'-TCG(N_2-N_4)CGA-3] (Marmorstein and Harrison 1994; Li and Kolattukudy 1997; Fernandes et al. 1998; Ehrlich et al. 2002). Promoter analysis also identified similar palindrome DNA sequence in the 5'- untranslated regions of *TSF1*, *OXR1*, *EfPKS1*, and *PRF1*, that may also be involved in transcriptional regulation. Northern-blot analysis also uncovered that not all of the clustered genes were coordinately regulated through the putative transcriptional regulator, TSF1, implying the requirement of other transcriptional regulators whose coding sequences are not linked to the ESC gene cluster. However, deletion of the *TSF1* gene resulted in fungal mutants that completely fail to accumulate any detectable *TSF1* transcript and ESCs, indicating the TSF1, likely acts as a core transcriptional activator, playing an essential role in ESC biosynthesis. Deletion of the *TSF1* gene influenced expression of the *RDT1*, *EfPKS1*, and *PRF1* gene considerably, yet had little effect for transcriptional suppression of the *ECT1* and *OXR1* genes.

Similarly, it is not yet clear whether or not the putative *EfHP1-4* ORFs close to the ESC cluster are also involved in ESC biosynthesis even though the expression of some of these genes was regulated by nitrogen starvation and affected by the deletion of *EfPKS1* or *TSF1* gene. Thus, the function of the ESC genes other than *EfPKS1* and *TSF1* in the biochemical pathway leading to ESC production can not be conclusively determined until each of the genes is inactivated. Expression of the ESC clustering genes in an *EfPKS1* null mutant reveals a transcriptional feedback inhibition, in which deletion of the *EfPKS1* gene suppressed expression of the *RDT1*, *TSF1*, *PRF1*, and *ECT1* genes in the cluster. Expression of a copy of the functional *EfPKS1* gene in a respective null mutant restored their expression to the extent comparable to those of their wild-type progenitor, further supporting the requirement of the *RDT1*, *TSF1*, *PRF1*, and *ECT1* genes for ESC biosynthesis.

In a prior study, deletion of the *EfPKS1* gene gave rise to a remarkable reduction in conidiation in addition to deficiency in the ESC production. Similar to the *EfPKS1*-disrupted mutants, the TSF1 null mutants were also severely disrupted in conidial production (data not shown). The connections between production of secondary metabolites and cell development and/or differentiation have also been investigated in other filamentous fungi (Adams and Yu 1998; Calvo et al. 2002; Yu and Keller 2005; Brodhagen and Keller 2006). Interestingly, examination of the 5'-untranslated regions revealed the presence of the consensus MRAGGGR motifs in the promoter regions of the *RDT1*, *EfPKS1*, and *ECT1* genes, which are presumably involved in the binding for a conidiation-specific Bristle (BrlA)-like transcriptional activator as described in *A. nidulans* (Adams et al. 1998). In addition, the consensus CATTCY motifs, recognized by the AbaA transcriptional activator (Andrianopoulos and Timberlake 1994), are found in the *RDT1*, *OXR1*, *EfPKS1*, and *ECT1* gene promoters. AbaA is activated by BrlA and is

involved in conidiphore development of *A. nidulans* (Andrianopoulos and Timberlake 1994). It is possible that the proteins or intermediates involved in the ESC biosynthesis and conidial formation might be coordinately regulated, at lease in part, by common transcriptional regulators in a complex and intertwined networks that include the G-protein/cAMP/protein kinase A- and the mitogen-activated protein (MAP) kinase-mediated signaling cassettes as described in other filamentous fungi (Calvo et al. 2002).

Expression a functional *TSF1* gene cassette in the respective null mutants failed to fully restore conidiation, ESC production, or fungal pathogenesis. It is very unlikely that failure to completely restore the mutant phenotypes was attributed to the transformed *TSF1* gene. Sequence analysis of the transformed *TSF1* gene cassette revealed no nucleotide substitutions or insertions (data not shown). Northern-blot analysis also showed a normal expression of the *TSF1* gene in the recombinant transformants comparable to those of wild type. The failure of *TSF1* to complement ESC production, conidiation, and virulence, when controlled by its endogenous promoter, was somewhat surprising and highlighted the requirement of specific regulation for the function of *TSF1* in particular and all other genes in the cluster in general.

As summarized in Fig. 5-8, I propose a regulatory model for the possible linkage of ESC biosynthesis and environmental cues. *Elsinoë fawcettii* responds to various external signals, such as light, pH, and nutrients. Subsequently, membrane receptors relay the external stimuli into fungal cells to elicit the global signaling pathways, such as G protein-mediated signaling pathways to release various secondary messengers. These second messengers, such as cAMP, Ca^{2+}, 1,4,5-trisphosphate (IP_3), or others, in turn trigger global transcription factors that directly or indirectly activate *TSF1*. Since the *EfPKS1*- and *TSF1*- disrupted mutants resulted in a

reduced conidiation, it is very likely that biosynthesis of ESC and fungal conidiation are coordinately regulated.

Table 5-1. Elsinochrome (ESC) biosynthetic gene cluster and adjacent *EfHP1-4* genes encoding hypothetical proteins in *Elsinoë fawcettii*

Gene (accession number)	Length (bp)	Intron no. (size in bp)	Amino acids	Closest blast match proteins and species (accession no.)	Amino acid similarity (%)	*E*-value
RDT1 (EU401704)	851	1 (47)	267	Hypothetical protein, *Botryotinia fuckeliana* (EDN21223)	224/264 (84%)	6e-110
				1,3,8-trihydroxynaphthalene reductase, *Alternaria alternata* (BAA36503)	221/261 (84%)	1e-108
TSF1 (EU401705)	3075	4 (49, 50, 43, and 53)	959	Hypothetical protein, *Phaeosphaeria nodorum* (EAT80399)	558/956 (58%)	0
				Transcription factor Cmr1, *Cochliobolus heterostrophus* (ABI81496)	659/1032 (63%)	0
OXR1 (EU401706)	744	1 (45)	232	Malate:quinone oxidoreductase, *Pseudomonas syringae* (AAY36032)	65/163 (39%)	1.1
EfPKS1 (EU086466)	6631	1 (52)	2192	Fungal type I polyketide synthase, *Bipolaris oryzae* (BAD22832)	1656/2197 (75%)	0
PRF1 (EU401707)	705	1 (111)	197	Hypothetical protein, *Phaeosphaeria nodorum* (EAT80391)	147/178 (82 %)	1e-67
				Probable prefoldin subunit 3, *Neosartorya fischeri* (EAW23892)	151/190 (79 %)	2e-57
ECT1 (EU414198)	1563	1 (45)	505	Hypothetical protein, *Phaeosphaeria nodorum* (EDN17791)	273/522 (52 %)	2e-82
				Highly affinity nitrate transporter, *Aspergillus terreus* (EAU39122)	260/513 (50 %)	3e-69

Table 5-1. (Continued)

Gene (accession number)	Length (bp)	Intron no. (size in bp)	Amino acids	Closest blast match proteins and species (accession no.)	Amino acid similarity (%)	*E*-value
EfHP1 (EU414199)	993	1 (57)	311	Hypothetical protein, *Chaetomium globosum* (EAQ88529)	71/147 (48%)	8e-06
EfHP2 (EU414200)	1158	0	385	Putative cation/multidrug efflux pump, *Marinomonas* sp. (EAQ67386)	44/94 (46%)	1.0
EfHP3 (EU414201)	819	1 (69)	249	Hypothetical protein, *Coprinopsis cinerea* (EAU83036)	60/117 (51%)	3e-06
EfHP4 (EU414202)	423	1 (54)	122	Isoleucyl-tRNA synthase, *Bacillus subtilis* (CAB13417)	39/80 (48%)	0.26

Table 5-2. Promoter analysis of genes in the elsinochrome (ESC) biosynthetic gene cluster of *Elsinoë fawcettii*

Gene		*RDT1*	*TSF1*	*OXR1*	*EfPKS1*	*PRF1*	*ECT1*	*EfHP1*	*EfHP2*	*EfHP3*	*EfHP4*
TATA		1	0	0	1	0	1	1	1	0	1
CCAAT or CAAT		7	7	7	9	8	2	7	3	4	0
AreA or WC1/WC2 GATA		3	4	1	8	5	1	1	6	0	7
pH regulatory GCCA (A/G)G		0	1	1	4	1	1	0	1	1	1
BrlA (A/C)(A/G)AGGG (A/G)		1	0	0	3	0	1	0	0	0	0
AbaA CATTC (C/T)		3	0	3	2	0	2	0	0	0	0
Palin-drome	TCG(N_{2-4})CGA	0	1, (N_3)	1, (N_3)	2, ($N_{2\ \&\ 3}$)	2, ($N_{3\ \&\ 4}$)	0	0	0	0	0
	CGG(N_{3-11})CCG	0	2, ($N_{5\ \&\ 11}$)	2, ($N_{8\ \&\ 9}$)	1, (N_{10})	1, (N_3)	0	0	0	0	0

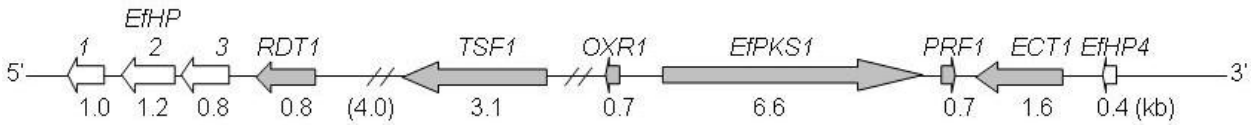


Figure 5-1. Elsinochrome (ESC) biosynthetic gene cluster. The 30-kb DNA region harboring 10 genes is shown. The putative genes include *RDT1* (reductase 1), *TSF1* (transcription factor 1), *OXR1* (oxidoreductase 1), *EfPKS1* (polyketide synthase 1), *PRF1* (prefoldin 1), *ECT1* (transporter 1). The nucleotide sequence of the 5' end flanking region contains three ORFs designated *EfHP1*, *EfHP2*, and *EfHP3*, and the 3' end contains one ORF designated *EfHP4*.

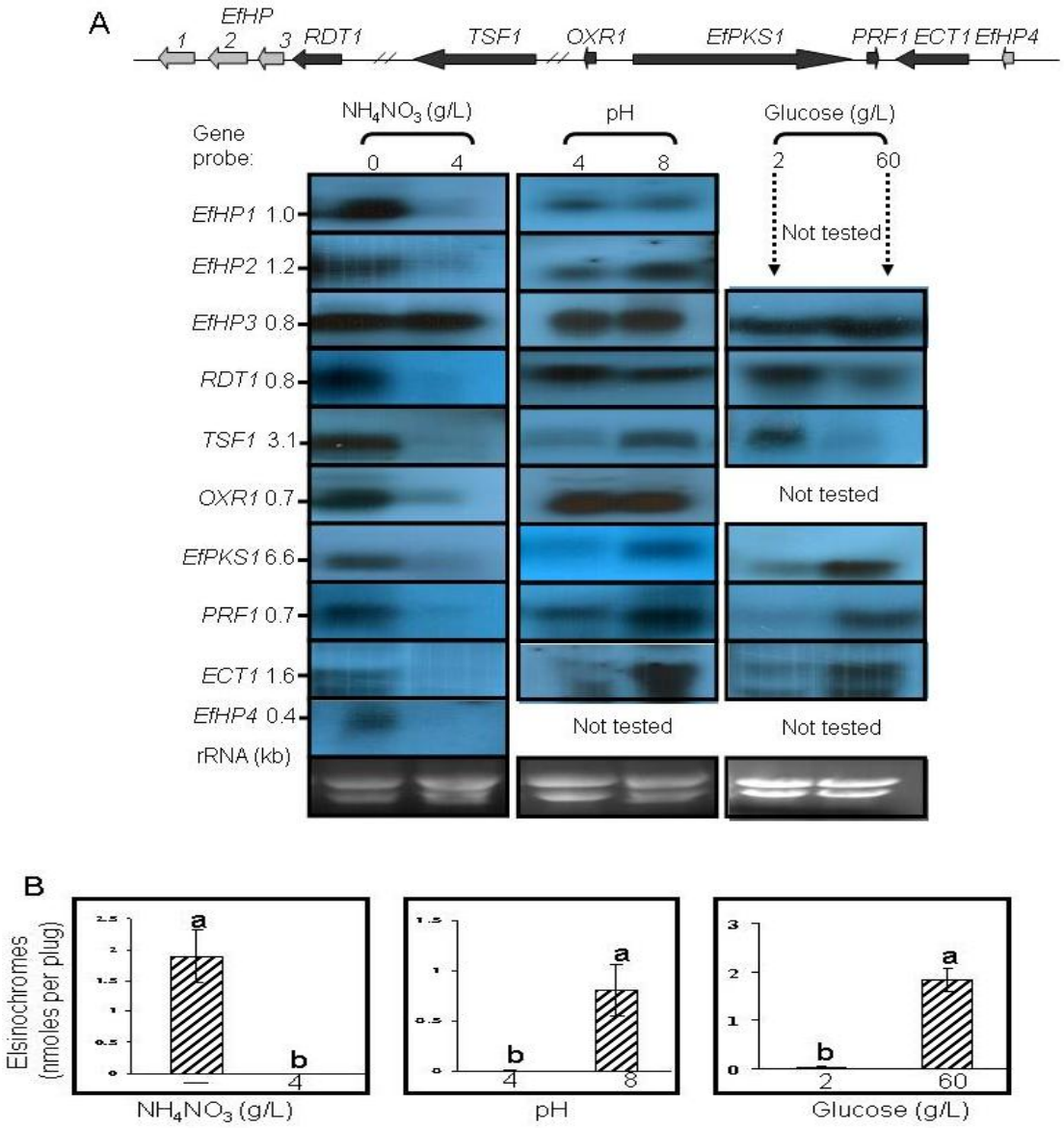


Figure 5-2. Northern-blot analysis of gene expression and elsinochrome (ESC) production in response to nitrogen, pH, and glucose in *Elsinoë fawcettii*. Fungal isolate was grown on complete medium containing different amounts of glucose, or on minimal medium containing different concentrations of ammonium nitrate or buffered to various pH as indicated under continuous light. A) Fungal RNA was isolated from 7-day-old cultures, electrophoresed in an agarose gel containing formaldehyde, transferred onto a nylon membrane, and hybridized to the gene-specific probes. Ribosomal RNA (rRNA) stained with ethidium bromide is shown for relative loading of the samples. B) ESCs were extracted with 5 N KOH and measured at A_{480}. The amounts of ESCs were determined by reference to a regression line that was established using ethyl acetate-purified ESCs. ESC data represent the means of two different experiments with four replicates of each treatment. Vertical bars represent standard deviation. Means followed by the same letter are not different at judged by Duncan's multiple range test at $P < 0.0001$.

