

Pathogen profile

***Elsinoë fawcettii* and *Elsinoë australis*: the fungal pathogens causing citrus scab**

KUANG-REN CHUNG*

Citrus Research and Education Center, and Department of Plant Pathology, Institute of Food and Agricultural Sciences (IFAS), University of Florida, 700 Experiment Station Road, Lake Alfred, FL 33850, USA

SUMMARY

Elsinoë fawcettii and *E. australis* are important pathogens of citrus. Both species are known to produce red or orange pigments, called elsinochrome. Elsinochrome is a nonhost-selective phytotoxin and is required for full fungal virulence and lesion formation. This article discusses the taxonomy, epidemiology, genetics and pathology of the pathogens. It also provides a perspective on the cellular toxicity, biosynthetic regulation and pathological role of elsinochrome phytotoxin.

Taxonomy: *Elsinoë fawcettii* (anamorph: *Sphaceloma fawcettii*) and *E. australis* (anamorph: *S. australis*) are classified in the Phylum Ascomycota, Class Dothideomycetes, Order Myriangiiales and Family Elsinoaceae.

Host range: *Elsinoë fawcettii* causes citrus scab (formerly sour orange scab and common scab) on various species and hybrids in the Rutaceae family worldwide, whereas *E. australis* causes sweet orange scab, primarily on sweet orange and some mandarins, and has a limited geographical distribution.

Disease symptoms: Citrus tissues infested with *Elsinoë* often display erumpent scab pustules with a warty appearance.

Toxin production: Elsinochrome and many perylenequinone-containing phytotoxins of fungal origin are grouped as photosensitizing compounds that are able to absorb light energy, react with oxygen molecules and produce reactive oxygen species, such as superoxide and singlet oxygen. Elsinochrome has been documented to cause peroxidation of cell membranes and to induce rapid electrolyte leakage from citrus tissues. Elsinochrome biosynthesis and conidiation are coordinately regulated in *E. fawcettii*, and the environmental and physiological inducers commonly involved in both processes have begun to be elucidated.

INTRODUCTION

Citrus scab (formerly sour orange scab or common scab) is caused by *Elsinoë fawcettii* Bitancourt and Jenkins, and is one of the most important foliage fungal pathogens in many citrus-producing areas worldwide. Citrus scab affects the fruit, leaves and twigs of many susceptible cultivars of citrus, including lemons, grapefruit and many tangerines and their hybrids, causing external blemishes and reducing the acceptability of the fruit for the fresh market. Citrus scab seldom reduces yield, but may reduce the value of the fruit by as much as 50% if uncontrolled. Sweet orange scab, caused by *Elsinoë australis* Bitancourt and Jenkins, affects the fruit but not the leaves of most sweet orange [*C. sinensis* (L.) Osbeck] and some mandarin (*C. reticulata* Blanco) cultivars. *Elsinoë australis* is common in humid citrus-growing areas of Argentina, Bolivia, Brazil, Ecuador, Paraguay and Uruguay, but its presence has not been confirmed elsewhere. The introduction and establishment of *E. australis* in a new area could have quarantine implications for the marketing of fresh fruit.

Isolates of both *E. fawcettii* and *E. australis* grow slowly in culture, often producing red or brown pigments. The pigments have been identified as a nonhost-selective, perylenequinone-containing phytotoxin, called elsinochrome. The production of elsinochrome is a prerequisite for full fungal virulence and lesion formation. The cellular toxicity is highly dependent on the production of reactive oxygen species (ROS), such as superoxide and singlet oxygen, under aerobic and illuminated conditions. This article summarizes information on the taxonomy, host ranges and life cycles of *E. fawcettii* and *E. australis*. It also presents the current state of our understanding of the mode of action, biosynthesis and regulation of elsinochrome, as well as its role in fungal pathogenesis from molecular and genetic perspectives.

TAXONOMY AND CHARACTERISTICS

Both *Elsinoë fawcettii* Bitancourt and Jenkins (anamorph: *Sphaceloma fawcettii* Jenkins), causing citrus scab, and

*Correspondence: Email: krchung@ufl.edu

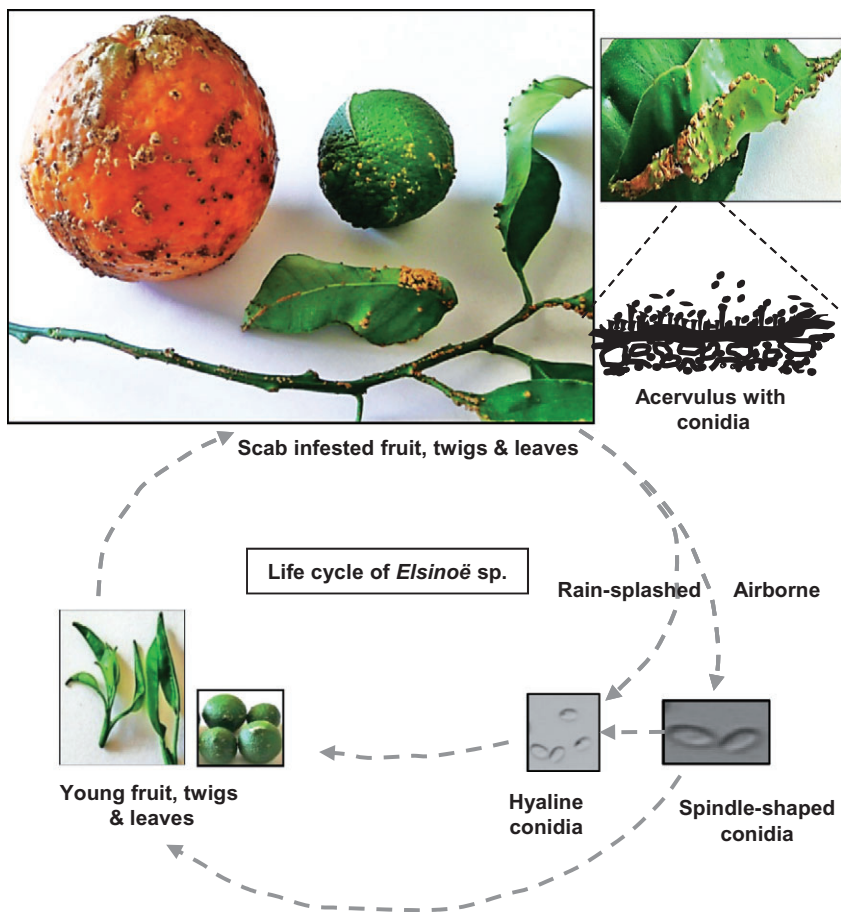


Fig. 1 Disease cycle of citrus scab caused by *Elsinoë fawcettii* on fruit, twigs and leaves, showing corky lesions with erumpent scab pustules.

