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Transcriptional regulation of elsinochrome phytotoxin biosynthesis by an EfSTE12 activator in the citrus scab pathogen *Elsinoë fawcettii*

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ABSTRACT

Elsinochrome (ESC), produced by the citrus pathogen *Elsinoë fawcettii*, is a nonhost-selective, light-dependent, polyketide-derived phytotoxin and plays a crucial role for full virulence. The biosynthesis of ESC is regulated by a wide array of environmental stimuli and is primarily governed by the pathway-specific TSF1 transcription regulator whose coding gene is clustered with the *EfPKS1* gene encoding a polyketide synthase and other biosynthetic genes in the genome. In this report, an *EfSTE12* gene, encoding a polypeptide resembling the yeast STE12 transcription factor, was cloned and characterized to play a role, independent of TSF1, for ESC production in *E. fawcettii*. The loss-of-function mutant, specifically disrupted at the *EfSTE12* locus, displays reduced ESC accumulation, elevated activities for pectinase and proteolytic enzymes but unaltered in conidiation and fungal pathogenicity. Impairment of the *EfSTE12* gene decreased the abundance of the *EfPKS1* but not the *TSF1* gene transcript. In contrast, expression of the *EfSTE12* gene appears normal in the *EfPKS1* or *TSF1* disruptants. The results indicate that EfSTE12 is functioning for ESC biosynthesis by directly activating the biosynthetic genes without regulating the pathway-specific TSF1 regulator. The defective phenotypes were fully reverted when a functional copy of *EfSTE12* was re-introduced into the disrupted mutant. A hypothetical model underlying intertwined regulatory pathways *via* TSF1, EfSTE12, and other potent transcriptional activators led to the ESC biosynthesis and conidiation is described.

Introduction

Elsinochrome (ESC) produced by many *Elsinoë* fungal species is structurally and functionally related to fungal toxins such as cercosporin produced by a number of *Cercospora* spp., phleichrome produced by *Cladosporium* spp., altertoxin I produced by *Alternaria* spp., hypericin produced by *Hypericum* spp., and hypocrellin A produced by *Hypocrella bambusae* (Daub *et al.* 2005). Structurally, all of these compounds contain a core perylenequinone chromophore. Functionally, they are photosensitizers owing to their abilities to absorb light energy, react with oxygen, and produce toxic reactive oxygen species

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(ROS) (Yamazaki *et al.* 1975; Daub 1982). *Elsinoë fawcettii* causes scab disease in citrus worldwide, affecting fruit, leaves, and twigs of many citrus cultivars, resulting in brownish necrotic lesions with a scabby or corky appearance.

ESC contains several tautomers differing only in side chains. The chemical structures and physical properties of ESC have long been studied in detail (Weiss *et al.* 1965; Lousberg *et al.* 1969, 1970; Meille *et al.* 1989; Mebius *et al.* 1990), yet its biological function acting as a phytotoxin was elucidated only recently (Liao & Chung 2008a). The cellular toxicity of ESC was showed to be light-dependent and apparently mediated by the production of ROS such as superoxide and singlet oxygen (Liao & Chung 2008a). Functional inactivation of the *E. fawcettii* polyketide synthase-coding gene *EfPKS1* created fungal mutants that were completely defective in ESC production and conidial formation and exhibited lower virulence to citrus compared with the wild-type progenitor (Liao & Chung 2008b). The studies concluded that ESC plays a crucial role in fungal pathogenesis even though there was no direct correlation between the levels of ESC production in culture and pathogenicity among the isolates (Wang *et al.* 2009a). Biosynthesis of ESC by *E. fawcettii* is regulated by a complex, interconnected network and was influenced by a wide range of environmental and nutritional conditions (Wang *et al.* 2009b). Light is not only required for the toxicity of ESC but also critical for initiation of ESC biosynthesis. Early studies by feeding *Elsinoë* spp. with the radio isotope-labeled substrate indicated that ESC was synthesized using a fungal polyketide pathway by condensation of acetate and malonate monomers (Chen *et al.* 1966; Kurobane *et al.* 1981). In fungi, the genes involved in the biosynthesis and regulation of secondary metabolites are often clustered in the genome (Hoffmeister & Keller 2007). It appears to be true for the biosynthesis of ESC by *E. fawcettii* as well. Apart from the *EfPKS1* gene, the *TSF1* gene encoding a polypeptide containing dual Cys_2His_2-type zinc finger and GAL4-like Zn_2Cys_6 binuclear cluster DNA-binding signatures has also been demonstrated to be required for ESC biosynthesis (Chung & Liao 2008). Sequence analysis of the regions flanking *EfPKS1* and *TSF1* found additional putative genes encoding polypeptides resembling reductase, oxidoreductase, prefoldin, and membrane transporter that are likely involved in the biosynthesis, regulation, and accumulation of ESC.