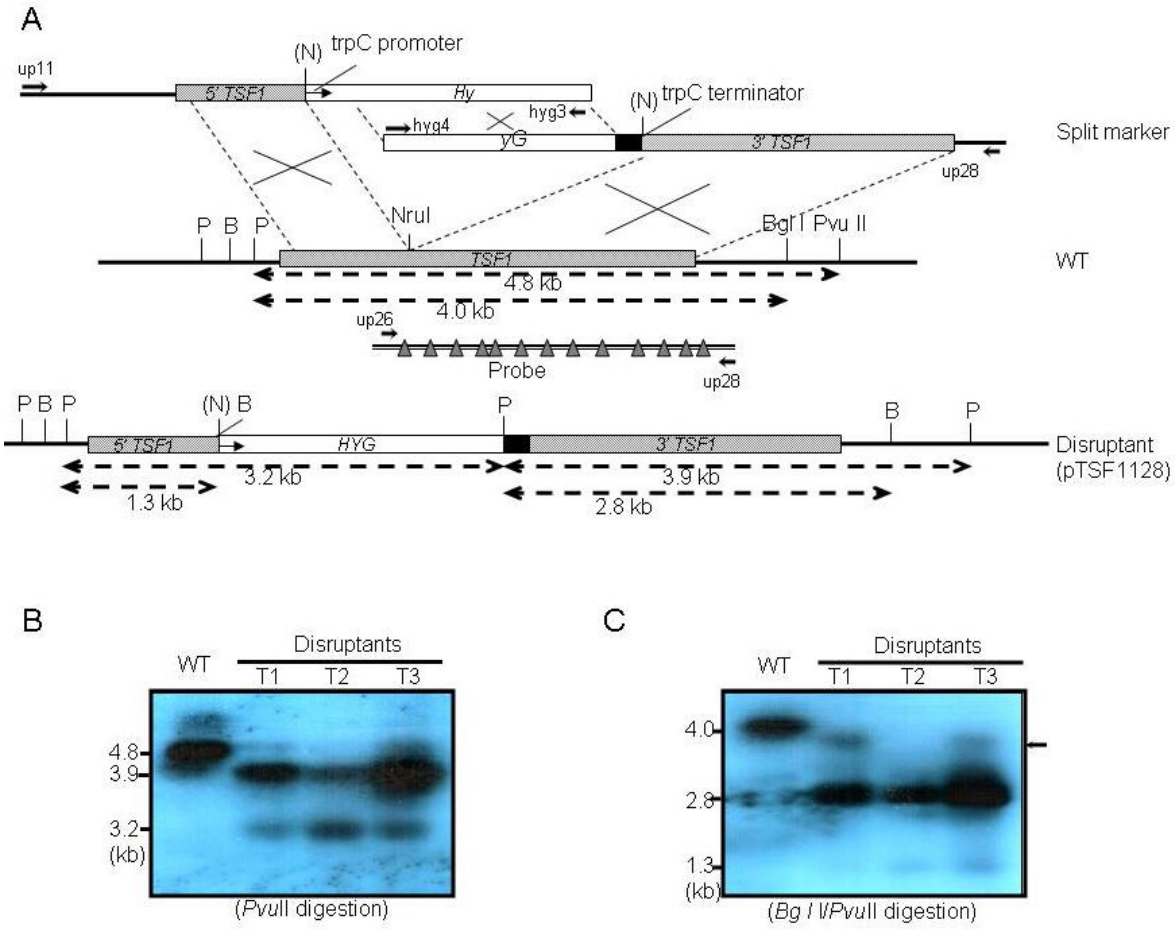


Figure 5-3. Targeted gene disruption of *TSF1* encoding a putative transcription regulator which contains dual Cys_2His_2 type zinc finger and GAL4-like Zn_2Cys_6 binuclear cluster DNA-binding domain using a split-marker strategy in *Elsinoë fawcettii*. A) Restriction maps of the *TSF1* gene in the genome of wild type (WT) and Δtsf1-disrupted mutant. Two truncated *TSF1* fragments fused with an overlapping *HY*/*YG* (hygromycin resistance gene cassette) were amplified by PCR with Oligonucleotide primers (up11 paired with hyg3; up28 paired with hyg4) as indicated. Note: drawing is not to scale. B, *Bgl*I; N, *Nru*I; P, *Pvu*II. B) and C) Southern-blot analysis of genomic DNA from wild type (WT) and three Δtsf1-disrupted mutants (T1, T2 and T3). Fungal DNA was digested with *Pvu*II (B) or *Bgl*I/*Pvu*II (C), electrophoresed, blotted onto a nylon membrane and hybridized with *TSF1* gene specific probe amplified by primers, up 26/up28, as indicated in A. Hybridizing patterns indicate disruption of the *TSF1* gene.

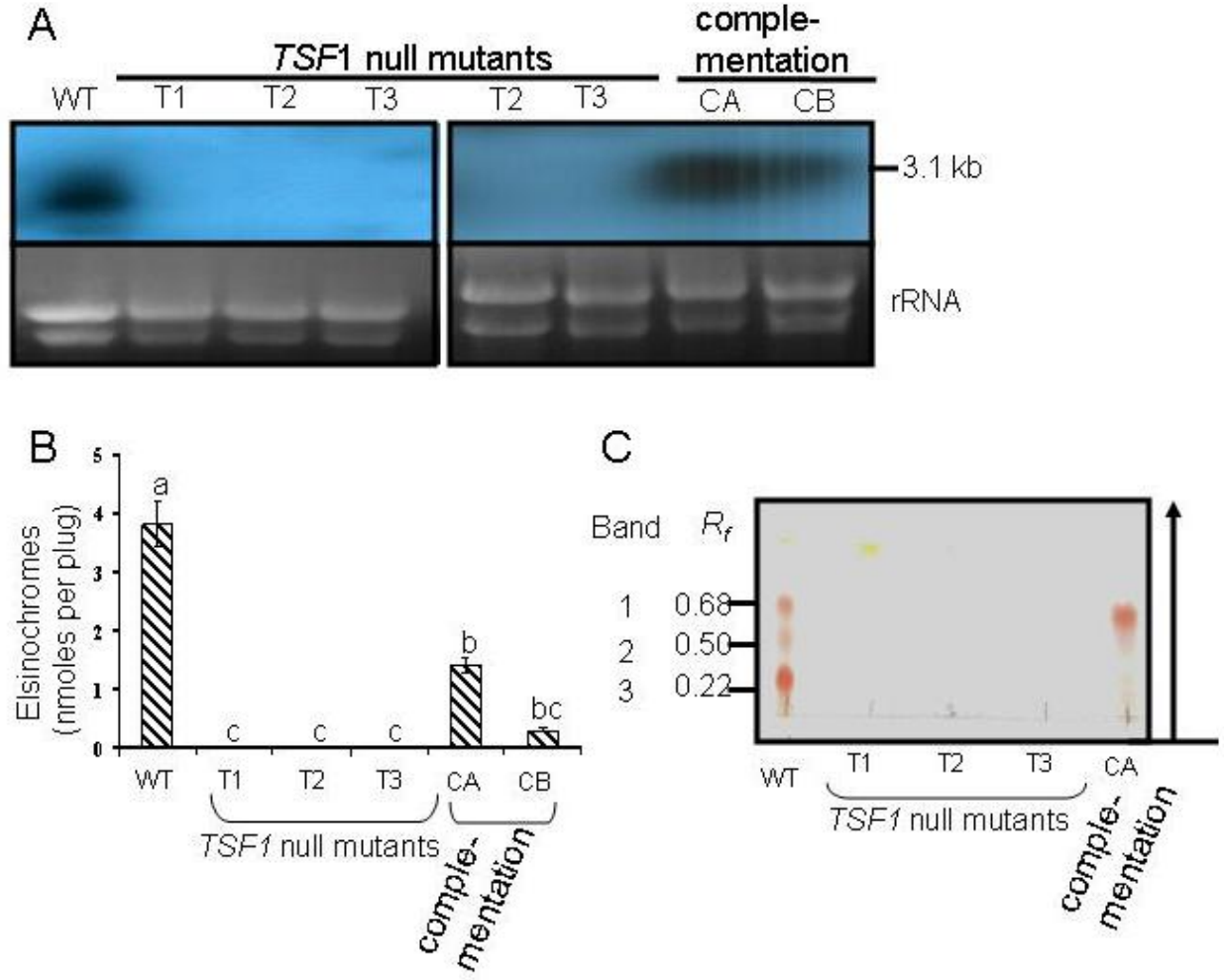


Figure 5-4. *TSF1* gene expression and ESC production in *TSF1* disruptants. A) Northern-blot analysis of fungal RNA purified from wild type (WT), three putative Δtsf1-disrupted mutants (T1, T2 and T3), and two *TSF1*-complemented strains (CA and CB) of *Elsinoë fawcettii*. RNA was denatured in a formaldehyde-containing gel, blotted to a nylon membrane, and hybridized to a DNA probe (probe amplified by primers, up26/up28 in Fig. 5-3A). Gel stained with ethidium bromide indicates relative loading of the RNA samples. Sizes of hybridization bands are indicated in kilobase pairs. B) Quantitative analysis of ESCs produced by wild type, *TSF1* disruptants and complemented strains. Fungal isolates were grown on PDA under continuous light for 7 days. ESC pigments were extracted with 5N KOH from agar plugs covered with fungal hyphae and measured at A_{480}. The concentrations of ESCs were calculated by referring to a regression line. Data represent the means of two different experiments with four replicates of each treatment. Vertical bars represent standard deviation. Means followed by the same letter are not different at judged by Duncan's multiple range test at $P < 0.0001$. C) TLC analysis of ESCs produced by wild type, three Δtsf1-disrupted mutants, and complemented strain (CA).

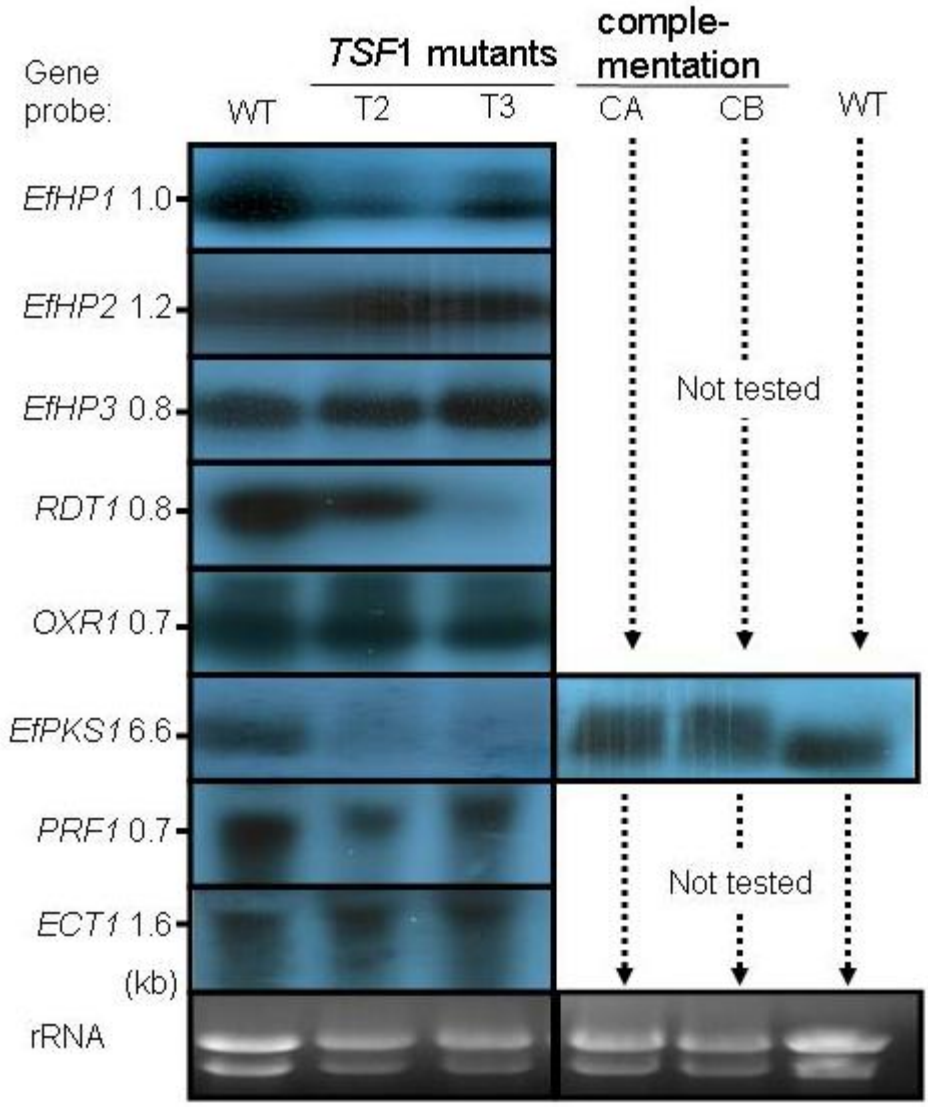


Figure 5-5. Northern-blot hybridization indicates that the Δtsf1-disrupted mutants failed to accumulate the EfPKS1 and reduce production of *RDT1*, *PRF1* and *EfHP1* transcripts. Ethidium bromide-stained rRNA indicates the relative loading of the samples. Total RNA was isolated from fungal strains grown on potato dextrose agar under continuous light for 7 days, electrophoresed in formaldehyde-containing gels, blotted onto nylon membranes and hybridized to the probes as indicated at 50 °C.

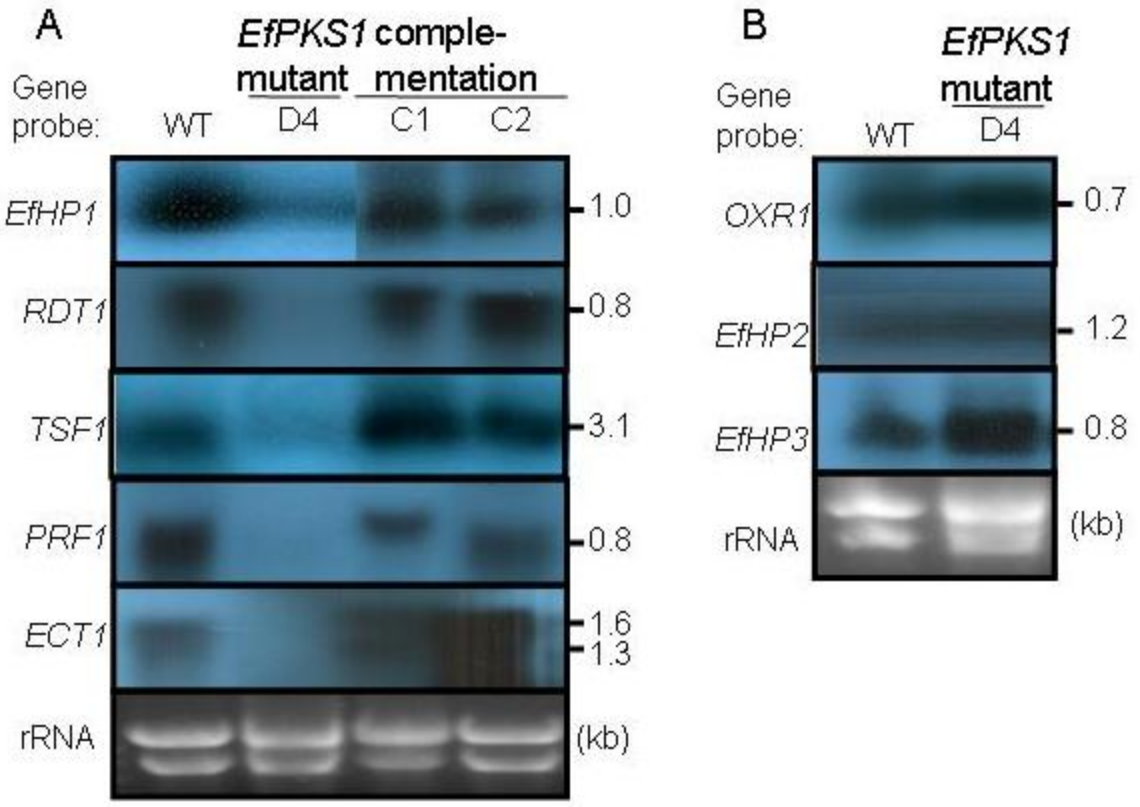


Figure 5-6. Northern-blot hybridization indicates a feedback inhibition of the ESC clustering genes in *Elsinoë fawcettii*. Expression of the *EfHP1*, *RDT1*, *TSF1*, *PRF1*, and *ECT1* genes in the *EfPKS1* disrupted mutant, D4, was dramatically repressed. Functional complementation by introducing a copy of *EfPKS1* into a null mutant yielded two fungal strains (C1 and C2) with a restored gene expression. Total RNA was isolated from fungal strains grown on potato dextrose agar under continuous light for 7 days, electrophoresed in formaldehyde-containing gels, blotted onto nylon membranes and hybridized to the probes as indicated. Ethidium bromide-stained rRNA is shown to indicate the relative loading of the samples.