E. australis Bitancourt and Jenkins (anamorph: *S. australis* Bitancourt and Jenkins), causing sweet orange scab, belong to Kingdom Fungi, Phylum Ascomycota, Class Dothideomycetes, Subclass Dothideomycetidae, Order Myriangiales and Family Elsinoaceae. The causal agent of 'Tryon's scab' was once described as *Sphaceloma fawcettii* var. *scabiosa* and has been regrouped with *S. fawcettii* (Timmer *et al.*, 1996). The teleomorph (sexual stage) of both *Sphaceloma* species was found only in Brazil (Bitancourt and Jenkins, 1936a, b) and its role in the onset of disease remains largely unknown. *Elsinoë fawcettii* (5–6 μm \times 10–12 μm) produces smaller ascospores (sexual spores) compared with those produced by *E. australis* (12–20 μm \times 15–30 μm). Both species produce single-celled, hyaline, elliptical conidia (asexual spores) with sizes of around 3–4 μm \times 4–8 μm . Conidia can be propagated through budding. *Elsinoë fawcettii*, but not *E. australis*, also produces dark-pigmented and spindle-shaped conidia (Fig. 1) on scab lesions, which may germinate to form hyaline conidia (Timmer *et al.*, 1996; Whiteside, 1975). *Elsinoë fawcettii* has been documented to form hyphae within hyphae, called intrahyphal hyphae or endohyphae (Kim and Hyun, 2007). In axenic culture, both *E. fawcettii* and *E. australis* grow slowly, lack extensive aerial

hyphae and form colonies often with red or brown pigments. Fungal colonies and pigments vary substantially among isolates, providing no reliable traits for distinguishing *E. fawcettii* from *E. australis*. The two species can be separated by host range and by molecular analysis of random amplified polymerase DNA (RAPD) polymorphism and of the amplified internal transcribed spacer (ITS) of ribosomal DNA digested with various restriction endonucleases (Hyun *et al.*, 2001; 2007; Tan *et al.*, 1996; Timmer *et al.*, 1996).

HOST RANGE AND PATHOGENETIC DIVERSITY

Elsinoë fawcettii has a number of pathogenic variants that are able to attack various citrus species and hybrids in the Rutaceae family. Citrus cultivars, such as 'Temple' and 'Murcott' tangors, 'Minneola' tangelos [*Citrus reticulata* Blanco \times *C. sinensis* (L.) Osbeck], lemons [*C. limon* (L.) Burm. F.] and grapefruit (*C. paradisi* Macf.), are most susceptible to *E. fawcettii*. Rootstocks, such as rough lemon (*C. jambhiri* Lush), sour orange (*C. aurantium* Lush) and 'Rangpur' lime (*C. limonia* Osbeck), are also susceptible to *E. fawcettii*. *Elsinoë fawcettii* has also been reported to affect ornamental citrus and rootstocks such as calamondins

(*C. madurensis* Lour.) and *Poncirus trifoliata* (L.) Raf. *Elsinoë fawcettii* rarely attacks Persian lime (*C. latifolia* Tanaka), kumquats [*Fortunella margarita* (L.) Swingle], pummelos (*C. maxima* Burm. f. Merr.), citrons (*C. medica* L.) or limes (*C. aurantifolia* Christm. Swingle).

Early studies based on field observations and pathogenicity assays led to the recognition of two different biotypes of *E. fawcettii* (Whiteside, 1978). One type infected the leaves and fruit of citrus cultivars, including lemon, rough lemon, grapefruit, sour orange, mandarins and some cultivars of tangelos. This biotype also affected the fruit, but not the leaves, of sweet orange, and thus was designated as the 'Florida Broad Host Range (FBHR)' pathotype (Timmer *et al.*, 1996). The second biotype was reported to affect only lemon, rough lemon, Cleopatra mandarin and grapefruit, and was designated as the 'Florida Narrow Host Range (FNHR)' pathotype. In addition, Tyron's pathotype (formerly *S. fawcettii* var. *scabiosa*), reported only in Australia, primarily affects lemon and rough lemon rootstock seedlings, as well as Cleopatra mandarin, but not sour orange. A new 'Lemon' pathotype affecting only rough lemon was also found among isolates collected from Australia (Timmer *et al.*, 1996). Pathogenicity assays with the field isolates collected from Florida or other countries revealed that some isolates could not be grouped with any of the described pathotypes of *E. fawcettii* (Hyun *et al.*, 2009; Wang *et al.*, 2009a), implicating the presence of novel pathogenic variants or cryptic subspecies. Isolates of *E. fawcettii* have very complex diversities of host range.

In contrast with *E. fawcettii*, *E. australis* affects the fruit, but not the leaves, of most sweet orange and mandarin cultivars. Sweet orange scab is primarily restricted to South America, including Argentina, Bolivia, Brazil and Paraguay (Timmer *et al.*, 1996). A novel 'Natsudaikai (*Citrus natsudaikai* Hayata)' pathotype of *E. australis* that is nonpathogenic to sweet orange has been identified recently (Hyun *et al.*, 2001).

SYMPTOMS

Elsinoë spp. grow inter- and intracellularly within host cells. Citrus scab symptoms vary among cultivars and are highly dependent on the age of the tissue when infected. Scab lesions can be seen on leaves as early as 4 days after infection, and on fruit around 7 days after infection. The lesions emerge as tiny spots, often with an irregular and rough appearance. As the host tissues expand, the affected areas become elevated and form erumpent scab pustules comprising fungal and host tissues (Fig. 1). Electron microscopy reveals the formation of dense cork layers beneath the lesions (Kim *et al.*, 2004). The raised warts, probably induced by hormone imbalance, often show light yellow or brown pigments in young tissues, and these darken as the tissues age or are colonized by other microorganisms. The

affected leaves may develop lesions with warty or protuberant pustules emerging from one side of the leaf and a correspondingly depressed area on the opposite side. Severely affected leaves often become deformed and scabby. Similar scabby lumps are also formed on twigs and fruit. The affected grapefruit or sweet oranges tend to have a flatter corky appearance.

LIFE CYCLE AND EPIDEMIOLOGY

Elsinoë fawcettii overwinters in scab pustules that are formed in leaves, fruit and twigs (Fig. 1), whereas *E. australis* probably survives solely in fruit. Both species propagate primarily via conidia, which are produced in acervuli on the edge of scab pustules. The pathological role of ascospores is less certain. *Elsinoë* spp. are mainly dispersed by rain or water splash of conidia, although dispersal via wind is also possible. The severity of citrus scab is principally dependent on the frequency and duration of humidity, as well as the inoculum source (Agostini *et al.*, 2003). In general, scab incidence is more severe in low-lying areas with frequent wetting, compared with elevated areas. At optimum temperature (24–28°C), a short wetting period (2–3 h) is sufficient to induce conidia formation, germination and infection (Agostini *et al.*, 2003; Whiteside, 1975). Citrus leaves are more susceptible to *Elsinoë* spp. soon after they develop from the bud, and become tolerant to infection 7–10 days after emergence. Fruit are vulnerable to infection 2 months after petal fall. Once infected, the pathogen reproduces rapidly inside the lesions and initiates new infection if environmental conditions are conducive and if susceptible hosts with young tissues are available. Infection by *Elsinoë* spp. occurs mainly during the spring flush and sporadically during the summer flush. Although scab occurring during summer flushes seldom causes severe damage, fungal inocula can build up for the following year and may result in an endemic problem.

