ABSTRACT

BESELI, AYDIN. Identification and Characterization of Cercosporin Resistance Genes in *Cercospora* Species. (Under the direction of Dr. Margaret E. Daub).

Cercospora species produce the photoactivated, active-oxygen-producing toxin cercosporin. Because cercosporin has broad-spectrum toxicity, we are interested in understanding toxin resistance mechanisms in *Cercospora* species. A suppressive subtractive hybridization library was recovered of genes differentially regulated between the cercosporin-resistant wild-type and a toxin-sensitive mutant deficient for a transcription factor required for resistance. We hypothesize that some of the 185 genes recovered are involved in cercosporin resistance. Q-PCR analysis of a subset of 66 genes (selected based on their putative function) was tested under conditions of cercosporin toxicity in a cercosporin-sensitive *C. nicotianae* transporter mutant exposed to cercosporin. Of the 66 genes tested, six were found to be significantly upregulated. These included genes for two transporters, two hypothetical proteins, hydroxynicotine oxidase, and cyanide hydratase. Transformation of these genes into the cercosporin-sensitive fungus, *Neurospora crassa* confirmed increase in cercosporin resistance for three of the genes. By contrast, gene disruption mutants for these genes were not altered in cercosporin resistance as compared to wild type. However, expression analysis of the disruptants revealed that other genes were complementing the effect of disrupted genes. Other than the genes selected from induction with cercosporin toxicity, library genes that are the homologs of *Rhodobacter sphaeroides* singlet oxygen resistance genes were also characterized. However, no function in cercosporin resistance was documented.

Identification and Characterization of Cercosporin Resistance Genes in *Cercospora* Species

by
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DEDICATION

To my family, who have always supported and encouraged me all my life.

BIOGRAPHY

Aydin Beseli was born in Istanbul, Turkey to his parents Ali Halit and Meral Beseli. Aydin graduated from Robert College in 2003 and came to the U.S. with his brother, Sinan, to attend the University of Wisconsin – Madison. Aydin earned his Bachelor's of Science degrees in Genetics and Biochemistry from Wisconsin. Upon graduation in 2007, Aydin moved to Raleigh, North Carolina to attend North Carolina State University for his Ph.D studies. Aydin spent six valuable years in the lab of Dr. Margaret Daub gaining research and teaching experience.

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CHAPTER 1: Literature Review

Cercospora Diseases

Cercospora fungi represent highly successful and destructive plant pathogens that cause disease on a wide range of host plants world-wide. The genus Cercospora belongs to the phylum Ascomycota, class Dothideomycetes, order Capnodiales, and family Mycosphaerellaceae. Fungi in this genus do not have a known sexual phase (Wang et al., 1998). Individual species of *Cercospora* are usually host specific, but collectively they infect remarkably diverse hosts including bothmonocot and dicot angiosperms, gymnosperms, and even some early diverging plants such as ferns. A taxonomic analysis of the genus identified 659 species with 548 species identified in the United States (Aptroot, 2006). *Cercospora* fungi cause disease on many of the major agricultural crops including sugar beet, corn, soybean, coffee, and peanut, as well as vegetable and ornamental crops (Farr et al., 1989).

Infection Process

The infection process of *Cercospora* fungi follows a common cycle with differences depending on host, fungal species, plant age, genotype, leaf age, temperature, light, and humidity. Cercospora infection begins in spring or early summer when conidia from propagules of the fungus that survived the winter in plant debris are dispersed onto the leaves of young plants. Cercospora conidia vary from hyaline to pale to medium green to brown.

They are long, straight or curved and have septa. *Cercospora* fungi do not form fruiting bodies. However conidia emerge from stroma on short dark conidiophores as a group (Horst, 2013). New conidia constantly grow on new tips and detach easily and travel long distances by wind.

Cercospora fungi are considered hemibiotrophs. With the right conditions, conidia attach to the leaves of the host plants and germinate. In the first stages of infection, they behave like biotrophs. Most *Cercospora* species, such as *C. zeae-maydis*, infect leaves through stomata. According to the research by Hun Kim et al., *C. zeae-maydis* has a blue-light photoreceptor called CRP1 that is required for stomatal tropism and infection in *C. zeae-maydis* (Kim et al., 2011). When the germ tube of *C. zeae-maydis* reaches a stomate, it differentiates into multilobed infection structures similar to appressoria, from which the infectious hyphae penetrate mesophyll tissues.

Studies on *C. zeae-maydis* documented that spores of *C. zeae-maydis* germinated within 24 hours after inoculation at 22-30 ^{0}C, in 12 hours light/dark cycle, when plants were exposed to 12 hours of mist (Beckman and Payne, 1982). With the same conditions, fungi formed abundant appressoria over stomata 4-5 days after inoculation (dai) and penetration through stomata occurred 6-7 dai into the mesophyll tissues of maize (Beckman and Payne, 1982).

After a period of colonization in the intercellular spaces between the cells within the leaf, *Cercospora* fungi are converted into a necrotrophic growth habit and cause expanding, necrotic lesions with the help of a secreted toxin called cercosporin. These lesions often

expand to blight the leaf tissue, and decrease the photosynthetic capacity and subsequent yield is also decreased significantly.

In mature maize plants, chlorotic spots formed at 9 days, elongated discolored streaks at 12 days and necrotic and sporulating lesions at 16-21 days. Young corn plants are also susceptible and develop sporulating lesions 3-4 days earlier than mature plants (Beckman and Payne, 1982). During colonization in leaf tissue, the fungi produce stroma that give rise to conidiophores producing conidia. These conidia can serve as secondary inoculum to infect neighboring plants after dispersal by wind or rain splash (Ward et al., 1999). Towards the end of the summer, infected leaves fall to the ground where conidia overwinter to reinitiate the disease cycle the following year.

Cercosporin

Cercosporin is produced mainly by species of *Cercospora*. Studies also reported that some fungi in the genus *Mycosphaerella*, the close relative of *Cercospora*, also produce cercosporin (Moreno, 2011). Cercosporin was first isolated in 1957 from the soybean pathogen *C. kikuchii* (Kuyama, 1957), but it was not until 1971 that cercosporin's structure, stereochemistry and function were described (Lousberg, et al., 1971). Production of cercosporin depends heavily on the environmental conditions as well as the genus (some species, such as *C. arachidicola, C. zeina, and C. sojina*, which are not known to synthesize cercosporin) (Fore et al., 1988; Goodwin and Dunkle, 2010).

Role of Cercosporin in Disease

Studies have shown that cercosporin, secreted by many species of *Cercospora*, significantly enhances the amount and severity of disease. The first evidence is the presence of cercosporin in infected plant parts, indicating its production during infection. Studies have also shown membrane damage and electrolyte leakage in infected leaves of sugar beets by *C. beticola*, consistent with the activity of the toxin in infected plant parts (Daub and Ehrenshaft, 2000). In addition, cercosporin is activated by light, and symptom development on coffee and sugar beet was shown to be significantly decreased under shaded areas. The most compelling evidence was the study done with mutants lacking the ability to produce cercosporin. The *ctb1* mutants are disabled in their ability to produce cercosporin and produced fewer lesions and did not cause the necrotic blighting symptom characteristic of wild type infection (Choquer et al., 2005).

Cercosporin and Photosensitizers

Cercosporin is classified as a photosensitizer, because it requires light for its toxicity (to ‘‘sensitize’’ cells) (Daub, 1982b). The discovery of photosensitizers dates back to the twentieth century (Raab, 1900). Oscar Raab’s studies on common dyes such as acridines and xanthenes showed that these dyes are highly toxic to Paramecia only when exposed to visible light in the presence of oxygen, and because of that these dyes are called photo-sensitizers (compounds that “sensitized” the protozoa with visible light). Now it is known that many organic molecules with diverse structure such as commonly used dyes, acridine orange,

methylene blue, and rose bengal or natural products such as flavins and porphyrins, or plant parts and products such as chlorophyll, coumarins, thiophenes, and acetylenes, are activated by light and thus called photosensitizers (Heitz and Downum 1995).

Studies on mice and bacteria in the 1970s verified that cercosporin is a photosensitizer (Yamazaki et al., 1975). In 1979, studies documented that cercosporin kills plant cells more with increasing light intensity (Macri and Vianello, 1979). Additional information that protection against cercosporin was possible with antioxidants (Macri and Vianello, 1979) further proves the activity of cercosporin as a photosensitizer. Furthermore it was shown that the wavelengths of light needed to kill plant cells in the presence of cercosporin were the same as the absorption spectrum of cercosporin, which provides additional evidence that cercosporin, rather than a light-responsive plant pathway or event, is responsible for the cell death (Daub, 1982b).

Cercosporin and Perylenequinone Photosensitizers

Cercosporin has a specific five ring structure that puts it into a specific group called perylenequinones which are a group of reactive oxygen species (ROS) generating photosensitizers. There are several perylenequinones important in medical or agricultural uses as well as having important functions in nature (Daub et al., 2013). The perylenequinones used in medicine are typically used in photodynamic tumor therapy where they can target tumor cells for laser irradiation to destroy tumors. Hypocrellins, produced by *Hypocrella bambusae* (=*Pseudonectria bambusae*) are an example of perylenequinones that

are used in photodynamic tumor therapy as well as for their antiviral properties. They are also commonly used in China as a medicine for rheumatoid arthritis, gastric diseases, and skin diseases related to fungal infections (Diwu, 1995). The perylenequinone Calphostin C is a potent inhibitor of protein kinase C and has also been investigated for use in cancer therapy (Wu et al., 1989). Another medicinal perylenequinone is hypericin which is found in the medicinal herb St. John's Wort (*Hypericum perforatum*) that is used as an herbal remedy for depression (Sandmann and Boger, 1989).

In agricultural applications, elsinochromeA, produced by *Stagonospora convolvuli* used as biocontrol agent against two species of bindweed weed (Ahonsi et al., 2005). Some perylenequinones are also known to be used by protozoa in their light avoidance response (Terazima et al., 1999).

There are several perylenequinones that are identified in fungal species, such as shiraiachromes from *Shiraia bambusicola* (Wu, 1989) or stemphyltoxin produced by *Stemphylium botryosum* (Davis and Stack, 1991). *Elsinoë*, *Cladosporium* and *Alternaria* species also produce several perylenequinones, including alteichin, altertoxins I, II, and III, alterlosin I and II (Stack et al., 1986; Glaeser et al., 2011).

Despite the large variety of perylenequinones, the majority of the characterized perylenequinones are found in plant pathogenic fungi (Daub et al., 2013). All fungal perylenequinone producers are found in the phylum Ascomycota, and mostly in the class Dothidiomycetes with the exception of some of the lichens (Mathey and Lukins, 2001).

Most of the fungal perylenequinones have a core 4,9-dihydroxy-3,10-perylenequinone composition that is important in their activity (Guedes and Eriksson, 2007), and they only differ in their side chains giving them variability in their solubility in organic solvents. These compounds are bright red in color and they turn into yellow-green in color when they are reduced. They emit a red fluorescence and the reduced form gives off an intense green fluorescence (Weiss et al., 1987). Perylenequinones such as altertoxins from *Alternaria* spp. and alteichin, and stemphyltoxins from *Stemphylium botryosum* are yellow to orange colored, because they are partially reduced forms of the other perylenequinones. Despite their reduced structure, these compounds have similar photoactive, ROS-generating activity (Hartman et al., 1989; Ostry, 2008).

Mode of Action of Perylenequinones and Cercosporin

Cercosporin is a nonspecific toxin that is toxic to almost any organism. One of the main reasons for this is its mode of action. As all photosensitizers, cercosporin generates reactive oxygen species. In the presence of light, cercosporin is converted first to the electronically excited singlet state and then to a long-lived triplet state. Triplet-state photosensitizers may sometimes react directly with biomolecules such as lipids (Girotti, 1990). However, most toxicity is caused by reaction of excited triplet-state photosensitizers with molecular oxygen. This reaction can happen in two ways. In the presence of reducing substrates, excited triplet-state photosensitizers can be reduced and react with oxygen by electron transfer reactions. These reactions are called ‘‘type I’’ reactions and they result in

the production of free radical forms of ROS, such as superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), or hydroxyl radical ($OH^{\cdot}$). In the ‘‘type II’’ reaction, photosensitizers react directly with oxygen, without the reducing substrates, through an energy transfer reaction to form the non-radical, but highly reactive singlet oxygen ($^1O_2$).

Both type I and type II reactions (production of superoxide ($O_2^{\cdot -}$) and singlet oxygen ($^1O_2$) were observed in reactions of oxygen with fungal perylenequinones, including cercosporin, hypocrellin A and B, elsinochrome A and D, calphostin C, hypomycin B, erythroaphin, and hypericin (Guedes and Eriksson, 2007). However the type II reaction is observed more commonly with the fungal perylenequinones than the type I. For example, $^1O_2$ quantum yields ($^1O_2$ yield per quantum of light absorbed) of cercosporin, elsinochrome A, and hypocrellins A and B was measured at 0.81–0.97, 0.98, 0.82, and 0.76, respectively (Daub et al., 2000; Diwu and Lown, 1993; Dobrowolski and Foote, 1983; Zhang et al., 2009). Efficiency of binding of the photosensitizer to the substrate often changes the mode of toxicity (type I vs. type II) (Ito, 1981).

Photosensitizers and their byproducts are toxic to a variety of macromolecules. Although it is very reactive, $^1O_2$ has a short life span (3 μs) (Skovsen et al., 2005) and does not diffuse long distances in cells. Because of that, in cells, the damage from photosensitizers correlates with their localization. For example, hydrophobic photosensitizers, as they can penetrate through cell membranes easier, have been shown to be more toxic than hydrophilic photosensitizers that have similar $^1O_2$ quantum yields in vitro (Moan et al., 1998). Photosensitizers such as porphyrins, thiazine and xanthene dyes are

excellent membrane photosensitizers as these lipid-soluble photosensitizers localize in membranes (Emiliani and Delmelle 1983; Ito, 1983). $^1O_2$ generated by these photosensitizers commonly breaks down membranes by adding unsaturated double bonds to lipids. Photosensitizers that localize in the cytoplasm or nucleus can damage proteins and nucleic acids. For example, acridines can localize in nucleus and bind to nucleic acids and cause DNA damage (Boulanger et al., 2005). Of the four nuclotides, guanine is the most susceptible to ROS. In proteins, sulfur-containing or aromatic amino acids are most susceptible.

All perylenequinone toxins characterized to this day are lipid-soluble and can penetrate through cellular membranes and localize in cellular compartments, excluding nuclei, such as lysosomes, endoplasmic reticulum, and golgi (Daub and Briggs, 1983; Diwu and Lown, 1990; Miller, 1995). Although the common core of 4,9-dihydroxy-3,10-perylenequinone is essential for ROS generation, the differences in side chains affect the solubility and yield of ROS production (Hudson et al., 1997).

Several studies show that cercosporin is a membrane-bound toxin that kills cells by causing peroxidation of membrane lipids. Membrane damage was documented in leaf tissues treated with cercosporin or in Cercospora-infected leaves (Steinkamp et al., 1979; Steinkamp et al., 1981). Also rapid ion leakage was shown in cells treated with cercosporin (Daub, 1982b).

Biosynthesis of Cercosporin

Biosynthesis of cercosporin shows similarities with many other perylenequinones. Several environmental and physiological factors influence the amount of toxin production. One of the most important abiotic factors is light. In the absence of light, cercosporin production is suppressed completely, whereas introduction of light immediately triggers its biosynthesis. The production can be altered solely by alternating light and dark periods, and rings of cercosporin production can be observed on growing mycelium (Ehrenshaft and Upchurch, 1991).

Using ammonium as the sole nitrogen source inhibits cercosporin production, whereas calcium promotes its production in *C. nicotianae*; a change in pH has little effect on production of cercosporin (You et al., 2008).

For investigating the biosynthesis of cercosporin, fungal strains were fed 14C-labeled acetate, and the distribution of radioisotopes in the recovered cercosporin was analyzed using nuclear magnetic resonance and mass spectrometry (Okubo et al, 1975). The findings from this work showed that cercosporin is produced via a polyketide mode of synthesis. The biosynthesis starts by reduction and decarboxylation of malonyl-CoA into a two carbon unit which then reacts with acetyl-CoA. The chain of carbon elongates by addition of two carbon units from a malonylketo group with each iterative decarboxylation and condensation cycle until a linear nine carbon polyketide, pentaketide, is formed. The pentaketide structure undergoes a series of oxidation, hydration, and methylation reactions to yield a cyclized

polyhydroxynaphthalene. The final form is accomplished by dimerization of the two identical units.

Cercosporin Biosynthetic Gene Cluster

The genes involved in the biosynthesis and modification of many secondary metabolites in fungi are organized in clusters (Daub and Chung, 2009). Cercosporin biosynthesis genes were identified by generating cercosporin deficient mutants through restriction enzyme-mediated insertion (REMI) mutagenesis (Chung et al., 2003). Several fungal polyketide synthases (PKSs) were recovered through the screening of cercosporin-deficient mutants, and the first cercosporin biosynthetic gene (CTB1) encoding a fungal PKS was identified. To confirm the role of CTB1 in cercosporin biosynthesis, the CTB1 gene in *C. nicotianae* was disrupted, and *ctb1* mutants were shown to lack cercosporin production. Furthermore, the expression of this gene was shown to correlate with cercosporin-producing conditions such as the presence of light (Choquer et al., 2005). CTB1 has similar domains to those in PKS proteins involved in fatty acid synthesis except that CTB1 lacks β-ketoreductase (KR), dehydratase (DH) and enolyreductase (ER) domains (Choquer et al., 2005). CTB1 contains a ketosynthase (KS), an acyltransferase (AT), a thioesterase (TE), and two acyl carrier protein (ACP) domains (Choquer et al., 2005). With these domains, CTB1 synthesizes the polyketomethylene backbone of cercosporin. The consecutive steps to get the final product such as successive ring closure, oxidation, hydration, and methylation reactions are catalyzed by other gene products in the CTB1 cluster (Choquer et al., 2005).

To identify other genes in the cluster, a chromosome walking strategy was used. Eight genes were identified. These include two *O*-methyltransferases (CTB2 and the N terminus of CTB3), a monooxygenase (CTB3 C terminus), an MFS transporter (CTB4), three oxidoreductases (CTB5, 6 and 7) and a Zn(II)Cys6 transcriptional regulator (CTB8) (Choquer et al., 2005).

The Zn(II)Cys6 transcriptional activator, CTB8, regulates the activity of the CTB genes, and it is induced under cercosporin-producing conditions such as the exposure of light. The disruption of *CTB8* gene blocks the expression of all of the core cercosporin cluster genes (Chen et al., 2007).

As previously mentioned, cercosporin biosynthesis was assumed to start with reduction and decarboxylation of malonylketo group of malonyl-CoA into a two carbon unit, and condensation of this group with acetyl-CoA (starter group) (Okubo et al., 1975) by the function of the polyketide synthase encoded by *CTB1*. Several domains in CTB1 work on this structure to form the polyketomethylene backbone of cercosporin. First the malonyl-CoA subunit is predicted to attach to the ACP domains of CTB1 by formation of phosphopantotheine (PPT) (Rawlings et al., 1989; Watanabe and Ebizuka, 2004). The AT domain of CTB1 aids in transferring PTT to the acetate unit from acetyl-CoA. The KS domain of CTB1 then condenses the carbons from the malonyl group and acetyl-CoAs by decarboxylation. Lastly, the condensed malonyl keto group is reduced. The condensation and incorporation of two carbons from malonyl-CoA subunit repeats until formation of the nine carbon polyketide.

The next step after forming the polyketide is cyclization to get the aromatic ring of cercosporin. This step would likely be catalyzed by the function of the TE domain in CTB1. The FAD/FMN-dependent monooxygenases/oxidoreductases, CTB3 and CTB5, are proposed to have role in polyketide oxidations during ring closure. Oxidoreductases/hydrogenases, CTB6 and CTB7, are proposed to have role in hydration, and CTB2 and/or CTB3 was proposed to have role in methylation at the C2 and C11 of cercosporin (Chen et al., 2007).

The MFS transporter, CTB4, is likely responsible for cercosporin export (Chen et al., 2007). Two other transporter genes, CFP and ATR1, have also been shown to play a role in transportation of cercosporin. However these transporters are not in the cluster (Amnuaykanjanasin and Daub, 2009; Callahan et al., 1999).

Resistance Mechanisms to Perylenequinones and $^1O_2$

Cercosporin is a perylenequinone photosensitizer generating ROS. As with other perylenequinone photosensitizers, resistance against cercosporin is not fully understood. Studies on cellular resistance mechanisms against perylenequinone photosensitizers such as cercosporin are of considerable interest. Knowledge in this area is not only important for engineering resistant crop plants with cercosporin resistance genes and for better management of Cercospora diseases, but also for applying this knowledge to resistance against other perylenequinones.

Due to their mode of action, perylenequinones have almost universal toxicity. Studies showed that they are toxic to plants, mice, bacteria, oomycetes, and viruses as well as to most fungi (Balis and Payne 1971; Daub 1987; Fajola 1978; Hudson et al., 1997; Yamazaki et al., 1975). The main cause of toxicity is the production of $^1O_2$. Resistance mechanisms against free radical forms of ROS, such as superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), or hydroxyl radical ($OH^{\cdot}$) are well characterized as they are common products of cellular metabolism. $^1O_2$, however, is only generated in photosynthetic organisms, as a byproduct of photosynthesis.

$^1O_2$ Quenchers and Antioxidant Enzymes

Several $^1O_2$ quenchers have been characterized (Bellus, 1979). Carotenoids are one of the most effective quenchers of cellular $^1O_2$. Carotenoid pigments are mainly found in the photosynthetic cells as $^1O_2$ is produced constantly in these cells as a byproduct of photosynthesis (Young, 1991). Carotenoids are not only efficient in quenching $^1O_2$, but also quench the activated triplet state of perylenequinones. The importance of carotenoids in cercosporin protection was documented in a study that showed that the only variety of rice, ‘‘Louisiana red rice,’’ that was resistant to *C. oryzae*, was the one with the most carotenoid production (Batchvarova et al., 1992). Out of four cultivars, Louisiana red rice was resistant, ‘Lemont’ and ‘Leah’ had lower levels of resistance and ‘Labelle’ was completely susceptible to the toxin. By testing the amount of carotenoid and susceptibility of cell suspensions and

calli from each of the rice cultivars, the level of carotenoids in each cultivar was correlated with host plant resistance

To better determine to role that carotenoids play in cercosporin resistance, organisms with different levels of carotenoid production were tested to see if carotenoids have any direct effect on cercosporin resistance. *Cercospora* fungi produce high levels of β-carotene, especially in the early stages of their life cycle (12μg/g dry weight) (Weete, 1980). *Neurospora crassa* also produce similar amounts of carotenoids, however this fungus is not resistant to cercosporin. Two oomycetes (Zygomycota, Mucoromycotina), *Phycomyces blakesleeanus* and *Phycomyces parasitica* produce comparatively fewer carotenoids, and are not resistant to cercosporin (Daub, 1982a). When the cercosporin sensitivity of carotenoid-deficient mutant strains of different fungi was compared, carotenoid-deficient mutants created through targeted gene disruption of the gene encoding phytoene dehydrogenase were significantly more sensitive to cercosporin than were carotenoid-producing wild type isolates. However, there was not a linear relationship between the cercosporin sensitivity and the amount of carotenoids produced (Daub and Payne, 1989).