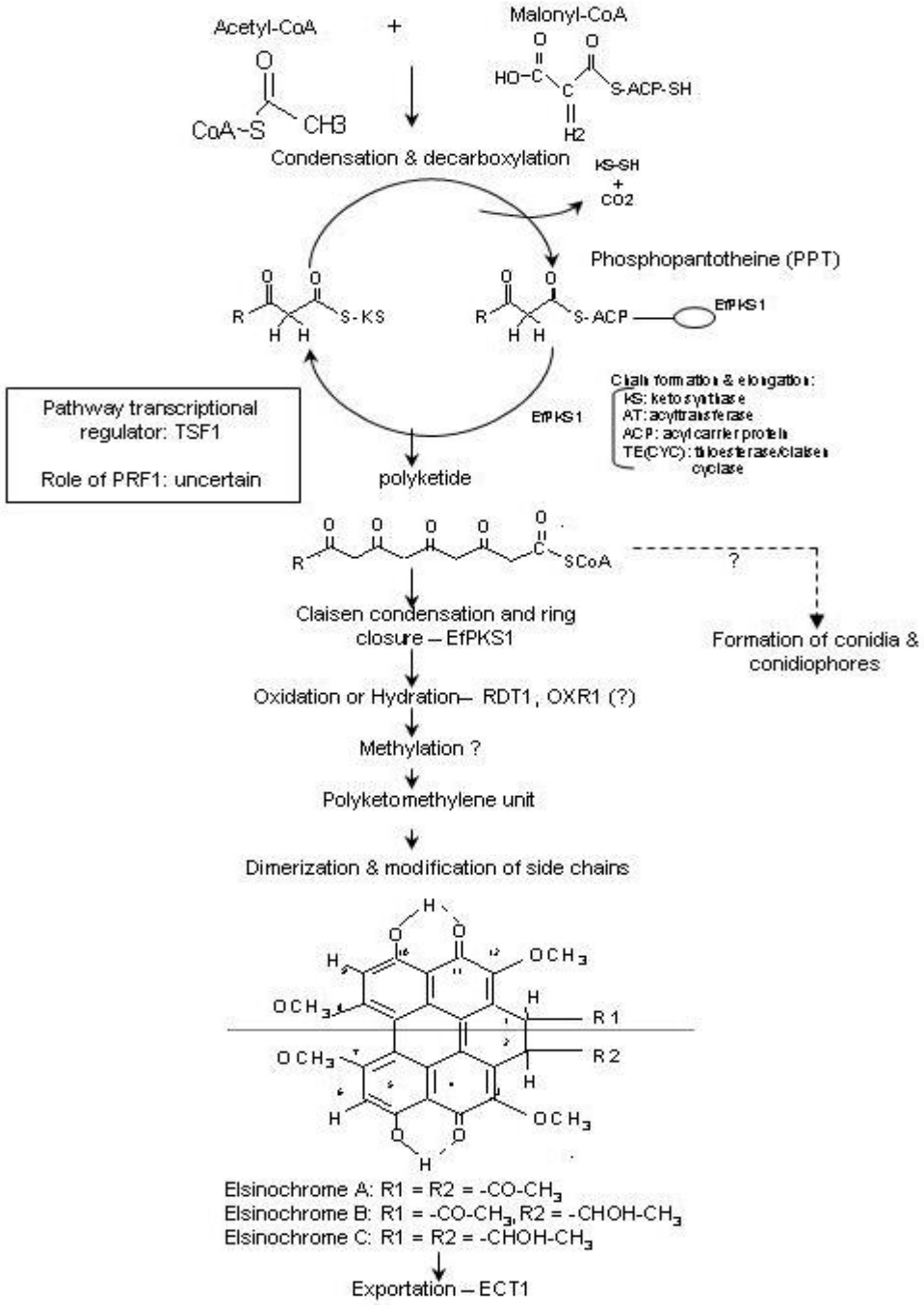


Figure 5-7. Hypothetical reaction pathway for the formation of elsinochromes (ESCs) by function of the ESCs biosynthetic clustering genes. The TSF1 transcription factor is proposed to regulate expression of the clustered genes. The early precursors (acetyl-CoA and Malonyl-CoA) are incorporated into a growing polyketide chain, which is released and cyclized by the function of EfPKS1 as stated in the text. The products of *RDT1* and *OXR1*, are thought to be required for post-polyketide-synthesis steps, whereas the actual function of PRF1 remains unknown. ECT1 is assumed to be responsible for ESC exportation.

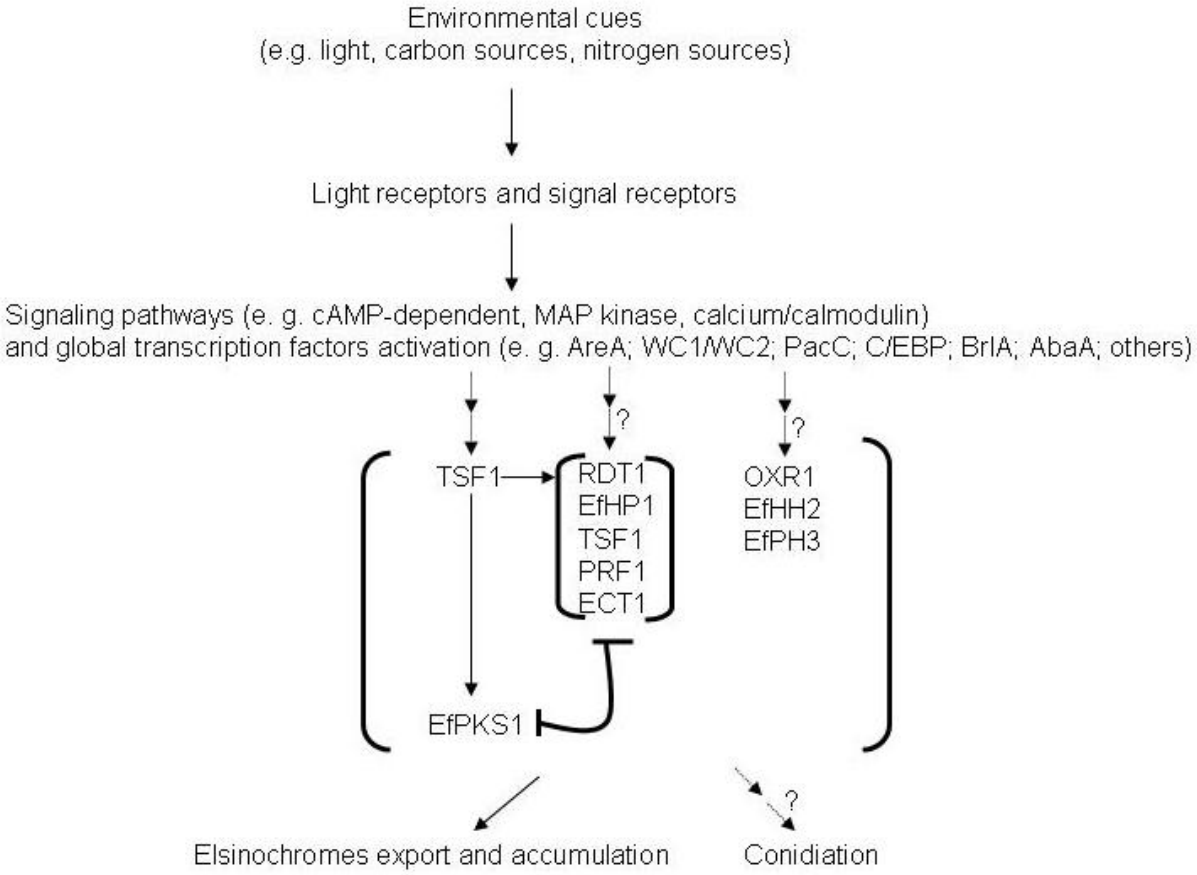


Figure 5-8. Hypothetical signal transduction and regulatory controls for ESC biosynthesis and accumulation. The biosynthetic pathway is assumed to be mainly regulated by pathway-specific transcriptional regulator, TSF1, that is presumably activated by a wide range of global regulatory factors in response to various environmental cues as described in text.

CHAPTER 6
GENETIC AND PATHOLOGICAL DETERMINATIONS OF *Elsinoë fawcettii* FIELD ISOLATES FROM FLORIDA

Elsinoë fawcettii, the causal pathogen of citrus scab, induces superficial corky lesions on the affected tissues. Production of elsinochrome (ESC) phytotoxins has been shown to play an essential role for *E. fawcettii* virulence and lesion development. In this study, 52 field-collected *E. fawcettii* isolates from Florida citrus growing areas were examined for ESC production and pathogenicity to three index citrus hosts: rough lemon, grapefruit, and sour orange. All pathogenic isolates were found to produce ESCs in cultures and/or *in planta*, further confirming the important role of ESCs in fungal virulence. Several field-collected isolates of *E. fawcettii* displayed different pathogenicity, host range, and ESC production, apparently differing from the other isolates. One field isolate, Ef41, was completely non-pathogenic to rough lemon, grapefruit, and sweet orange, and did not produce ESCs. Ef41 failed to express the *EfPKS1* gene and differed considerably from other *E. fawcettii* isolates on the basis of phylogenetic analysis inferred from ITS region. Moreover, as assays on detached rough lemon and grapefruit leaves, co-inoculation of a low virulence isolate attenuated disease severity caused by the highly virulent isolate.

Introduction

Citrus scab caused by *Elsinoë fawcettii* is widely distributed in humid citrus-growing areas worldwide. The development of superficial corky lesions induced by this pathogen leads to a significant economic loss for the fruit intended for fresh market. Many phytopathogenic *Elsinoë* species have been reported to produce elsinochromes (ESCs) (reviewed by Weiss, et al. 1987). Limited work has been done to demonstrate the linkages between ESC production and fungal pathogenesis. ESCs presumably react with oxygen upon illumination and generate superoxide and singlet oxygen, which have been previously shown to be highly toxic to cells and tissues of

citrus and tobacco (Liao and Chung 2008a). Furthermore, the non-ESC producing *E. fawcettii* mutants, derived from disruption of the *EfPKS1* gene, exhibited a reduced virulence to citrus (Liao and Chung 2008b), further confirming the important role of ESCs in fungal pathogenesis. However, ESC toxins have never been isolated from *Elsinoë*-induced lesions. *Elsinoë* species or *Elsinoë fawcettii* isolates are not readily distinguishable by morphological traits (M. Priest unpublished data; Fantin 1988; Fortes, 1989; Timmer et al. 1996). Molecular approaches such as restriction analysis of the amplified internal transcribed spacer (ITS) of ribosomal DNA digested with several endonucleases and sequence analysis of the ITS have been applied to differentiate the closely related species of *Elsinoë*, such as *E. fawcettii*, *E. australis,* and *Sphaceloma fawcettii* var. *scabiosa* (Tan et al. 1996). The internal transcribed spacer 1 (ITS 1) between 18S rDNA and 5.8S rDNA is relatively less conserved among species, and has been demonstrated to be a useful marker for differentiating inter-relationships of fungal strains, species, and genera (Duncan et al. 1998; Brookman et al. 2000). For *E. fawcettii*, the random amplified polymorphic DNA (RAPD) technique has been proven as a useful and easy tool for identifying *Elsinoë* species and the isolates from different geographic areas (Tan et al. 1996). In Florida, two pathotypes of *E. fawcettii*: Florida Broad Host Range (FBHR) and Florida Narrow Host Range (FNHR) have been described on the basis of host range (Whiteside 1978; Timmer et al. 1996). The FBHR pathotype primarily affects the leaves and fruit of grapefruit (*C. paradisi* Macf.), lemon (*C. limon* (L.) Burm. F.), rough lemon (*C.jambhiri* Lush), Murcott and Temple tangors (*C. sinensis* (L.) Osbeck x *C. reticulata* Blanco) and sour orange (*C.aurantium* L.), and the fruit of sweet orange (*C. sinensis*). By contrast, the FNHR pathotype affects all of the above except sour orange, Temple tangor, and sweet orange. Except for differences in host range, FBHR and FNHR pathotypes are not distinguishable morphologically and genetically.

In the present study, I surveyed 52 *E. fawcettii* isolates from citrus groves in Florida and found that many of the isolates accumulated red or orange pigments in culture. In this chapter, close relationships between fungal virulence and ESC production *in vitro* and *in planta* were elucidated. Investigation of their host ranges and taxonomic relationships among isolates of *E. fawcettii* suggests the presence of novel pathotypes or phylogenetic species of *E. fawcettii* in Florida.

Materials and Methods

Fungal Isolates and Growth Conditions

All fungal isolates of *Elsinoë fawcettii* Bitancourt & Jenkins (anamorph: *Sphaceloma fawcettii* Jenkins) were collected from citrus-growing areas in Florida and were kindly provided by L. W. Timmer (University of Florida). The origins of these isolates are indicated in Table 6-1. Fungal isolates were maintained on potato dextrose agar (PDA, Difco, Sparks, MD) under constant fluorescent light at 25 °C. Other media used in this study include a complete medium (CM) containing 1 g $Ca(NO_3)_2 \cdot 4H_2O$, 0.2 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.15 g NaCl, 10 g glucose, 1 g yeast extract, and 1 g casein hydrolysate per liter (Jenns et al. 1989), and a 10% skim milk medium (Difco). For ESC production, fungal isolates were grown on thin PDA plates (5 mL in 100 x 15 mm Petri dish plates) for 10 days under light. Fungal isolates were grown on agar plate overlaid with a piece of sterile cellophane when DNA or RNA isolation was desired.

Extraction and Analysis of Elsinochromes (ESCs)

ESC toxins were extracted from fungal cultures and analyzed by TLC as described in Chapter 2. Quantitative analysis of ESC produced *in vitro* was performed by extracting agar plugs with fungal hyphae with 5 N KOH and measuring absorbance at 480 nm. To extract ESCs from affected rough lemon leaves, leaf spots with necrotic lesions were cut, pooled, and extracted twice with ethyl acetate at 4 °C for 16 h in the dark. Organic solvent was collected, air-

dried at room temperature, suspended in small volumes of acetone, and analyzed by spectrophotometry at A_{480} and TLC. The concentration of ESCs was calculated by reference to a regression line that was established using pure cercosporin (Sigma-Aldrich) as standard and was expressed as cercosporin equivalents.

Production and Germination of Conidia

Conidia were prepared by the method described by Timmer and colleagues (1996) with modifications. Approximately, 0.03 g of fungal hyphae was ground with a disposable pestle in 4 mL Fries medium (Fries, 1978), placed on a Petri dish (15 x 90 mm), and incubated at 25 °C for 2 days in the dark to trigger conidial formation. Plates were washed with sterile, distilled water, flooded with distilled water (pH 7.0) and incubated for additional 12-15 hr in the dark. Conidia on the bottom of the plate were washed once with distilled water, scraped, re-suspended after centrifugation (6000 rpm, 10 min), and passed through a filter to remove mycelia. Conidia were counted and measured with the aid of a hemocytometer using a microscope at x 200 magnification. Germination of conidia was determined 2 days post inoculation on detached rough lemon and grapefruit leaves as described below and was calculated in proportion to the total number of conidia recovered from the inoculated spot.

Pathogenicity Assays

Assays for fungal pathogenicity were conducted on detached rough lemon (*Citrus jambhiri* Lush), grapefruit (*Citrus paradisi*), and sour orange (*Citrus aurantium*) leaves inoculated with conidial suspension. Rough lemon is susceptible to all *Elsinoë* pathotypes of citrus (Timmer et al. 1996). Conidial suspension (1µL, 1 x 10^5 mL^{-1}) was carefully placed on the leaf surface and the inoculated leaves were incubated in a mist chamber under constant fluorescence light for lesion formation.