CHEMICAL CONTROL

Citrus scab causes severe external blemishes on fruit and decreases their economic value. The disease must be controlled if the fruit are destined for the fresh market. Citrus scab is manageable with appropriate fungicide spray programmes that achieve sustained profitability. In Florida, two or three fungicide applications are required for scab control. The first spray is often applied when new flushes emerge in the early spring, and this is followed by a second application after petal fall and a third application approximately 3 weeks thereafter. If inoculum is low in a given area, the first spray can be disregarded. Copper fungicides, benomyl, ferbam, thiram, captafol and the sterol biosynthesis-inhibiting fungicides—although some are not registered for use in citrus, and others have been discontinued—are effective for scab control. However, frequent applications of a

fungicide may facilitate the occurrence of the fungicide-resistant strains. Benomyl-resistant strains of *E. fawcettii* are widespread in Florida (Whiteside, 1980) and probably in many of the citrus-growing areas worldwide (Leki, 1994; Tyson and Fullerton, 2001). Apart from fungicides, the avoidance of overhead sprinkler irrigation and the planting of susceptible cultivars on higher ground or in well-circulated areas may substantially reduce scab incidence.

ELSINOCHROME PIGMENTS PRODUCED BY *ELSINOË* SPECIES

Many *Elsinoë* species have long been known to produce red or yellow pigments in culture (Fig. 2). The water-insoluble pigment, named 'elsinochrome', was first isolated from cultures of *Elsinoë randii* Jenkins and Bitanc. (anamorph: *Sphaceloma randii*) and characterized by Weiss *et al.* (1957). The chemical structure of elsinochrome was independently elucidated to be a derivative of 1,2-dihydrobenzo-perylenequinone (Fig. 2). The chemical and physical properties of elsinochrome have been studied in detail by several research groups (Lousberg *et al.*, 1969; 1970a; Mebius *et al.*, 1990; Meille *et al.*, 1989; Shirasugi and Misaki, 1992; Weiss *et al.*, 1965). Elsinochrome consists of various derivatives (A, B, C and D) that have common phenolic quinones and vary

only in their side-chains (Weiss *et al.*, 1987). Each elsinochrome contains interchangeable tautomers (Fig. 2). Elsinochromes A, B and C, containing a two-fold rotation axis with highly symmetrical side-groups, are red pigments. Elsinochrome D, probably derived from 'C' by the formation of a methylenedioxy ring, was identified and characterized from the pigment mixture of *Elsinoë annonae* Bitanc. and Jenkins (Kurobane *et al.*, 1981; Lousberg *et al.*, 1970b; Shirasugi and Misaki, 1992).

Some *E. fawcettii* isolates produce the main A, B or C derivatives, accompanied by small amounts of other pigments, whereas other isolates accumulate large amounts of 'B' or 'C' with little or no 'A' produced. Elsinochrome can be extracted from fungal mycelia with acetone or ethyl acetate. The separation of 'A', 'B' and 'C' derivatives can be achieved by thin-layer (Fig. 3a) or high-performance liquid chromatography using various adsorbents (Weiss *et al.*, 1965). In addition to *Elsinoë* spp., elsinochrome has been reported to be produced by some isolates of the bamboo pathogen *Hypocrella bambusae* (Berk. and Broome) Sacc. and the bindweed biocontrol fungus *Stagonospora convolvuli* Dearness and House LA39 strain (Ahonsi *et al.*, 2006; Ma *et al.*, 2003). The latter is genetically distinct from *Septoria* sp. (Pfirter *et al.*, 1999). A previously described 'phycaron' pigment produced by a fungus, which was mistakenly identified as *Phyllosticta caryae* Peck., was later rec-

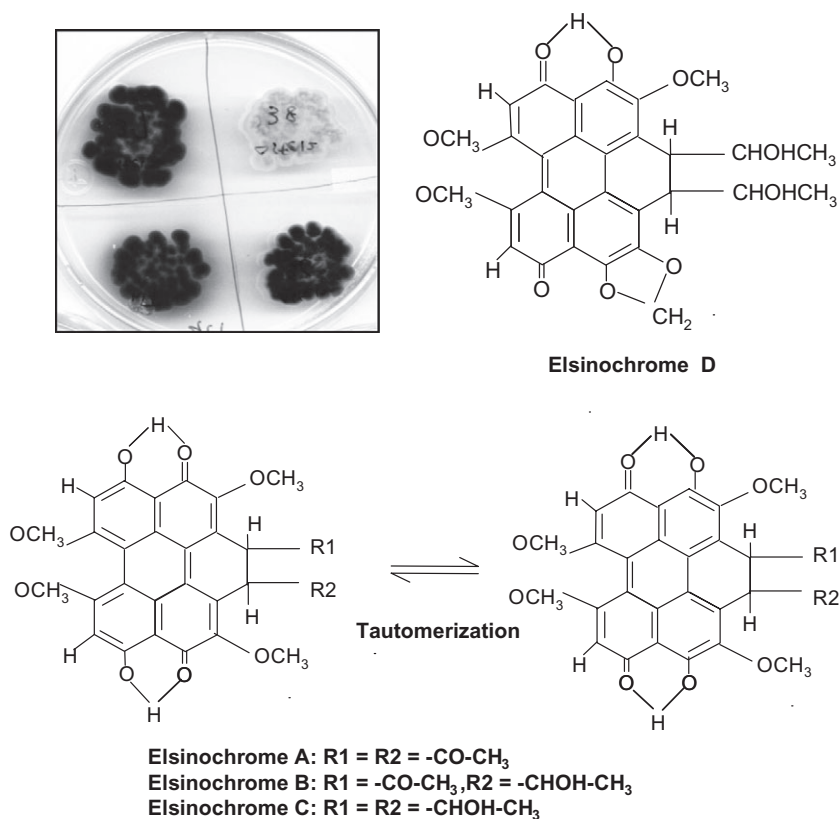


Fig. 2 Accumulation of red- or yellow-pigmented elsinochrome phytotoxin by the isolates of *Elsinoë fawcettii*. The fungal cultures were grown on potato dextrose agar in the light for 4 weeks. The chemical structures and tautomer of elsinochrome (1,2-dihydrobenzo-perylenequinone) A–D are also shown.

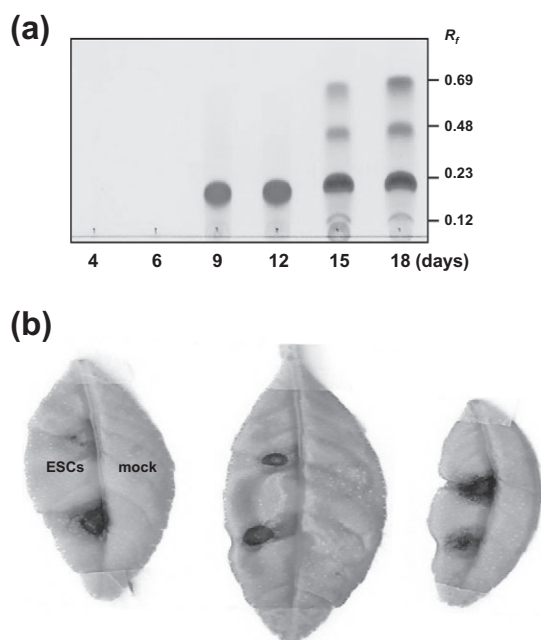


Fig. 3 (a) Thin-layer chromatography (TLC) analysis of elsinochrome produced by *Elsinoë fawcettii*, causing citrus scab. Elsinochrome was separated on silica gel using a solvent containing chloroform and ethyl acetate (1 : 1, v/v). (b) Induction of necrotic lesions on citrus leaves treated with elsinochrome (ESC). The mock controls were treated with acetone alone.

ognized to be elsinochrome. The producing fungus is actually an asexual stage of *Elsinoë randii* Bitancourt and Jenkins (Jenkins and Bitancourt, 1938). Moreover, *Pyrenochaeta terrestris* (H.N. Hansen), a fungal pathogen causing pink root disease of onions, was once reported to synthesize elsinochrome (Kurobane *et al.*, 1981). However, the identity of the producing fungus remains questionable because only one field-collected isolate was able to produce the red pigments.