In a further attempt to determine the effect of carotenoids in cercosporin resistance, three carotenoid inhibitors, norflurazon, mevinolin and B-ionone, were used to inhibit the production of carotenoids in *C. nicotianae*. Carotenoid synthesis in *C. nicotianae* was not affected by any of the inhibitors, whereas synthesis in *N. crassa* was inhibited, suggesting that *C. nicotianae* has mechanisms to protect the production of carotenoids as it is the crucial part of its life cycle (Daub and Payne, 1989). As the inhibitors did not inhibit carotenoid

production, targeted gene disruption of the gene encoding phytoene dehydrogenase was generated in *C. nicotianae*. These mutants were deficient in β-carotene production, however they were not more sensitive to cercosporin (Ehrenshaft et al., 1995).

Localization is an important factor in resistance as research shows that carotenoids protect *N. crassa* conidia from methylene blue and toludine blue, photosensitizers that target plasma membranes, but not against acridine orange which targets the nucleus (where carotenoids are not localized) (Ito, 1978). It is also reported that in plants, localization of carotenoids is in chloroplasts and in other plastids, but not in cell walls or cytoplasm. Localization of carotenoids might be the reason for their inability to protect plant cells from toxic effect of cercosporin and other photosensitizers (Briton, 1982).

To be able to detect the localization of carotenoids, carotenoid content was measured in the protoplasts of *C. nicotianae* and *N. crassa*. Similar amounts of carotenoids were found in both species, suggesting that carotenoids in these species are localized in the protoplast, but not in other parts of the cells such as cell walls (Daub and Payne, 1989). Furthermore the sensitivity of the protoplasts of both species was similar, but as the protoplasts regenerated walls, *C. nicotianae* regains its high levels of resistance, while *N. crassa* remained sensitive. Thus it is concluded that, based on the localization of carotenoids in *C. nicotianae*, carotenoids are not the cell wall resistance factor. To further support this hypothesis, *N. crassa* isolates were transformed with carotenoid genes from *C. nicotianae* to over-express the genes, and no increase in resistance to cercosporin was detected (Daub and Payne, 1989).

Other than carotenoids, some phenols, amines, and peptides as well as the amino acids cysteine, histidine, methionine, and tryptophan have also been documented as $^1O_2$ quenchers (Wilkinson et al., 1995). In proteins, sulfur-containing or aromatic amino acids are most susceptible to singlet oxygenIn addition, antioxidant enzymes such as superoxide dismutase, catalase, and peroxidases can protect against free radical oxygen species generated by photosensitizers. However, data to show that these antioxidant enzymes are involved in cercosporin resistance is not concordant. For example, tobacco cells with elevated levels of catalase and superoxide dismutase activity were not more resistant to cercosporin (Hughes et al., 1984). However sugarbeet plants transformed for increased expression of superoxide dismutase had increased tolerance to cercosporin (Tertivanidis et al., 2004). Furthermore, transcription of superoxide dismutase and catalase was shown to increase with the addition of cercosporin, although no increase in resistance was correlated with increased gene expression (Williamson and Scandalios 1992).

In an effort to characterize and identify additional mechanisms of resistance, five mutants sensitive to and one mutant resistant to cercosporin (control) were used to examine changes in sensitivity to cercosporin toxicity when reducing agents were exogenously added. In the absence of cercosporin, no light sensitivity was observed in the mutants. However when cercosporin was added at concentrations as low as 0.1 μM, growth of the five mutants was suppressed, and increasing light intensity from 3 to 40 μE m^{-2} s^{-1} increased cercosporin sensitivity significantly ($p=0.05$). However when ascorbate, cysteine, or reduced glutathione were added along with cercosporin, these five mutants were less sensitive. The analysis

showed that, in all of the six mutants, endogenous levels of β-carotene, ascorbate, cysteine, reduced glutathione, or protein thiols were constant with or without cercosporin toxicity. These results led to the conclusion that these reducing agents are important singlet oxygen quenchers, but are not specifically used by *Cercospora* fungi to protect against cercosporin (Jenns and Daub, 1995).

The effect of the Cpd1 protein, a FAD pyridine nucleotide reductase similar to GSH reductase and thioredoxin reductase, on cercosporin toxicity was tested to find if this protein from *Saccharomyces cerevisiae* is involved in cercosporin resistance. When Cpd1 was over-expressed in yeast and in tobacco, resistance was significantly increased (Panagiotis et al., 2007; Ververidis et al., 2001).

Vitamin B6 is a very effective $^1O_2$ quencher. *C. nicotianae*, which is resistant to cercosporin, has 2-3 fold more B6 vitamers content than the cercosporin-sensitive fungi *N. crassa* and *Aspergillus flavus* (Herrero and Daub, 2007). Furthermore, *C. nicotianae* mutants lacking a functional vitamin B6 biosynthetic gene lose their resistance to cercosporin (Ehrenshaft et al., 1999). However, as vitamin B6 is an essential growth regulator, further characterization of it role in cercosporin protection is challenging as B6-deficient mutants are lethal.

Transporters

Transporter proteins export and import a variety of molecules in and out of cells (Higgins, 1992; Locher et al., 2002; Dahl et al., 2004). In fungal systems, there are two major families of transporters, which are the ATP binding cassette (ABC) and major facilitator superfamily (MFS) transporters (Higgins, 1992; Pao et al., 1998). Both of these transporter families have a common structure consisting of a set of transmembrane (TM) domains arranged in two homologous halves (Jones and George, 2004; Pao et al., 1998; Paulsen et al., 1996). The domain of ABC transporter that is characteristic of them is the ATP-binding cassette, where energy for substrate translocation comes from hydrolysis of ATP. Both small and big macromolecules can be translocated by ABC transporters. MFS transporters do not have ABC domains. Instead of using energy from hydrolysis of ATP, translocation is made possible by a chemiosmotic ion gradient. Only small molecules, however, can be translocated through these transporters (Pao et al., 1998).

Membrane transporters have an important role in resistance against cercosporin toxicity (Callahan et al., 1999; Hayashi et al., 2002; Ververidis et al., 2001). The proposed mechanism of resistance is through the excretion of the toxin out of the cells by using these transporters. Both MFS and ABC transporters have been reported to have activity against cercosporin. The first transporter identified in *Cercospora* fungi was *C*ercosporin *F*acilitator *P*rotein (CFP). It is an MFS transporter originally identified in the soybean pathogen *C. kikuchii* by examining differences in transcript levels in the presence and absence of light (Ehrenshaft and Upchurch, 1991). When CFP was disrupted in *C. kikuchii*, CFP disruptants

had less resistance to cercosporin, were less virulent on soybean and also produced less cercosporin (Callahan et al., 1999). CFP was also transformed into a cercosporin-sensitive fungus, *Cochliobolus heterostrophus*, and the transformants gained resistance to cercosporin (Upchurch et al., 2002). Furthermore, tobacco plants transformed with *CFP* had reduced frog-eye lesion sizes when inoculated with *C. nicotianae* (Upchurch et al., 2005).

ATR1 is an ABC transporter that was characterized as having a role in cercosporin resistance (Amnuaykanjanasin and Daub, 2009). Similar with *CFP*, *ATR1* was disrupted in *C. nicotianae* and the disruptants lost their resistance to cercosporin and their cercosporin production decreased significantly.

The expression of another MFS transporter from *C. nicotianae*, CTB4, which is in the cercosporin biosynthetic cluster, was inhibited by disruption of the CTB8 transcriptional regulator required for expression of all CTB genes. Surprisingly, resistance to cercosporin was not altered in these mutants (Chen et al., 2007), suggesting that CTB4 does not have a role in resistance.

Several other transporters from different organisms have also been characterized to provide resistance. One of the membrane transporters characterized to have a role in cercosporin resistance is the yeast protein Snq2p. *Snq2p* encodes an ABC transporter protein, which confers resistance to cercosporin when overexpressed (Ververidis et al., 2001). Another transporter that confers resistance is Bcmfs1 from *Botrytis cinerea*. *Bcmfs1* encodes an MFS transporter that when disrupted in *B. cinerea* increases sensitivity of the fungus to cercosporin (Hayashi et al., 2002).

Photosensitizer Degradation Mechanisms

Another method of resistance against photosensitizers is their enzymatic degradation. Some insects such as swallowtail caterpillars that feed on furanocoumarin containing plants initiate a cytochrome P450-mediated degradation of the photosensitizing furanocoumarins (Li et al., 2003). During a search for biocontrol agents against the sugar beet pathogen *C. beticola*, several fungi in the phylum Basidiomycota were found. Several of these fungi that were effective against *C. beticola* produced laccase, a phenol oxidoreductase (Caesar-TonThat, 2009). Effectiveness of laccase in cercosporin degradation was also shown in *in vitro* analysis. Laccase from *Pleurotus ostreatus* catalyzed cercosporin degradation *in vitro* and protected *Escherichia coli* and sugar beet leaves from cercosporin toxicity.

In another study, 244 bacterial strains from 12 genera and 23 species were screened for cercosporin-degrading activities (Mitchell et al., 2002). Cercosporin degradation was seen mostly in isolates of *Xanthomonas*, *Pseudomonas*, and *Ralstonia* (all plant associated species). All 32 isolates of *Xanthomonas campestris pv zinnia* (a pathogen of Zinnia) in the study could degrade about 90% of cercosporin by modifying cercosporin into xanosporic acid (a non-toxic form) (Mitchell et al., 2002). An oxidoreductase was identified to be responsible for this degradation through complementation of non-degrading mutants of *X. campestris*. Furthermore, Southern blot analysis showed a correlation between cercosporin-degrading activity and presence of the gene (Taylor et al., 2006). However transformation of this gene into non-degrading bacteria and plants in an effort to confer resistance through cercosporin degradation did not confer resistance. A plausible explanation for this

observation might be that other enzymes or other factors (localization, other substrates or cofactors) may be required in addition to this gene product for the complete cercosporin-degrading activity.

Reductive Detoxification

Several studies have shown that reduced cercosporin is a poor generator of $^1O_2$. Cercosporin that was reduced with strong reducing agents such as zinc or dithionite produced as little as 2% of normal levels of $^1O_2$ (Daub et al., 2000; Leisman and Daub, 1992). This finding led to the hypothesis that one of the most significant resistance mechanisms of *Cercospora* species is that they keep cercosporin in its reduced, non-toxic form inside the hyphae. The reduced form of cercosporin inside the hyphae in resistant fungi (*Cercospora* and *Alternaria*) was confirmed by fluorescence and confocal microscopy by examining differences in fluorescence emission (Daub et al., 2000). Throughout the studies, it was also shown that the reduced cercosporin fluoresces much stronger than non-reduced form (with fluorescence intensities of 80–160 times greater). This finding supports the hypothesis that light energy absorbed by reduced cercosporin is released via fluorescence rather than transfer to O_2 for ROS production.

Resistant (*Cercospora* species and *Alternaria alternata*) and sensitive fungi (*Aspergillus flavus*, *N. crassa*, and two *Penicillium* species, *Penicillium camembertii* and *Penicillium caseicolum*) were also used to test if resistance is correlating with the reducing power of the membranes. Several tetrazolium dyes differing in a wide range of redox

potentials were added to growth. It was documented that cercosporin-resistant fungi can reduce more dyes than sensitive fungi, particularly those with more negative redox potentials (Sollod et al., 1992).

The role of cercosporin reduction in resistance was also confirmed through studies of a FAD-dependent pyridine nucleotide reductase identified in yeast. In support of the hypothesis that reduction power is linked to cercosporin resistance, this gene was overexpressed in yeast. Overexpression provided increased resistance to cercosporin and other photosensitizers (Ververidis et al., 2001).

Cercosporin Autoresistance Genes from *Cercospora* Fungi

Cercosporin is considered as a virulence factor playing an important role in symptom development and disease severity. Due in part to its mode of action, there are not many organisms that have resistance to cercosporin. However, *Cercospora* species produce and secrete millimolar concentrations of cercosporin without any observable sensitivity. These fungi are also resistant to other photoactivated compounds that generate singlet oxygen (Daub and Ehrenshaft, 2000). Because of this, *Cercospora* spp. became a model for identifying resistance genes.

One of the first attempts to identify genes in *Cercospora* involved in cercosporin resistance was to generate cercosporin-sensitive mutants of *C. nicotianae* and isolate the genes via mutant complementation using a library from a wild type *C. nicotianae* (Jenns and Daub 1995; Jenns et al., 1995). With these studies, three genes were identified to be required

for resistance. Two of these genes encode enzymes involved in the pyridoxine (vitamin B6) pathway and the third gene encoded a transcription factor.

A. Pyridoxine Biosynthetic Genes

Complementation of five distinct cercosporin-sensitive mutants with sequences from wild type *C. nicotianae*, identified two genes, *PDX1* and *PDX2* that encode enzymes in a previously unknown pathway for pyridoxine (vitamin B6) biosynthesis (Ehrenshaft and Daub 2001; Ehrenshaft et al., 1999). Although there are vitamins such as vitamins C and E, with known antioxidant defenses, until this finding, vitamin B6 was not known to have antioxidant activity, but only to be involved in transamination reactions as a co-factor. After this discovery, however, pyridoxine and its vitamers were assayed and found to quench $O_2^{\cdot -}$ and $^1O_2$ and also have general antioxidant activity (Bilski et al., 2000; Denslow et al., 2005).

Further analysis on vitamin B6 showed that *Cercospora nicotianae* have 2 to 3 fold higher amounts of vitamin B6 than do cercosporin-sensitive fungi, *Aspergillus flavus* and *N. crassa* suggesting that elevated B6 levels may be an important defense mechanism (Herrero and Daub 2007). Studies in other systems also showed a link to the amount of B6 vitamers in a cell and antioxidant defense. For example, this vitamin is important in protecting eyes from $O_2^{\cdot -}$ mediated eye damage in diabetics and to prevent oxidation of lipids and proteins in blood caused by H_2O_2 and high glucose (Jain et al., 2002).

PDX1 and *PDX2* genes were used to transform tobacco to test if overexpression of B6 vitamers would increase resistance to cercosporin and *Cercospora* diseases (Herrero and Daub 2007). Unfortunately, none of the transgenic lines with *C. nicotianae PDX1* and *PDX2*

genes showed elevated resistance to cercosporin or increased production of vitamin B6. A major reason for this might be that as vitamin B6 is involved in many important activities in cells, and thus this pathway might be tightly regulated at the transcriptional level. In support of this hypothesis, gene expression analysis showed that the endogenous B6 biosynthetic genes were down-regulated in the transgenic plants (Herrero and Daub 2007). Thus, although increased levels of B6 may protect plants against cercosporin toxicity, further research is needed to be able to elevate levels of this vitamin in plants.

B. CRG1

The third gene recovered from the mutant complementation study encodes for a binuclear zinc cluster transcription factor that was called *CRG1* (*c*ercosporin *r*esistance *g*ene 1) (Chung et al., 2003). *crg1* mutants show significantly reduced resistance to cercosporin, but are able to confer resistance to other $^1O_2$ generating photosensitizers. These mutants also had reduced cercosporin production.

In fungi, zinc binuclear transcription factors regulate a variety of processes such as mycotoxin synthesis and multiple drug resistance (Mamnun et al., 2002; Payne and Brown, 1998). In the absence of CRG1, the fungus loses its resistance to cercosporin, thus it was hypothesized that CRG1 regulates genes involved in cercosporin resistance. To identify the genes regulated by CRG1, a suppressive subtractive hybridization library was constructed between the wild-type *C. nicotianae* and a *crg1* null mutant (Herrero et al., 2007). A total of 185 ESTs were recovered from two libraries, a forward library (down-regulated in *crg1*) and

reverse (down-regulated in wild type). The libraries recovered from this study were predicted to include genes involved in cercosporin resistance. The EST sequences in the libraries were blasted against nucleotide databases to identify putative homologs in other organisms. These EST sequences were then divided into different functional groups according to their homology. These functional groups included the resistance mechanisms previously identified, such as transporters, gene products with reducing power, antioxidants and quenchers.

Homologs of many of the previously characterized cercosporin resistance genes were also found in these libraries. One example is CFP, the MFS transporter involved in cercosporin resistance (Callahan et al., 1999; Choquer et al., 2007). Another transporter gene found in the library is ATR1, an ABC transporter. This transporter was also characterized later to have a role in resistance through disruption analysis in *C. nicotianae* (Amnuaykanjanasin and Daub, 2009).

Some of the genes in the cercosporin toxin biosynthetic cluster were also shown to be regulated by CRG1 and recovered in the subtractive libraries. These are CTB2, and CTB5. Finally, some EST sequences in the library were not aligned with any other sequences or aligned with sequences that are not characterized; these were annotated as hypothetical genes (Herrero et al., 2007).

Conclusion

Fungal diseases of crop plants are a significant threat to global food security. Improving host resistance is one of the most cost-effective and environmentally sound strategies for sustainable disease management. *Cercospora* species are highly successful and destructive fungal plant pathogens that cause disease on a wide range of host plants world-wide. The toxin, cercosporin, produced by most species of *Cercospora* is playing an important role in infection. Due mainly to the mode of action of this toxin, it is almost universally toxic, except to the fungi producing it. In the next three chapters, I present my research to characterize putative genes in the subtractive library to identify genes in *Cercospora* fungi that have a role in resistance against cercosporin. The ultimate goal of these studies is to use these candidate genes to transform host plants for cercosporin and resistance to *Cercospora*.

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CHAPTER 2: Membrane Transporters in Cercosporin Biosynthesis and Self-resistance in *Cercospora nicotianae*

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Abstract

Studies have documented the importance of membrane transporters in self-resistance of fungi in the genus *Cercospora* to the light activated, active-oxygen-producing toxin cercosporin they produce for infection of host plants. Transporters identified in a subtractive cDNA library between a *Cercospora nicotianae* wild type and a mutant, *crg1*, severely attenuated in cercosporin biosynthesis and self-resistance, were characterized, including two ABC transporters (CnATR2, CnATR3), an MFS transporter (CnMFS2), a uracil transporter, and a zinc transport protein. Phylogenetic analysis demonstrated that CnATR2 belongs to the ABC-C (multidrug resistance-associated protein [MRP]) subfamily, whereas CnATR3 is classified in the ABC-G (pleiotropic drug resistance-like [PDR]) subfamily. MFS2 groups with dityrosine transporters. Quantitative RT-PCR analysis of gene expression under conditions of cercosporin toxicity showed significant increased expression of *CnATR2*, suggesting a role in cercosporin resistance, whereas the other transporter genes were not up-regulated. Transformation and expression of *CnATR2* into the cercosporin-sensitive fungus *Neurospora crassa* provided increased resistance to cercosporin toxicity. Targeted gene disruption of *CnATR2* in the wild type *C. nicotianae*, however, did not decrease cercosporin resistance. Production of cercosporin as well as sporulation and pathogenicity were also unaffected in *C. nicotianae atr2* disruption mutants. Analysis of expression of other transporters in the *atr2* mutant under conditions of cercosporin toxicity showed significant induced expression of *c*ercosporin *f*acilitator *p*rotein (CFP), encoding an MFS transporter

previously implicated in cercosporin resistance. We conclude that CnATR2 can provide protection against cercosporin toxicity. However, up-regulation of other genes in *C. nicotianae* can compensate for a loss of resistance in *atr2* mutants.

1. Introduction

Toxins are commonly produced by many filamentous fungi for a variety of functions. One well studied group of toxins are the photoactivated perylenequinones, produced by several fungal plant pathogens including species of *Alternaria*, *Cercospora*, *Cladosporium*, and *Elsinoë* (Daub and Chung, 2009; Daub et al., 2013). Perylenequinone toxins produced by these pathogens are red in color and have a similar structure and mode of action. These toxins act by absorbing light energy, resulting in conversion to an energetically activated triplet state. This activated triplet molecule then reacts with oxygen and results in the generation of reactive oxygen species such as singlet oxygen ($^1O_2$) and superoxide ($O_2^{-\cdot}$), which damage host cells.

Of the perylenequinone toxins, cercosporin, produced by *Cercospora* species, has been the most characterized (Daub and Chung, 2009; Daub et al., 2013). The importance of cercosporin in pathogenesis has been demonstrated by studies with toxin-deficient mutants. Also, observation of increased pathogenicity in the presence of light required for toxin activation further documents the activity of this toxin. The production of reactive oxygen species by cercosporin results in broad-spectrum toxicity to plants, animals, and microorganisms, leading to investigations of toxin self-resistance in *Cercospora* as a source of genes to engineer resistance in host crops.

Studies to identify the resistance genes in *Cercospora* species began with the isolation and characterization of *C. nicotianae* mutants selected for sensitivity to cercosporin (Jenns and Daub, 1995; Jenns et al., 1995). Complementation of the cercosporin-sensitive

phenotype led to the identification of a gene encoding a zinc cluster transcription factor named CRG1 (*c*ercosporin *r*esistance *g*ene 1) (Chung et al., 2003). The *crg1*-null mutant (205C3) (Chung et al., 2003) is highly sensitive to cercosporin and also reduced in cercosporin production. To identify putative cercosporin-resistance genes regulated by CRG1, a subtractive cDNA library was generated between the *C. nicotianae* wild type and the *crg1* null mutant (Herrero et al., 2007). The resulting library contained 185 expressed sequence tags (ESTs) representing genes altered in their regulation between wild type and the *crg1* mutant. ESTs from the subtraction library were sequenced, classified into functional categories, and characterized as putative resistance genes based on known mechanisms of cercosporin resistance. These include reductive detoxification of cercosporin (Daub et al., 1992), antioxidants and quenchers of both superoxide and singlet oxygen (Ehrenshaft et al., 1999; Ehrenshaft and Daub, 2001), and efflux of cercosporin out of the cell by membrane transporters (Amnuaykanjanasin and Daub, 2009; Callahan et al., 1999; Choquer et al., 2007).

Two genes in the *Cercospora* cercosporin biosynthetic cluster were also found in the library. This cluster, called *C*ercosporin *T*oxin *B*iosynthesis (CTB), consists of eight genes encoding a polyketide synthase (CTB1), two methyltransferases (CTB2 and CTB3), three oxidoreductases (CTB5, CTB6 and CTB7), a membrane transporter (CTB4) and a transcription factor (CTB8) (Chen et. al, 2007). In an effort to characterize the possible role of CTB gene products in cercosporin resistance, a *C. nicotianae* CTB8 transcription factor null mutant, that has decreased expression of all of the core cercosporin cluster genes, was

tested for cercosporin resistance but no significant change in cercosporin resistance was documented (Chen et. al., 2007). Thus the possible role of CTB genes in cercosporin resistance is not clear. Eight genes encoding membrane transporters were identified in the subtractive library.