Enzymatic Activity Assays

Assays for enzymatic activities were determined as described (You and Chung 2007). Glucose in the complete medium (CM) was substituted by appropriate carbon sources (1% citrus pectin, pH4.5 or pH7.6; 1% polygalacturonic acid, pH4.5 or pH7.6; 0.5% carboxymethyl cellulose; 10 g glucose plus 0.1% 16-hydroxyhexodecanoic acid (cutin monomer)) for induction of the respective enzyme. Skin milk used for proteolytic activity assays was prepared by mixing (3:22, v:v) solution A (10% skim milk dissolved in 0.05 M phosphate buffer, pH 6.8) and solution B (Agar medium containing 0.23% yeast nitrogen base) after sterilization.

Extracellular activities CWDEs (cell wall degradation enzymes) were evaluated by measuring the amounts of reducing sugar that was released from 1% polygalacturonic acid (PGA), 1% citrus pectin or 0.5% carboxymethyl-cellulose (CMC) and reacted with dinitrosalicylic acid (DNS) reagent under alkaline conditions (Bailey et al. 1993). Fungal isolates were grown on a modified CM in which glucose was substituted by an appropriate polysaccharide for 10 days. Five 5-mm agar plugs bearing fungal mycelia were cut and placed in 0.1M sodium phosphate buffer containing 1% PGA (pH 4.5 or pH 7.6), 1% citrus pectin (pH 4.5 or pH 7.6), or 0.5% CMC (pH 5). After incubation at 50 °C for 1 hour, an equal volume of DNS reagent was added, boiled at 95 °C for 5 min, and absorbance was measured at A_{540nm}. The regression line and correlation coefficient ($r^2 > 0.98$) were established using glucose as a standard. One unit of enzyme activity is defined as 1 nmole of glucose liberated from the substrate per minute.

Extracellular cutinase activities were determined by formation of a yellow color after reacting with 50 mM paranitrophenyl butyrate (PNPB) in 0.1M sodium phosphate buffer (pH 5) for 1 hour and measured at A_{405nm} (Stahl and Schäfer 1992). Fungal isolates were grown on a modified CM containing 0.1% 16-hydroxyhexadecanoic acid (HHDA, dissolved in 1% sodium

acetate) as a sole carbon source for 8 days for induction before the enzymatic assays. One unit of cutinase released is 1 nmole *p*-nitrophenol produced per minute.

Proteolytic activities were determined by formation of clear zones on 10% skim milk plates. Fungal mycelia were ground in sterile water, and spread onto an agar plate. After incubation at 28 °C for 4 days, the sizes of clear zones were measured.

PCR-Restriction Fragment Length Polymorphism (RFLP) and Sequence Analysis of ITS and β-tubulin Genes

The ITS regions of fungal rDNA were amplified by PCR with primer ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') as described by White et al. (1990). A 600-bp DNA fragment, encompassing entire ITS 1, 5.8S and partial 18S of rDNA, was amplified. Partial β-tubulin gene (~ 500 bp) of fungal isolates were amplified with primer sets Bt1R (5'-GACGAGATCGTTCATGTTGAACTC-3') and Bt1F (5'-TTCCCCCGTCTCCACTTCTTCATG-3') as described (Glass and Donaldson 1995). The PCR amplification reactions were carried out in a Peltier thermal cycler (MJ. Research, INC., Watertown, MA). The DNA was amplified by GoTaq® Flexi DNA polymerase (Promega, WI) in 50 μL reaction containing 0.2 μM of each of the primer set and 50 ng of genomic DNA. Thermal cycling parameters for amplification of both ITS and β-tubulin include a single cycle of 95 °C for 2 min, followed by 35 cycles of 45 sec at 95 °C, 45 sec at 56 °C, and 1 min at 72 °C and a final step at 72 °C for 5 min. The amplified DNA fragments were digested with 4-bp recognition sequence restriction endonucleases, electrophoresed in 2% agarose gel, stained with ethidium bromide, visualized, and photographed under ultraviolet light. The amplified ITS fragments were cleaved with *Hae*III, *Msp*I, or *Taq*αI, whereas the amplified β-tubulin gene fragments were cut with *Alu*I, *Dpn*I, *Hae*III, *Msp*I, *Nla*IV, or *Taq*αI. For direct sequencing, the DNA fragments were amplified by an Expand High Fidelity PCR system (Roche Applied

Science). Otherwise, all fragments were cloned into pGEM-T easy vector (Promega, Madison, WI) for sequencing analysis from both directions at Eton Bioscience (San Diego, CA). Similarity was determined by Emboss MATCHER Program (Sanger Institute, Hinxton, Cambridge, UK.). The ITS 1 sequences were aligned with published sequences and phylogenetic analyses were performed using DS Gene v2.5 program (Accelrys Inc., San Diego).

Random Amplified Polymorphic DNA (RAPD)

RAPD fragments were amplified with a 10-mers primer, OPX-7 (5'-GAGCGAGGCT-3'), OPX-13 (5'-ACGGGAGCAA-3'), or MtR 1 (5'-GTAAAGGGGG-3'), by Taq DNA polymerase (GenScript, Piscataway, NJ). The cycling profile for amplification includes an initial cycle of 94 °C for 3 min, immediately followed by 35 cycles of 94 °C for 30 sec, 36 °C for 45 sec, 72 °C for 4 min and a final step of 72 °C for 7 min. The amplified DNA fragments were separated by electrophoresis in 2% agarose gel.

Miscellaneous Methods of Processing Nucleic Acids

Plasmid DNA was purified using a Wizard DNA purification kit (Promega) from transformed *Escherichia coli* DH5α bacterial cells. Fungal RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA). Standard procedures were used for electrophoresis and Northern-blot hybridization. DNA probes used for hybridization were labeled with digoxigenin (DIG)-11-dUTP (Roche Applied Science) by PCR with gene-specific primers. The manufacturer's recommendations were followed for probe labeling, hybridization, post-hybridization washing and immunological detection of the probe using a CSPD chemofluorescent substrate for alkaline phosphatase (Roche Applied Science).

Results

Morphological Examination

Isolates of *Elsinoë fawcettii* were first examined for potential morphological variations using light or dissection microscopes. All isolates produced filamentous mycelia, and formed nodular/bulbous mycelia after a prolonged incubation (e.g., Fig. D-1). Pleomorphic colonies were observed among isolates when cultured on PDA or CM culture (Fig. D-2 and data not shown). All isolates had a limited mycelial extension, which were slightly raised in the center. Some isolates (e.g., Ef12, Ef17, Ef29, Ef37, and Ef48) produced colonies that were dark red with a little fluffy velvet-like mycelia, while others produced pale colonies (e.g., Ef30 and Ef49) covered with or without fluffy mycelia. All isolates produced hyaline conidia, showing elliptical to obclavate, and nonseptate cells (data not shown). The isolate Ef41 produced smaller conidia having the means size of 3.4 x 1.9 µm (length x width), while other isolates produced conidia with the mean size 4.1-5.1 x 2.2-2.7µm (length x width).

Pathogenicity Assays

As rough lemon is susceptible to all *Elsinoë* spp. (Timmer et al. 1996), fungal pathogenicity was primarily evaluated in this citrus cultivar. In total, 52 ***E. fawcettii*** isolates cultured from different citrus cultivars and geographic locations of citrus-growing areas in the state of Florida were evaluated for pathogenicity on detached rough lemon leaves (Table 6-1). All isolates produced substantial numbers of conidia, ranging from 0.1-50 x 10^6 conidia per mL. As assayed on detached rough lemon leaves, all isolates except one (Ef41) incited necrotic lesions on young leaves (7-10 days after flushing) and exhibited a reduced virulence to varying degrees when inoculated onto older leaves (14-18 days after flushing, data not shown). Isolate Ef41 failed to induce any necrosis on rough lemon, while isolates Ef10 and Ef29 had a weak virulence on rough lemon. Pathogenicity of nine isolates (Ef10, 12, 17, 29, 30, 37, 41, 48, and 49)

was also assessed after inoculation onto detached grapefruit and sour orange leaves (Table 6-1). Isolates Ef10 and Ef29 were weakly virulent to either grapefruit or sour orange and isolate Ef41 was nonpathogenic to either citrus cultivar. Other isolates were weakly or moderately virulent to grapefruit or sour orange (Table 6-1).

Production of ESC Toxin in Culture

ESCs produced by *E. fawcettii* isolates was extracted from agar plugs bearing fungal mycelium with 5N KOH and quantified by measuring absorbance at 480 nm. As shown in Table 6-1, production of ESC toxins was variable among isolates, ranging from 1.4 to 16.6 nmoles per agar plug. Noticeably, several isolates (Ef1, 15, 25, 26, 27, and 47) produced little or no ESCs in axenic cultures despite being pathogenic to rough lemon. i.e., there was no relationship between *in-vitro* production of ESCs and lesion formation on rough lemon.

Extraction of ESCs from Scab Lesions

To determine if ESCs were accumulated during fungal colonization, 23 field isolates of *E. fawcettii*, including five isolates (Ef1, 25, 27, 41and 47) that produced no ESCs in cultures, were inoculated onto detached rough lemon leaves. ESCs were then extracted from a pooled sample of 20 necrotic lesions of each isolate, and analyzed by TLC. ESCs with different banding profiles and intensities were extracted from lesions induced by 22 isolates tested (Fig. 6-1). Isolate Ef41 did not incite any visible lesions on rough lemon leaves and accumulated no detectable ESCs. Isolates Ef1, Ef25, Ef27and Ef47 produced no detectable levels of ESCs in cultures, but apparently produced ESCs *in planta* and induced characteristic scab lesions on rough lemon leaves (Fig. 6-1; Table 6-1). Judging from band width and intensity, band 1 (R_f 0.68) appeared to be the major compound of the leaf extracts, whereas band 3 (R_f 0.22) was the most abundant product of the cultural extracts. Spectrophotometric scanning indicated that the acetone extracted pigments from scab lesions on rough lemon leaves displayed a strong absorbance at 460 nm with

two minor peaks at 530 and 570 nm, resembling those purified from *E. fawcettii* cultures as described in Chapter 2.

Further Characterization of a Nonpathogenic *Elsinoë fawcettii* Isolate

In addition to assays for fungal pathogenicity and ESC production, nine *E. fawcettii* isolates, representing nonpathogenic (Ef41), weakly virulent (Ef10, Ef17, Ef29, and Ef49) and highly virulent isolates (Ef12, Ef30, Ef37, and Ef48), were further examined for potential genetic variations. As shown in Table 6-1, isolates that were pathogenic to rough lemon were also pathogenic to grapefruit. All isolates except Ef41 produced scab pustules on young leaves (7 days after flushing) of rough lemon and grapefruit and exhibited a reduced virulence to varying degrees on older leaves (14 days after flushing). These nine *E. fawcettii* isolates were not all pathogenic on sour orange. Only three isolates, Ef12, Ef17, and Ef48, of nine isolates produced severe pustule lesions on sour orange.

Alterations in Hydrolytic Enzyme Activities

To determine if fungal hydrolytic enzymes are required for pathogenicity of *E. fawcettii*, the extracellular activities of proteolytase, cellulase, pectinase, polygalacturonase and cutinase were determined among nine isolates described above. Isolate Ef41 had no detectable proteolytic activity, whereas isolates Ef10, Ef17, Ef29, and Ef49 with lower virulence had lower proteolytic activity compared to the pathogenic isolates Ef12, Ef30, Ef37, and Ef48 (Fig. 6-2A). Similarly, isolates Ef12, Ef30, Ef37, and Ef48 displayed higher cellulase activity than other isolates tested (Fig. 6-2B). All *E. fawcettii* isolates tested exhibited high levels of pectolytic activities when grown in a medium containing citrus pectin as a sole carbon source (pH 4.5), but the overall pectolytic activities decreased considerably at pH 7.6 (Fig. 6-2C). When grown in a medium containing polygalacturonic acid as a sole carbon source, all isolates had no or little polygalacturonase activity when the medium was buffered to pH 7.6. By contrast, isolate Ef41

exhibited marked polygalacturonase activity when the medium was buffered to pH 4.5 (Fig. 6-2D). There was no significant difference in cutinase activities among all isolates tested, despite that isolate Ef41 showed slightly but not significantly lower cellulase activity (Fig. 6-2E).

Production of ESC and Expression of the *EfPKS1* gene in the Nonpathogenic Isolate (Ef41)

Of 52 isolates examined, only Ef41 isolate failed to infect rough lemon, grapefruit, or sour orange and produced no ESC either in culture or *in planta* (Table 6-1). Thus, expression of the polyketide synthase-coding gene, *EfPKS1*, that has been shown to be required for ESC biosynthesis (Liao and Chung, 2008b) was examined in Ef12 and Ef41 isolates by Northern blot analysis.

As shown in Fig. 6-2F, accumulation of the *EfPKS1* transcript was normal in the ESC-producing isolate Ef12. The *EfPKS1* gene transcript was undetectable in isolate Ef41, which may account for no ESC production.

Co-inoculation of Two *E. fawcettii* Isolates Reduces Lesion Formation

To test if the isolates with low virulence will compete with the isolates with high virulence, various combinations of two *E. fawcettii* isolates were co-inoculated onto detached rough lemon and grapefruit leaves and lesion formation was assessed.

Co-inoculation of conidial suspension of isolate Ef12 with either isolate Ef29 or Ef49 resulted in a repressed symptom development on both citrus cultivars compared to those inoculated with the Ef12 isolate alone (Fig. 6-3). When isolate Ef12 alone was inoculated onto rough lemon or grapefruit, over 65% of the total spots inoculated developed severe scab lesions covered with numerous pustules (level 4-5). When isolate Ef12 was co-inoculated with isolate Ef29 or Ef49, less than 14% of the inoculated spots on either rough lemon or grapefruit leaves developed severe scab lesions. Co-inoculation of isolate Ef12, Ef29 or Ef49 with isolate Ef41 did not alleviate symptom development (Fig. 6-3).

Molecular Evaluation of *E. fawcettii* Isolates

E. fawcettii isolates cultured from Florida citrus growing areas displayed variations in virulence to rough lemon, grapefruit, and sour orange and ESC production. To determine if such variations also occur at the genetic level, molecular approaches, including PCR-RFLP, RAPD, and sequence analysis of internal transcribed spacer (ITS) region and the conserved gene (β-tubulin), were used to further characterize *E. fawcettii* isolates with differ virulence.

As assessed by PCR-RFLP of the amplified ITS rDNA fragment (~600 bp) cleaved with either *Hae*III, *Msp*I, or *Taq*αI, isolate Ef41 apparently had DNA banding profiles that were clearly distinguishable from those of other isolates tested (Fig. 6-4). Polymorphisms of the amplified β-tubulin gene fragments digested with *Hae*III or *Taq*αI, also separated isolate Ef41 from other *E. fawcettii* isolates (Fig. 6-5). The amplified tubulin cleaved with *Alu*I, *Dpn*I, *Msp*I, or *Nla*IV resulted in similar profiles among *E. fawcettii* isolates (Fig. 6-5). Furthermore, isolate Ef41 was apparently different from other *E. fawcettii* isolates when randomly amplified DNA fragments using a 10-mer primer OPX-7, OPX-13, or MtR 1 were compared (Fig. 6-6).

The amplified ITS 1 region from isolates Ef12, Ef29, Ef41, and Ef49 was sequenced and compared with the published sequences in the database (National Center for Biotechnology Information) to determine their phylogenetic relationships. The ITS sequences obtained from isolates Ef29 and Ef49 were identical, showing 100% identity, while Ef12 had 90% identity to those of *E. fawcettii* (EFU28058) and *Sphaceloma fawcettii* (SFU28059). Isolate Ef41 displayed lowest ITS sequence similarity and apparently was separated from other *E. fawcettii* isolates of citrus (Fig. 6-7). Furthermore, partial β-tubulin gene sequences of isolates Ef12 and Ef41 were compared with the published sequences in the database. The relative identity of fragments is shown in Table 6-2, indicating that isolate Ef41 shared higher nucleotide identity with isolate Ef12 (85.8%), whereas Ef12 displayed varying similarity of β-tubulin gene sequence to other

fungi including *Aspergillus nidulans* (88.9%), *Cercospora beticola* (87.0%), *Mycosphaerella graminicola* (86.7%), *Neurospora crassa* (90.1%), and *Neosartorya fischeri* (87.7%) (Table 6-2).