TOXICITY OF ELSINOCHROME: A NONHOST-SELECTIVE PHYTOTOXIN

The toxicity of elsinochrome to Gram-negative bacteria has long been recognized to be dependent on both light and oxygen (Weiss *et al.*, 1957). Elsinochrome structurally resembles the perylenequinone phytotoxins produced by a number of fungal species. These toxins include: aspergillin produced by *Aspergillus niger* (Lund *et al.*, 1953); alteichin, altertoxin, alterlosin and stemphytoxin produced by *Alternaria* spp. (Davis and Stack, 1991; Stack *et al.*, 1986; Stierle and Cardellina, 1989) and *Stemphylium botryosum* (Davis and Stack, 1991); cercosporin produced by many *Cercospora* spp. (Daub *et al.*, 2005) and *Scolicotrichum graminis* (Tabuchi *et al.*, 1994); hypomycin A produced by *Hypomyces* spp. (Liu *et al.*, 2001); hypocrellin A/B

produced by *Hypocrella bambusae*; shiraiachromes produced by another bamboo pathogen, *Shiraiia bambusicola* (Wu *et al.*, 1989); and phleiochrome, calphostin C, cladochrome and entisophleiochrome produced by *Cladosporium* spp. (Arnone *et al.*, 1988; Weiss *et al.*, 1987; Yoshihara *et al.*, 1975). All of these compounds contain a core 3,10-dihydro-oxy-4,9-perylenequinone chromophore. They are grouped as photosensitizing reagents because of their ability to sensitize cells to visible light and to produce ROS, such as superoxides (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH \cdot) and/or singlet oxygen (1O_2) (Daub, 1982; Daub and Chung, 2009; Daub *et al.*, 2005; Yamazaki *et al.*, 1975). Oxygen and light are pivotal determinants for the photodynamic function and toxicity of these compounds. On illumination, photosensitizing compounds are converted to a stable electronically excited state, called the triplet state. The activated photosensitizers are able to react with oxygen, primarily via two different mechanisms, and generate toxic ROS (Daub and Chung, 2009; Dobrowolski and Foote, 1983; Girotti, 1990; Spikes, 1989). The triplet photosensitizers react with oxygen molecules by transferring a hydrogen atom or electron from reducing substrates, and produce reduced oxygen species including O_2^- (Type I reaction). The activated photosensitizers are also able to transfer energy directly to oxygen molecules to produce the electronically reactive 1O_2 (Type II reaction). Both 1O_2 and O_2^- are capable of inducing the oxidation of fatty acids, membranes, proteins/enzymes, sugars and nucleic acids, and hence are very toxic to cells (Daub *et al.*, 2005). These photodynamic reactions often result in cell death.

The phytotoxic effect of elsinochrome towards host and nonhost plant cells *in vitro* and *in vivo* has been documented recently to be light dependent (Liao and Chung, 2008a). Elsinochrome purified from an isolate of *E. fawcettii* is highly toxic to citrus (host) and tobacco (nonhost) cells in the light. The toxicity of elsinochrome has been demonstrated to be mediated through the production of ROS, which could damage cell membranes and cause electrolyte leakage from the cells. The incubation of citrus leaf discs with elsinochrome in the light induced a steady increment of electrolyte leakage (Liao and Chung, 2008a). The toxicity was greatly alleviated by adding 1O_2 quenchers, such as carotenoid carboxylic acid (bixin), 1,4-diazabicyclo-octane (dabco), reduced glutathione or ascorbate (vitamin C). Application of elsinochrome alone to citrus leaves produced necrotic lesions resembling those caused by the fungal pathogen (Fig. 3b). Moreover, lesion development induced by elsinochrome could be inhibited by the addition of bixin, dabco or ascorbate, but not by α -tocopherol (Liao and Chung, 2008a). Elsinochrome is a highly effective 1O_2 producer with a quantum yield (0.83–0.98 per quantum of light absorbed) comparable with that of two photosensitizing compounds, haematoporphyrin and cercosporin, that are well known 1O_2 generators (Aminian-Saghafi *et al.*, 1992; Ma *et al.*, 2003; Zhang *et al.*,

2009). The addition of β -carotene, a potent quencher, prevents $^1\text{O}_2$ production considerably. Elsinochrome has also been documented experimentally to generate O_2^- *in vitro*, and this accumulation was suppressed by superoxide dismutase (SOD), a scavenger of O_2^- , but was not suppressed by the $^1\text{O}_2$ quencher, dabco (Liao and Chung, 2008a). In addition, elsinochrome is a substantial hydroperoxide producer after irradiation with visible light (Arnone *et al.*, 1988). These studies demonstrate that elsinochrome functions as a photosensitizing compound that produces $^1\text{O}_2$ and O_2^- and exerts toxic effects on plant cells. Similar to many photosensitizing compounds, elsinochrome has anti-tumour activities. Elsinochrome and its derivatives have been shown to generate significant amounts of $^1\text{O}_2$ and to induce apoptosis of human colorectal carcinoma cells, as well as primate embryonic stem cells, in a light-dependent manner (Ma *et al.*, 2003; Zhang *et al.*, 2009).

BIOSYNTHESIS AND REGULATION OF ELSINOCHROME

The production of elsinochrome under laboratory conditions is growth phase dependent and is highly regulated by a variety of environmental and physiological factors (Wang *et al.*, 2009b). Light has been demonstrated to be the most essential factor, not only for toxicity, but also for elsinochrome production. Elsinochrome is primarily produced when the fungus is incubated in light, and its production is markedly suppressed when the fungus is grown in continuous darkness or in a light–dark cycle. In addition to this light response, *E. fawcettii* also produces large quantities of elsinochrome when grown under alkaline conditions. In contrast, pH has little effect on the production of cercosporin toxin by *Cercospora nicotianae* (You *et al.*, 2008). *Elsinoë fawcettii* has been shown to accumulate large quantities of elsinochrome in the absence of nitrogen, or when glucose or mannitol is used as the sole carbon source. The accumulation of elsinochrome is suppressed completely when ammonium chloride or ammonium nitrate is used as the sole nitrogen source. The presence of Ca^{2+} , Co^{2+} or Li^+ decreases elsinochrome production by *E. fawcettii*. In contrast, ions including K^+ , Na^+ , Cu^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} and Fe^{3+} have been shown to stimulate elsinochrome production to various levels, depending on the concentration of the compound tested. Metal ions could affect the production of fungal toxins by controlling the expression of the genes whose products are required for toxin biosynthesis. In contrast, calcium is required for cercosporin production (Chung, 2003; You *et al.*, 2008). The production of elsinochrome is also affected by antioxidants (Wang *et al.*, 2009b). Dabco or bixin increases elsinochrome production, whereas butylated hydroxyanisole at 5 mM inhibits elsinochrome production. Ascorbate, chlorogenic acid, catechin and gallic acid increase substantially elsinochrome production. Pyridoxine (vitamin B6), cysteine, α -tocopherol