Membrane transport proteins efflux a wide array of molecules across membranes (Svein et al., 2004). In toxin-producing fungi, transporters are critical in the efflux of endogenously-produced toxins. In filamentous fungi, there are two main families of transporters: the ATP Binding Cassette (ABC) family and the Major Facilitator Superfamily (MFS) transporters (Higgins, 2007; Pao et al., 1998). Both of these transporter families have common transmembrane (TM) domains arranged in two homologous halves (Jones and George, 2002). ABC transporters also contain an ATP-binding cassette, which can transport both small molecules and macromolecules. Translocation is facilitated by energy from the ATP molecule which becomes bound to the ABC transporter during the efflux process (Higgins, 1992). MFS transporters use chemiosmotic ion gradients for the translocation of small molecules (Pao et al., 1998).

Previous studies with two of the subtractive library transporters showed that the ABC transporter ATR1 (*A*BC *Tr*ansporter 1) (Amnuaykanjanasin and Daub, 2009) and the MFS transporter CFP (*c*ercosporin *f*acilitator *p*rotein) (Callahan et al., 1999) play an important role in cercosporin self-resistance in *Cercospora* species. These findings were based on analysis of *Cercospora* mutants and gene expression in cercosporin-sensitive organisms. By contrast, the MFS transporter CTB4, found in the cercosporin biosynthetic cluster, does not appear to

play a role in cercosporin resistance as *ctb4* mutants retain normal resistance to cercosporin (Choquer et al., 2007). Here we report characterization of the five remaining predicted transporter genes recovered in the subtractive library between *C. nicotianae* wild type and the cercosporin-sensitive *crg1*-null mutant (Herrero et al., 2007): *CnATR2* (*A*BC *Tr*ansporter 2), *CnATR3* (*A*BC *Tr*ansporter 3), *CnMFS2* (*MFS* transporter 2), a uracil transporter, and a zinc transport protein.

2. Materials and Methods

2.1 Fungal strains, culture conditions, and plasmids

All strains of *Cercospora nicotianae* including the wild type (WT) strain ATCC18366 were maintained on complete medium (CM) agar or potato dextrose agar (PDA; Difco, Sparks, MD) as previously described (Jenns and Daub, 1995). *Neurospora crassa* was maintained on Vogel's medium (Vogel, 1956).

Cloning, DNA plasmid isolation, restriction enzyme analysis, and ligation used standard molecular techniques (Deininger, 1990). Standard PCR was performed using Taq polymerase (Denville Scientific Inc., NJ; Invitrogen, Carlsbad, CA) or High Fidelity Taq polymerase (Invitrogen) and gene-specific primers (Table 1). The plasmids pGEM-T Easy (Promega, Madison, WI) or pCB1636 (Sweigard et al., 1997), which harbors the *hph* gene encoding for hygromycin B phosphotransferase, were used as recipient vectors for construction of recombinant plasmids for cloning, sequencing and/or fungal transformation. *Escherichia coli* strain DH5-α was used to maintain all plasmids.

2.2 Cloning and sequencing of transporter genes

Different methods were performed to clone and sequence the full-length genomic sequences of the transporter genes based on partial EST sequences recovered in the subtractive library between *C. nicotianae* wild type and the *crg1* mutant (Herrero et al., 2007) . Cloning of *CnATR2* and *CnMFS2* was performed using inverse PCR (Keim, 2004). For genes encoding the uracil transporter, the zinc transport protein and CnATR3, primers specific to homologs from the closely related maize pathogen *Cercospora zeae-maydis* (http://www.jgi.doe.gov/) were designed to amplify and sequence full-length copies of *C. nicotianae* transporter genes. Intronic regions in *C. nicotianae* were identified from genomic sequences in *C. zeae maydis,* and Genescan (Burge, 1998) was used to confirm the locations of introns.

DNA sequencing was performed by Eton Biosciences Inc. (San Diego, CA) and Macrogen USA (Rockville, MD). Sequence analyses were performed using the following programs: BLAST (the US National Center for Biotechnology Information); ExPASy Proteomics tools (Swiss Institute of Bioinformatics, Geneva, Switzerland); NEB cutter V2.0 (New England BioLabs, Beverly, MA); and Search Launcher software (Baylor College of Medicine). Protein homologies were identified using protein BLAST. Nucleotide and amino acid sequences of *CnATR2, CnATR3, CnMFS2,* zinc transport protein gene and the uracil transporter gene are in the GenBank database with the accession numbers GU646036.1, KC959476, GU646037, KC959477, AF306523.1, respectively.

2.3 Phylogenetic analysis of transporter genes

CnATR2, CnATR3 and CnMFS2 sequences were searched for similarity to known proteins using tBLASTx (the US National Center for Biotechnology Information). Putative introns were predicted using similarity search results and the presence of the GT/AG 5' and 3' intron splicing sites (Jacobs and Stahl, 1995) and were digitally removed. Deduced amino acid sequences of CnATR2, CnATR3 and CnMFS2 were aligned with known ABC and MFS transporters in the NCBI database using CLUSTALX package 1.81 (Thompson et al., 1997). Phylogenetic analysis was performed using the Neighbor Joining Method in the CLUSTAL X. The phylogenetic trees were viewed and edited with NJPLOT (Thompson et al., 1997).

2.4 Gene expression under conditions of cercosporin toxicity

Disrupted mutants for CnATR1 (*atr1*) (Amnuaykanjanasin and Daub, 2009) and for CnATR2 (*atr2*) were used to quantify gene expression under cercosporin toxicity conditions. Homogenized mycelial plugs were used to inoculate 100 ml of PDB, and cultures were grown on a shaker (250 rpm) at room temperature for 3 days under dark conditions. Cultures were then treated with either 25 µM cercosporin or with 1.25% acetone (used to solubilize cercosporin). After cercosporin or acetone treatment, cultures were incubated under high light intensities (55-65 $\mu E.m^{-2}sec^{-1}$) for 1 or 3 hours before harvesting mycelia for RNA extraction and quantitative RT-PCR (q-RTPCR) analysis.

2.5 RNA extraction

Total RNA was extracted from lyophilized and homogenized mycelia with TRI-REAGENT (Sigma-Aldrich, St Louis, MO) following the manufacturer's recommendation with some modifications. Total nucleic acids were purified by phenol-chloroform extraction and ethanol precipitation following standard protocols. To obtain highly purified RNA, a LiCl and ethanol precipitation steps were performed, after which samples were treated with DNAse (Turbo DNA-free^{TM} Kit (Ambion, Austin, TX) according to the manufacturer's recommendations.

2.6 Quantitative RT-PCR analysis

Synthesis of cDNA was carried out using Taqman Reverse Transcription Reagents (Applied Biosystems, Somerville, MA) according to the manufacturer's recommendations, and a SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used for q-RTPCR reactions. The primers used to amplify each gene are shown in Table 1, and the cycling parameters for cDNA synthesis and q-RTPCR reactions were those previously used by Herrero et al., (2007). Single amplicons were identified using a melting point analysis protocol (60–90 ^{0}C every 0.5 ^{0}C for 1 second). The q-RTPCR reactions were performed in triplicate, and negative controls included: i) the use of RNA as a template to check for gDNA contamination in samples, and ii) a water control. Each sample was normalized against the actin control, and fold-change relative to no-cercosporin was calculated according to the $2^{-\Delta\Delta}C_{\mathrm{T}}$ method (Livak and Schmittgen, 2001; Schmittgen et al., 2000; Winer, 1999).

2.7 Expression of CnATR2 in N. crassa

The fungal transformation plasmid pTxA-1 (Amnuaykanjanasin and Daub, 2009) containing the *hph* resistance gene under the control of a *TrpC* promoter (HygR cassette) was used to insert the *CnATR2* gene under the control of the *Pyrenophora tritici-repentis* constitutive *ToxA* promoter (Lorang et al., 2001). A full-length copy of *CnATR2* was amplified using primers 14c-start2 (containing *Xba*I) and 14c-3end (Table 1). This PCR fragment was digested with *Xba*I, and ligated to the pTxA-1 that was digested with *Xba*I and *Sma*I. The resulting plasmid (pTxA-14c) was sequenced to confirm the presence of an intact *CnATR2* sequence.

The pTxA-14c plasmid was transformed into *N. crassa* protoplasts using polyethylene glycol (PEG)-mediated transformation. Protoplast generation and transformation was carried out as previously described with some modifications (Vollmer and Yanofsky, 1986). The cell-wall lysing mix consisted of 1.5% (m/v) of Sigma-Aldrich's lysing enzymes and 1.5% (v/v) of β-glucuronidase (Sigma-Aldrich, St. Louis, MO) in 100ml of 1M sorbitol. For transformation, a total of 2 X 10^7 protoplasts were incubated with 10 μg of plasmid DNA and regenerated in the presence of 100 μg/ml hygromycin. Single hygromycin (hyg)-resistant colonies that formed after 2-3 days were transferred to new plates and were continuously maintained under selection (100 μg/ml hygromycin) for subsequent manipulations. Transformation and presence of *CnATR2* was confirmed in hyg-resistant colonies by PCR screening using *CnATR2*-specific primers ATR2-F1 and ATR2-R1 (Table

1). An NaOH DNA extraction method from Wang *et al*. (1993) was used to extract gDNA for PCR analysis.

2.8 Screening of N. crassa CnATR2 transformants for cercosporin resistance

The *Cn*ATR2 transformants confirmed by PCR analysis were tested for cercosporin resistance on split petri plates. Each half-plate contained Vogel's medium supplemented with either 10 µM cercosporin or 0.5% acetone. Mycelial plugs (6 mm) of the *CnATR2*-transformants or wild type *N. crassa* were inoculated onto each side of the split plates, and incubated at room temperature under continuous fluorescent light (55-65 $\mu E.m^{-2}sec^{-1}$). Radial growth of colonies was measured 16 hours after inoculation, and % growth on cercosporin was calculated relative to acetone controls on the same plate. Each transformant was replicated five times, and the experiment was repeated two times. The mean value of all 10 samples are shown in the graph with standard error bars. Significant differences between wild type and each transformants were calculated by using Dunnett's t Tests (Dunnett, 1955).

For gene expression analysis total RNA was extracted from lyophilized and homogenized mycelia of 5 randomly chosen transformants, and q-RTPCR analysis was conducted as described in sections *2.5* and *2.6*. An ATR2-F and ATR2-R primer set was used for amplification of *ATR2* (Table 1). Each sample was normalized against the *N. crassa*-specific tubulin control. The fold changes were calculated relative to WT *N. crassa* according to the $2^{-\Delta\Delta}C_T$ method (Livak and Schmittgen, 2001; Schmittgen et al., 2000; Winer, 1999).

2.9 Targeted gene disruption in C. nicotianae

For targeted disruption of the *CnATR2* gene in wild type *C. nicotianae*, a split-marker recombination technique was modified as previously described (You et al., 2009). The 5' (from 200 bp upstream to 400 bp downstream of start codon) and 3' (from 900 bp downstream to 500 bp upstream of stop codon) *CnATR2* sequences were amplified from *C. nicotianae* gDNA by PCR with *CnATR2*-specific primers containing restriction enzyme linkers (Table 1). The two primer sets used to amplify the 5' and 3' ends of *CnATR2* were: ATR2-5'-F1 w/ *Apa*I, ATR2-5'-R1 w/ *Apa*I and ATR2-3'-F1 w/ *Eco*RI, ATR2-3'-R1 w/ *Sac*I. The PCR products were digested with the appropriate restriction enzymes (ATR2-5' digested with *Apa*I; ATR2-3' digested with *Eco*RI and *Sac*I) for cloning into the receptor plasmid pCB1636 (Sweigard et al., 1997). Plasmid pCB1636 was first digested with *Apa*I and ligated with *Apa*I digested *ATR2-5*'. Recombinant plasmids isolated from this step were selected based on the insert's correct orientation, and subsequently digested with *Eco*RI and *Sac*I and ligated with *ATR2*-3' to obtain a CnATR2 disruption construct (Fig. 1). Using this construct as a template (Fig. 1), two different overlapping PCR fragments were amplified using primers (Table 1) specific to the *CnATR2* sequence and the HygR cassette (split marker 1: ATR2-5'-F1 and HYG-split 5A; split marker 2: ATR2-3'-R1 and HYG-split 3S). Each split marker PCR fragment was sequenced to confirm their identity.

Protoplasts of *C. nicotianae* were isolated and transformed as previously described (Amnuaykanjanasin and Daub, 2009; Ehrenshaft, 1995) except that cultures were grown in PDB. For transformation, $1x10^7$ protoplasts were incubated with 10 µg of each of the two

split marker PCR fragments in the presence of spermidine (3mM) and heparin (0.5 mg/ml). Transformants were originally selected in medium containing 125 µg/ml of hygromycin. Hyg-resistant colonies were transferred to PDA amended with 125 µg/ml hygromycin, and were transferred a minimum of 5 transfers to fresh selection plates to ensure stability of transformation.

CnATR2 disruption was confirmed by PCR analysis using primer sequences shown in Table 1. Genomic DNA was extracted from lyophilized mycelia that were grown in PDB amended with 125 µg/ml hygromycin using the EZNA Fungal DNA Mini kit (Omega Bio-tek, Norcross, GA).

2.10 Screening of atr2 disruption mutants for cercosporin sensitivity, production, sporulation, and pathogenicity

Transformants confirmed to have a disrupted copy of *CnATR2* were screened for cercosporin sensitivity as described above for *N. crassa* sensitivity tests, except that radial growth of colonies was measured 3 days after the inoculation. For assaying cercosporin production, disruptants were grown under cercosporin-producing conditions in PDA under constant light (14 µE m^{-2} s^{-1}) at room temperature. Cercosporin was extracted with 5N KOH and concentrations were measured as previously described (Jenns et al., 1989). For sporulation, cultures were grown on V8 agar (30% V8 juice, 0.045M $CaCO_3$, 2% agar) at 18 ^{0}C. To induce conidiation, the cultures were brushed with a sterile paint brush after 4 days and incubated under same conditions for 4 additional days. To recover spores, plates were

flooded with water and brushed gently. Spores were quantified using a hemacytometer. For inoculations, spores collected from 2 disruptants (#17and #74) and wild type *C. nicotianae* were diluted to a final concentration of 3.3 X 10^4 cells/ml, and 50 ml of spore solution was atomized onto the upper leaves of 3 month-old 'Burley 21' tobacco plants (2 plants per strain). Plants were covered with plastic bags for 4 days to provide high humidity conditions. Disease ratings were taken 2, 4 and 7 weeks after inoculation.

3. Results

3.1 Sequence analysis of transporters

Full-length sequences were obtained for five transporter genes: *CnATR2*, *CnATR3*, *CnMFS2*, and the genes encoding the uracil transporter, and zinc transport protein. *CnATR2* has a 3926 bp genomic sequence, a 3864 bp open reading frame (ORF), and has a single intron. The 1288 amino acid CnATR2 protein consists of two similar halves, and each half is arranged as a transmembrane segment $(TMS)_6$ – nucleotide-binding domain (NBD) (Fig. 2), similar to most eukaryote transporters (Lamping et al., 2010). *CnATR2* (GenBank accession # GU646036.1) had high homology to a *Mycosphaerella populorum* multidrug resistance protein (MRP) (EMF13380.1, 79% identical in 1284 amino acids), a *Pseudocercospora* (*Mycosphaerella*) *fijiensis* MRP-type ABC transporter (EME84780.1, 75% identical in 1271 amino acids), and a *Zymoseptoria tritici* ABC transporter (XP_003854389.1, 69% identical in 1228 amino acids) (Table 2).

ATR3 had a 4717 bp genomic sequence, 4404 bp ORF and 6 introns (GenBank accession # KC959476). As with CnATR2, the protein consists of two similar halves with transmembrane and nucleotide binding domains (Fig. 2). The protein has high homology to an *M. fijiensis* ABC transporter (PDR type) (EME78227.1, 64% identical in 1461 amino acids), *Dothistroma septosporum* ABC transporter-like protein (EME40339.1, 63% identical in 1435 amino acids), *Z. tritici* ABC transporter (XP003848348.1, 63% identical in 1432 amino acids), and an *M. populorum* ABC transporter 1 (EMF09059.1, 62% identical in 1422 amino acids).

CnMFS2 (GenBank accession # GU646037) had a 1625 bp genomic sequence that consists of a 1464 bp ORF with three introns. The putative protein size is 488 amino acids with 12 TM domains arranged in two similar halves (Fig. 2). The protein has high homology to a *D. septosporum* hypothetical protein (EME45897.1, 72% identical in 456 amino acids), two *Colletotrichum* MFS transporters (ELA34042.1 and EFQ27918.1, both at 66% in 443 amino acids), an *Aspergillus oryzae* MFS transporter (XP_001823281.2, 59% identical in 464 amino acids), and an *Aspergillus flavus* putative bicyclomycin resistance protein (XP_002378643.1, 62% identical in 466 amino acids).

The zinc transport protein had an 1865 bp gDNA sequence, and a 1761 bp ORF with one intron (GenBank accession # KC959477). It includes the Zinc (Zn^{2+})-Iron (Fe^{2+}) Permease (ZIP) Family (TC 2.A.5) domain with eight putative transmembrane spanners that have been shown to transport Zn^{2+} as well as Fe^{2+} in some cases (Marchler et al., 2013). The protein has high homology to an *A. flavus* putative ZIP zinc transporter (XP_002373107.1,

53% identical in 504 amino acids), *A. oryzae* ZIP zinc transporter, (XP_001817943.2, 52% identical in 504 amino acids) and *Neosartorya fischeri* putative zinc transporter (XP_001267053.1, 53% identical in 511 amino acids).

The uracil transporter gene had a 1176 bp gDNA sequence and lacks introns (GenBank accession # AF306523.1). The protein has high homology to an *M. populorum* uridine permease Fui1 (EMF17950.1, 86% identical in 383 amino acids), *Pyrenophora tritici-repentis* uracil permease (XP_001936272.1, 75% identical in 376 amino acids) and *Z. tritici* hypothetical protein (XP_003857354.1, 79% identical in 372 amino acids). The gene includes solute binding domains found in Solute Carrier (SLC) families 5 and 6 and in nucleobase-cation-symport-1 (NCS1) transporters. Proteins with the SLC5 domain are involved in co-transport of Na^+ with sugars, amino acids, inorganic ions or vitamins (Wright et al., 2004). The SLC6 domain is commonly found in Na^+/Cl^{-2} dependent plasma membrane transporters (neurotransmitters) (Kristensen et al., 2011). NCS1 transporters are essential secondary active transporters for nucleobases, hydantoins, and vitamins. and work by using a sodium ion, or a proton as a mechanism of energization (Weyand et al., 2008). Various transporters with NCS1 transporter -like family domains are common in fungi such as *Aspergillus nidulans, A. fumigatus, C. albicans and Saccharomyces cerevisiae* (Pantazopoulou and Diallinas, 2007).

3.2 Phylogenetic analysis of CnATR2, CnATR3, and CnMFS2

ABC and MFS transporters have been identified in previous studies to have roles in cercosporin production and resistance (Daub and Chung, 2009; Daub et al., 2013), thus we compared CnATR2, CnATR3, and CnMFS 2 to these and other ABC and MFS transporters using phylogenetic analysis (Figs. 3 & 4, Supplemental Table 1). For the ABC transporter analysis, the two ABC domains of each transporter were used due to their greater degree of conservation as compared to transmembrane domains. In the analysis, "ABC1" and "ABC2" refer to the first and second ATP-binding cassette domains from the N-terminus and C-terminus, respectively (Fig. 2). Overall, phylogenetic analysis showed a clear separation between the ABC-G (or PDR, pleiotropic drug resistance-like) subfamily and the ABC-C (or MRP, multidrug resistance-associated protein) subfamily (Fig. 3). In the PDR (ABC-G) group, all the ABC1 domains were clustered with 95% bootstrap support, and all the ABC2 domains were grouped together with 100% bootstrap support (Fig. 3A). With the exception of the slime mold *Dictyostelium discoideum* AbcG14 domains, which grouped with the fungi, the non-fungal ABC proteins, including *Arabidopsis thaliana* PDR4, *D. discoideum* AbcG16, and *Oryza sativa* PDR9, fell into a separate branch within each of the ABC1 or ABC2 clusters. ATR3 clustered with the fungal PDR proteins, along with two previously characterized transporters involved in cercosporin resistance, the *C. nicotianae* ATR1 (Amnuaykanjanasin and Daub, 2009) and the yeast ScSnq2p (Servos et al., 1993) (Fig. 3A).

For the MRP-like (ABC-C) group, the ABC1 and ABC2 domains of fungal transporters were clustered with considerably lower bootstrap support (Fig. 3B). CnATR2

clustered with the MRP-like group for both the ABC1 and ABC2 domains. No previously characterized transporters with roles in cercosporin production or resistance were found in the MRP-like transporter group.

The MFS transporter MFS2 in *C. nicotianae* clustered with dityrosine transporters (Fig. 4, Supplemental Table 1) including the *S. cerevisiae* Dtr1p that has been experimentally verified for transport of bisformyl dityrosine into the surface of ascospores (Felder et al., 2002). Filamentous fungal and yeast dityrosine transporters were clearly separated in the tree with strong bootstrap support (Fig. 4). Two MFS transporters previously shown to play a role in cercosporin resistance, CFP (*c*ercosporin *f*acilitator *p*rotein) from *Cercospora kikuchii* and *C. nicotianae* (Callahan et al., 1999, Amnuaykanjanasin and Daub, 2009) and Mfs1 from *Botrytis cinerea* (Hayashi et al., 2002), were clustered together in a distinct group with 100% bootstrap support. These proteins share the ability to transport cercosporin. CnCTB4, the MFS transporter found within the cercosporin biosynthetic cluster, is also shown.

3.3 Expression of transporters under conditions of cercosporin toxicity

The expression of the five transporters along with the previously characterized *CnCFP* was assayed under conditions of cercosporin toxicity by treating the cercosporin-sensitive *atr1* mutant (Amnuaykanjanasin and Daub 2009) with cercosporin under high light conditions. Both *CnCFP* and *CnATR2* were strongly induced when cercosporin toxicity was induced (Fig 5). *CnCFP* was induced more than 50 fold after both 1 and 3 hours of treatment, and *CnATR2* was upregulated 9-16-fold. By contrast, there was no induction of *CnMFS2*,

CnATR3, the uracil transporter or the zinc transport protein. Based on these gene expression results, *CnATR2* was chosen for further characterization.

3.4 Ability of CnATR2 to impart resistance in N. crassa

The ability of CnATR2 to impart cercosporin resistance was tested by transforming the cercosporin-sensitive fungus *N. crassa* to express CnATR2. Colonies were selected on medium containing hygromycin, and putative transformants were screened for the presence of *CnATR2* by PCR. PCR-positive transformants were assayed for resistance to cercosporin by measuring radial growth on cercosporin-containing medium relative to growth on control medium. Cercosporin at 10 µM inhibits radial growth of *N. crassa*, resulting in approximately 30% of the radial growth on medium lacking cercosporin (Fig. 6). Of the 23 PCR-positive transformants tested, six were found to be significantly more resistant to cercosporin than wild type ($P < 0.05$). Mean resistance of the remaining transformants varied from 40% to 60%.

Five randomly choosen *ATR2* transformants screened for resistance were selected to analyze the expression of *ATR2*. Quantitative RTPCR analysis of *ATR2* expression in all five of the *ATR2* transformants showed high levels of expression (between 100- to 400-fold), except *ATR2* transformant #8 (40-fold). *ATR2* transformant #8 was not more resistant to cercosporin, correlating with low expression. However, transformant # 21 (also not resistant) had the highest expression. Thus there was no correlation between *ATR2* expression and resistance of the transformant.