Discussion

ESCs produced by *E. fawcettii,* due to the production of toxic oxygen species upon irradiation, have been shown to be highly toxic to the cells and tissues of citrus and tobacco (Liao and Chung 2008a) and also essential for full virulence of *E. fawcettii* (Liao and Chung 2008b). Analysis of ESC production and fungal pathogenicity on three index hosts of citrus (rough lemon, grapefruit, and sour orange) revealed a marked variation among *E. fawcettii* isolates cultured from Florida. Evidence presented in this study indicated that the majority of *E. fawcettii* isolates produced ESCs in axenic culture (~90%, Table 6-1), but the production was varied widely with isolate.

All *E. fawcettii* isolates that were pathogenic to rough lemon produced ESCs in culture and/or *in planta*, in spite of lacking of a relationship between the amounts of ESCs accumulated in culture and *in planta*. The amounts of ESCs produced by an individual fungal isolate in culture or *in planta* did not account for the severity of scab disease induced by the same isolate. A survey of 52 isolates revealed that four isolates (Ef1, Ef25, Ef27, and Ef47) accumulated no detectable level of ESCs in culture, but provoked characteristic scab lesions and accumulated substantial amounts of ESCs in rough lemon leaves, implicating the presence of signals or substrates from the plant for ESC production. The Ef41 isolate that was not pathogenic to rough lemon, grapefruit, or sour orange, was unable to produce any detectable ESCs. Since ESCs are extremely toxic upon reaction with oxygen molecules under light, this study further confirmed the critical role of ESCs for lesion formation and fungal pathogenesis, consistent with the previous finding from molecular analysis (Liao and Chung 2008b). As all pathogenic *E. fawcettii* isolates produced varying amounts of ESCs *in planta*, it is tempting to speculate that a trace

amount of ESCs, continuously produced by the pathogen, might be sufficient to facilitate fungal invasion and colonization to its hosts.

The occurrence of citrus scab on various citrus species and cultivars has been reported in different countries of the world, primarily based on field observation. Yet, little study has been done toward delineating the host range and distribution of the species and pathotypes of citrus scab fungi. The "Florida Broad Host Range (FBHR) and the "Florida Narrow Host Range (FNHR)" described in Florida based on host range are not distinguishable genetically (Whiteside 1988; Timer et al. 1996). A recent study described a novel *E. fawcettii* pathotype that was only pathogenic to Natsudaidia (*C. natsudaidai* Hayata) and Kinkoji (*C. obovoidea* Hort ex Tak.) fruit in Korea (Hyun et al. 2001).

As is evident from the present study, it seems likely that additional pathotypes or new species of *Elsinoë* might be present in Florida citrus-growing areas other than FBHR and FNHR. The Ef10 and Ef29 isolates, originally cultured from infected SunCha Shakat mandarin and Swingle citrumelo, respectively, were only weakly virulent to grapefruit, rough lemon, and sour orange. The Ef17 isolate, originally cultured from infected Temple leaves, appeared to be highly virulent to sour orange, yet induced moderate or mild scab lesions on rough lemon and grapefruit. The Ef12 isolate was highly virulent to the three citrus hosts tested, but displayed low similarity of the ITS 1 sequence to those of *E. fawcettii*. The Ef41 isolate, also cultured from infected Temple leaves, was apparently different from other field isolates, in terms of pathogenicity, morphology, toxin production, phylogenic variations, and other genetic variations and thus, likely represents a novel phylogenic or biological species. Although the Ef41 isolate failed to attack the leaves of three index citrus cultivars, it does not necessarily indicate that Ef41 is an *E. fawcettii* saprophyte. Similar pathotypes that were nonpathogenic to rough lemon, grapefruit, and

sour orange have also been reported from field-collected isolates in Argentina (Timmer et al. 1996). However, the Argentina isolates had different ITS RFLP banding profiles from that of Ef41 isolate. Since only a limited number of isolates were tested in this study, it is impossible to conclusively determine if Ef41, Ef29, and Ef49 are different pathotypes of *E. fawcettii* or simply represent novel cryptic *Elsinoë* species that have not yet been characterized in citrus. More field isolates need to be tested and a broader range of citrus cultivars are required to be inoculated to understand the complexity of all of the pathotypes of *E. fawcettii* in Florida.

Table 6-1. Pathogenicity and production of elsinochromes (ESCs) *in vivo* and in *planta* by different field isolates of *Elsinoë fawcettii*

Fungal isolates (No. and names)		Origins[1]	Disease severity[2] detached-leaf			ESC production (nmole per plug or spot)	
# (Ef)	Name		RL	GF	SO	*In vitro*[3]	*In planta*[4]
1	Duda-Imk-1	Temple	+++	nd[5]	nd	0	0.5
2	Duda-Imk-2	Temple	++++	nd	nd	3.0 ± 1.3	nd
3	Duda-Imk-5	Temple	++++	nd	nd	8.8 ± 0.4	nd
4	Eus-1	Murcott	+++++	nd	nd	3.2 ± 1.2	nd
5	Eus-2	Murcott	+++	nd	nd	1.8 ± 0.6	nd
6	Eus-3	Murcott	+++++	nd	nd	1.6 ± 0.2	nd
7	Eus-4	Murcott	+++	nd	nd	2.6 ± 0.2	nd
8	Lkpcd-2	Temple	+++++	nd	nd	4.0 ± 0.6	nd
9	Navarro-3	Grapefruit	+++++	nd	nd	5.0 ± 0.2	8.9
10	Scsk	Sun Shu Shakat	+/-	+	+	12.6 ± 3.0	0
11	Navarro-6	Grapefruit	+++++	nd	nd	6.2 ± 0.2	11.6
12	FtPierce-3a	Temple	++++	++	+++	6.4 ± 3.0	nd
13	FtPierce- 4b	Temple	+++	nd	nd	5.6 ± 0.6	nd
14	FtPierce-3	Temple	+++	nd	nd	6.8 ± 1.2	nd
15	Carrizo	Carrizo citrange	+++	nd	nd	0.2 ± 0.0	0.9
16	Irrec-3	Murcott	+++	nd	nd	5.6 ± 1.6	nd
17	R-36	Temple	+	++	++++	1.4 ± 0.0	1.1
18	Labelle	Lemon	++	nd	nd	16.6 ± 1.2	nd
19	Ftmead4	Grapefruit	++++	nd	nd	5.6 ± 0.6	nd
20	Duda-Imk-3	Temple	++++	nd	nd	4.4 ± 0.7	nd
21	Ss-Imk-3	Temple	++++	nd	nd	3.0 ± 0.6	nd
22	Ss-Imk-1	Temple	++++	nd	nd	5.8 ± 0.6	2.2
23	Tbl-La-2	Temple	++++	nd	nd	6.2 ± 0.8	6.0
24	Rl-2	Rough lemon	++++	nd	nd	7.8 ± 0.8	4.6
25	Russel-15	Temple	+++++	nd	nd	0	2.9
26	Smoak 1	Temple	+++++	nd	nd	0.7 ± 0.2	7.7
27	Ss Imk 1	Tahiti lime	+++++	nd	nd	0	2.6
28	Stl-Lv-d	Temple	+++	nd	nd	6.2 ± 1.2	1.7
29	Citro-1	Swingle citrumelo	+/-	+	+/-	2.6 ± 0.6	0.9
30	Eus-Lv-1	Murcott	++++	++++	+/-	3.8 ± 0.4	nd
31	Eus-1	Temple	++	nd	nd	7.9 ± 0.8	nd
32	Ftmead 3	Grapefruit	+++++	nd	nd	3.0 ± 0.4	nd

Table 6-1. (Continued)

Fungal isolates (No. and names)		Origins[1]	Disease severity[2] detached-leaf			ESC production (nmole per plug or spot)	
# (Ef)	Name		RL	GF	SO	*In vitro*[3]	*In planta*[4]
33	FtPierce-1	Temple	+++++	nd	nd	5.4 ± 0.8	nd
34	FtPierce-4c	Temple	+++++	nd	nd	5.8 ± 0.6	nd
35	Imk-Br-1a	Grapefruit	+++	nd	nd	5.2 ± 0.8	nd
36	Imk-Br-1b	Grapefruit	+++	nd	nd	5.6 ± 0.4	nd
37	Imk-Br-1c	Grapefruit	++++	++++	+/-	6.2 ± 1.2	nd
38	Imk-br-2	Grapefruit	+++	nd	nd	5.2 ± 0.6	nd
39	SWFREC-Lv-1	Murcott	++++	nd	nd	5.2 ± 0.6	4.9
40	Imk-Br-3a2	Grapefruit	+++	nd	nd	3.8 ± 0.8	nd
41	SWFREC- Imk-1	Temple	-	-	-	0	0
42	Imk-Br-3c	Grapefruit	+++	nd	nd	3.2 ± 0.4	2.6
43	Tbl-La-1	Temple	++++	nd	nd	6.4 ± 0.4	7.9
44	Imk-Br-3e	Grapefruit	+++	nd	nd	7.2 ± 0.2	nd
45	Imk-Br-4d	Grapefruit	+++	nd	nd	5.6 ± 0.4	nd
46	Lefond b	Grapefruit	+++	nd	nd	6.0 ± 1.4	nd
47	Lefond d	Grapefruit	+++	nd	nd	0	1.1
48	Manatee w-2	Temple	++++	+++	++++	5.2 ± 0.6	nd
49	Cbl-Wh-1	Unknown	++	++	+/-	6.5 ± 0.4	1.2
50	Manatee w-5	Temple	++++	nd	nd	5.0 ± 0.2	4.7
51	Avonpark m2	Unknown	++++	nd	nd	5.8 ± 0.6	7.5
52	Avonpark m3	Unknown	++++	nd	nd	4.8 ± 0.6	2.1

[1]Citrus species or cultivars (*Citrus* spp.). Temple (*C. temple*), Murcott (*C. reticulate* x *C. sinensis*), Grapefruit (*C. paradisi*), Sun Shu Shakat (*C. sinesis*), Carrizo citrange (*C. sinensis* x *Poncitrus trifoliate*), Lemon (*C. limon*), Rough lemon (*C. jambhiri*), Tahiti lime (*C. aurantifolia*), Swingle citrumelo (*C. paradisi* x *P. trifoliata*), sour orange (*C. aurantium*).

[2]Pathogenicity assays were performed on detached leaves (7-10 days after flushing) inoculated with conidial suspension (1×10^5 mL^{-1}). Inoculated leaves were kept in a moist chamber under constant florescent light for 15 days. Disease severity was rated on a scale, - for no lesion formation; +/-, chlorotic reaction only, no scab pustules; +, lesions with minor necrosis, and +++++, lesions with severe necrosis. RL, rough lemon; GF, grapefruit; SO, sour orange.

[3]Fungal isolates were cultured on PDA under constant light for 10 days and elsinochromes (ESCs) were extracted with 5N KOH and measured at A_{480}. Data are the means and standard errors of four replicates containing 12 agar plugs of each sample.

[4]Conidial suspension was inoculated onto detached rough lemon leaves and incubated for 14 days. In total, 20 spots (2-mm in diameter) were cut from various leaves, pooled, and soaked in ethyl acetate. ESCs were separated on TLC plate (Fig. 6-1) and quantified using a regression line established with known concentrations of ESCs and cercosporin.

[5]nd, not determined

Table 6-2. Sequence identity (%) of fungal partial β-tubulin gene

Fungus[1]	Ef12	Ef41	AN	CB	MG	NC	NF	SS	VD
EF12	-	85.8[2]	88.9	87.0	86.7	90.1	87.7	75.0	76.0
EF41	-	-	84.1	84.5	84.5	85.0	82.6	71.0	74.1
AN	-	-	-	87.7	85.2	90.8	92.5	75.4	76.3
CB	-	-	-	-	88.1	88.1	88.1	74.2	74.2
MG	-	-	-	-	-	86.7	85.5	74.0	73.8
NC	-	-	-	-	-	-	89.1	77.5	79.9
NF	-	-	-	-	-	-	-	75.8	75.7
SS	-	-	-	-	-	-	-	-	80.3
VD	-	-	-	-	-	-	-	-	-

[1]Partial β-tubulin genes of *Elsinoë fawcettii* isolates Ef12 and Ef41 were amplified and sequenced. Partial β-tubulin genes from other fungi were obtained from Genbank with accession numbers. AN, *Aspergillus nidulans* (XM_653694); CB, *Cercospora beticola* (AY856373); MG, *Mycosphaerella graminicola* (AJ310917); NC, *Neurospora crassa* (XM_952576); NF, *Neosartorya fischeri* (XM_001264677); SS, *Sclerotinia sclerotiorum* (AY12374); VD, *Verticillium dahliae* (DQ266135)

[2]Nucleotide identity (%) was analyzed by emboss MATCHER program (Sanger Institute, Hinxton, Cambridge, UK.). Approximately, 485-bp sequences from each organism were aligned and compared for identities (Fig. D-3).

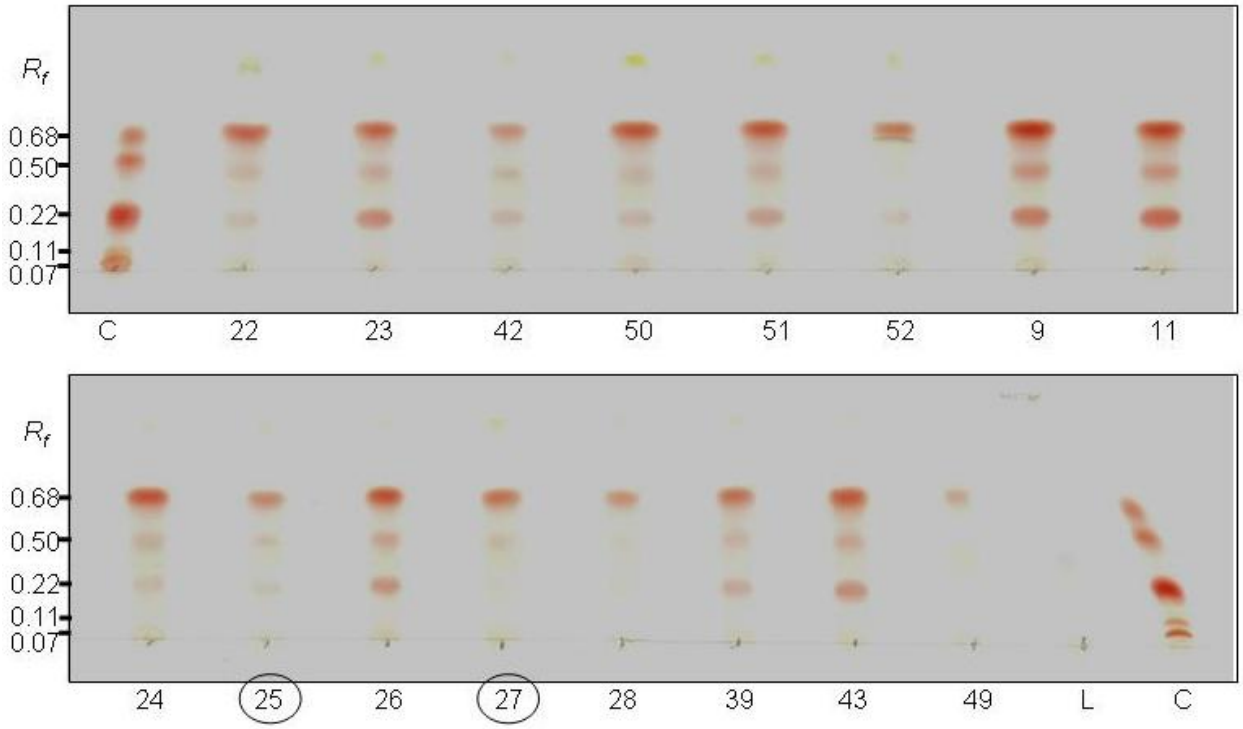


Figure 6-1. Thin-layer chromatography analysis (TLC) of ESC phytotoxins (the red/orange pigments) extracted from affected rough lemon leaves inoculated with different isolates of *Elsinoë fawcettii*. Fungal isolates were inoculated onto detached rough lemon leaves and incubated for 15 days for lesion development in a moist chamber. Leaf lesions were pooled and soaked in ethyl acetate. ESCs were recovered by suspending in small volumes of acetone and separated on TLC plates with a solvent system containing chloroform and ethyl acetate (1:1, v:v). ESCs extracted from cultural filtrate (C) of CAL WH-1 isolate showing five distinct bands at R_f as indicated were used as positive controls (Chapter 2), whereas negative controls were the extracts of rough lemon leaves (L) inoculated with water only. Fungal isolates that did not produce detectable ESCs in culture are circled.