(vitamin E), vanillic acid, caffeic acid and reduced glutathione have little or no effect on elsinochrome accumulation. Phenolic antioxidants also inhibit ochratoxin A production by *Aspergillus* spp. (Palumbo *et al.*, 2007; Passone *et al.*, 2005). Ascorbate enhances aflatoxin biosynthesis by *Aspergillus parasiticus*, yet caffeine, flavonoids, gallic acid and phenolics repress its biosynthesis (Holmes *et al.*, 2008; Patel *et al.*, 1990). How antioxidants affect elsinochrome production remains unknown at this point. Some antioxidants may alter the environmental and physiological conditions that normally would be conducive for elsinochrome biosynthesis; others may modulate signal transduction networks that lead to elsinochrome biosynthesis. Alternatively, some compounds may have direct effects on gene expression or enzyme activity of dedicated steps in the elsinochrome biosynthetic pathway.

All known hydroxyperylenequinones of fungal origin are polyketide derivatives containing two highly symmetrical structures. Their biosynthesis via phenol coupling of two polyhydroxynaphthalenes has been well studied (Weiss *et al.*, 1987). The synthesis of elsinochrome in *Elsinoë* spp. also apparently occurs via a fungal polyketide pathway, a process resembling fatty acid biosynthesis (Kennedy *et al.*, 1999). The biosynthesis of elsinochrome was first investigated by the analysis of culture filtrates after feeding *Elsinoë* spp. with radioisotope-labelled $1\text{-}^{14}\text{C}$ acetate and examining the subsequent products (Chen *et al.*, 1966). The study revealed that the 4,9-dihydroxyperylene-3,10-quinone chromophore of elsinochrome A is indeed derived from 4,4',5,5'-tetrahydroxy-1,1'-dinaphthyl by oxidation of phenols. In addition, ^{14}C formate was incorporated only into the methoxy groups of elsinochrome. Chen *et al.* (1966) postulated that elsinochrome A is synthesized by iterative decarboxylation to condense one acetate and six malonate units, followed by hydroxylation, methylation and dimerization. The biosynthesis of elsinochromes C and D was also investigated by a separate group, using dually labelled $2\text{-}^{13}\text{C}$ and $2\text{-}^2\text{H}_3$ acetates (Kurobane *et al.*, 1981). The analysis of the distribution of radioisotope in elsinochrome C and acetate in elsinochrome 'D' by ^{13}C and ^2H NMR spectrometry also confirmed that the carbon skeleton of elsinochrome is formed from a naphthalenoid heptaketide intermediate, followed by oxidative coupling of phenols. Elsinochrome D (Fig. 2), containing a methylenedioxy ring, is formed from 'C' by dehydroxylation between the C-3 methoxy and C-4 hydroxy moieties (Kurobane *et al.*, 1981).

MOLECULAR ANALYSES OF THE ELSINOCHROME BIOSYNTHETIC GENES

Further support for the notion that elsinochrome is synthesized via a polyketide pathway comes from evidence of a gene that encodes a type-I fungal polyketide synthase (PKS), and was cloned and characterized from an isolate of *E. fawcettii* (Liao and

Chung, 2008b). The cloned gene, designated *EfPKS1*, encodes a polypeptide containing all of the functional domains characteristic of fungal nonreducing PKSs (Yang *et al.*, 1996). These include a β -keto-synthase domain, an acyltransferase domain, two acyl carrier protein domains and a thioesterase/Claisen cyclase domain. Thus, *EfPKS1* seemingly functions to condense acetate and malonate molecules by iterative decarboxylation, chain elongation of the polyketide and formation of aromatic rings from polyketomethylenes (Watanabe and Ebizuka, 2004). Northern blotting revealed that the expression of *EfPKS1* is upregulated on exposure to light, in the presence of large amounts of glucose, during nitrogen starvation or at alkaline pH—all conditions highly conducive to elsinochrome accumulation. The insertion of a bacterial hygromycin phosphotransferase gene (*HYG*) cassette under the control of the *Aspergillus nidulans trpC* gene promoter within the *EfPKS1* gene, via double crossover recombination, resulted in fungal mutants that completely failed to accumulate any detectable elsinochrome (Liao and Chung, 2008b). The study concluded that the translational product of *EfPKS1* is required for elsinochrome biosynthesis. The introduction and expression of a functional copy of *EfPKS1* in a null mutant fully restored its capacity for elsinochrome production, thus conclusively demonstrating that fungal PKS is essential for the biosynthesis of elsinochrome.

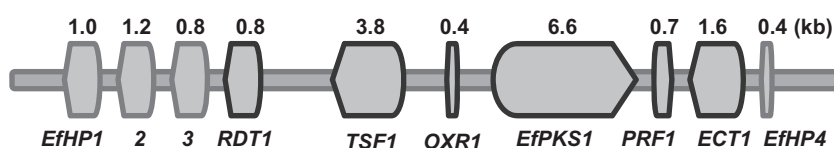
The genes required for the biosynthesis of secondary metabolites in filamentous fungi often reside in a cluster (Keller and Hohn, 1997; Keller *et al.*, 2005). A DNA sequence analysis upstream and downstream of the *EfPKS1* locus was conducted to determine whether any genes adjacent to *EfPKS1* might also be involved in elsinochrome production in *E. fawcettii* (Chung and Liao, 2008). The combination of sequencing and chromosomal walking strategies further identified nine open reading frames (ORFs), designated *EfHP1*, *EfHP2*, *EfHP3*, *PRF1*, *ECT1*, *TSF1*, *RDT1*, *OXR1* and *EfHP4*, in addition to *EfPKS1* (Fig. 4). Some of

these may have roles in elsinochrome biosynthesis. A *PRF1* gene, located immediately downstream from *EfPKS1*, encodes a polypeptide showing similarity to prefoldin protein subunit 3. An *ECT1* gene, located further downstream of *PRF1*, possesses a translational product highly similar to fungal nitrate transporters. A *TSF1* gene, which is located upstream and transcribed divergently from *EfPKS1*, encodes a putative protein having strong similarity to fungal transcriptional factors that contain a Cys₂His₂-type zinc finger and a GAL4-like Zn₂Cys₆ binuclear cluster. A *RDT1* gene, located further upstream from *TSF1*, encodes a polypeptide similar to a number of fungal reductases, such as 1,3,8-trihydroxynaphthalene (T₃HN) reductase that is involved in melanin biosynthesis (Kimura and Tsuge, 1993; Tsuji *et al.*, 2003). A small ORF, designated *OXR1*, was found between *EfPKS1* and *TSF1*. The predicted *OXR1* protein, rich in proline, displays low similarity (39%) to bacterial malate:quinone oxidoreductases. The *EfHP1* product shows low similarity (46%) to cation/multidrug efflux pumps in *Marinomonas* spp. The *EfHP1*, *EfHP3* and *EfHP4* genes encode hypothetical proteins with unknown functions.