3.5 atr2 disruption mutants of C. nicotianae

A split marker strategy was used to generate *C. nicotianae* disruption mutants by homologous recombination. A total of 191 hyg-resistant colonies were screened to confirm *CnATR2* disruption using PCR. Primer sets from the HygR marker sequences, genomic regions outside the split marker sequences, and genomic sequences within the deleted region (Table 1) were used to confirm disruption as shown in Fig. 7. A total of nine transformants were confirmed as being disrupted for *CnATR2*.

The nine *atr2*-disruption strains were tested for cercosporin sensitivity by growing them on cercosporin-containing medium in the light. Wild type and two transformed, but not disrupted, strains were used as controls. Results are shown in Fig.8. There was no statistically significant difference in cercosporin resistance between the wild type, non-disrupted transformants, or *atr2* disruptants in radial growth on cercosporin.

As other transporters (ATR1, CFP) have shown a dual role in both cercosporin resistance and production, six of the *atr2* disruptants were selected for assay of cercosporin production (Fig. 9A) as compared to wild type. One *atr2* disruptant (#174) produced significantly less cercosporin than wild type, however, there was no significant difference between cercosporin production between wild type and the other *atr2* transformants indicating that CnATR2 is not involved in cercosporin production. Four *atr2* disruptants, one non-disruptant and wild type *C. nicotianae* were tested for changes in sporulation (Fig. 9B). Average production of spores ranged from 1- 2.4 X 10^6 spores/plate, and there was no significant difference in the number of spores produced by the strains. Disruptant strains #

17 and 174 were then selected for pathogenicity assays on tobacco. Symptoms caused by disruptant strains did not differ from those caused by wild type *C. nicotianae* (data not shown).

3.6 Expression of other transporters and cercosporin biosynthetic genes in atr2 disrupted mutants

Expression of *CnATR2* in *N. crassa* demonstrated that CnATR2 can provide cercosporin resistance, however, *atr2* mutants of *C. nicotianae* were not more sensitive to cercosporin than wild type. We thus assayed for expression of other transporters previously shown to impart cercosporin resistance (CnATR1, CnCFP) as well as expression of the other library transporters to determine if they are up-regulated in the *atr2* mutant background (Fig. 10A). Expression of *CnATR1, CnMFS2, CnATR3,* the uracil transporter, and the zinc transport protein was not increased. By contrast, *CnCFP* expression was strongly increased, over 700-fold at 1 hour and over 170-fold at 3 hours. These results suggest that the lack of sensitivity of the *atr2* mutant may be due to compensation by over-expression of *CnCFP*. The subtractive library of putative cercosporin resistance genes also includes two genes from the cercosporin biosynthetic pathway: CTB2, encoding an *O*-methyltransferase, and CTB5, encoding an O_2, FAD/FMN-dependent oxidoreductase (Daub and Chung, 2009; Herrero et al., 2007). Analysis of expression of cercosporin biosynthetic genes in the *atr2* mutant, showed significant up-regulation of CTB5 as well as CTB7, a second FAD/FMN-dependent

oxidoreductase (Fig. 10B); expression of CTB2 and the other biosynthetic genes including the CTB4 transporter were not altered.

4. Discussion

Several membrane transporters have been shown to play a role in resistance to the toxin cercosporin. These include the ABC transporter ATR1 (Amnuaykanjanasin and Daub, 2009) and the MFS transporter CFP (Callahan et al., 1999), both found in a subtractive library between cercosporin-resistant wild type and cercosporin-sensitive *crg1* mutant (Herrero et al., 2007). Other studies also have confirmed the importance of transporters in resistance to cercosporin and to structurally related compounds. The yeast ABC transporter Snq2p increased cercosporin resistance in yeast when overexpressed (Ververidis et al., 2001). MFS transporters from *B. cinerea* (Bcmfs1) and *Mycosphaerella graminicola* (MgMfs1) have also been shown to provide resistance to cercosporin and to camptothecin, a quinone alkaloid produced by *Camptotheca acuminata* (Hayashi et al., 2002; Roohparvar et al., 2007). The role of transporters has also been shown in medical studies using the structurally-similar photoactivated perylenequinone hypericin, where increased expression of ABC transporters in cancer cells reduced utility of hypericin as an anti-cancer agent (Jendzelovsky et al., 2009). Our subtractive library of putative cercosporin resistance genes included seven genes encoding membrane transporters (Herrero et al., 2007), two of which (ATR1, CFP) have previously been characterized (Amnuaykanjanasin and Daub, 2009; Callahan et al., 1999; Choquer et al., 2007). Cloning and bioinformatics analysis of the remaining five transporter

genes identified two additional ABC transporters (CnATR2, CnATR3), an MFS transporter (CnMFS2), a uracil transporter, and a zinc transport protein.

As both ABC and MFS transporters have previously been implicated in cercosporin resistance and production, we conducted a phylogenetic analysis of ABC and MFS transporters from this study as well as those previously implicated in cercosporin resistance. Phylogenetic analysis of the ABC transporters showed that CnATR3 as well as the previously characterized *C. nicotianae* ATR1 and *S. cereviseae* Snq2p clustered with the PDR (pleiotropic drug-resistance) subfamily. The two ABC domains of CnATR3 cluster most closely with the domains of AtrE from the human fungal pathogen *Trichophyton rubrum*; AtrE is involved in drug resistance to terbinafine, 4-nitroquinoline 1-oxide, and ethidium bromide (Fachin et al., 1996). The ABC2 domain of CnATR3 also clusters with Snq2p as well as with the *Schizosaccharomyces pombe* BFR1 and the *S. cerevisiae* PDR10. BFR1 confers brefeldin A and cycloheximide resistance (Nagao et al., 1995). PDR10 has not been shown tobe involved in resistance, but rather in maintenance of plasma membrane asymmetry of budding yeast (Rockwell et al., 2009). In our study, *CnATR3* expression was unchanged under conditions of cercosporin toxicity, thus it is not clear what role CnATR3 may play in *C. nicotianae.* The two ABC domains of the previously characterized ATR1 clustered within their own group, and clustered with ABC domains of the ATR-B transporters in the Leotiomycetes *Monilinia fructicola* and *B. cinerea*. ATR-B transporters from these fungi confer resistance to many major classes of fungicides such as myclobutanil and propiconazole (Schnabel et al., 2003) as well as some natural toxic compounds such as

camalexin (Stefanato et al., 2009). ATR1's role in cercosporin resistance also makes this group of ABC transporters strong candidates for toxin resistance (Amnuaykanjanasin and Daub, 2009).

Of the genes in this study or those known to be involved in cercosporin resistance or production, only CnATR2 clustered with the MRP (multidrug resistance-associated protein-like) transporters. The ABC domains of CnATR2 cluster with those of *M. populorum* MRP3, *M. fijiensis* MDR, and *Z. tritici* ABC transporters. These fungi are in the Dothidiomycetes, the main taxonomic group in which perylenequinone-producers are found (Daub and Chung, 2009; Daub et al., 2013), and *M. fijiensis* is resistant to cercosporin (unpublished results). Our results demonstrated that *CnATR2* can impart cercosporin resistance to *N. crassa* and raises the possibility that the homologous *Mycosphaerella* and *Zymoseptoria* transporters may have a similar resistance role.

At total of 19 MFS transporters, four of which were previously shown to be involved in cercosporin production or resistance, were used to construct the phylogenetic tree of MFS transporters. Bcmfs1 from *B. cinerea* (Hayashi et al., 2002) and CFP from *C. nicotianae* and *C. kikuchii* (Callahan et al., 1999) clustered together in a separate group. These results might indicate that these transporters have a unique role in cercosporin resistance, as two different fungi have very similar proteins involved in resistance. CnMFS2, the transporter protein from the library, closely clusters with a *Dothistroma septosporum* transporter protein. *D. septosporum* (teleomorph = *Mycosphaerella pini*) is in the class Dothidiomycetes and produces the photoactivated toxin dothistromin (de Wit et al., 2012; Stoessl et al., 1990).

These two closely related transporters in two different fungi producing two different photoactivated toxins might be an indication of a role in toxin resistance. However, they also cluster with dityrosine transporters, involved in diverse roles in cells including yeast spore formation (Felder et al., 2002) and in protein localization and zinc transport (Salazar et al., 2009). Finally, CnCTB4, the transporter found in the cercosporin biosynthetic cluster, has been shown to be regulated by CRG1, a regulator of cercosporin biosynthesis (Daub et al., 2013). However, *CTB4* was not induced under cercosporin toxicity, consistent with previous studies documenting no role for CTB4 in cercosporin resistance (Daub et al., 2013).

To test the role of these transporters in cercosporin resistance, we assayed for changes in expression when the cercosporin-sensitive *atr1* transporter mutant was exposed to cercosporin toxicity. Only two of the transporter genes were upregulated. The previously characterized *CFP* (Callahan et al., 1999) was induced almost 100-fold at both time points tested (1 and 3 hours). *CnATR2* was also induced approximately 10-fold at both time points. There was no induction of *CnATR3,* the uracil transporter, or the zinc transport protein, and *CnMFS2* was down-regulated at 1 hour. These results demonstrate that not all transporters are involved in complementing ATR1's role in resistance against cercosporin, and that resistance to cercosporin is likely mediated by specific transporters including the previously characterized CFP and ATR1.

Up-regulation of *CnATR2* in response to cercosporin toxicity led us to further characterize its role in resistance. We first tested the ability of CnATR2 to increase cercosporin resistance in the cercosporin-sensitive fungus *N. crassa*. Under the conditions of

the assays, growth of wild type *N. crassa* is inhibited by about 70% whereas growth of the resistant *C. nicotianae* is inhibited under these assay conditions by about 40%. Assay of *N. crassa* ATR2 transformants identified six that had significantly more resistance to cercosporin than wild type, confirming the ability of CnATR2 to impart cercosporin resistance. Not all of the transformants, however, had significantly increased resistance. Random insertion of transgenes into the fungal genome is known to affect gene expression (Smith et al., 2008), thus lack of resistance may reflect differences in transgene expression. To test this hypothesis, expression of ATR2 in selected *N. crassa* transformants was analyzed. All of the transformants had high expression of the transgene, and differences in resistance did not correlate with expression. We hypothesize that ATR2 imparts resistance, but that the effect is small. This hypothesis is supported by the observation that all 23 transformants grew better on cercosporin than wild type, although the differences were only significant for six of the transformants.

The role of CnATR2 in *C. nicotianae* was then characterized by creating disruption mutants. Previous studies with *atr1* and *cfp* transporter mutants in *Cercospora* confirmed that mutants were more sensitive to cercosporin and also showed decreases in cercosporin production, sporulation and pathogenicity. When *CnATR1* was disrupted in *C. nicotianae*, cercosporin resistance was decreased by 20-30% (Amnuaykanjanasin and Daub, 2009). In *Cercospora kikuchii cfp* disruptants, resistance was decreased 40-45% (Callahan et al., 1999). Production of cercosporin was also significantly decreased in both *cfp* and *atr1* disruptants, and *cfp* disruptants were shown to be less pathogenic. These results suggest that

these two transporter genes have an important role in the regulation of cercosporin biosynthesis as well as resistance against cercosporin. In contrast to the results obtained with *atr1* and *cfp* disruptants, there was no difference between the *atr2* disruptants and wild type *C. nicotianae* or the non-disrupted transformants in either cercosporin resistance or production of cercosporin. Sporulation and pathogenicity were also unaffected.

The discrepancy between the ability of CnATR2 to impart cercosporin resistance to *N. crassa* and the lack of increased sensitivity of *C. nicotianae atr2* mutants led us to test if induction of other transporters compensates for the *atr2* mutation, as is seen in the *atr1* mutant where both CnCFP and CnATR2 are induced under conditions of cercosporin toxicity. We tested expression of *CnATR1, CnATR3, CnCFP, CnMFS2,* and the uracil transporter and zinc transfer protein genes in the *atr2* mutant under conditions of cercosporin toxicity. Of these genes, only *CnCFP* was induced, but it was strongly induced (200-700-fold). Given CFP's documented role in cercosporin resistance, we hypothesize that upregulation of *CnCFP* compensates for mutations in both *CnATR1* and *CnATR2*. Further, the induction of *CnCFP* in the *atr2* mutant lends support to the importance of CnATR2 in cercosporin resistance.

In addition to induction of transporters in the *atr2* mutant, we also tested induction of genes (*CTB*) in the cercosporin biosynthetic pathway. We have hypothesized that resistance genes may be found within the biosynthetic cluster. However, gene disruption experiments in *C. nicotianae* have not shown decreases in resistance in *ctb* mutants (Chen et al., 2007), suggesting that these genes are not involved in resistance. Based on our results with *atr1* and

atr2 mutants that suggest that upregulation of other resistance genes may compensate for these mutations, we decided to test whether *ctb* genes were upregulated in these mutants. In the *atr1* mutant, all genes in the biosynthetic cluster (*CTB1-8*) were up-regulated (data not shown), perhaps due to a response to cercosporin deficiency caused by the loss of ATR1. Cercosporin production is not affected in the *atr2* mutant, but we found that two of the genes, *CTB5* and *CTB7* were up-regulated. These two genes encode putative FAD/FMN^- or NADPH dependent oxidoreductases (Chen et al., 2007), and reduction of cercosporin is a major mechanism of cercosporin resistance (Daub et al., 2013; Daub et al., 1992). It is possible that CTB5 and CTB7 may contribute to cercosporin resistance but, as with CnATR2, mutations are compensated for by upregulation of other genes. Further research will be needed to better define these enzymes and their function.

In summary, we have identified five novel genes encoding transporters in our subtractive library of genes putatively involved in *C. nicotianae* resistance to cercosporin. Assay of induction under conditions of cercosporin toxicity led us to characterize the ABC transporter CnATR2. We show that CnATR2 can impart cercosporin resistance to the cercosporin-sensitive fungus *N. crassa.* Lack of phenotype of *C. nicotianae atr2* mutants may be due to compensation by up-regulation of other resistance genes including the previously characterize CFP transporter. Further, upregulation of *CFP* in the absence of CnATR2 supports the importance of CnATR2 in cercosporin resistance. We conclude that multiple transporters including CnCFP, CnATR1, and CnATR2 are involved in resistance to cercosporin. This work confirms a core role of transporters in cercosporin resistance. This

work, combined with previous documentation of cercosporin reduction and of chemical quenchers in cercosporin resistance (Daub et al., 2013), demonstrates that cercosporin resistance in *Cercospora* is mediated by multiple mechanisms that are carefully regulated and redundant to ensure resistance.

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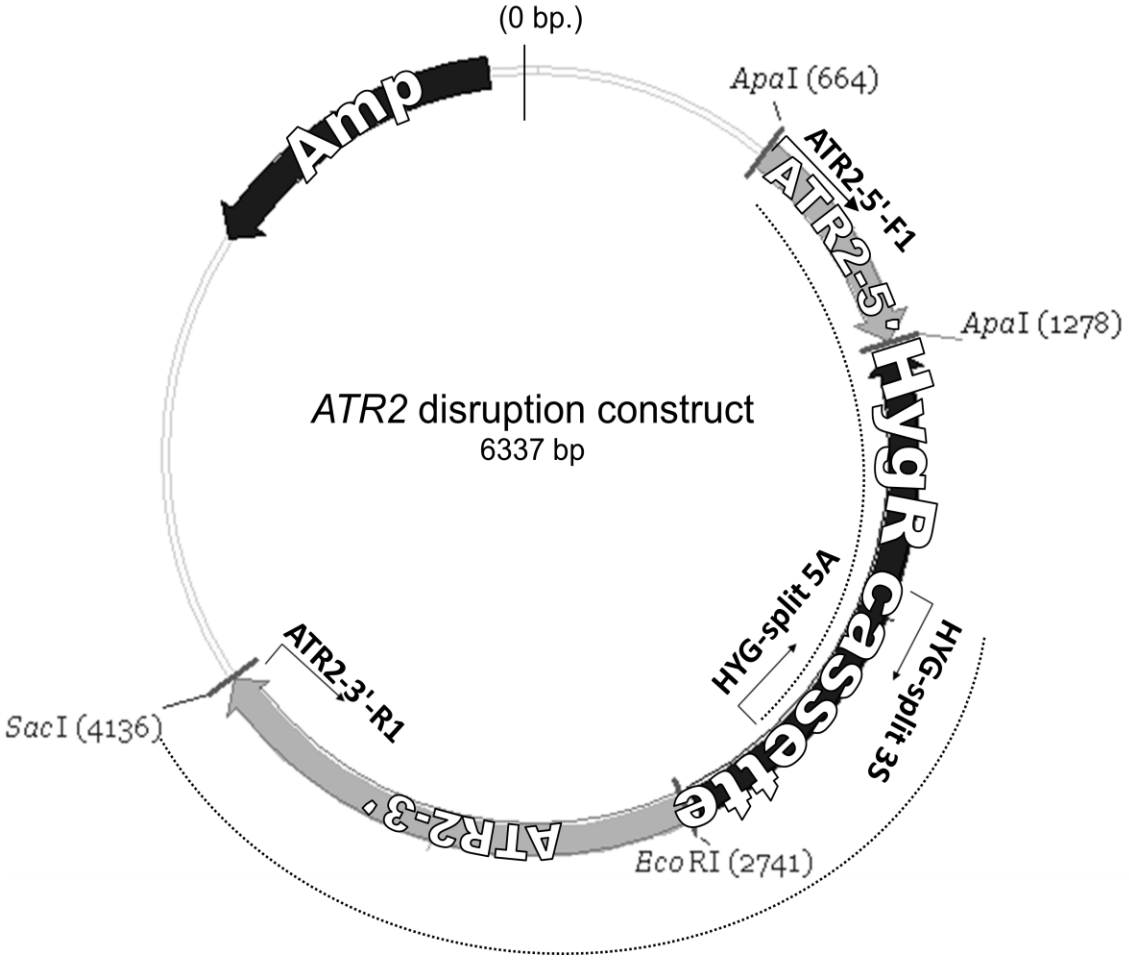


Figure 1. Construct for *C. nicotianae atr2* disruption. The CnATR2 disruption construct was engineered by amplifying the 5' and 3' regions of *ATR2* from *C. nicotianae* genomic DNA and cloning them on the either side of the hygromycin resistance (HygR) cassette in pCB1636 (Amnuaykanjanasin and Daub, 2009). The 5' *ATR2* fragment is 600bp and 3' *ATR2* fragment is 1.5kb. Arrows represent the primers (shown in Table 1) used to amplify the two split marker PCR fragments (shown as dotted lines) from the vector. Restriction sites are also shown with location relative to 0 bp.

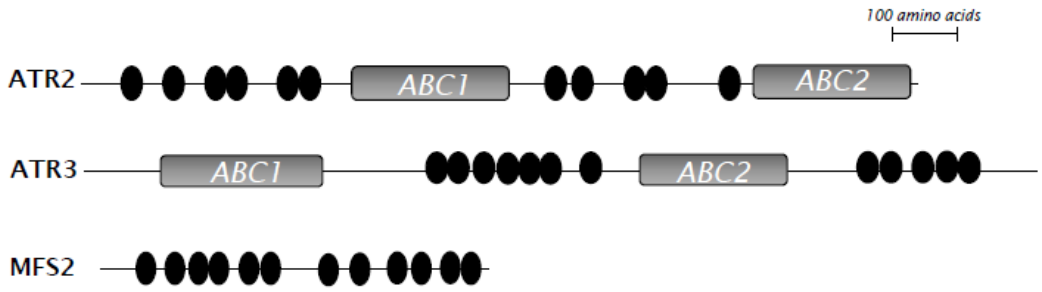
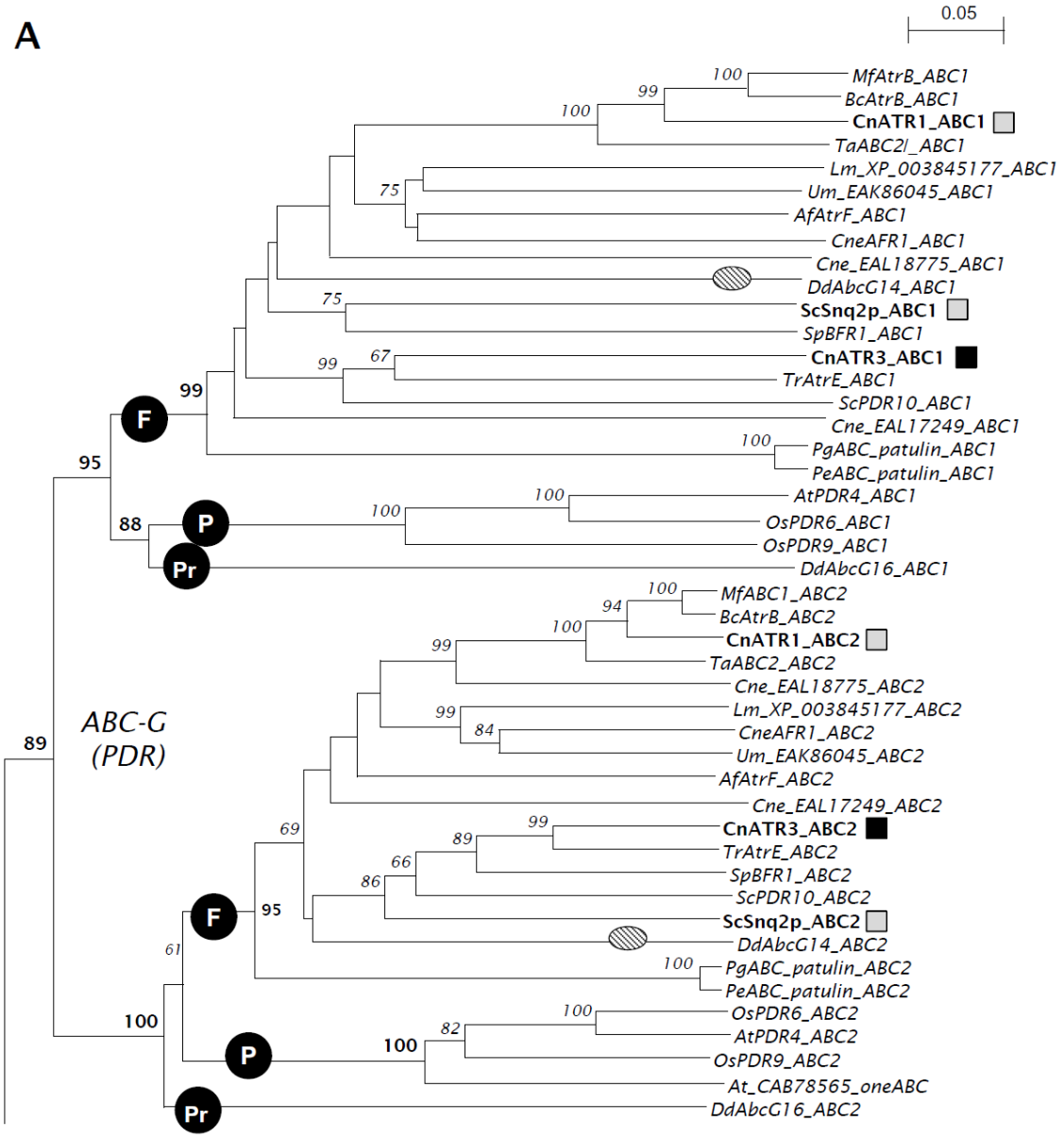


Figure 2. Conserved domain organization of CnATR2, CnATR3, and CnMFS2. ATP-binding cassettes and transmembrane domains are shown as rectangles and ovals, respectively.