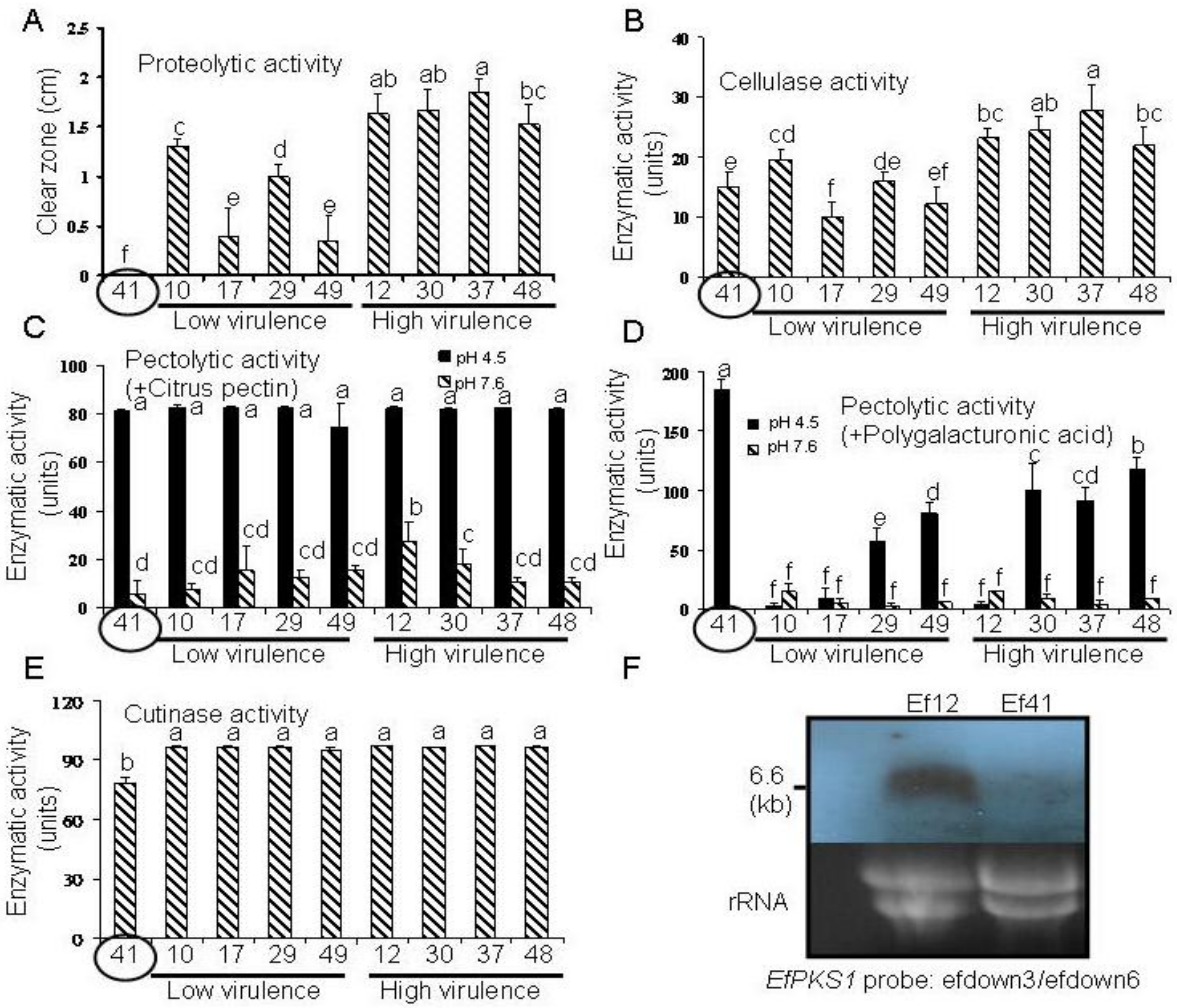


Figure 6-2. Determination of extracellular enzyme activities: A) proteolysase; B) cellulase; C) pectolytic activities (mainly pectin lyase, pH 4.5 and pectin methyl esterase, pH 7.6); D) pectolytic activities (primarily endo- and exo-PGs, pH 4.5 and pectate lyase, pH7.6); and E) cutinase by *Elsinoë fawcettii* isolates. Fungal isolates were grown on CM with inducers [5% skim malk; 0.5% CMC; 1% PG; 1% citrus pectin, or 0.1% HHDA] as appropriate for 4-10 days and enzymatic activities were measured either by diameter of clear zone (proteolytic activity) or by spectrophotometry after reaction with appropriate chromogens (cellulase, pectolytic, and cutinase activities). Each column represents the mean value of enzymatic activity from two independent experiments with at least three replicates. Vertical bars represent standard deviation. Means followed by the same letter are not different at judged by Duncan's multiple range test at $P < 0.0001$. Details in measuring enzymatic activities are described in the text. F) Northern-blot analysis of fungal RNA purified from isolate Ef12 and Ef41 of *Elsinoë fawcettii*. RNA was denatured in a formaldehyde-containing gel, blotted to a nylon membrane, and hybridized to a DNA probe (probe 3) generated from *EfPKS1* gene of *E. fawcettii* CAL WH-1 isolate as described in chapter 4. Gel stained with ethidium bromide indicates relative loading of the RNA samples. Sizes of hybridization bands are indicated in kilobase pairs.

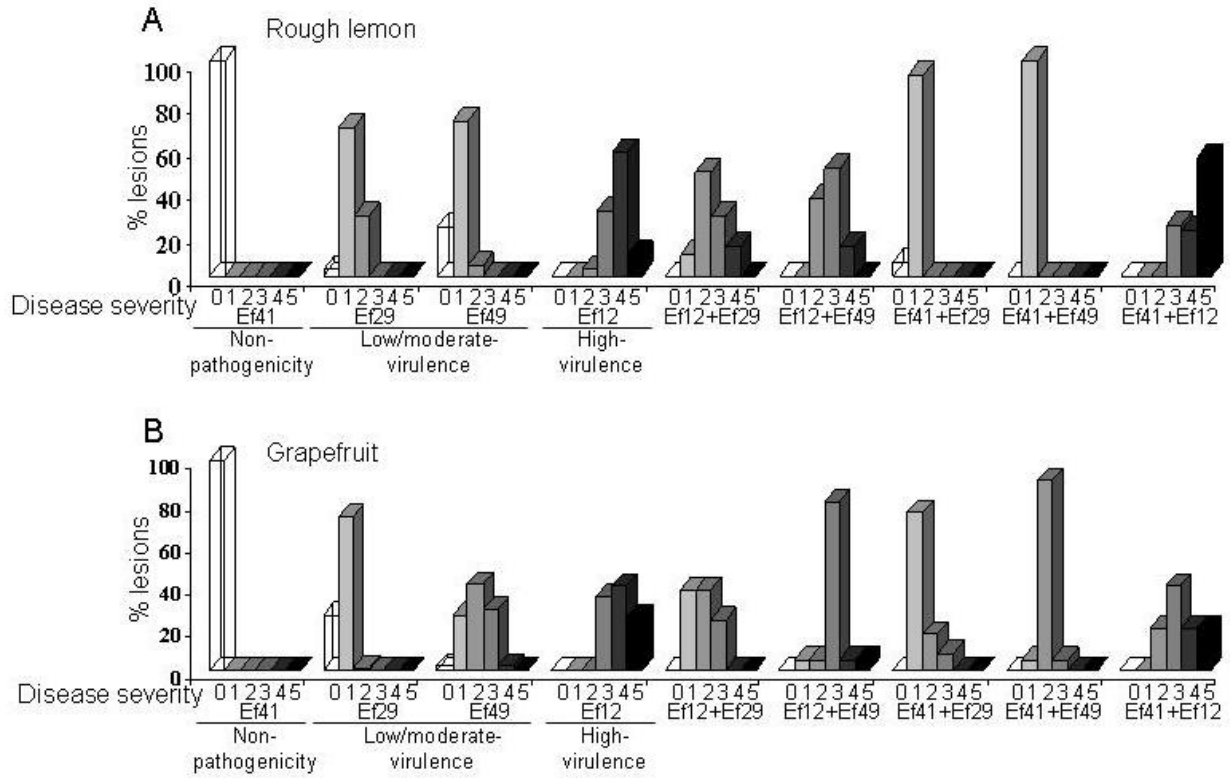


Figure 6-3. Alleviation of symptom development by co-inoculation of two *Elsinoë fawcettii* isolates. Conidial suspensions (1 µL, 1 x 10^5 mL^{-1}) of a non-pathogenic isolate (Ef41), two low/moderate-virulent isolates (Ef29 and Ef49), and a high-virulent isolate (Ef12) of *Elsinoë fawcettii* were placed alone or in combinations onto detached leaves of rough lemon (A), and grapefruit (B) (14 days after emergence). Inoculated leaves were incubated in a moist chamber. Formation of scab lesions was recorded 12 days post inoculation. The controls were inoculated with water only. Disease severity is rated on a scale, 0 for no lesion formation; 1, chlorotic reaction only, no scab pustules; 2, lesions with minor pustules; 3, scab pustules present, shower forming; 4, scab formed on all sites; and 5, lesions with severe pustules. % leaves with symptoms was calculated from over 100 leaves inoculated in three independent experiments.

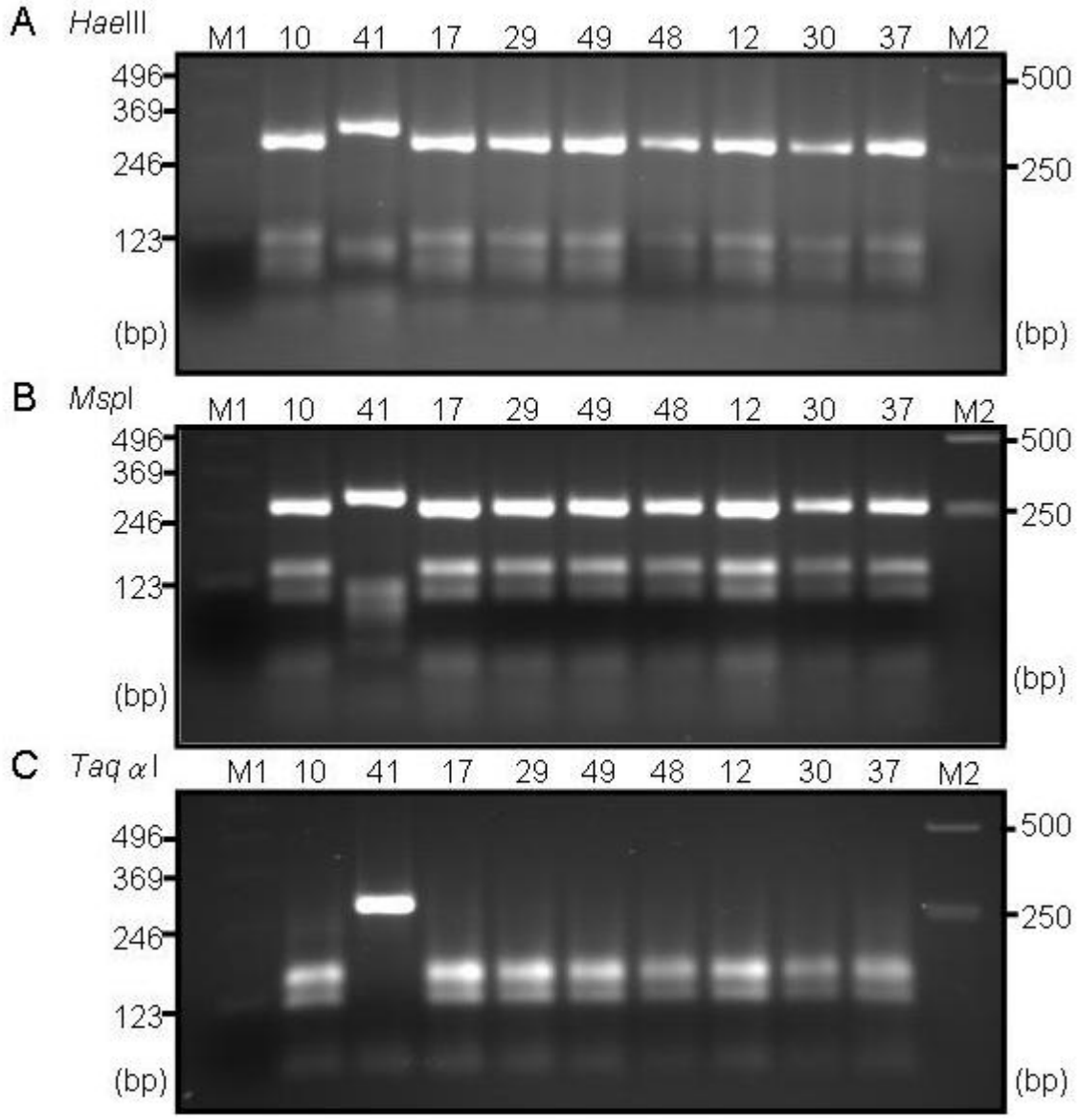


Figure 6-4. Electrophoresis of the amplified internal transcribed spacer (ITS) rDNA fragments after cleaved with A) *Hae*III; B) *Msp*I; C) *Taq*αI on 2% agarose gels. The numbers of *Elsinoë fawcettii* isolates are marked upon the lanes. M1, the 123 DNA ladder; M2, 1 kb ladder (Promega). Isolate Ef41 showed the different patterns among all three restriction enzyme digestions compared to the remaining isolates.