Northern blot analysis revealed that the accumulation of transcripts of the identified genes does not completely correspond to the conditions conducive for elsinochrome production (Chung and Liao, 2008). All putative ORFs, except *EfHP3*, were upregulated under nitrogen starvation and in response to light. *TSF1*, *EfPKS1*, *PRF1*, *ECT1* and *EfHP2* were preferentially expressed under alkaline conditions, whereas pH had no effect on the expression of *EfHP3*, *EfHP4* or *OXR1*. Glucose promoted the accumulation of *EfPKS1*, *PRF1*, *ECT1* and *OXR1* gene transcripts and suppressed the expression of *TSF1*. Expression of the *RDT1*, *EfHP1*, *EfHP2* and *EfHP4* genes was apparently not regulated by glucose.

To determine whether the genes clustered with *EfPKS1* are also required for the biosynthesis and regulation of

Elsinochrome Toxin Biosynthetic Gene Mini-Cluster



EfPKS1:

Involved in condensation, chain elongation and cyclization

TSF1:

Transcription regulator

OXR1:

Involved in oxidation and hydroxylation

RDT1:

Involved in oxidation and hydroxylation

ECT1:

Involved in ESC exportation

PRF1:

Unknown function

EfHP1–4:

Hypothetical proteins

Fig. 4 Schematic diagram showing the elsinochrome (ESC) biosynthetic gene cluster with predicted functions identified in *Elsinoë fawcettii*. This figure shows data reprinted from fig. 1 of Chung and Liao (2008) [reprinted with permission from the Society for General Microbiology (UK)].

elsinochrome, the *TSF1* gene was inactivated by targeted gene disruption and the mutants were characterized in detail. Similar to the *EfPKS1* null mutant, the *TSF1* null mutant failed to accumulate any detectable elsinochrome in axenic cultures (Chung and Liao, 2008). Although *TSF1* is situated next to the *EfPKS1* gene in the genome, and is absolutely required for elsinochrome production, its expression profiles did not fully correlate with those of *EfPKS1* under certain conditions. However, *TSF1* was observed to regulate the expression of the *RDT1*, *EfPKS1*, *PRF*, *ECT1* and *EfHP1* genes. Interestingly, impairment of *EfPKS1* also led to a marked reduction in transcripts of the *RDT1*, *TSF1*, *PRF1*, *ECT1* and *EfHP1* genes, most probably as a result of a feedback inhibition effect. *TSF1* or *EfPKS1* had no effect on the expression of the *OXR1*, *EfHP2* or *EfHP3* genes. Nevertheless, *TSF1* acts as a core transcriptional activator and plays a profound role in elsinochrome biosynthesis. Although we could not demarcate precisely the elsinochrome biosynthetic gene cluster, the determination of gene expression patterns in the *EfPKS1*- or *TSF1*-disrupted mutants suggested that some newly identified genes are needed for elsinochrome production. However, the function of the elsinochrome genes, other than *EfPKS1* and *TSF1*, in the biochemical pathway leading to elsinochrome production cannot be conclusively determined until each of the genes is functionally inactivated.

A recent study has further revealed that the *E. fawcettii* *EfSTE12* gene, encoding a polypeptide analogue to the yeast STE12 transcription factor, plays a regulatory role in the biosynthesis of elsinochrome (Yang and Chung, 2010). *EfSTE12* is not clustered with the elsinochrome biosynthetic genes. In *Saccharomyces cerevisiae*, transcriptional activation of STE12 is induced by a pheromone response mitogen-activated protein kinase (MAPK) pathway, and is required for the regulation of the genes involved in developmental and physiological functions (Madhani *et al.*, 1999; Schwartz and Madhani, 2004). Disruption of the *EfSTE12* gene led to a reduced expression of the *EfPKS1* gene, but not the *TSF1* gene. It appears that *EfSTE12* bypasses the pathway-specific *TSF1* regulator and activates the biosynthetic genes directly.

A PROPOSED ROUTE LEADING TO THE PRODUCTION OF ELSINOCHROME

Elsinochrome is synthesized via a pathway resembling the biosynthesis of fatty acids and fungal polyketides. Thus, the assembly of the polyketide backbone of elsinochrome is probably accomplished by the function of *EfPKS1* (Fig. 5). One proposal suggests that *EfPKS1* iteratively catalyses decarboxylation between acetyl-CoA and malonyl-CoA subunits for polyketide chain elongation. In each condensation cycle, the malonyl-CoA subunit is first attached to the acyl carrier protein domains of *EfPKS1* to form a phosphopantetheine (PPT) complex, which

accepts the acetate unit from acetyl-CoA via the function of the acyltransferase domain. The β -keto-synthase domain of *EfPKS1* catalyses the condensation to produce a linear polyketide chain. In each cycle, the keto group of malonyl-CoA is reduced and results in the integration of two carbons into the polyketide chain. Once the chain is completed, the polyketide is unattached from the complex, probably via the function of the thioesterase/Claisen cyclase domain. The released polyketide undergoes cyclization to form an aromatic ring, and proceeds via serial modification steps to produce the heptaketide backbone of elsinochrome. As elsinochrome has a symmetrical structure, two identical heptaketides are fused to form a core 1,2-dihydrobenzo-perylene ring structure, which can then be successively modified to produce the various derivatives of elsinochrome. Some of these reactions may be cooperatively carried out, at least in part, by the products of *RDT1*, *OXR1* and *EfPKS1*.

The *PRF1* gene, embedded within the elsinochrome cluster, produces a protein displaying high similarity to prefoldin protein subunit 3. The prefoldin protein comprises multiple subunits, and forms part of a molecular chaperone system that binds and stabilizes newly synthesized polypeptides to ensure appropriate folding in eukaryotes and archaea (Vainberg *et al.*, 1998). Although the expression of the *PRF1* gene coincided with the conditions conducive for elsinochrome production and was markedly repressed in the *EfPKS1* or *TSF1* null mutants, the actual function of the polypeptide of the *PRF1* gene still remains uncertain. If the *PRF1* gene product is actually involved in elsinochrome production, it is very possible that the PRF1 protein functions to stabilize some of the biosynthetic enzymes required for elsinochrome production. As prefoldin is a hexamer containing two α and four β subunits; additional prefoldin subunits, whose coding genes may not immediately link to the elsinochrome biosynthetic gene cluster, are required to fulfill the chaperone function. In addition, no methyltransferase-coding gene exists within the biosynthetic gene cluster, even though elsinochrome has four methyl groups at positions C3, C7, C8 and C12 (Fig. 5). Apparently, the identified gene cluster does not contain the entire entourage of genes responsible for elsinochrome biosynthesis. Once elsinochrome is synthesized, it must be exported outside the fungal cells, which is probably accomplished by the *ECT1* transporter, to avoid toxicity.