Figure 3. Phylogenetic analysis of ABC transporters. Fungal, plant, protist, and animal branches are noted in black circles as F, P, Pr, and A, respectively. Transporters from other taxomonic groups within those branches (e.g. *Dictyostelium* transporter clustering within the fungal group) are noted by hatched ovals. Transporters in this study as well as those previously shown to play a role in cercosporin resistance or production are shown in bold. CnATR2 and CnATR3 are marked by black squares, and the two previously reported cercosporin efflux pumps CnATR1 and ScSnq2p are marked by gray squares. CnATR1, CnATR3, and ScSnq2p grouped with the subfamily ABC-G (PDR) transporters (panel AB). CnATR2 grouped with ABC-C (MRP) transporters (panel B). Bootstrap analysis was performed with 1,000 replicates, and values of > 60% are given at the nodes. A bacterial ABC transporter, *Bacillus subtilis* BceA, was used as the outgroup. Protein names and accession numbers for each sequence are shown in supplemental Table 1.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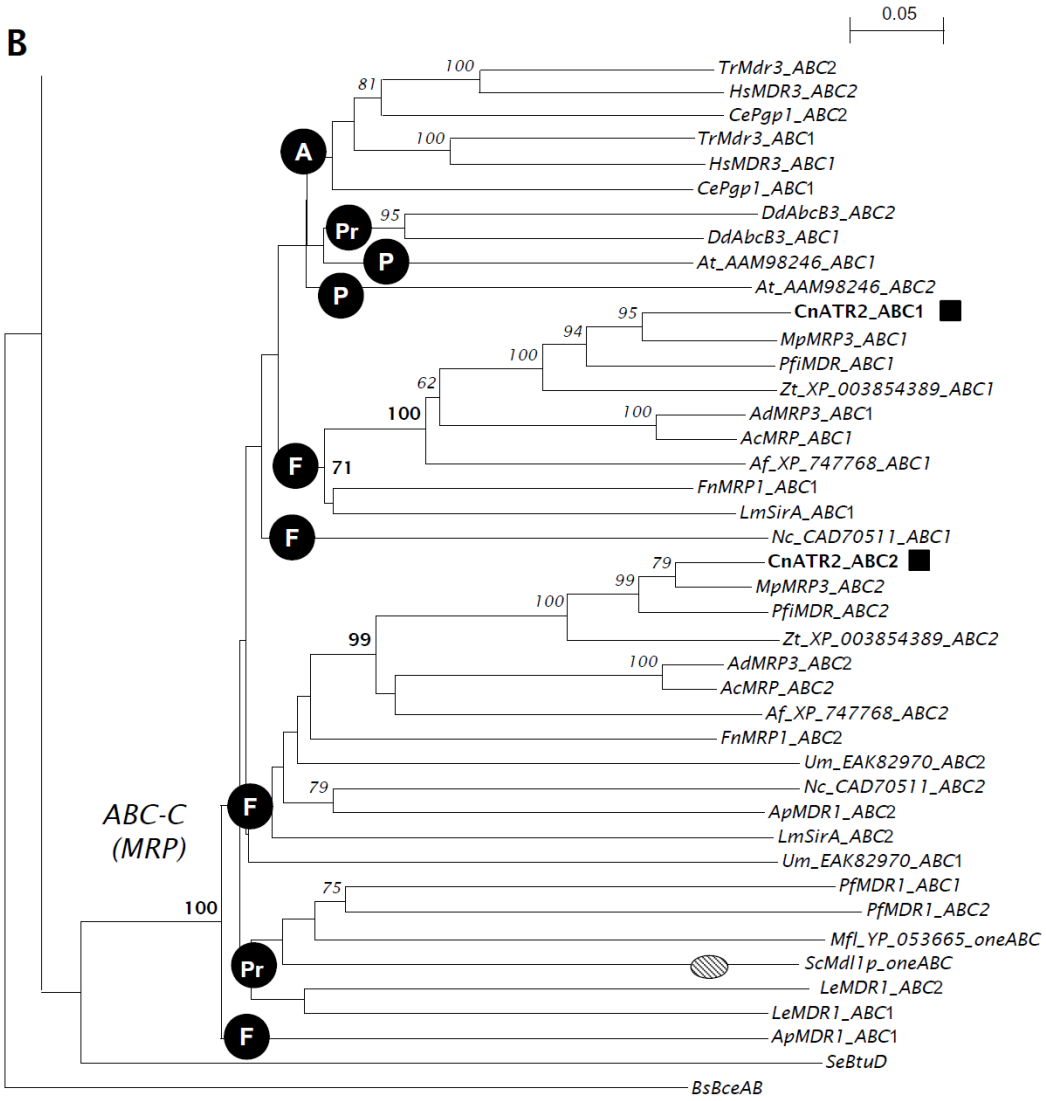
BcAtrB_ABC1
CnATR1_ABC1
TaABC2/_ABC1
Lm_XP_003845177_ABC1
Um_EAK86045_ABC1
AfAtrF_ABC1
CneAFR1_ABC1
Cne_EAL18775_ABC1
DdAbcG14_ABC1
ScSnq2p_ABC1
SpBFR1_ABC1
CnATR3_ABC1
TrAtrE_ABC1
ScPDR10_ABC1
Cne_EAL17249_ABC1
PgABC_patulin_ABC1
PeABC_patulin_ABC1
AtPDR4_ABC1
OsPDR6_ABC1
OsPDR9_ABC1
DdAbcG16_ABC1
MfABC1_ABC2
BcAtrB_ABC2
CnATR1_ABC2
TaABC2_ABC2
Cne_EAL18775_ABC2
Lm_XP_003845177_ABC2
CneAFR1_ABC2
Um_EAK86045_ABC2
AfAtrF_ABC2
Cne_EAL17249_ABC2
CnATR3_ABC2
TrAtrE_ABC2
SpBFR1_ABC2
ScPDR10_ABC2
ScSnq2p_ABC2
DdAbcG14_ABC2
PgABC_patulin_ABC2
PeABC_patulin_ABC2
OsPDR6_ABC2
AtPDR4_ABC2
OsPDR9_ABC2
At_CAB78565_oneABC
DdAbcG16_ABC2
ABC-G
(PDR)
F
P
Pr
F
P
Pr


B
0.05
TrMdr3_ABC2
HsMDR3_ABC2
CePgp1_ABC2
TrMdr3_ABC1
HsMDR3_ABC1
CePgp1_ABC1
DdAbcB3_ABC2
DdAbcB3_ABC1
At_AAM98246_ABC1
At_AAM98246_ABC2
CnATR2_ABC1
MpMRP3_ABC1
PfiMDR_ABC1
Zt_XP_003854389_ABC1
AdMRP3_ABC1
AcMRP_ABC1
Af_XP_747768_ABC1
FnMRP1_ABC1
LmSirA_ABC1
Nc_CAD70511_ABC1
CnATR2_ABC2
MpMRP3_ABC2
PfiMDR_ABC2
Zt_XP_003854389_ABC2
AdMRP3_ABC2
AcMRP_ABC2
Af_XP_747768_ABC2
FnMRP1_ABC2
Um_EAK82970_ABC2
Nc_CAD70511_ABC2
ApMDR1_ABC2
LmSirA_ABC2
Um_EAK82970_ABC1
PfMDR1_ABC1
PfMDR1_ABC2
Mfl_YP_053665_oneABC
ScMdl1p_oneABC
LeMDR1_ABC2
LeMDR1_ABC1
ApMDR1_ABC1
SeBtuD
BsBceAB
A
Pr
P
P
F
F
F
Pr
F
ABC-C
(MRP)

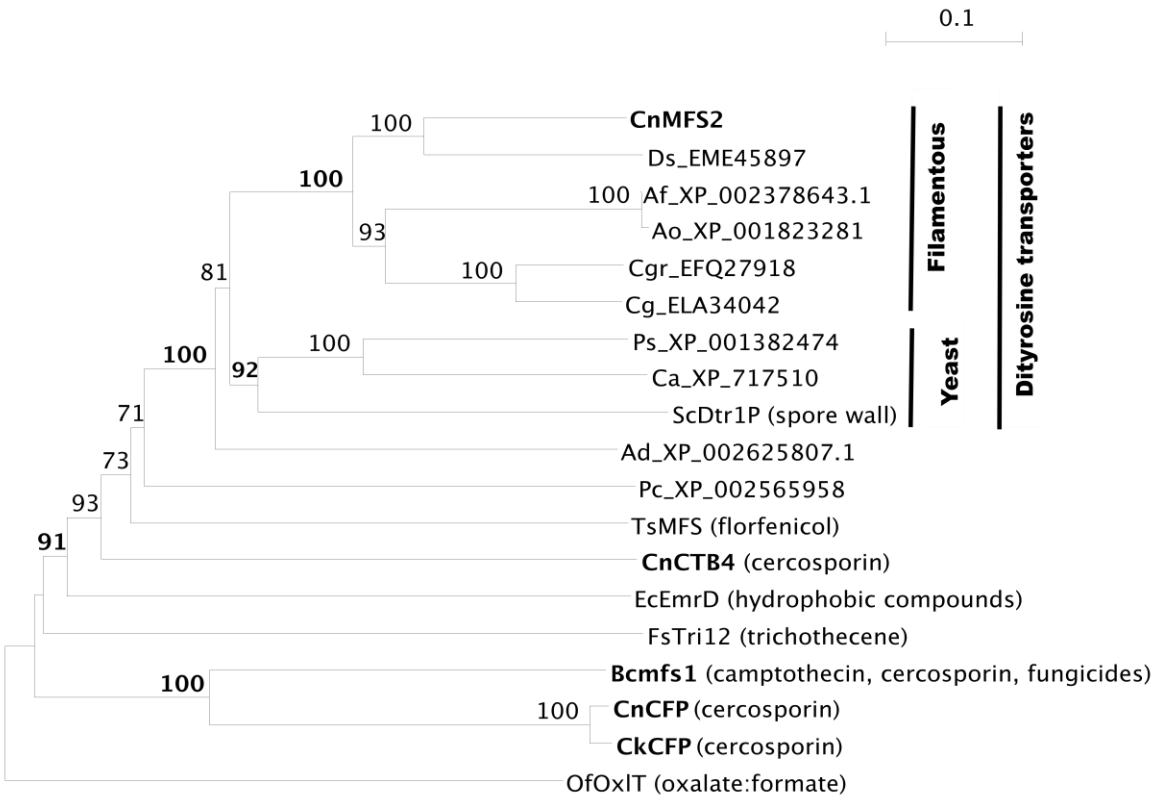


Figure 4. Phylogenetic analysis of MFS transporters. The phylogenetic tree included known fungal MFS transporters, with target compounds, when known, indicated in parenthesis. Transporters in this study as well as those previously shown to be involved in cercosporin production or resistance are shown in bold. CnMFS2 clusters with the group of dityrosine transporters. There was a clear divide between dityrosine transporters in yeast and filamentous fungi. Two transporters previously shown to be involved in cercosporin resistance and export (BfMfs1, CFP) clustered together. CnCTB4, the transporter found in the cercosporin biosynthetic cluster is also shown. Bootstrap analysis was performed with 1,000 replicates, and values of > 60% are given at the nodes. A bacterial MFS transporter, *Oxalobacter formigenes* OxlT, was used as the outgroup in this tree. Protein names and accession numbers for each sequence are shown in supplemental Table 1.

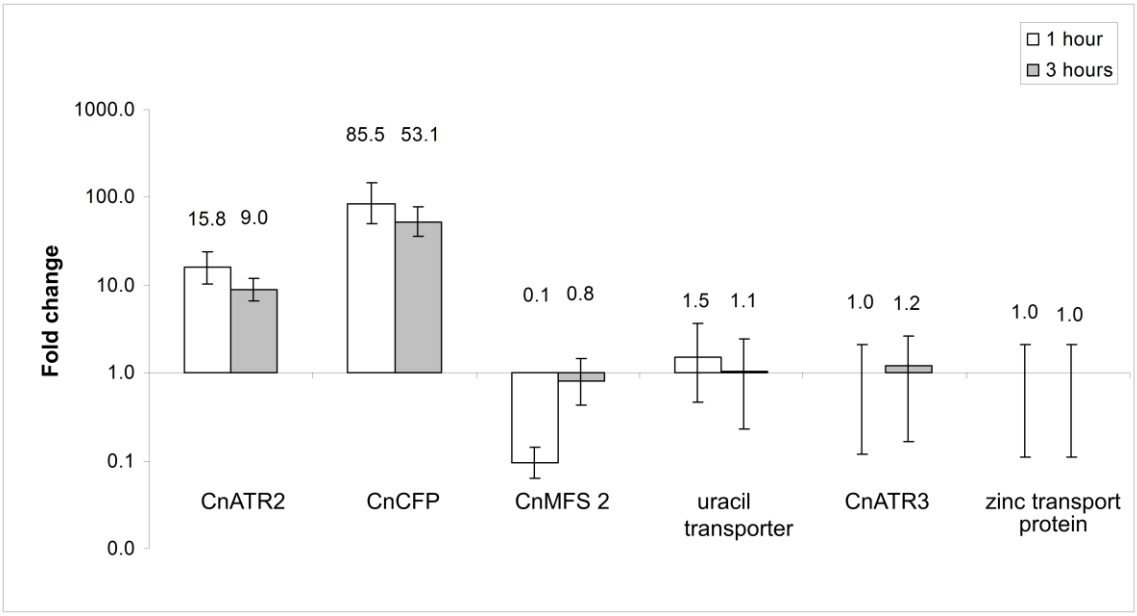
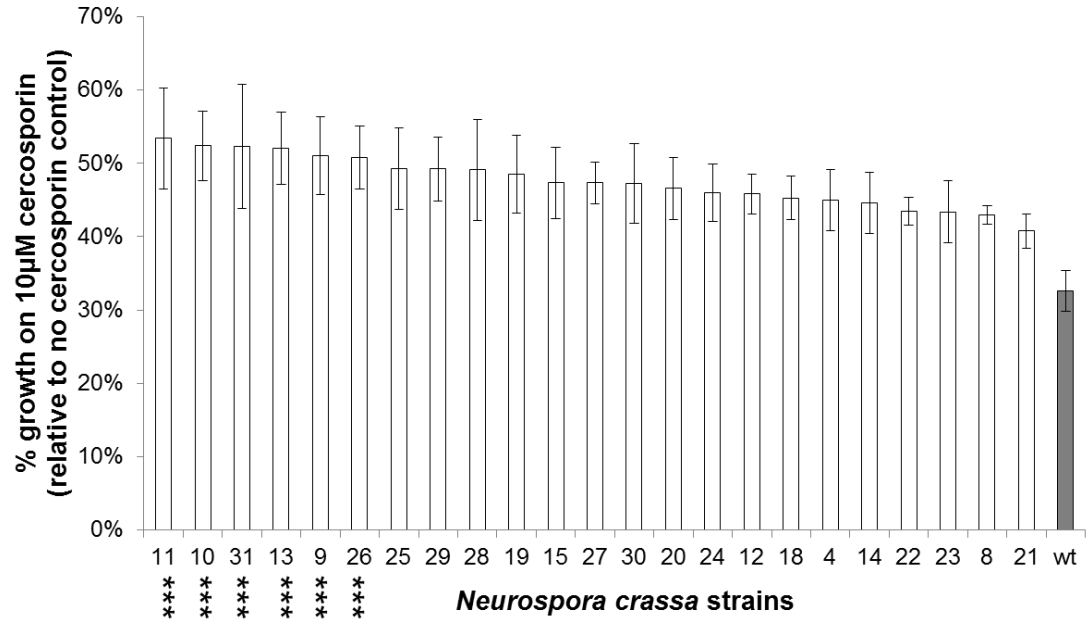


Figure 5. Quantitative RT-PCR analysis of gene expression of transporter genes in the *C. nicotianae atr1* mutant treated with cercosporin in the light. Each sample was normalized against the actin control, and fold-change relative to no-cercosporin was calculated according to the $2^{(-\Delta\Delta C(T))}$ method (Livak and Schmittgen, 2001). Data represent the mean of two independent experiments each with 3 technical replications. The mean values are included at the top of each bar. Error bars represent 95% confidence intervals

A



B

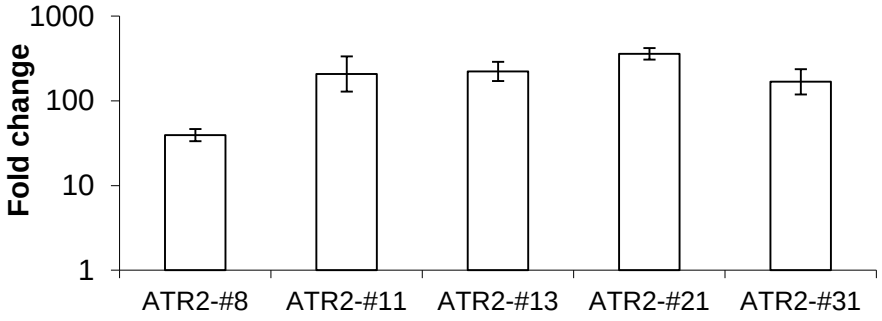
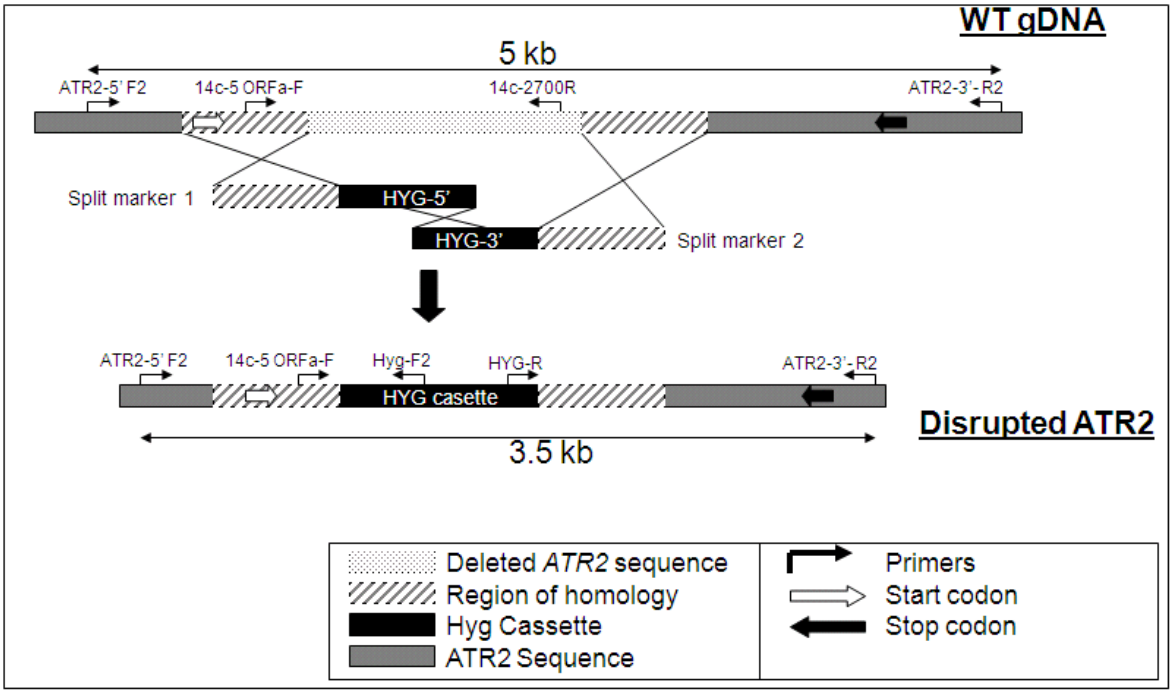


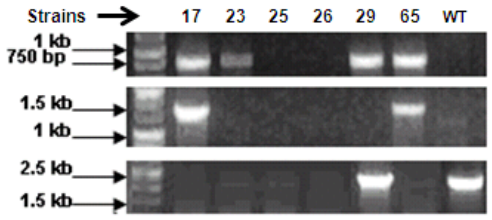
Figure 6. (A) Cercosporin resistance of *Neurospora crassa* wild type (grey bar) and *CnATR2*-transformed strains (white bars). Data are the results of two independent experiments with 5 replications each. Strains marked with three asterisks (***) have significantly greater resistance than wild type ($P < 0.05$). Error bars represent standard error. (B) Quantitative RT-PCR analysis of gene expression of ATR2 transformants #8, 11, 13, 21 and 31. Each sample was normalized against the tubulin control, and fold-change relative to wild type *Neurospora crassa* control (no transgene) was calculated according to the $2^{(-\Delta\Delta C(T))}$ method (Livak and Schmittgen, 2001). Error bars represent 95% confidence intervals.

Figure 7. Targeted gene disruption of *CnATR2* in *C. nicotianae* wild type (WT) using a split-marker recombination method (You et al., 2009). (A) Diagrams of *C. nicotianae ATR2* genomic DNA, split marker PCR fragments, and resulting disruption sequence. Hatched bars show regions of homology between genomic DNA and split marker PCR fragments. Homologous recombination results in an intact HygR cassette replacing a region (dotted bar) of the *CnATR2* sequence. Primers used to confirm disruption are shown. (B) Example of PCR amplification to confirm disruption. Primers and expected band sizes are shown in the table. Top gel image: primer set A; middle gel image: primer set B; Bottom gel image: primer set C. Lane 1: 1 kb marker; lanes 2-7: hyg-resistant transformants; lane 8: WT. Transformants in lanes 2 and 7 (# 17, # 65) were confirmed as disruptants due to the presence of the 780 bp (top gel) and 1700 bp (middle gel) bands and lack of the wild type 2500 bp (bottom gel). One transformant (lane 6) contained both the disrupted sequences and an intact wild type sequence. Seven additional disruptants (not shown) were identified from a total of 191 hyg-resistant transformants screened.