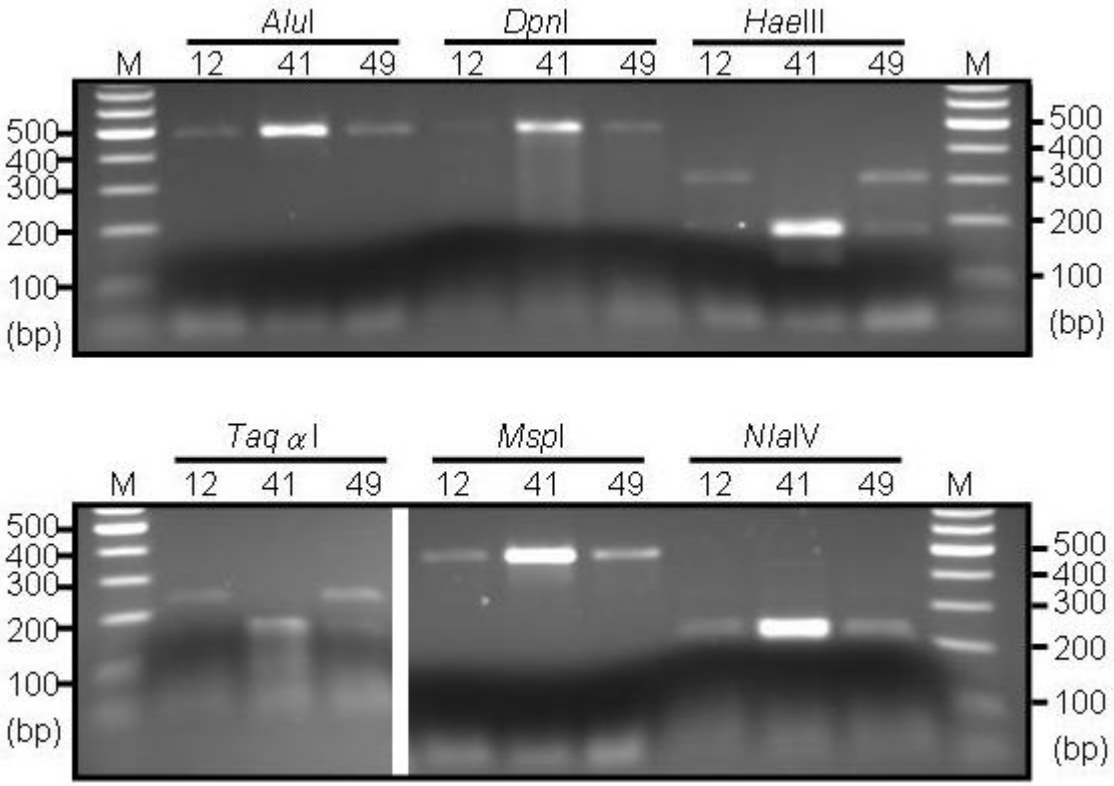


Figure 6-5. Electrophoresis of the amplified partial β-tubulin genes (~500bp) digested with six different 4-bp recognition sequence restriction enzymes on 2% agarose gels. The numbers of *Elsinoë fawcettii* isolates are marked upon the lanes. M, the 100 bp DNA ladder. The pattern of isolate Ef41 differed from the *Hae*III and *Taq*αI digestion patterns of the other two remaining isolates, Ef12 and Ef49.

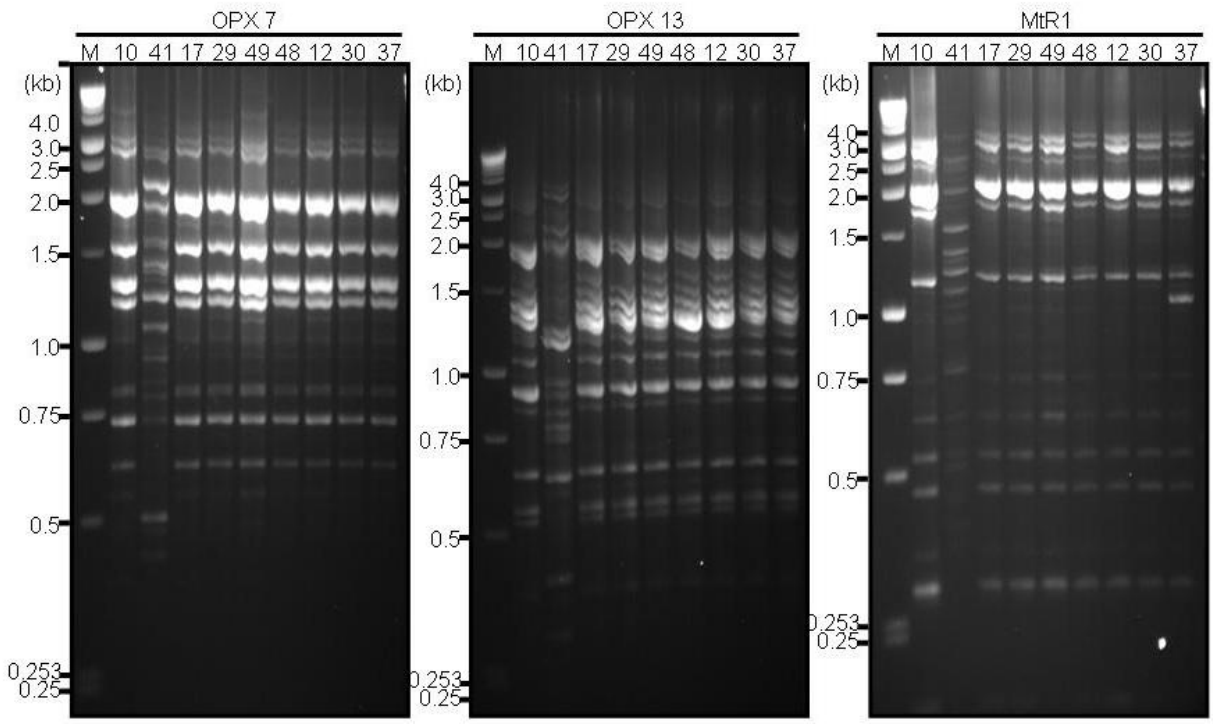


Figure 6-6. Electrophoresis of the randomly amplified polymorphic DNA fragments (RAPD) on 2% agarose gels. The numbers of *Elsinoë fawcettii* isolates are marked upon the lanes. M, 1 kb ladder (Promega). 10-mer primers, OPX-7, OPX-13, or MtR 1 was used to randomly amplify DNA from the isolates. Isolate Ef41 showed the different patterns from the remaining isolates in all three amplifications.

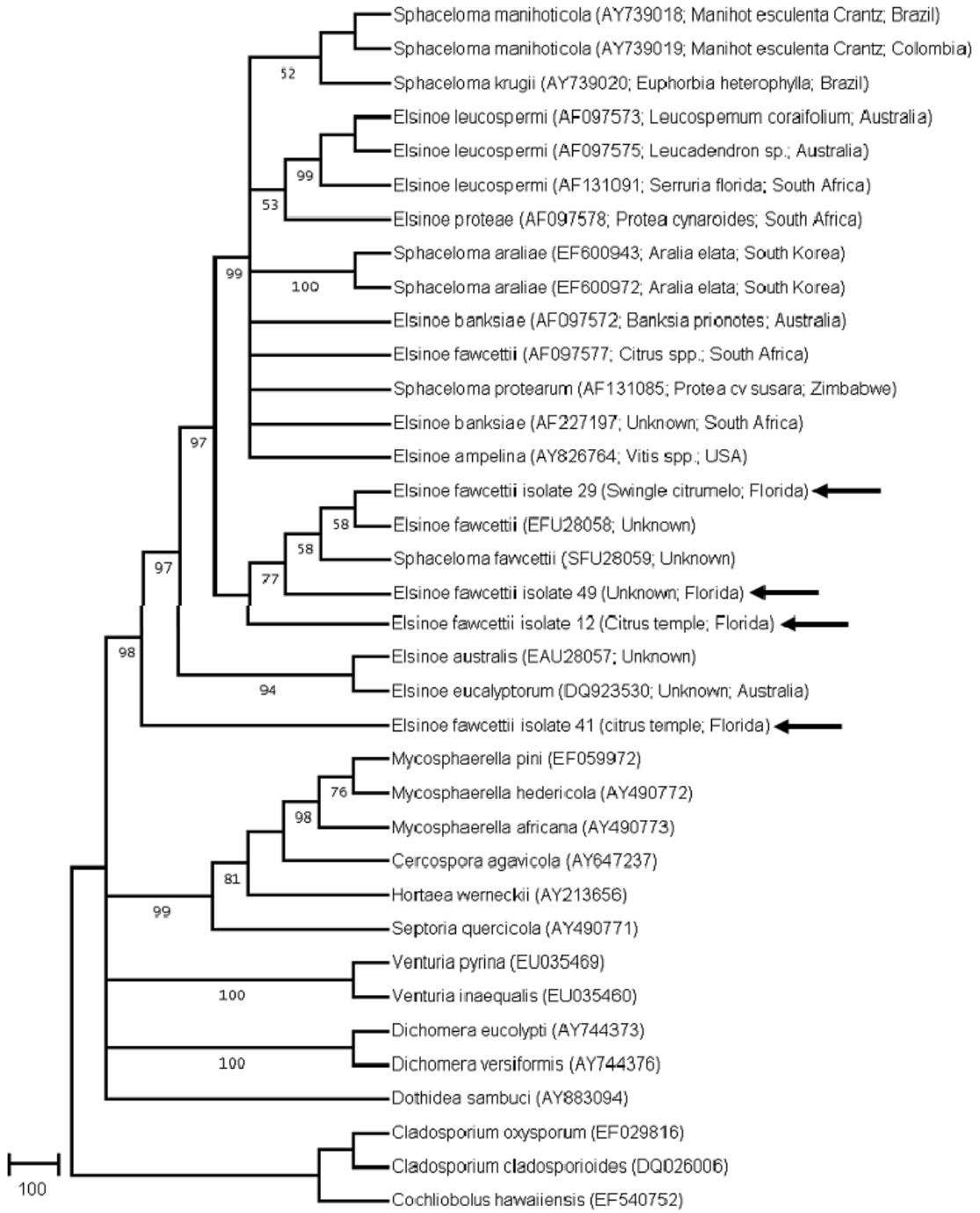


Figure 6-7. Comparison of the internal transcribed spacer 1 (ITS 1) sequences between *Elsinoë fawcettii* Florida isolates and other *Elsinoë* spp. and other species belonging to the order of Loculoascomycetes via phylogenetic analysis. The phylogenetic tree was generated by using DS Gene v2.5 program with 100 bootstrap replicates. *E. fawcettii* Florida isolates Ef12, Ef29, Ef41, and Ef49 were sequenced in this study. Accession numbers for all sequences obtained from GenBank, original-collected host of *Elsinoë spp*, and location of *Elsinoë spp*. were indicated in parentheses.

APPENDIX A
SUPPLEMENTAL DATA FOR CHAPTER TWO

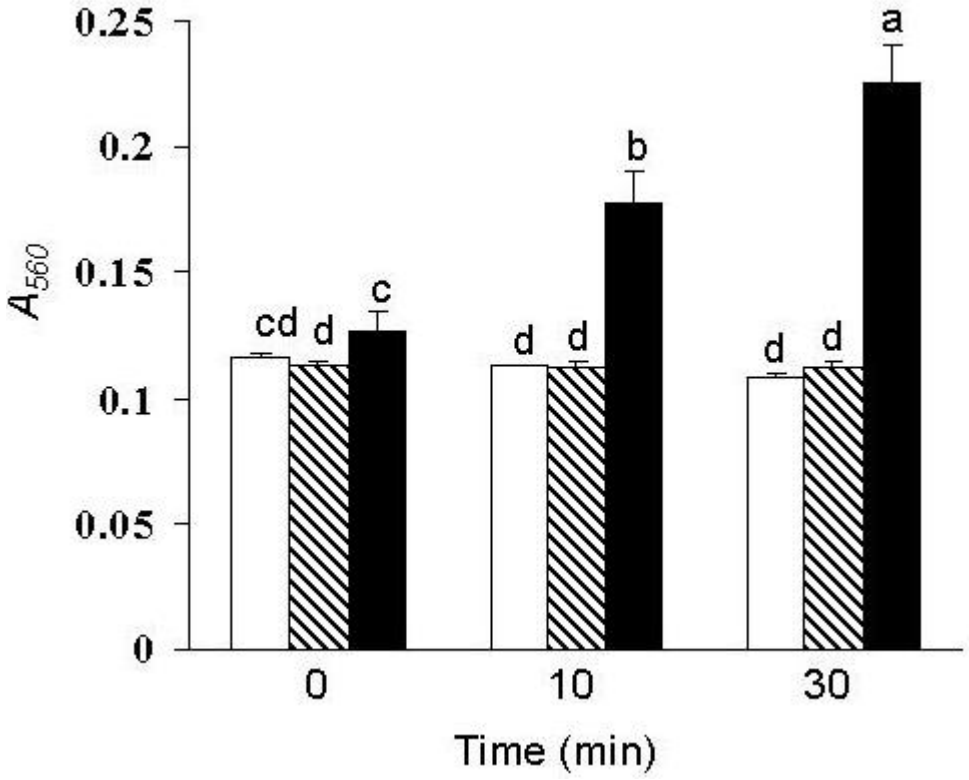


Figure A-1. Spectrophotometric absorbance of elsinochromes (ESCs) treated with 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer (open bars), ESCs treated with nitrotetrazolium blue chloride (NBT) (hatched bars), or ESCs treated with both MOPS and NBT (closed bars) at 560 nm. Vertical bars represent standard deviation. Means followed by the same letter are not different at judged by Duncan's multiple range test at $P < 0.0001$.

APPENDIX B
SUPPLEMENTAL DATA FOR CHAPTER THREE

Table B-1. Effects of ions on ESC production on CM by *E. fawcettii*

Treatment	Concn (mM)	Mean colony dai. (mm) ± SEM	ESC (nmoles per plug), mean ± SEM
none	-	10.31± 0.24	2.72 ± 0.32
$CuCl_2 \cdot 2H_2O$	0.1	nd[1]	nd
	1.0	9.34 ± 0.41	6.94 ± 0.36
	10.0	nd	nd
$FeCl_3$	0.1	10.22 ± 0.21	3.84 ± 0.22
	0.2	10.97 ± 0.12	3.00 ± 1.24
	0.5	10.97 ± 0.45	3.70 ± 1.52
	1.0	9.72 ± 0.12	3.56 ± 1.08
	2.0	10.19 ± 0.65	5.60 ± 0.70
	10.0	nd	nd
KCl	50.0	nd	nd
	100.0	10.56 ± 0.24	3.08 ± 0.16
$MgCl_2 \cdot 6H_2O$	50.0	nd	nd
	100.0	9.56 ± 0.60	2.78 ± 0.72
$MnCl_2 \cdot 4H_2O$	0.2	10.31 ± 0.52	1.46 ± 0.56
	1.0	9.81 ± 0.31	4.48 ± 0.56
	5.0	9.81 ± 0.38	5.68 ± 1.00
	10.0	10.50 ± 0.46	5.92 ± 0.18
	100.0	nd	nd
NaCl	50.0	nd	nd
	100.0	10.44 ± 0.60	4.94 ± 0.22
$ZnCl_2$	0.1	nd	nd
	1.0	11.00 ± 0.43	5.52 ± 0.38
	10.0	nd	nd

[1]nd, not determined

APPENDIX C
SUPPLEMENTAL DATA FOR CHAPTER FOUR

Table C-1. Sequences of primers

Name	Sequences
efup 1	5’-GCACTCCTGACCCGATTGGA-3’
efup 5	5’-CCGATCTACGCTCCTTACCACGCTG-3’
efpks 1	5’-CTCCACGTGAAGCGGCACAGAC-3’
efpks 5	5’-GGATCAGTCTGTGCCGCTTCACGTGG-3’
efdown 5	5’-CGGTAGTTGTGCTGGATGCGACGAG-3’
efdown 8	5’-CCGAAACGCCCTTGGTTCAAAGATG-3’
ef down 10	5’-CATCATCGGCGGTTGGTCAGCAGG-3’

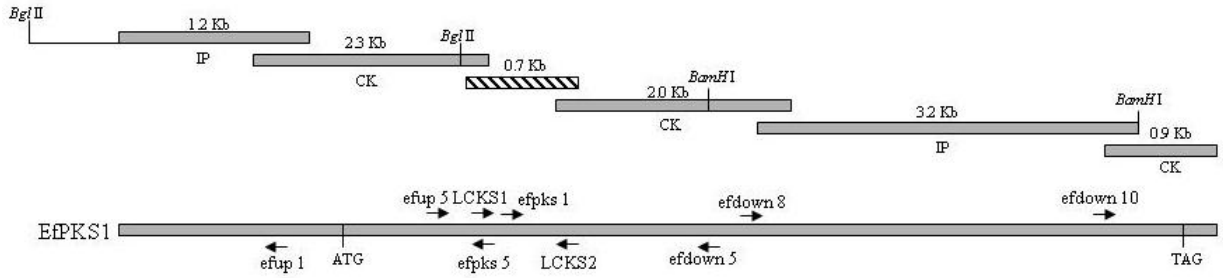


Figure C-1. Schematic describing the strategies to obtain full-length of *EfPKS1*. The amplified DNA fragment (hatched bar, about 700 bp) by two degenerate oligonucleotides (LCKS1/LCKS2) was first used for extension of 5' and 3' franking region of *EfPKS1* gene. Chromosome walking (CW) and inverse PCR (IP) were used to obtain full-length of *EfPKS1*. Arrowheads indicate relative positions of primers used for chromosome walking (efpks 1, efpks 5, and efdown 10) and inverse PCR (efup 1/efup 5 and efdown 5/efdown 8). Restriction enzyme sites used for inverse PCR were indicated. Drawing is not to scale.