CONNECTIONS BETWEEN ELSINOCHROME AND CONIDIATION

In filamentous fungi, the production of secondary metabolites is often closely associated with cell development or differentiation (Adams and Yu, 1998; Brodhagen and Keller, 2006; Calvo *et al.*, 2002; Yu and Keller, 2005). Similarly, elsinochrome production and the process of conidiation may have an intimate relationship

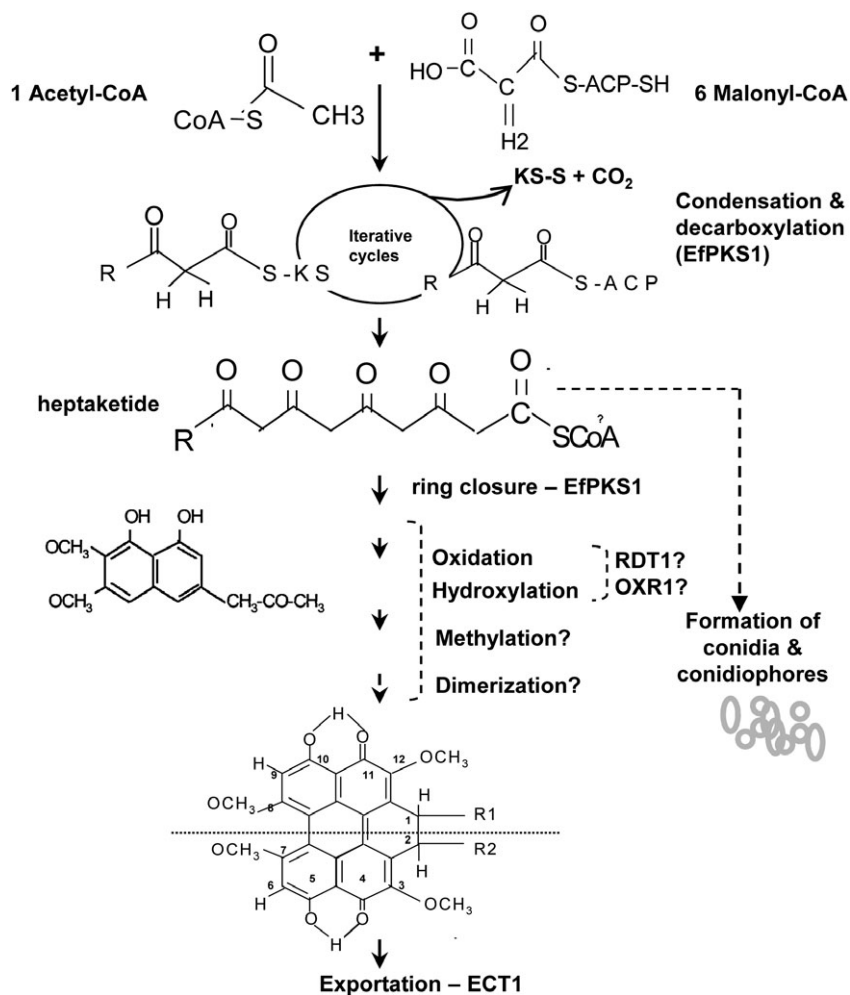


Fig. 5 Proposed biosynthetic pathway and involvement of the gene products leading to the formation of elsinochrome and conidia by *Elsinoë fawcettii*.

in *Elsinoë* spp. In addition to complete obliteration of elsinochrome production, the *E. fawcettii* mutants disrupted in *EfpKS1* or *TSF1* also failed to produce conidia (Chung and Liao, 2008; Liao and Chung, 2008b). Conidiation and elsinochrome production can be genetically reverted to wild-type levels by expressing a functional *EfpKS1* gene in a null mutant. Surprisingly, *TSF1* restored elsinochrome production, but not conidiation, in a *TSF1* null mutant (Chung and Liao, 2008). Although EfsTE12 was required for the regulation of elsinochrome biosynthesis, it was apparently totally dispensable for conidiation (Yang and Chung, 2010).

Environmental and physiological cues that trigger both conidiation and the production of secondary metabolites are very probably perceived and transduced by common signalling pathways in fungi. Environmental factors, such as light, carbon/nitrogen sources and ambient pH, have long been known to regulate both the developmental differentiation and biosynthesis of secondary metabolites in fungi (Calvo *et al.*, 2002). The genetic mechanisms connecting both processes are complex, but

are beginning to unfold through molecular studies. An involvement of a G-protein/cyclic adenosine monophosphate/protein kinase A-mediated pathway and many cellular regulators has been documented in both asexual sporulation and mycotoxin production in *Aspergillus* spp. (Calvo *et al.*, 2002; Tsitsigiannis and Keller, 2006). Similarly, the MAPK and cyclin-dependent kinase pathways have also been documented to regulate both conidiation and the production of cercosporin in *Cercospora zea-maydis* (Shim and Dunkle, 2003) and fumonisins in *Fusarium verticillioides* (Shim and Woloshuk, 2001), respectively.

The coordinate regulation of conidiation and the production of elsinochrome via similar regulatory pathways, as described in *Aspergillus* species, remains to be proven in *Elsinoë* spp. However, the *EfpKS1*, *TSF1* and *PRF1* promoters contain one or multiple 5'-(A/C)(A/G)AGGG(A/G)-3' motifs that serve as binding sites for the conidial formation-related Bristle (BrlA) transcription activator of *A. nidulans* (Adams *et al.*, 1998). Each of the promoter regions of the *RDT1*, *OXR1*, *EfpKS1* and *ECT1* genes has a consensus sequence, 5'-CATT(C/T)-3', which acts as a

binding site for the AbaA transcription activator involved in conidiophore development in *A. nidulans* (Andrianopoulos and Timberlake, 1994). In *A. nidulans*, AbaA is regulated by BrlA and both are required for conidiation. Thus, elsinochrome or intermediates generated during the course of biosynthesis may be involved directly in conidial formation by *Elsinoë* spp.

GENETIC DETERMINATION OF FUNGAL PATHOGENICITY

Many phytopathogenic fungi are capable of producing nonhost-selective, light-activated perylenequinone toxins. For example, cercosporin produced by many *Cercospora* species has been documented to play a profound role in plant diseases (Callahan *et al.*, 1999; Chen *et al.*, 2007; Choquer *et al.*, 2005, 2007; Dekkers *et al.*, 2007; Shim and Dunkle, 2003). However, the role of other perylenequinone toxins required for fungal

pathogenesis and for the onset of plant diseases has not yet been investigated widely. Purified elsinochrome has been documented to be toxic to suspension-cultured citrus and tobacco cells (Liao and Chung, 2008a). Elsinochrome also caused lipid peroxidation and severe electrolyte leakage of host leaves after irradiation, owing to the generation of ROS. Elsinochrome could be obtained from necrotic lesions on *Elsinoë*-affected citrus leaves (Wang *et al.*, 2009c), indicating its production during the course of disease development. Furthermore, the application of $^1\text{O}_2$ quenchers, such as bixin, ascorbate and reduced glutathione, or of O_2^- scavengers, such as α -tocopherol, L-cysteine and SOD, mitigated the cellular toxicity of elsinochrome (Liao and Chung, 2008a). Most intriguingly, elsinochrome alone induced necrotic lesions on citrus leaves on exposure to visible light, and the development of such lesions could be impeded by the co-application of $^1\text{O}_2$ quenchers. These results imply an important role of elsinochrome in the formation of necrotic lesions.