A



B



	Expected size (bp)	
Primer Sets used for screening *atr2* Strains	Disruptant	WT
A) ATR2-5' F2 + Hyg-F2	780	0
B) ATR2-3'-R2 + HYG-R	1700	0
C) 14c-5 ORFa-F+ 14c-2700R	0	2500

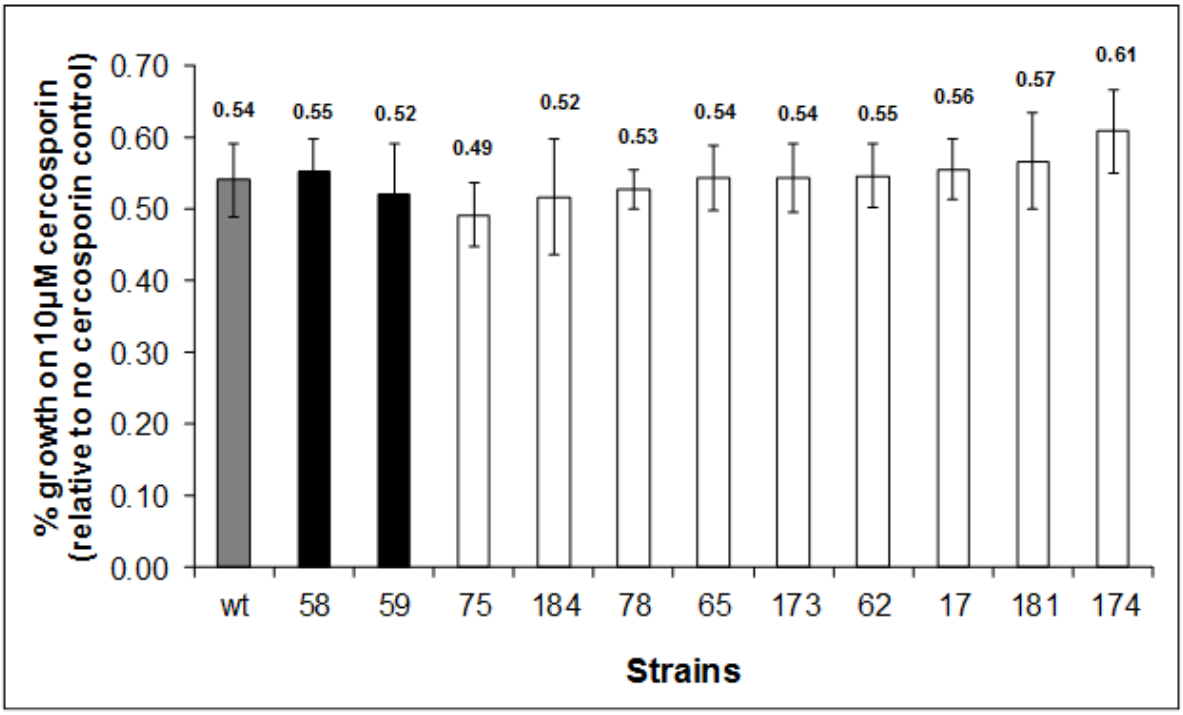
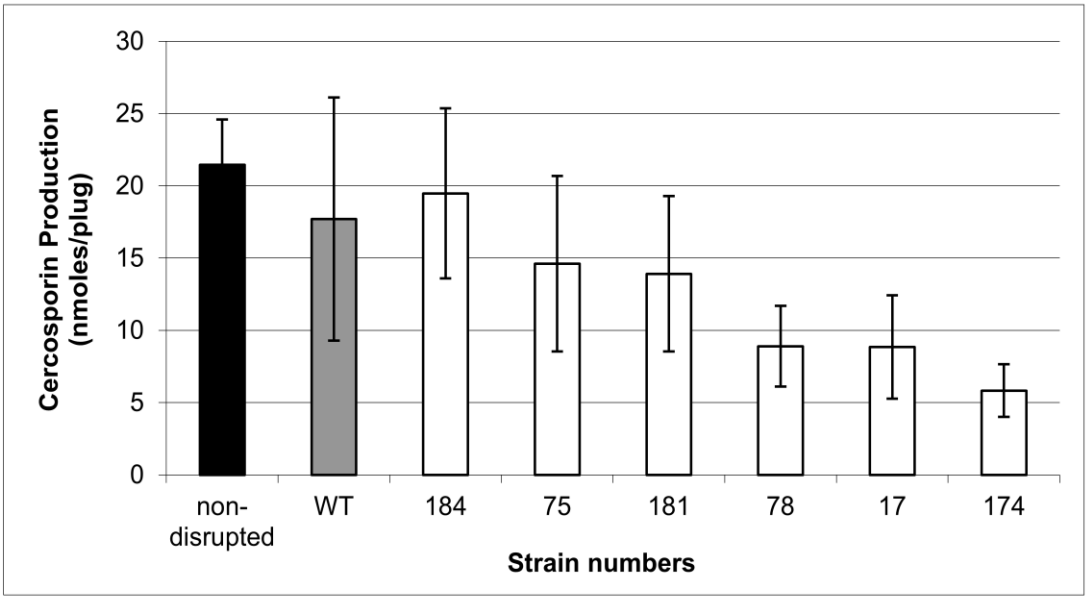


Figure 8. Cercosporin resistance of *C. nicotianae* wild type (grey bar), *CnATR2* non-disrupted transformants (black bars) and disrupted strains (white bars). Data are the results of 10 replications each and the mean of two separate experiments. The mean values are included at the top of each bar. Error bars represent upper and lower confidence intervals ($P < 0.05$). There were no significant differences in cercosporin sensitivity between wild type, non-disrupted hyg-resistant transformants and *atr2* disruptants.

Figure 9. Cercosporin and spore production by *atr2* disruptant mutants. (A) Cercosporin production by *atr2* disruptants (white bars) relative to wild type (grey bar) and a transformed, but non-disrupted strain (black bar). Cercosporin was extracted in 5 N KOH from 7-day old colonies grown on potato dextrose agar, and quantified by measuring absorbance of the KOH extract at 480 nm. Data are results of 2 experiments with four replicates per strain per experiment. Error bars represent upper and lower confidence intervals ($P < 0.05$). Disrupted mutant # 174 produced significantly less cercosporin than wild type, however all other disruptants showed no reduction in cercosporin production according to the paired- T test analysis with 95% confidence interval. (B) Sporulation assay of wild type (grey bar), CnATR2 non-disrupted transformant (black bars) and *atr2*-disrupted strains (white bars). Bars represent mean spore number per petri dish. Data are the average of 7 plates. Each data point is reported as mean (± SE).

A


Cercosporin Production (nmoles/plug)
30
25
20
15
10
5
0
non-disrupted
WT
184
75
181
78
17
174
Strain numbers

B

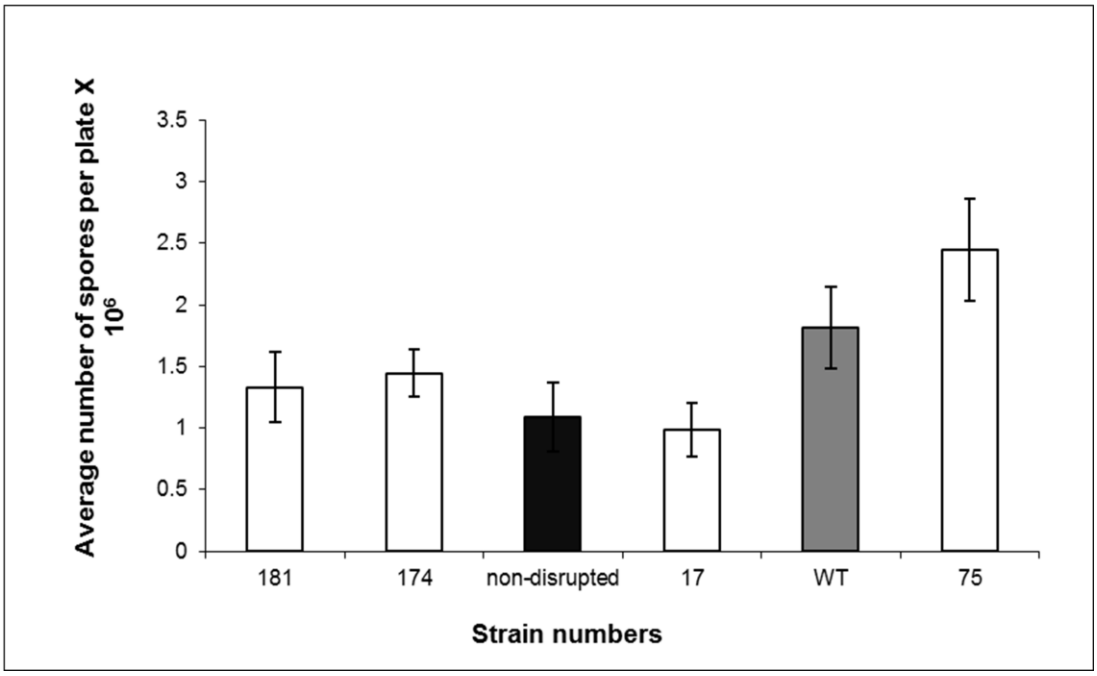
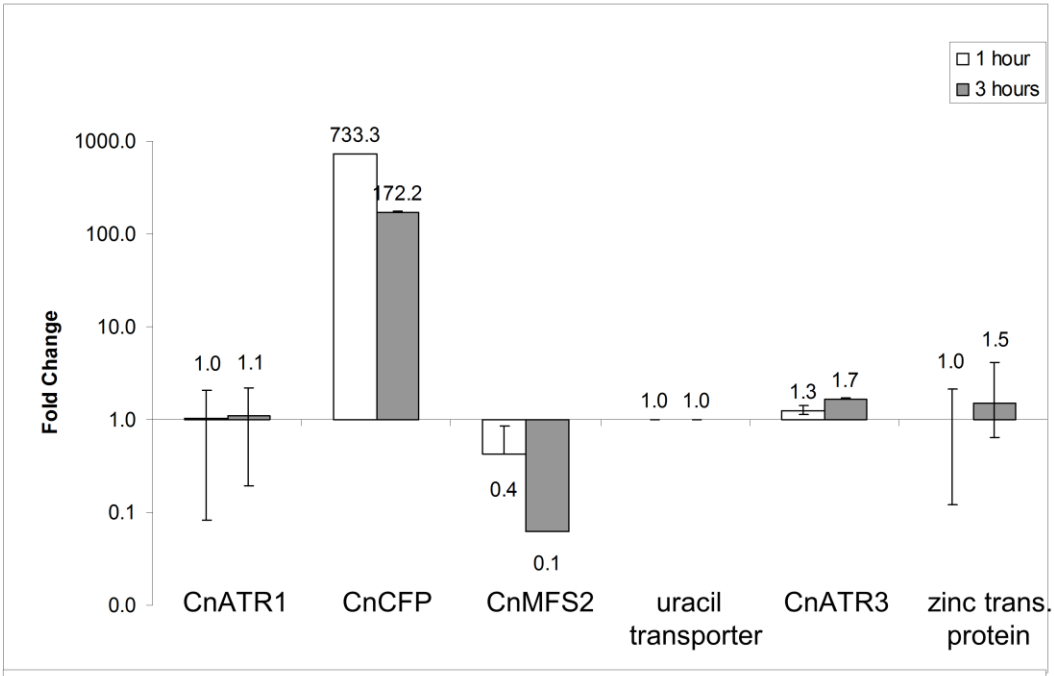

Average number of spores per plate X 10^6
3.5
3
2.5
2
1.5
1
0.5
0
181
174
non-disrupted
17
WT
75
Strain numbers

Figure 10. Quantitative RT-PCR analysis of gene expression in *atr2* mutant (disruptant #17) treated with cercosporin in the light. Each sample was normalized against the actin control, and fold-change relative to no-cercosporin treatment was calculated according to the $2^{(-\Delta\Delta C(T))}$ method (Livak and Schmittgen, 2001). (A) Expression of transporter genes. Data are the mean of two independent experiments. The mean values are included at the top of each bar. Error bars represent 95% confidence intervals. (B) Expression of genes in the cercosporin biosynthetic cluster (CTB genes). Data are the mean of two independent experiments. Error bars represent 95% confidence intervals.

A


1 hour
3 hours
Fold Change
1000.0
100.0
10.0
1.0
0.1
0.0
733.3
172.2
1.0 1.1
0.4
0.1
1.0 1.0
1.3 1.7
1.0 1.5
CnATR1
CnCFP
CnMFS2
uracil transporter
CnATR3
zinc trans. protein

B

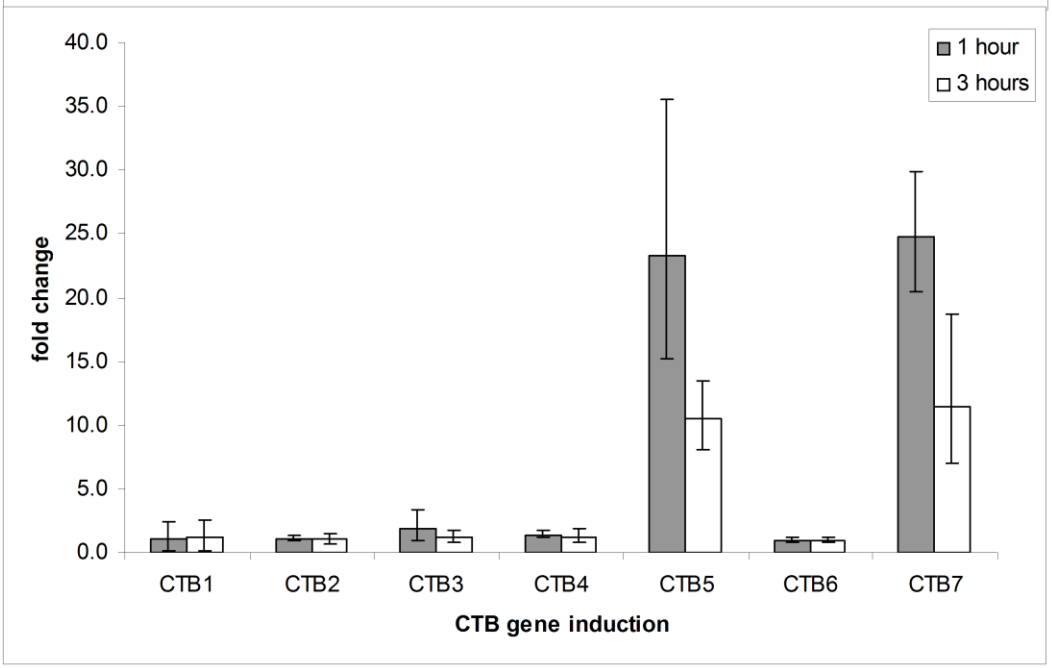

1 hour
3 hours
fold change
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
CTB1
CTB2
CTB3
CTB4
CTB5
CTB6
CTB7
CTB gene induction

Table 1: Primer sequences[1]

Cn**ATR2 disruption construct cloning**	
ATR2-5'-F1w/ ApaI	ATTATA**GGGCCC**TCCTGGGCATTGTTCGATCCTCTAC
ATR2-5'-R1w/ ApaI	ATTATA**GGGCCC**ATGAGAACAGTCGTGATGTATGCGAGAAC
ATR2-3'-F1w/ EcoRI	ATTATA**GAATTC**TCCACTCTACCAAGGCTCTGTTCG
ATR2-3'-R1w/ SacI	ATTATA**GAGCTC**ACCTGGTCGGCAGTGTTCTCATC
Amplification of split markers	
ATR2-5'-F1	TCCTGGGCATTGTTCGATCCTCTAC
ATR2-3'-R1	ACCTGGTCGGCAGTGTTCTCATC
HYG-split3S	CGTTGCAAGACCTGCCTGAA
HYG-split5A	GGATGCCTCCGCTCGAAGTA
Screening *atr2* –disrupted strains	
ATR2-5' F2	TGTCTTGGTGGCGTCGCTAG
ATR2-3'- R2	ATCCACTTCAGGGTCGAGCATG
Hyg-F2	TGAACCATCTTGTCAAACGACAC
HYG-R	TGTCGGGCGTACACAAATCG
14c-2700R	GATCGTTTGCTTGAGCTTCGAG
ATR2-F1	ACAGGAAGTCACCCATCGTTACC
14c-5 ORFa-F	CTGGAGGTCTGAAGGATTACTG
Cn***ATR2*-expressing construct**	
14c-start2 w/ Xba	GC**TCTAGA**ACCTCGACATGGGTGACTACA
14c-3end	GCTTTCTGAAGTACTGCGACTGC
Screening the presence of Cn***ATR2* in *N. crassa* transformants**	
ATR2-F1	ACAGGAAGTCACCCATCGTTACC
ATR2-R1	AACGACGAGACCGAGCTTCC
Amplification of cDNA for q-RT PCR	
ATR1-F	GTCGCTTGCACCGTCTACTG
ATR1-R	CATGGTAGCCTTCTGCTTCTCA
ATR2-F	GATGCTCGAGAGGTACATCAG
ATR2-R	AACGACGAGACCGAGCTTCC
MFS2-F	AGCCGGCAATGTCACACTTAC
MFS2-R	TCAAGGCGCAGTATGGATCTT
CFP-F	ACGCCGGAACTGTCTTATGC
CFP-R	TGCGGTGTGTCGAGTGTGA
ATR3-F	TGTCATTACCGGCTCCATGTT
ATR3-R	TGCTGCTGCACATAGCCTGT
Uracil-F	GGAGCCAAATATCACCTGAGCTT
Uracil-R	CGCCCCAAATCCCAAAAG
Zinc-F	CCAGCCCTCAGGTGGAGA
Zinc-R	TGAGTGCTCGCCTTTACCAC

[1] Bold and underlined sequences represent restriction enzymes targets

Table 2: Homologs of transporters in the library

CnATR2	**Accession number**	**% identity in total amino acids aligned**
Mycosphaerella populorum multidrug resistance protein (MRP)	EMF13380.1	79% identical in 1284 amino acids
Mycosphaerella fijiensis MRP-type ABC transporter	EME84780.1	75% identical in 1271 amino acids
Zymoseptoria tritici ABC transporter	XP_003854389.1	69% identical in 1228 amino acids
CnATR3	**Accession number**	**% identity in total amino acids aligned**
Mycosphaerella fijiensis ABC transporter (PDR type)	EME78227.1	64% identical in 1461 amino acids
Zymoseptoria tritici ABC transporter	XP003848348.1	63% identical in 1432 amino acids
Dothistroma septosporum ABC transporter-like protein	EME40339.1	63% identical in 1435 amino acids
Mycosphaerella populorum ABC transporter 1	EMF09059.1	62% identical in 1422 amino acids
CnMFS2	**Accession number**	**% identity in total amino acids aligned**
Dothistroma septosporum hypothetical protein	EME45897.1	72% identical in 456 amino acids
Colletotrichum MFS transporter	ELA34042.1	66% identical in 443 amino acids
Colletotrichum MFS transporter	EFQ27918.1	66% identical in 443 amino acids
Aspergillus oryzae MFS transporter	XP_001823281.2	59% identical in 464 amino acids
Aspergillus flavus putative bicyclomycin resistance protein	XP_002378643.1	62% identical in 466 amino acids
The zinc transport protein	**Accession number**	**% identity in total amino acids aligned**
Aspergillus flavus putative ZIP zinc transporter	XP_002373107.1	53% identical in 504 amino acids
Aspergillus oryzae ZIP zinc transporter	XP_001817943.2	52% identical in 504 amino acids
Neosartorya fischeri putative zinc transporter	XP_001267053.1	53% identical in 511 amino acids
The uracil transporter gene	**Accession number**	**% identity in total amino acids aligned**
Mycosphaerella populorum uridine permease Fui1	EMF17950.1	86% identical in 383 amino acids
Pyrenophora tritici-repentis uracil permease	XP_001936272.1	75% identical in 376 amino acids
Zymoseptoria tritici hypothetical protein	XP_003857354.1	79% identical in 372 amino acids

Supplemental Table S1. ABC and MFS Transporters used in phylogenetic analysis and their GenBank accession numbers.

ABC transporters in Fig. 3	Protein	Accession number
AdMRP3_ABC1, _ABC2	*Ajellomyces dermatitidis* MRP3	**XP_002623024.1**
ApMDR1_ABC1, _ABC2	*Aureobasidium pullulans* MDR1	**AAD00580.1**
AfAtrF_ABC1, _ABC2	*Aspergillus fumigatus* AtrF	**XP_747642.1**
AtPDR4_ABC1, _ABC2	*Arabidopsis thaliana* PDR4	**NP_180259.1**
At_AAM98246_ABC1, _ABC2	*Arabidopsis thaliana* ABC transporter	**AAM98246.1**
At_CAB78565_oneABC	*Arabidopsis thaliana* ABC transporter	**CAB78565.1**
BsBceAB	*Bacillus subtilis* BceAB	**NP_390916.1**
CePgp1_ABC1, _ABC2	*Caenorhabditis elegans* Pgp1	**CAA46190.1**
CnATR1_ABC1, _ABC2	*Cercospora nicotianae* ATR1	**ACD42872.1**
CnATR3_ABC1, _ABC2	*Cercospora nicotianae* ATR3	**KC959476**
CneAFR1_ABC1, _ABC2	*Cryptococcus neoformans* AFR1	**CAD21006.1**
Cne_EAL18775_ABC1, _ABC2	*Cryptococcus neoformans* CNBI0360,	**EAL18775.1**
Cne_EAL17249_ABC1, _ABC2	*Cryptococcus neoformans* CNBN0760	**EAL17249.1**
DdAbcB3_ABC1, _ABC2	*Dictyostelium discoideum* AbcB3	**AAL74250.1**
DdAbcG14_ABC1, _ABC2	*Dictyostelium discoideum* AbcG14	**AAL91499.1**
DdAbcG16_ABC1, _ABC2	*Dictyostelium discoideum* AbcG16	**AAL91501.1**
FnMRP1_ABC1, _ABC2	*Filobasidiella neoformans* MRP1	**AAC49890.1**
HsMDR3_ABC1, _ABC2	*Homo sapiens* MDR3	**NP_061338.1**
LeMDR1_ABC1, _ABC2	*Leishmania enriettii* MDR1	**Q06034.1**
Lm_ XP_003845177_ABC1, _ABC2	*Leptosphaeria maculans* ABC transporter	**XP_003845177**
LmSirA_ABC1, _ABC2	*Leptosphaeria maculans* SirA	**AAS92552.1**
MfAtrB_ABC1, _ABC2	*Monilinia fructicola* AtrB	**AAL80009.1**
Mfl_YP_053665_oneABC	*Mesoplasma florum* ABC transporter	**YP_053665.1**
MpMRP3_ABC1, _ABC2	*Mycosphaerella populorum* MRP3	**EMF13380**
Nc_CAD70511_ABC1, _ABC2	*Neurospora crassa* MRP	**CAD70511.1**

Supplemental Table S1 Continued

OsPDR6_ABC1, _ABC2	*Oryza sativa* PDR6	**CAD59571.1**
OsPDR9_ABC1, _ABC2	*Oryza sativa* PDR9	**BAD29210.1**
Pg_ABD66573_ABC1, _ABC2	*Penicillium griseofulvum* ABC transporter	**ABD66573**
Pe_ ABN48540_ABC1, _ABC2	*Penicillium expansum* ABC transporter	**ABN48540**
PfMDR1_ABC1, _ABC2	*Plasmodium falciparum* MDR1	**XP_001351787.1**
PfiMDR_ABC1, _ABC2	*Pseudocercospora fijiensis* MDR	**EME84780.1**
SeBtuD	*Serratia* sp. AS12 BtuD	**YP_004500529**
ScMdl1p_oneABC	*Saccharomyces cerevisiae* Mdl1p	**NP_013289.1**
ScPDR10_ABC1, _ABC2	*Saccharomyces cerevisiae* PDR10	**NP_014973.1**
ScSnq2p_ABC1, _ABC2	*Saccharomyces cerevisiae* Snq2p	**CAA47270**
SpBFR1_ABC1, _ABC2	*Schizosaccharomyces pombe* BFR1	**NP_587932.3**
TaABC2_ABC1,_ ABC2	*Trichoderma atroviride* ABC2	**AAX68676.1**
TrAtrE_ABC1, _ABC2	*Trichophyton rubrum* AtrE	**AAN28699**
TrMdr3_ABC1, _ABC2	*Takifugu rubripes* Mdr3	**AAO20901.1**
Um_EAK86045_ABC1, _ABC2	*Ustilago maydis* UM05642.1	**EAK86045**
Um_EAK82970_ABC1, _ABC2	*Ustilago maydis* UM05096.1	**EAK82970.1**
Zt_XP_003854389_ABC1, _ABC2	*Zymoseptoria tritici* ABC transporter	**XP_003854389.1**