APPENDIX D
SUPPLEMENTAL DATA FOR CHAPTER SIX

Table D-1. Germination of *Elsinoë fawcettii* isolates on detached leaves of rough lemon and grapefruit

Isolate no.	Pathogenicity[1]	Germination (% of conidia with germ tube/total conidia)[2]	
		Rough lemon	Grapefruit
41	none	0	0
29	low	4 ± 0.3	2 ± 0.1
12	high	33 ± 4.3	23 ± 3.0

[1]Pathogenicity assays were conducted on detached leaves as described in Table 6-1.
[2]Germination was evident by forming germ tube. For each isolate, 100 conidia were examined and the data show the means and standard errors of three independent experiments.

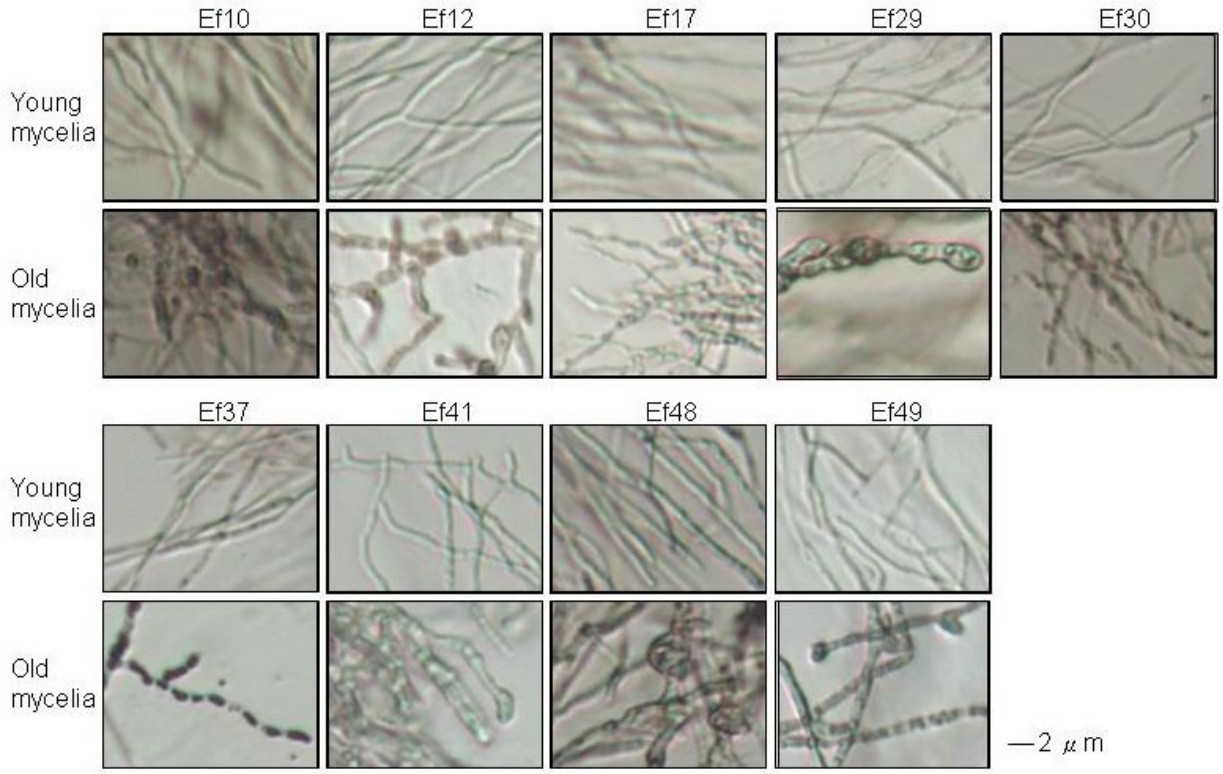


Figure D-1. *Elsinoë fawcettii* hyphae observed using a light microscope at x 200 magnification. Nonpathogenic isolate (Ef41), low virulence isolates (Ef10, Ef17, Ef29, and Ef49), and high virulence isolates (Ef12, Ef30, Ef37, and Ef48) of *Elsinoë fawcettii* were cultured on PDA under constant fluorescence light. Small pieces of young (4 day culture) and old (8 day culture) mycelia were removed from PDA, suspended in water and placed on slides for observation.

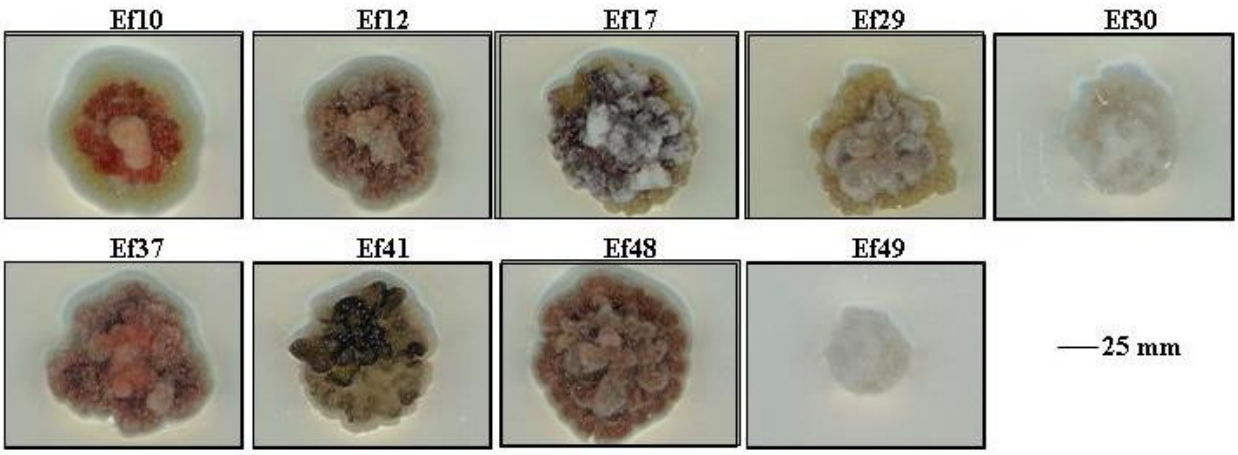


Figure D-2. Variation of fungal colony under a dissection microscope at x 25 magnification. Non-pathogenic isolate (Ef41), low virulence isolates (Ef10, Ef17, Ef29, and Ef49), and high virulence isolates (Ef12, Ef30, Ef37, and Ef48) of *Elsinoë fawcettii* were cultured on PDA media under constant fluorescence light for 10 days.

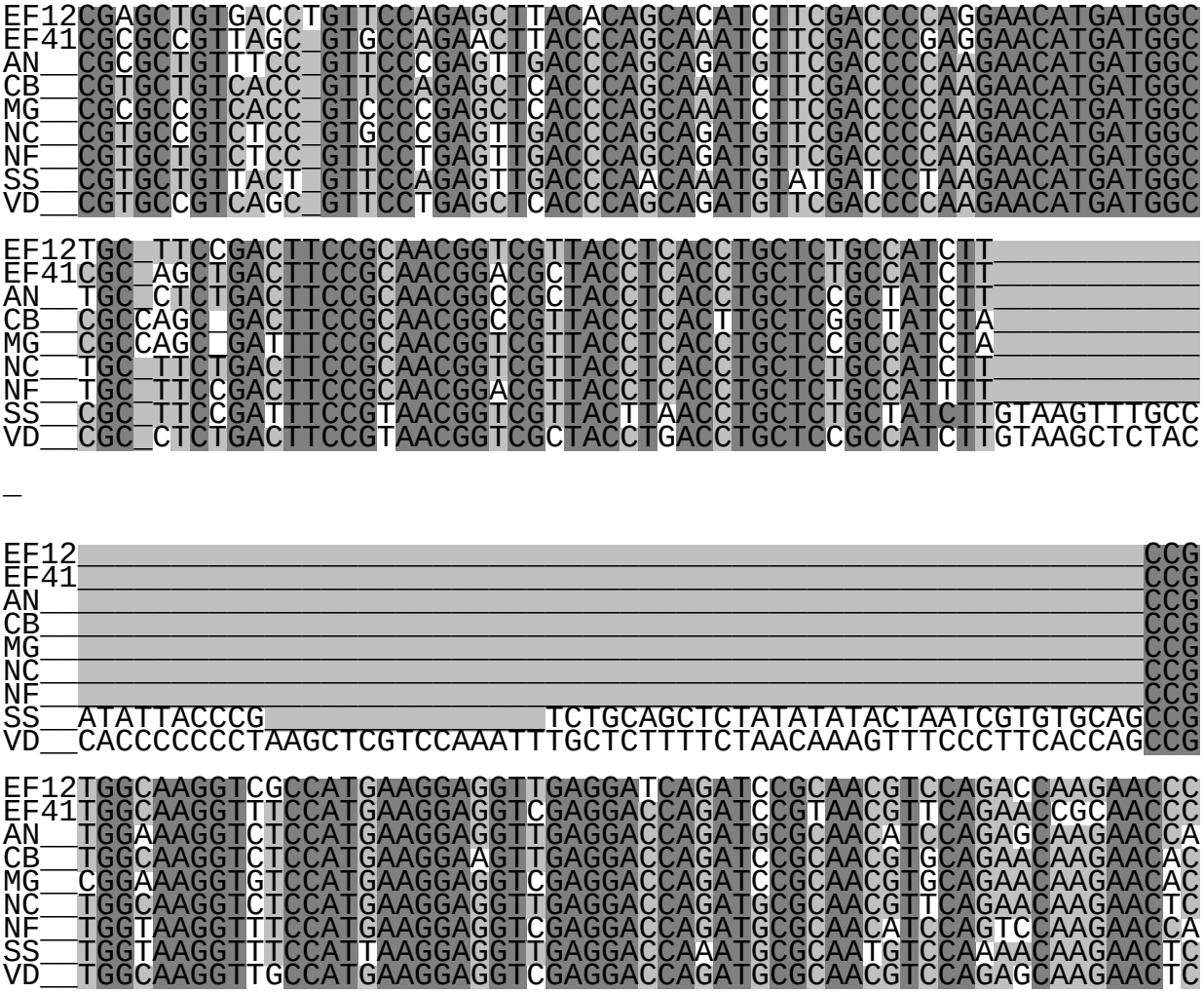


Figure D-3. Alignment of nucleotide sequences of fungal partial β-tubulin genes. The % identity between organisms were described in Table 6-2. Partial β-tubulin genes of *Elsinoë fawcettii* isolates, Ef12, and Ef41, were amplified by primer sets, Bt1F and Bt1R, and sequenced. Partial β-tubulin genes from other fungi were obtained from Genbank with accession numbers. AN, *Aspergillus nidulans* (XM_653694); CB, *Cercospora beticola* (AY856373); MG, *Mycosphaerella graminicola* (AJ310917); NC, *Neurospora crassa* (XM_952576); NF, *Neosartorya fischeri* (XM_001264677); SS, *Sclerotinia sclerotiorum* (AY12374); VD, *Verticillium dahliae* (DQ266135).

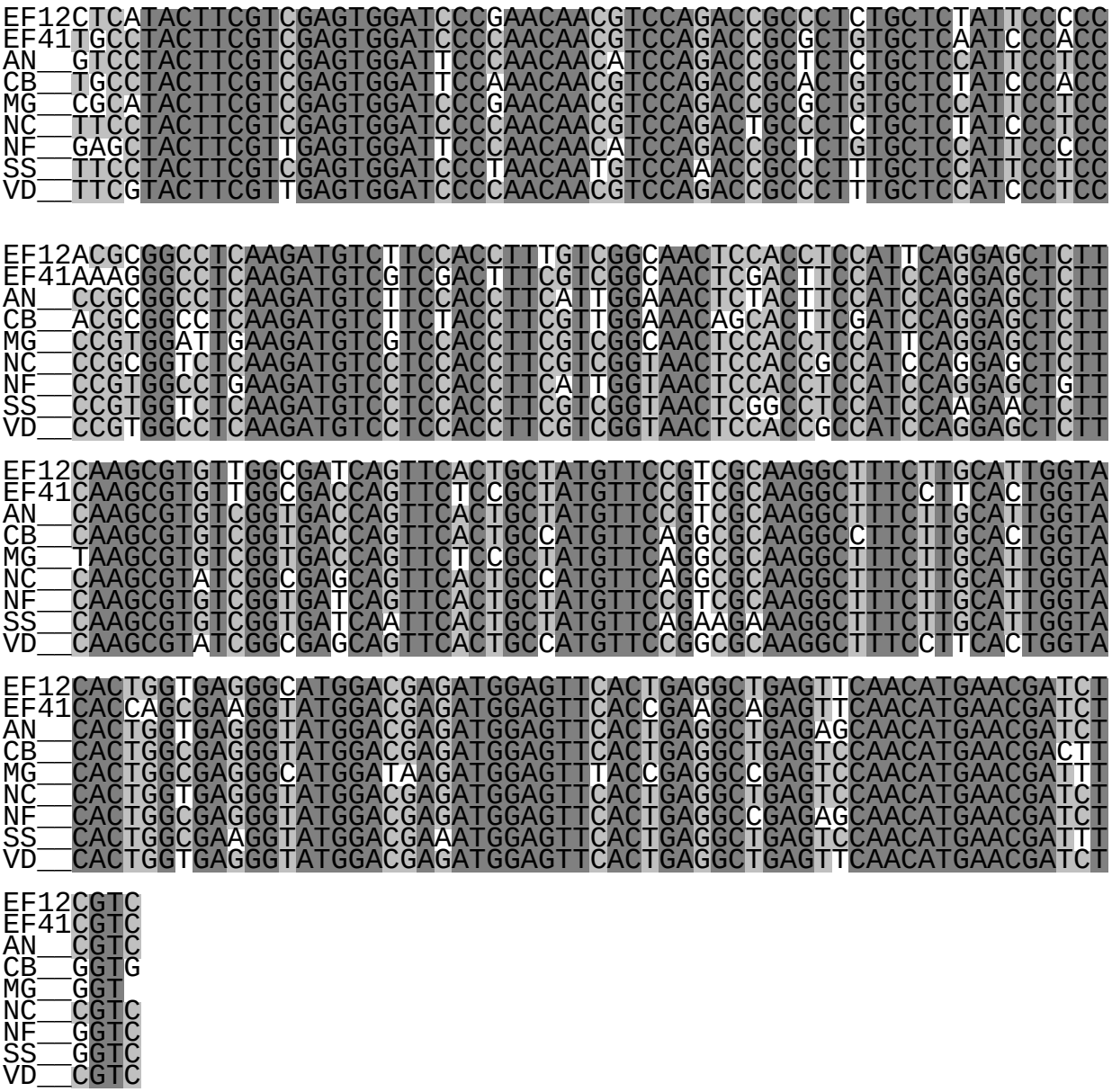


Figure D-3. (Continued)

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BIOGRAPHICAL SKETCH

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