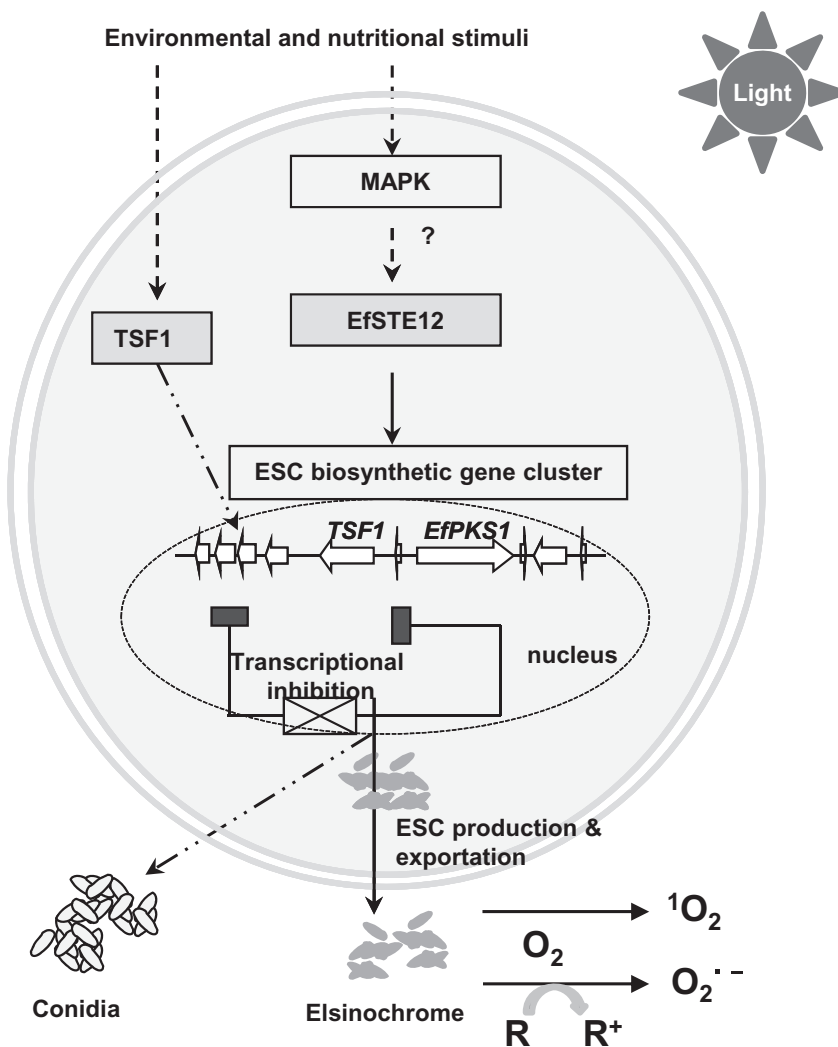


Fig. 6 Proposed regulatory pathways involving two transcriptional activators, TFS1 and EfSTE12, and leading to the production of elsinochrome (ESC) and conidia in *Elsinoë fawcettii*. On exposure to light, ESC assumes an activated triplet state which reacts with oxygen to produce superoxide (O_2^-) through a reducing substrate (R) or singlet oxygen ($^1\text{O}_2$) via an energy relay reaction. A cross indicates an inhibitory effect for ESC biosynthesis, presumably by blocking the expression of TFS1 and thus of the elsinochrome-biosynthetic genes. A filled rectangle indicates the inhibitory activity of expression of the ESC biosynthetic genes. TFS1 is also required for conidiation. The regulation of ESC biosynthesis by EfSTE12 bypasses TFS1 and is probably controlled by a mitogen-activated protein kinase (MAPK)-mediated signalling pathway.

Elsinochrome is a virulence determinant in *Elsinoë* spp., although no strong correlation exists between the levels of elsinochrome production in culture and pathogenicity among isolates (Wang *et al.*, 2009c). As stated above, both *EfPKS1* and *TSF1* null mutants failed to produce any detectable levels of elsinochrome. Fungal pathogenicity assayed on detached citrus leaves revealed that these null mutants defective in *EfPKS1* or *TSF1* induced fewer necrotic lesions compared with the number induced by wild-type or genetically reverted strains (Chung and Liao, 2008; Liao and Chung, 2008b). Both conidiation and fungal pathogenicity were fully restored in an *EfPKS1* null mutant on acquisition and expression of a functional *EfPKS1*. Although the *TSF1* complementation strain failed to fully restore conidiation, the fungus caused necrotic lesions on citrus leaves at a rate and magnitude that was indistinguishable from that of the wild-type. However, the *EfSTE12* null mutant, which is partially defective in elsinochrome production, is still fully pathogenic to citrus (Yang and Chung, 2010). The *EfSTE12* null mutant still produces elsinochrome, which might be at levels sufficient to facilitate fungal invasion.

CONCLUDING REMARKS

Elsinochrome produced by many *Elsinoë* spp. is a photosensitizing polyketide. Great strides are being made in the elucidation of its mode of action and in the identification of its biosynthetic and regulatory genes. Elsinochrome clearly is a critical virulence factor for *E. fawcettii* and, perhaps, for *E. australis* as well. As with many photoactivated toxins containing perylenequinones (Daub and Chung, 2009), elsinochrome toxin operates by absorbing light energy and generating ROS, which kills host cells. The production of elsinochrome may provide *Elsinoë* spp. with access to nutrients and allow the pathogen to thrive within host tissues. The analysis of elsinochrome-deficient mutants has confirmed an important role in normal disease development. Recent studies have also documented that the biosynthesis of elsinochrome is regulated by diverse environmental and physiological cues. Such regulation is mediated by the pathway-specific TSF1 transcription activator (Fig. 6). The *TSF1* gene is located upstream of the *EfPKS1* gene, and is clustered with several other genes whose products are implicated in the production of elsinochrome. Environmental signals could be transduced down a number of signalling pathways. For example, elsinochrome biosynthesis, but not conidiation, is regulated, at least in part, by the *EfSTE12* transcription factor, whose activity is probably controlled by a MAPK-mediated signalling pathway. Bypassing the TSF1 regulator, *EfSTE12* functions in elsinochrome biosynthesis by direct activation of biosynthetic genes. Elsinochrome is favourably produced by *E. fawcettii* grown in light, with large amounts of glucose, under nitrogen deprivation or at alkaline

pH. Other transcription regulators, such as the nitrogen-induced AreA (Marzluf, 1997), the light-regulated WC1/WC2 (Linden and Macino, 1997), the ambient pH-regulated PacC (Espeso *et al.*, 1997) and the conidial formation-related BrIA and AbaA (Adams *et al.*, 1998), might also be involved in elsinochrome biosynthesis and conidiation. Elsinochrome biosynthesis and conidial formation are very probably coordinately regulated, at least in part, by common transcriptional regulators in a complex and intertwined network. Alternatively, the proteins or intermediates involved in elsinochrome production might participate directly in conidia formation.

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