MFS transporters in Fig. 4	**Protein**	**Accession number**
Ad_ XP_002625807.1	*Ajellomyces dermatitidis* SLH14081	**XP_002625807.1**
Af_ XP_002378643.1	*Aspergillus flavus* MFS transporter	**XP_002378643.1**
Ao_XP_001823281	*Aspergillus oryzae* MFS transporter	**XP_001823281.1**
Bcmfs1	*Botrytis cinerea* Mfs1	**AAF64435.2**
Ca_ XP_717510.1	*Candida albicans* MFS transporter	**XP_717510.1**
Cg_ ELA34042	*Colletotrichum gloeosporioides* dityrosine transporter	**ELA34042**
Cgr_ EFQ27918	*Colletotrichum graminicola* MFS transporter	**EFQ27918**

Supplemental Table S1 Continued

CkCFP	*Cercospora nicotianae* CFP	**ACD42873.1**
CnCTB4	*Cercospora nicotianae* CTB4	**ABK64181.1**
CnCFP	*Cercospora kikuchii* CFP	**AAC78076.1**
CnMFS2	*Cercospora nicotianae* MFS2	**ADV35576.1**
Ds_EME45897	*Dothistroma septosporum* DOTSEDRAFT_22021	**EME45897**
EcEmrD	*Escherichia coli* EmrD	**WP_001335494**
FsTri12	*Fusarium sporotrichioides* Tri12	**AAK33071.1**
Pc_XP_002565958	*Penicillium chrysogenum* MFS transporter	**XP_002565958.1**
Ps_XP_001382474.2	*Pichia stipitis* MFS transporter	**XP_001382474.2**
OfOxlT	*Oxalobacter formigenes* oxalate:formate antiporter OxlT	**AAC43722**
ScDtr1p	*Saccharomyces cerevisiae* Dtr1p	**CBK39254.1**
TsMFS	*Talaromyces stipitatus* putative florfenicol exporter	**XP_002486985.1**

CHAPTER 3: Characterization of *Cercospora nicotianae* Hypothetical Proteins in Cercosporin Resistance

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Abstract

The photoactivated toxin, cercosporin, produced by *Cercospora* species, plays an important role in pathogenesis of this fungus to host plants. Cercosporin has almost universal toxicity to cells due to its production of singlet oxygen. For that reason, *Cercospora* species, which are highly resistant to their own toxin, are good candidates to identify cercosporin resistance genes. In previous research, the zinc cluster transcription factor *CRG*1 (*c*ercosporin *r*esistance *g*ene 1) was found to be crucial for *Cercospora* species' resistance against cercosporin, and subtractive hybridization analysis identified 185 genes differentially expressed between *Cercospora nicotianae* wild type (wt) and a *crg1* mutant. The focus of this work was to identify and characterize the hypothetical proteins that were identified in *Cercospora nicotianae* as potential resistance factors. Quantitative RT-PCR analysis of 21 genes encoding hypothetical proteins showed that two, *24cF* and *71cR*, were induced under conditions of cercosporin toxicity, suggesting a role in resistance. Transformation and expression of *24cF* and *71cR* in the cercosporin-sensitive fungus, *Neurospora crassa*, showed that *71cR* provided increased resistance to cercosporin toxicity, whereas no significant increase was observed in *24cF* transformants. Gene disruption was used to generate *C. nicotianae* 71cR mutants; these mutants did not differ from wt *C. nicotianae* in cercosporin resistance or production. Quantitative RT-PCR analysis showed induction of other resistance genes in the *71cr* mutant and may compensate for the loss of 71cR. Analysis of the 71cR sequence revealed that it has a domain in the DUF1348 family,

similar to limonene-1,2-epoxide hydrolase, SnoaL2 and SnoaL1 domain families. Proteins with all three domain families are involved in modification of toxic molecules. We hypothesize that 71cR may provide resistance by modification of cercosporin into a less toxic form.

1. Introduction

Cercospora species are fungal plant pathogens causing leaf spot and blight diseases on many major crops including corn, soybean, sugar beet, and coffee. *Cercospora* species cause significant losses in many countries world-wide, and control is strongly depending on usage of pesticides. Most *Cercospora* species produce a photoactivated perylenequinone toxin called cercosporin, which is a virulence factor (Daub and Ehrenshaft 2000). Mutants deficient in cercosporin production are much less virulent on their host plants, thus engineering cercosporin-resistant crop plants with cercosporin resistance genes may be an ecologically friendly method for controlling these damaging diseases.

Cercosporin is very toxic due to its mode of action. Cercosporin is converted into an energetically activated triplet state by absorbing light energy (Daub et al., 2013) . Photoactivated cercosporin reacts with oxygen, generating reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) and superoxide ($O_2^{-\cdot}$). Production of ROS leads to peroxidation of cell membrane lipids in host plants and can also damage nucleic acids, proteins and lipids in the target cells (Daub and Ehrenshaft 2000).

Because cercosporin has broad-spectrum toxicity due to its production of ROS, research has focused on identifying autoresistance genes in *Cercospora* species which can be transformed into crops to engineer cercosporin resistance (Daub et al., 2010). Characterization of *C. nicotianae* mutants selected for sensitivity to cercosporin (Jenns and Daub 1995; Jenns et al., 1995) led to the discovery of the zinc cluster transcription factor *CRG*1 (*c*ercosporin *r*esistance *g*ene 1) (Chung et al., 2003) required for autoresistance to

cercosporin. To identify putative resistance genes, a subtractive cDNA library was generated between the *C. nicotianae* wild type and a *crg1* mutant. From the library 185 differentially regulated expressed sequence tags (ESTs) were found that are candidates as possible resistance genes. The ESTs were classified into functional categories based on their homology to known sequences. These functional categories include ones known to be involved in cercosporin resistance including reductases (Daub et al., 1992), antioxidants and quenchers of ROS (Ehrenshaft et al., 1999), and membrane transporters (Amnuaykanjanasin and Daub 2009; Callahan et al., 1999; Choquer et al., 2007). Several of the library genes have been characterized for their role in cercosporin resistance. For example, two genes encoding transporters in the library, ATR1 and CFP, were characterized to have an important role in resistance (Amnuaykanjanasin and Daub 2009; Callahan et al., 1999); disruption of either of these genes caused the disruptant strains to be sensitive to cercosporin. CFP transformants of tobacco were not reduced in lesion numbers after inoculation with *C. nicotianae* but the size of lesions were significantly reduced, suggesting that CFP might be a useful gene for engineering plants for cercosporin resistance (Upchurch et al., 2005). In the case of ROS quenchers, vitamin B6 was shown to quench $^1O_2$ and be involved in defense against cercosporin (Bilski et al., 2000; Ehrenshaft et al., 1999). Studies to engineer tobacco to constitutively express *C. nicotianae* B6 biosynthetic genes (PDX1 and PDX2), however, resulted in no statistically significant increase in levels of the B6 vitamers, due to heavy regulation of this gene in plants (Herrero and Daub 2007).

The cercosporin biosynthetic cluster has also been the focus of studies for finding candidate cercosporin resistance genes (Chen et al., 2007). The CTB (*c*ercosporin *t*oxin *b*iosynthesis) cluster contains eight genes, two of which (CTB2 and CTB5) were also recovered in the subtractive library. The cluster includes a polyketide synthase (CTB1), methyltransferases (CTB2 and CTB3), oxidoreductases (CTB5, CTB6 and CTB7), a membrane transporter (CTB4) and a transcription factor (CTB8). Throughout the studies to test the role of each CTB gene products, increased sensitivity of the *ctb* mutants was not documented (Daub et al., 2013), a possible role of *CTB* genes in cercosporin resistance is not yet known.

In the library, apart from the genes in known functional categories, genes encoding hypothetical proteins whose functions are not yet characterized were also found. As cellular resistance to singlet oxygen is not well understood, we were interested in determining if any of these hypothetical proteins play a role in cercosporin resistance. The purpose of this paper was to identify and characterize the subtractive library genes encoding hypothetical proteins and assess their putative role in cercosporin resistance.

2. Materials and Methods

2.1 Fungal strains, culture conditions, and plasmids

All *Cercospora nicotianae* strains including the wild type (wt) strain ATCC18366 were maintained on potato dextrose agar (PDA; Difco, Sparks, MD) as previously described

(Jenns and Daub 1995). All *Neurospora crassa* strains including the wt strain ORS-6a (Fungal Genetics Stock Center) were maintained on Vogel's medium (Vogel 1956).

Cloning, DNA plasmid isolation, restriction enzyme analysis, and ligation used standard molecular techniques (Deininger 1990). Standard PCR was performed using High Fidelity Taq polymerase (OneTaq® DNA Polymerase, New England Biosciences) and gene-specific primers (Table 1). The plasmids pGEM-T Easy (Promega, Madison, WI) or pCB1636 (Sweigard et al., 1997), which harbors the *hph* gene encoding for hygromycin B phosphotransferase, were used as recipient vectors for construction of recombinant plasmids for cloning, sequencing and/or fungal transformation. *Escherichia coli* strain DH5-α was used to maintain all plasmids.

2.2 Domains of hypothetical proteins in the library

The EST sequences recovered in the subtractive library between *C. nicotianae* wild type and the *crg1* mutant (Herrero et al., 2007) were blasted against the *Cercospora zeae-maydis* genome database (http://www.jgi.doe.gov/) to obtain full-length sequences. The *C. zeae-maydis* sequences were used to find conserved domains using the Conserved Domain Database by the US National Center for Biotechnology Information (NCBI).

2.3 Gene expression under conditions of cercosporin toxicity

CnATR1 (*atr1*) disrupted *C. nicotianae* mutant (Amnuaykanjanasin and Daub, 2009) and two 71cR disrupted *C. nicotianae* mutants (#16 and 18) were used to quantify gene

expression under conditions of cercosporin toxicity. Homogenized mycelial plugs of *atr1* and *71cR* disruptants were grown in 100 ml of PDB on a shaker (250 rpm) at room temperature for 3 days under dark conditions. Cultures were then treated with either 25 μM cercosporin or with 1.25% acetone (used to solubilize cercosporin). After cercosporin or acetone treatment, cultures were incubated under high light intensities (55-65 μE.m-2sec-1) for 1 or 3 hours before harvesting mycelia for RNA extraction and quantitative RT-PCR (q-RTPCR) analysis.

Total RNA was extracted from lyophilized and homogenized mycelia with TRI-REAGENT (Sigma-Aldrich, St Louis, MO) following the manufacturer's protocol. Samples were treated with DNAse (Turbo DNA-free^{TM} Kit [Ambion, Austin, TX]).

2.4 Quantitative RT-PCR analysis

Synthesis of cDNA was carried out using Taqman Reverse Transcription Reagents (Applied Biosystems, Somerville, MA) according to the manufacturer's protocol, and a SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used for q-RTPCR reactions. The primers used to amplify each gene are shown in Table 1, and the cycling parameters for cDNA synthesis and q-RTPCR reactions were those previously used by Herrero et al (Herrero et al., 2007). Single amplicons were identified using a melting point analysis protocol (60–90 ^{0}C every 0.5 ^{0}C for 1 second). The q-RTPCR reactions were performed in triplicate, and negative controls included: i) the use of RNA as a template to check for gDNA contamination in samples, and ii) a water control. Each sample was

normalized against the *C. nicotianae*-specific actin control, and fold-change relative to no-cercosporin (acetone control) was calculated according to the $2^{-\Delta\Delta}C_T$ method (Livak and Schmittgen 2001; Schmittgen et al., 2000; Winer 1999).

2.5 Sequencing of full length hypothetical genes and cloning

Two different methods were performed to clone and sequence the full-length *C. nicotianae* genomic sequences of the two hypothetical protein genes, *71cR* and *24cF*, based on their partial EST sequences (Herrero et al., 2007). In the first method, primers specific to homologs from the closely related maize pathogen *Cercospora zeae-maydis* (http://www.jgi.doe.gov/) were designed to amplify and sequence full-length copies of *C. nicotianae* genes. The second method utilized inverse PCR to recover sequence information of the *C. nicotianae* non-coding regions (Keim et al., 2004). Intronic regions in *C. nicotianae* were identified from genomic sequences in *C. zeae maydis*, and Genescan (Burge 1998) was used to confirm the locations of introns.

The PCR amplicons were ligated into pGEM-T Easy (Promega, Madison, WI) and sent to Eton Biosciences Inc. (San Diego, CA) for DNA sequencing. Nucleotide and amino acid sequences of *71cR* and *24cF* are in the GenBank database with the accession numbers KJ126714 and KJ126715, respectively.

2.6 Expression of hypothetical genes in N. crassa

The fungal transformation plasmid pTxA-1 (Amnuaykanjanasin and Daub 2009) containing the *hph* resistance gene under the control of a *TrpC* promoter (HygR cassette) was used to insert the two hypothetical protein genes, 71cR and 24cF, under the control of the fungal constitutive promoter *ToxA*. A full-length copy of 71cR and 24cF were amplified, respectively, using primers 71cR-F-EcoRI and 71cR-R-HindIII (containing *EcoRI* and *HindIII* sites, respectively) for 71cR amplification and 24cF-F-EcoRI and 24cF-R-HindIII (containing *EcoRI* and *HindIII* sites, respectively) for 24cF amplification (Table 1-B). These PCR fragments were digested with *EcoRI* and *HindIII*, and ligated to the pTxA-1 that was digested with the same restriction enzymes. The resulting plasmids, pTxA -71cR and pTxA -24cF, were sequenced to confirm the presence of an intact *71cR or 24cF* sequence (Fig. 1).

The two plasmids were transformed into *N. crassa* protoplasts using polyethylene glycol (PEG)-mediated transformation. Protoplast generation and transformation was carried out as previously described with some modifications (Vollmer and Yanofsky 1986). The cell-wall lysing mix consisted of 1.5% (m/v) of Sigma-Aldrich's lysing enzymes and 1.5% (v/v) of β-glucuronidase (Sigma-Aldrich, St. Louis, MO) in 100ml of 1M sorbitol. For transformation, a total of 2 X 10^7 protoplasts were incubated with 10 µg of plasmid DNA and regenerated in the presence of 100 µg/ml hyg. Single hyg-resistant colonies that formed after 2-3 days were transferred to new plates and were continuously maintained under selection (100 µg/ml hyg) for subsequent manipulations. Transformation and presence of *71cR* or *24cF* was confirmed in hyg-resistant colonies by PCR screening using gene specific primers

(ToxA-Rev1 and 71cR-R, ToxA-Rev1 and 24cF-R, HYG-R and 71cR-F, HYG-R and 24cF-F) (Table 1-C). An NaOH DNA extraction method (Wang et al., 1993) was used to extract gDNA for PCR analysis.

2.7 Screening of N. crassa 71cR and 24cF transformants

N. crassa 71cR and *24cF* transformants confirmed by PCR analysis were tested for cercosporin resistance on split petri plates. Each half-plate contained Vogel's medium supplemented with either 10 µM cercosporin or 0.5% acetone. Mycelial plugs (6 mm) of the transformants or wild type *N. crassa* were inoculated onto each side of the split plates, and incubated at room temperature under continuous fluorescent light (55-65 $\mu E.m^{-2}sec^{-1}$). Radial growth of colonies was measured 21 hours after inoculation, and % growth on cercosporin was calculated relative to acetone controls on the same plate. Each transformant was replicated five times, and the experiment was repeated two times. For gene expression analysis total RNA was extracted from lyophilized and homogenized mycelia of 4 randomly chosen transformants, and q-RTPCR analysis was conducted as described in sections *2.3* and *2.4* using primers shown in Table 1-A. Each sample was normalized against the *N. crassa*-specific tubulin control.

2.8 Targeted gene disruption in C. nicotianae

The *71cR* gene was disrupted in wild type *C. nicotianae* using a split-marker recombination technique modified as previously described (You et al., 2009). The 5' (from

1517 bp upstream to 5 bp downstream of start codon) and 3' (from 443bp upstream to 43 bp downstream of stop codon) *71cR* sequences were amplified from *C. nicotianae* gDNA by PCR with *71cR*-specific primers containing restriction enzyme linkers (Table 1-D). The two primer sets used to amplify the 5' and 3' ends of *71cR* were: 71cR-5'-F-*Apa*I, 71cR-5'-R-*Apa*I and 71cR-3'-F-*SacI*, 71cR-3'-R-*Sac*I. The PCR products were digested with the appropriate restriction enzymes (71cR-5' digested with *Apa*I; 71cR-3' digested with *Sac*I) for cloning into the receptor plasmid pCB1636 (Sweigard et al., 1997). Plasmid pCB1636 was first digested with *Apa*I and ligated with *Apa*I digested *71cR*-5'. Recombinant plasmids isolated from this step were selected based on the insert's correct orientation, and subsequently digested with *Sac*I and ligated with *SacI* digested *71cR*-3' to obtain a 71cR disruption construct (Fig. 2). Using this construct as a template (Fig. 2), two different overlapping PCR fragments were amplified using primers specific to the *71cR* sequence and the HygR cassette (split marker 1: 71cR-5'-F and HYG-split 5A; split marker 2: 71cR-3'-R and HYG-split 3S) (Table 1-D). Each split marker PCR fragment was sequenced to confirm their identity.

Protoplasts of *C. nicotianae* were isolated and transformed as previously described (Ehrenshaft et al., 1995) except that cultures were grown in PDB. For transformation, $1x10^7$ protoplasts were incubated with 10 µg of each of the two split marker PCR fragments in the presence of spermidine (3mM) and heparin (0.5 mg/ml). Transformants were originally selected in medium containing 125 µg/ml of hyg. Hyg-resistant colonies were transferred to PDA amended with 125 µg/ml hyg, and were transferred a minimum of 5 transfers to fresh

selection plates to ensure stability of transformation. *71cR* disruption was confirmed by PCR analysis using primer sequences shown in Table 1-E.

2.9 Screening of 71cR disruption mutants for cercosporin sensitivity and production

Transformants confirmed to have a disrupted copy of *71cR* were screened for cercosporin sensitivity as described above for *N. crassa* sensitivity tests, except that radial growth of colonies was measured 3 days after inoculation on PDA supplemented with either 10 µM cercosporin or 0.5% acetone. Data shown are the result of two independent experiments with 12 replications each. For assaying cercosporin production, disruptants were grown on PDA under constant light (14 µE m^{-2} s^{-1}) at 25 ^{0}C for seven days. Cercosporin was extracted with 5N KOH and concentrations were measured at 480 nm as previously described (Jenns et al., 1989).

2.10 Characterization of 71cR

The Conserved Domain Database in the NCBI website (Marchler-Bauer et al., 2011) and Pfam (Punta et al., 2014), were used to find functional domains of the 71cR protein. The amino acid sequence of 71cR was subject to BLASTp (NCBI) searches to find the homologs in other organisms. The selected homologs of 71cR from BLASTp search along with the proteins with similar functional domains were used to construct a tree using CLUSTALW. The secondary structure of the amino acid sequence of 71cR was predicted using the PredictProtein server (Rost et al., 2004), Raptor software (Källberg et al., 2012),

Phyre2 (Kelley and Sternberg 2009) and PSIPRED (Buchan et al., 2013). The tertiary structure of the71cR amino acid sequence was checked using Raptor and Phyre2. PredictProtein was used for the solvent accessibility prediction, transmembrane helices and disulphide bridges.

To test the activity of the 71cR protein to modify cercosporin, 200 mg of freshly growing tissue of four 71cR transformants (#343, 344, 349, 350) were collected and grown in 5ml of Vogels medium for 5 days in dark at room temperature. Cercosporin was added to final concentration of 10 uM and incubated in the dark for 16hours. Mycelium was removed and the medium was analyzed by scanning in the range of 250 nm to 600 nm using a spectrophotometer. For control, 0.5% acetone instead of cercosporin was used. Two cercosporin-resistant 71cR transformants (#349 and 350) and wild type *N. crassa* were also observed using confocal microcospy. Cultures were grown and treated with cercosporin as described above. Mycelium was filtered and analyzed by examining the fluorescence spectrum using a Zeiss LSM 710 confocal microscope.

3. Results

3.1 Identification of the domains of genes in the library encoding hypothetical proteins

When the EST sequences of 21 hypothetical genes from the subtractive library (Herrero et al., 2007) were blasted against the *C. zeae-maydis* genome database each of them aligned with only one gene in *C. zeae-maydis* genome. A search on the Conserved Domain Database by NCBI using these *C. zeae-maydis* sequences revealed functional

domains for each of the hypothetical proteins, except two. Results are shown in Table 2. Most of the domains found are associated with membranes or cell walls such as phospholipid methyltransferase domain in 24cR and 84cR, cell wall mannosidase domain found in 49cR, chitin binding domain in 11sf, nucleotide sugar transporter domain in 15cF, membrane curvature sensing domain in 200sF and retinal pigment epithelial membrane protein in 56cR. Membrane binding domains (11sf and 15cF) and the membrane curvature sensing domain (200sF) may have a role in resistance by transporting cercosporin out of the cells. Other proteins contained DNA binding domains (77sR3 and 214sR3) that may have regulatory function. Reduction of cercosporin to a non-toxic form is a known resistance mechanism (Daub et al., 1992), and domains of two of the homologs (140sR5 and 55cR) have cytochrome domain, which might function in reduction. Another domain included a pyridoxal phosphate (PLP)-dependent enzyme; PLP is known to involve in singlet oxygen resistance (Ehrenshaft et al., 1999). A domain common in photoreceptor cells (207sF) was found, and is of interest due to cercosporin's photoactivity. Glutathione-dependent formaldehyde-activating enzyme domain (21cF) and pyrrolidone carboxyl peptidase (88sR) are associated with glutathione-dependent systems that are known for singlet oxygen resistance (Carmel-Harel and Storz 2000). Domains that catalyze the cleavage of HMG-CoA (84sF) or ring closure steps in the biosynthesis of polyketide antibiotics (71cR) might also have a novel role in modifying cercosporin. Some domains, such as domains characteristic of transposons (35cF) and a mating loci (32sR and 56cR) do not relate with any known resistance mechanisms.