The STE12 transcription activator is conserved in yeasts and fungi and is involved in diverse biological functions. In yeasts, STE12 was reported to regulate numerous genes related to cellular processes, including cell cycle, mating, cell fusion, filamentous growth, polarized growth, budding, stress, starvation, and signal transduction (Fraser *et al.* 2004; Ren *et al.* 2005). In *Saccharomyces cerevisiae*, transcriptional activation of STE12 is often triggered by a pheromone response mitogen-activated protein kinase (MAPK) pathway and often forms a dimer complex with another transcription factors or partners to positively or negatively regulate the downstream genes associated with a wide array of developmental and physiological functions (Madhani *et al.* 1999; Schwartz & Madhani 2004). STE12 homologs have also been shown to be important virulence determinant in the animal pathogens *Candida albicans*, *Candida glabrata*, *Cryptococcus neoformans*, and *Cryptococcus gattii* (Lo *et al.* 1997; Chang *et al.* 2000, 2001; Ren *et al.* 2006; Hoi *et al.* 2007) as well as in the plant pathogenic fungi including *Colletotrichum* spp. and *Alternaria brassicicola* (Park *et al.* 2002; Cho *et al.* 2009). In filamentous fungi, STE12 homologs were reported to be involved in appressorium-mediated penetration, infectious growth, sexual reproduction, regulation of cell surface protein biosynthesis, maintenance of cell-wall integrity, and pathogenicity (Vallim *et al.* 2000; Park *et al.* 2002, 2004; Tsuji *et al.* 2003; Hoi *et al.* 2007; Morita *et al.* 2007). The objectives of this present study are to determine if STE12 plays any roles in pathogenicity, ESC production, and other biological functions in the citrus fungal pathogen *E. fawcettii*.

Materials and methods

Biological materials

The wild-type CAL WH-1 strain of *Elsinoë fawcettii* Bitanc. and Jenkins (anamorph: *Sphaceloma fawcettii* Jenkins), used for cloning, transformation, and target gene disruption, was cultured from scab-affected calamondin (*Citrus madurensis* Lour) fruit in Florida and has been previously characterized (Liao & Chung 2008a,b; Chung & Liao 2008). The genetically modified *EfPKS1* and *TSF1* disruptants were reported in a previous study (Liao & Chung 2008b; Chung & Liao 2008). Media for growing *E. fawcettii* were either potato dextrose agar (PDA, Difco, Sparks, MD), minimal medium (MM), or protoplast regeneration medium (RMM) (Chung *et al.* 2002). The pH of media was adjusted with 0.1 M phosphate buffer as described elsewhere (You *et al.* 2007). For elsinochrome toxin biosynthesis, fungal mycelium was ground, spread on PDA, and incubated under continuous fluorescent light of intensity at 40 $\mu E\ m^{-2}\ s^{-1}$ at 28 °C. Screening and identification of elsinochrome-deficient mutants was carried out on thin PDA as previously described (Choquer *et al.* 2005; Liao & Chung 2008a). Fungal pathogenicity was assessed on detached rough lemon (*Citrus jambhiri* Lush) leaves inoculated with conidial suspension as previously described (Liao & Chung 2008b).

Cloning of the EfSTE12 *gene*

A 0.8-kb DNA fragment of the *EfSTE12* gene was obtained by PCR amplification with two degenerate primers ste12F (5′-aa gaaYtcHaaRaaRttYgaggaRgg-3′) and ste12R (5′-tcYtcRttggcRatRtaWgcRggYt-3′) that are complementary to the conserved STE12 amino acid sequences KNSKKFEE and PAYIANE of various yeasts and fungi. A chromosome library of *Elsinoë fawcettii* was constructed using the Universal GenomeWalker kit per the manufacturer's instructions (BD Biosciences, San Jose, CA). To walk upward and downward into unknown genomic regions, primers were synthesized to complement the known regions and used for two rounds of PCR amplification with adaptor primers supplied with the kit. DNA fragments amplified from the library by PCR were cloned into pGEM-T easy vector (Promega, Madison, WI) for sequencing analysis at Eton Bioscience (San Diego, CA). Open reading frame (ORF) and exon/intron positions were determined by alignments of genomic and cDNA sequences using the CLUSTAL W algorithm. Functional domains were identified through the PROSITE database using the ExPASy service. The *EfSTE12* sequence was deposited in the GenBank/EMBL/DDBJ database under accession no. GQ292875.