3.2 Expression of hypothetical genes under conditions of cercosporin toxicity

In order to define a possible role in cercosporin resistance, the 21 genes encoding hypothetical proteins were assayed for changes in expression under conditions of cercosporin toxicity. The cercosporin-sensitive *C. nicotianae atr1* mutant, deficient in the ATR1 ABC transporter involved in cercosporin resistance (Amnuaykanjanasin and Daub 2009), was treated with cercosporin under high-light conditions to induce toxicity, and gene expression was assayed by q-RTPCR. Genes *24cF* and *71cR* were upregulated 9.4 and 8.7 fold, respectively, three hours after cercosporin toxicity was induced (Fig. 3). None of the other 19 genes were upregulated under these conditions. Based on these gene expression results, *24cF* and *71cR* were chosen for further characterization.

3.3 Ability of 24cF and 71cR to impart resistance in N. crassa

The cercosporin-sensitive fungus *N. crassa* was transformed with *24cF* and *71cR* to test the ability of these genes to impart cercosporin resistance. Hyg-resistant colonies after transformation were screened for the presence of the genes by PCR. Out of a total of 84 and 93 hyg-resistant colonies of *71cR* and *24cF* transformants, 60 and 12, respectively, were confirmed to contain the intact transgene by PCR screening (data not shown). All 12 of the *24cF* and 27 of the *71cR* PCR-positive strains were assayed for resistance to cercosporin by measuring radial growth on cercosporin-containing medium relative to growth on control medium. Cercosporin at 10 µM inhibits radial growth of *N. crassa,* resulting in approximately 30% of the radial growth on medium lacking cercosporin (Fig. 4). Of the

randomly chosen 27 PCR-positive *71cR* transformants tested, eleven were found to be significantly more resistant to cercosporin than wild type ($P < 0.05$). However of the 12 PCR-positive *24cF* transformants tested, none of them was found to be significantly more resistant to cercosporin than wild type ($P < 0.05$).

Expression of the transgenes was assayed in selected transformants screened for resistance. Quantiative RTPCR analysis of *71cR* expression in the two resistant *71cR* transformants assayed (#349 and 350) showed high levels of expression (between 1000 to 10000 fold), whereas the two non-resistant 71cR transformants assayed (#343 and 344) showed either no expression or 100-fold increase (Fig. 5). Thus *71cR* expression correlated with cercosporin resistance. For *24cF*, high levels of expression were found in all four transformants tested (between 1000-10000 fold) (Fig.5). From these results we concluded that the 71cR protein can provide resistance to cercosporin toxicity, but that 24cF cannot.

3.4 Disruption of 71cR in C. nicotianae and phenotype of disruption mutants

A split marker strategy was used to generate *C. nicotianae* disruption mutants by homologous recombination. A total of 14 hyg-resistant colonies were screened to confirm *71cR* disruption by PCR analysis using primer sequences shown in Table 1-E. Primers 71cR-Rev3 and 71cR-inv5' that span the hyg resistance cassette were used to screen the disruptants; with these primers the wt band is 400bp and the band resulting from disruption is 2 kb (Fig. 6-A). A total of 12 transformants were confirmed as being disrupted for *71cR.* One had an ectopic integration of the split markers (#23) and one lacked evidence for

integration of the split marker (#15) (Fig. 6-B). All 12 of the disruptants and the strain with ectopic integration of the split markers (#23) were then screened using a 71cR locus-specific primer outside the split marker sequence (71cR-Forw 10) and a primer specific to hyg cassette sequence (Hyg-F2). Presence of the 1.7kb fragment confirmed the correct location of split marker integration for all 12 disruptants (Fig. 6-A). No band was seen on amplification of strain #23 (transformant with ectopic integration of the split markers) (Fig. 6-C).

Ten *71cR*-disruption strains were tested for cercosporin sensitivity by growing them on cercosporin-containing medium in the light. Wild type and a transformed but non-disrupted strain (#23) were used as controls. Results are shown in Fig.7. There was no statistically significant difference in cercosporin resistance between the wild type, the non-disrupted transformant (#23), or *71cR* disruptants in radial growth on cercosporin.

As other resistance proteins such as ATR1 and CFP have shown a dual role in both cercosporin resistance and production, *71cR* disruptants were also assayed for cercosporin production (Fig. 8) as compared to wild type and the non-disrupted transformant #23. Cercosporin production varied between the different strains, with the non-disrupted transformant #23 having the least production. None of the *71cR* disruptants produced significantly less cercosporin than wild type.

3.5 Expression of transporters and cercosporin biosynthetic genes in 71cR disrupted mutants under cercosporin toxicity

Expression of *71cR* in *N. crassa* demonstrated that 71cR can provide cercosporin resistance, however, *71cr* mutants of *C. nicotianae* were not more sensitive to cercosporin or produce less cercosporin than wild type. We thus assayed for expression of additional library genes previously shown to impart cercosporin resistance (*CnATR1*, *CnATR2* and *CnCFP*) as well as genes in the cercosporin biosynthetic cluster (*CTB* genes) to determine if they are up-regulated in the *71cR* mutant background (Fig. 9). Two 71cR disruptants (disruptant #16 and 18) were tested. Expression of *CnATR1* and *CnATR2* was not increased significantly in either of the disruptants. Expression of *CnATR1* was 1 fold at 1 and 3 hours in disruptants #16 and 18 except for 4 fold difference in 1 hour in disruptant #18. Expression of *CnATR2* was 5.5 and 2 fold, respectively, at 1 hour, and 4 and 2 fold, respectively, at 3 hours in disruptants #16 and 18. By contrast, *CnCFP* expression was strongly increased: 285 and 20 fold, respectively, at 1 hour, and 40- and 3- fold, respectively, at 3 hours in disruptants #16 and 18, respectively. These results suggest that the lack of sensitivity of the *71cR* mutant may be due to compensation by over-expression of *CnCFP*.

The subtractive library of putative cercosporin resistance genes also includes two genes from the cercosporin biosynthetic pathway: CTB2, encoding an *O*-methyltransferase, and CTB5, encoding an O_2, FAD/FMN-dependent oxidoreductase (Chen et al., 2007; Herrero et al., 2007). Thus we assayed expression of all genes in the cercosporin biosynthetic cluster in the *71cr* mutants under cercosporin toxicity. Two genes were

significantly up-regulated: *CTB5* (found in the library) as well as *CTB7*, encoding a second FAD/FMN-dependent oxidoreductase (Fig. 9). Expression of *CTB2* and the other biosynthetic genes including the CTB4 transporter were not altered. Up-regulation of *CTB5* and *CTB7* was less than that of CFP in both 71cR disruptant induced with cercosporin toxicity. *CTB5* was induced 10- and 6-fold in disruptants #16 and 18, respectively, at 1 hour, and 6- and 3-fold, respectively, at 3 hours in disruptants #16 and 18, respectively. For *CTB7*, fold-increase in disruptants #16 and 18 was 28- and 10-fold, respectively, at 1 hour and 6- and 7-fold, respectively, at 3 hours.

3.6 Bioinformatics analysis of 71cR

71cR is an intronless 457 bp sequence. We blasted the protein sequence against the NCBI protein database and found it to have high homology to hypothetical proteins from *Talaromyces stipitatus* (XP_002479782.1, 70% identical in 153 amino acids), *Coniosporium apollinis Sterfl.* (EON66350, 70% identical in 151 amino acids), *Bipolaris sorokiniana* (EMD69219.1, 68% identical in 151 amino acids), and *Pyrenophora teres* (XP_003300671.1, 68% identical in 151 amino acids). None of the 100 closest relatives to the 71cR protein from different fungal strains found by the BLASTp search (the least similar one, having 65% identical in 133 amino acids) have been characterized for function.

A search on the Conserved Domain Database in the NCBI website (Marchler-Bauer et al., 2011) showed a 131 amino acid portion of the 71cR protein aligns with a Nuclear Transport Factor 2 (NTF2-like) super-family domain. This result was confirmed with using

Pfam software (Punta et al., 2014), and the sequence was further identified as being in the DUF1348 family, one of the 24 families in the NTF2-like super-family. Of the 24 families in the NTF2-like super-family, 16 have known functions. The remaining eight families, including DUF1348, are not characterized (Punta et al., 2014).

As all of the close relatives of 71cR were hypothetical proteins, relationships between 71cR protein and proteins with known functions were analyzed in an attempt to identify a putative function for 71cR. A phylogenetic tree was generated by ClustelW2, using the amino acid sequences of 71cR, the *Talaromyces stipitatus* (XP_002479782.1) protein (closest homolog), and a bacterial protein (Protein Pfl3262 from *Pseudomonas fluorescens*) in the same domain family (DUF1348), along with 16 proteins from different organisms with known domains representing each of the characterized domains within the NTF2-like super family. This analysis (Fig. 10) showed that the 71cR protein from *C. nicotianae* forms a unique branch with the hypothetical protein from *Talaromyces stipitatus* and these two proteins cluster with the bacterial protein with the DUF1348 family domain (gi-118138474). This group of proteins are then grouped with a protein in the limonene-1,2-epoxide hydrolase domain family and then to proteins in the SnoaL2 and SnoaL1 domain families.

The secondary structure of 71cR was predicted using several different software programs. The results from PredictProtein (Rost et al., 2004) and Raptor software (Källberg et al., 2012) showed 6 antiparallel beta strands, two of them being relatively small, and 3 alpha helices. Phyre2 (Kelley and Sternberg 2009) predicted 6 beta strands and 6 alpha helices. The PSIPRED server (Buchan et al., 2013) predicted 4 beta strands and 4 alpha

helices. The prediction of locations of each of the beta strands, and alpha helices on the 71cR sequence were similar in all four programs. Based on all four software programs, the approximate locations of the main alpha helix structures are from 10th to 24th, 51st to 65th, 68th to 74th and 75th to 80th amino acids; The main beta sheets are from 81st to 92nd, 98th to 103th, 109th to 111th and 130th to 133th amino acids (Fig. 11).

The tertiary structure of the 71cR amino acid sequence was analyzed using Raptor and Phyre2. The predicted structure of 71cR protein is a half barrel structure with 7 beta sheets enclosing the alpha helix (Fig. 12). The alpha helix that is buried in the protein is composed of non-acidic amino acids. The two other alpha helices are located at the tip of the structure (Fig. 12). The predicted modal states that this protein forms a dimer to complete the barrel structure.

The Solvent Accessibility prediction using PredictProtein shows 3 possible polynucleotide binding regions and 11 protein binding regions (Ofran and Rost 2007) (Fig. 13). No transmembrane helices or disulphide bridges were found. As disulfide bridges are mainly found in secretory proteins, lysosomal proteins and extraspace of the membranes (Sevier and Kaise 2002) this protein was predicted as localized mainly in the cytosol.

3.7 Analysis of 71cR effect on cercosporin

Through the bioinformatic analysis, the 71cR amino acid sequence aligned with an uncharacterized domain, DUF1348, which is closely related to the limonene-1,2-epoxide hydrolase family and Snoal1 and SnoaL2 families. Limonene-1,2-epoxide hydrolases

catalyses the hydrolysis of toxic epoxides (Arand et al., 2003). SnoaL2 catalyzes the hydroxylation of the polyketide, nogalamycin (Beinker et al., 2006). Snoal1 is also involved in modification of nogalamycin by catalyzing aldol condensation (Sultana et. al., 2004). To test if 71cR is catalyzing a reaction to modify the structure of cercosporin, we assayed for changes in cercosporin in media and inside the cells. Resistant *N. crassa* 71cR transformants (#349 and 350), non-resistant *N. crassa* 71cR transformants (#343 and 344) and wt *N. crassa* were grown in liquid medium containing cercosporin, and the absorption spectrum of cercosporin in the medium was measured using a spectrophotometer. No differences were seen between the isolates (data not shown). In the second method, the hyphae of wt *N. crassa* and 71cR transformants #349 and 350 were observed using confocal microscopy and the cercosporin fluorescence emission spectrum in the hyphae was measured. There was no detectable difference in the cercosporin emission spectrum of the resistant transformants as compared to wild type *N. crassa* (data not shown).

4. Discussion

The 185 EST sequences recovered from the subtractive library provided a large set of candidate gene products that might have a role in cercosporin resistance (Herrero et al., 2007). Among this list are two transporters, ATR1 and CFP, that have been shown to have a role in resistance (Amnuaykanjanasin and Daub 2009; Callahan et al., 1999). In this paper, we characterized genes in the library encoding hypothetical proteins. To obtain the full length sequences, the EST sequences were blasted against the database of *C. zeae maydis*,

which is a close relative of *C. nicotianae*. Each EST sequence was aligned to one sequence in *C. zeae maydis*. The full length sequences of the *C. zeae maydis* homologs were used to identify functional domains (Table 2). The results revealed diverse functional domains that may be important in resistance.

To test the role of these hypothetical proteins in cercosporin resistance, we assayed for changes in expression when the *C. nicotianae* cercosporin-sensitive *atr1* transporter mutant was exposed to cercosporin toxicity. This mutant is sensitive due to a mutation in the ATR1 transporter involved in transport of cercosporin out of the cell (Amnuaykanjanasin and Daub 2009), thus it is a useful strain for assaying resistance gene expression. Only two of the 21 hypothetical protein-encoding genes, *24cF* and *71cR* were significantly induced. These results led us to further characterize their role in resistance.

To test the ability of these genes to increase cercosporin resistance, the cercosporin-sensitive fungus *N. crassa* was transformed with the complete gene sequences of *71cR* and *24cF* under the control of a constitutive promoter. Under the conditions of our cercosporin-sensitivity assay, growth of wild type *N. crassa* is inhibited by about 70% whereas growth of the resistant *C. nicotianae* is inhibited by about 40%. No significant increase in resistance was seen in any of the *24cF* transformants tested, however significant increases in cercosporin resistance were found in some *71cR* transformants, with inhibition as low as 20%. Resistance of the *71cR* transformants varied, with some showing no increase. This variation might be explained by the random integration of the insert and different levels of transgenic transcripts (Smith et al., 2008). To confirm that the resistance seen in 71cR

transformants was due to the transgene, expression of the *71cR* transgene was tested from randomly picked transformants; two that showed significant resistance and two that did not. The results showed that transformants that showed increased resistance had high expression of the gene (between 10,000-100,000 fold compared to wt expression), whereas the expression of the gene in the transformants that did not have increased resistance were not as high (little detectable expression in #344 and about 100-fold in #343). Thus for 71cR transformants, expression of cercosporin resistance correlated with 71cR gene expression. For 24cF, each of the transformant assayed had high levels of expression without imparting increases in resistance. Thus we concluded that 24cF does not have significant role in cercosporin resistance.

To further characterize the role of 71cR in cercosporin resistance, 71cR was disrupted in wt *C. nicotianae*, and resistance to cercosporin was assayed. The resistance of the disruptants to cercosporin was not significantly different than the wt *C. nicotianae*. The cercosporin production in 71cR disruptants was also compared to wt, and no difference in cercosporin production was observed. We hypothesized that the disruption of 71cR may be compensated for by induction of other resistance genes. We thus assayed two of the 71cR disruptants for expression of known resistance genes and for genes in the cercosporin biosynthetic pathway, when the disruptants were treated with cercosporin. Three genes, *CFP, CTB5 and CTB7*, were found to be induced by cercosporin toxicity. CFP is an MFS transporter previously characterized to have a role in cercosporin resistance (Callahan et. al., 1999). We have also shown that *CFP* is strongly induced by cercosporin toxicity in

cercosporin-sensitive *atr1* and *atr2* mutants of *C. nicotianae* (unpublished results), thus CFP induction may be a general response of *C. nicotianae* against cercosporin toxicity, perhaps by facilitating export of the toxin out of the cells. The two CTB genes encode oxidoreductases found in the cercosporin biosynthetic cluster (Chen et al., 2007; Daub et al., 2013). Oxidoreductase activity has been hypothesized to be involved in cercosporin resistance through reduction of cercosporin (Daub et al., 2013) and these genes are also induced in the cercosporin-sensitive *atr2* background (unpublished results). Mutants for CTB5 and CTB7 have not been shown to be altered in resistance; however, possible compensation by upregulation of other resistance genes in *ctb5* and *ctb7* mutants has not been investigated. Thus it remains possible that these two biosynthetic genes might also be involved in cercosporin resistance and induced as a general stress response against cercosporin.

The ability of 71cR to impart cercosporin resistance in *N. crassa* led us to further characterize this gene. Through the Conserved Domain Database in NCBI website (Marchler-Bauer et al., 2011) and Pfam (Punta et al., 2014), 71cR was shown to have an uncharacterized domain in the DUF1348 family in the NTF2 superfamily. These proteins have alpha helixes and beta sheets. The NTF2 superfamily proteins have cystatin-like folds with alpha helixes packed against coiled antiparallel beta-sheets (Fox et al., 2013; Murzin et al., 1995). The NTF2-like superfamily contains families with a common fold that results in a cone-like shape with a cavity inside. This superfamily includes very different sequences without any common sequence motif (Eberhardt et al., 2013). The NTF2-like superfamily contains both enzymatically active and non-enzymatically active proteins. SnoaL polyketide

cyclase, scytalone dehydratase, limonene-1,2-epoxide hydrolase and δ5-3-ketosteroid isomerase are examples of the enzymatically active group, which are typically intracellular (Eberhardt et al., 2013). Non-enzymatically active NTF2-like protein domains are mainly extracellular. The examples of proteins with non-enzymatically active NTF2-like domains are the C-terminus of calcium/calmodulin-dependent protein kinase II (Griffith et al., 2003), Mba1,a putative ribosome-binding receptor (Ott et al., 2006), proteins involved in DNA transfer during bacterial conjugation (Goessweiner-Mohr et al., 2013), and immunity proteins in the bacterial polymorphic toxin systems (Zhang et al., 2012).

The secondary and tertiary structures of 71cr were predicted using several programs (Buchan et al., 2013; Källberg et al., 2012; Kelley and Sternberg 2009; Rost et al., 2004). Results showed that the protein is likely to have antiparallel beta strands forming a half barrel structure with one of the main alpha helix structures buried inside this half barrel. The protein is predicted to form a dimer to complete the barrel structure. Solvent Accessibility prediction (Rost et al., 2004) shows 3 possible polynucleotide binding regions and 11 protein binding regions. As no transmembrane helices or disulphide bridges were found, this protein is predicted to be localized mainly in cytosol.

In an attempt to characterize 71cR through homology, the amino acid sequence of 71cR was blasted against the NCBI protein database and found to have high homology to hypothetical proteins from diverse fungi, with the sequence of the hypothetical protein in *Talaromyces stipitatus* (XP_002479782.1, 70% identical in 153 amino acids). All of the 71cR homologs identified in this search were hypothetical proteins, thus we conducted a

phylogenetic analysis with 71cR protein and including proteins with NFT2-like superfamily domains. The results confirmed relatedness to the *Talaromyces* sequence as well as to a *Pseudomonas* protein with a DUF1348 domain. These then grouped with proteins with a limonene-1,2-epoxide hydrolase domain and to Snoal domains.

Snoal domain-containing proteins from *Streptomyces nogalater* are polyketide cyclases involved in the modification of the polyketide antibiotic nogalamycin (Sultana et al., 2004). This homology, combined with the predicted cytosolic localization of the protein led us to hypothesize that 71cR may impart resistance by altering the structure of the polyketide cercosporin molecule. Efforts to document an alteration in cercosporin structure in resistant 71cR-expressing *N. crassa* transformants by assay of the cercosporin absorption and emission spectra from growth media and hyphae, however, did not identify any detectable changes. Further research is required to characterize the specific function of the 71cR protein in cercosporin resistance.

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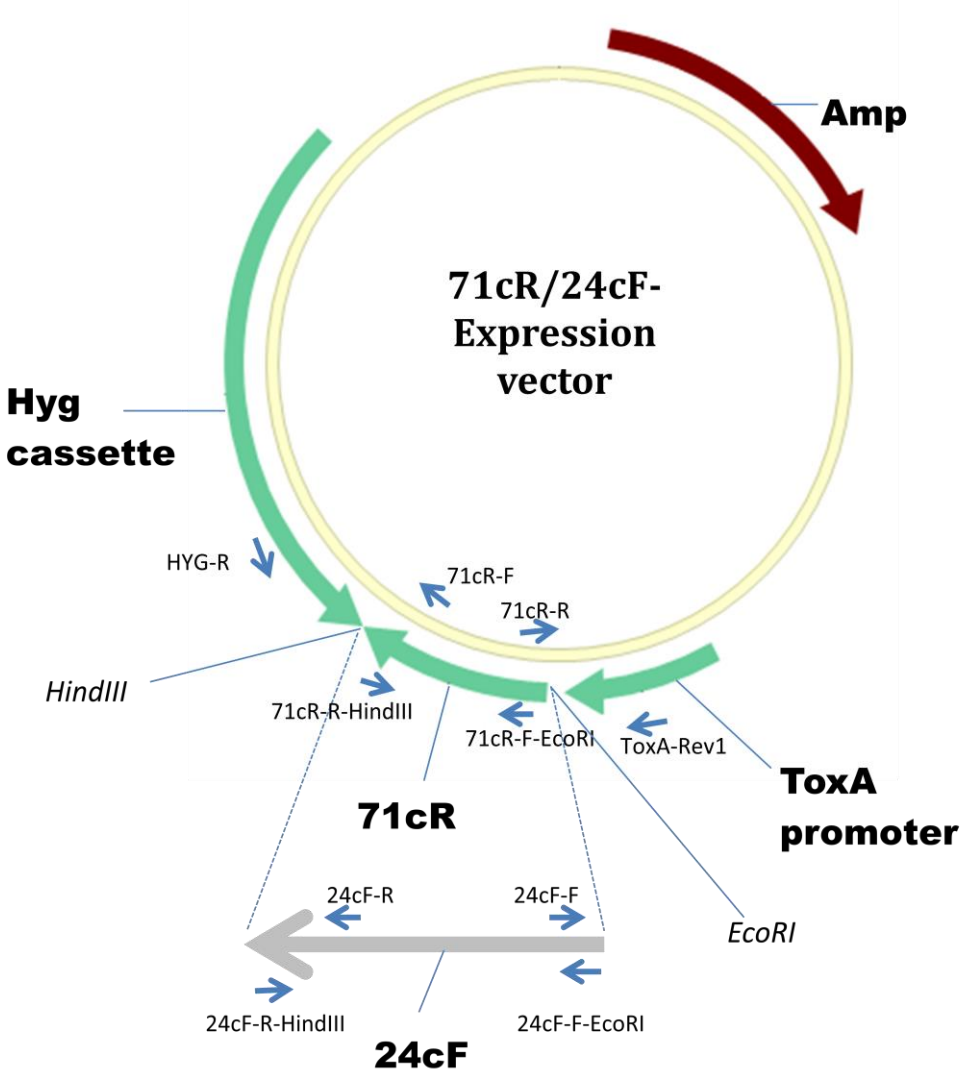

Figure 1. 71cR/24cF- Expression vector. pToxA plasmid was used to clone *71cR* or *24cF* full gene sequences from *C. nicotianae*. pTxA plasmid which has Hyg cassette and the Amp cassette for selection in fungi and *E. coli*, respectively and ToxA promoter from *Pyrenophora tritici-repentis*, was digested with EcoRI and HindIII. Each gene (71cR and 24cF) was amplified using gene specific reverse and forward primers with EcoRI and HindIII restriction enzyme sites incorporated them respectively and ligated into EcoRI and HindIII digested pTxA.

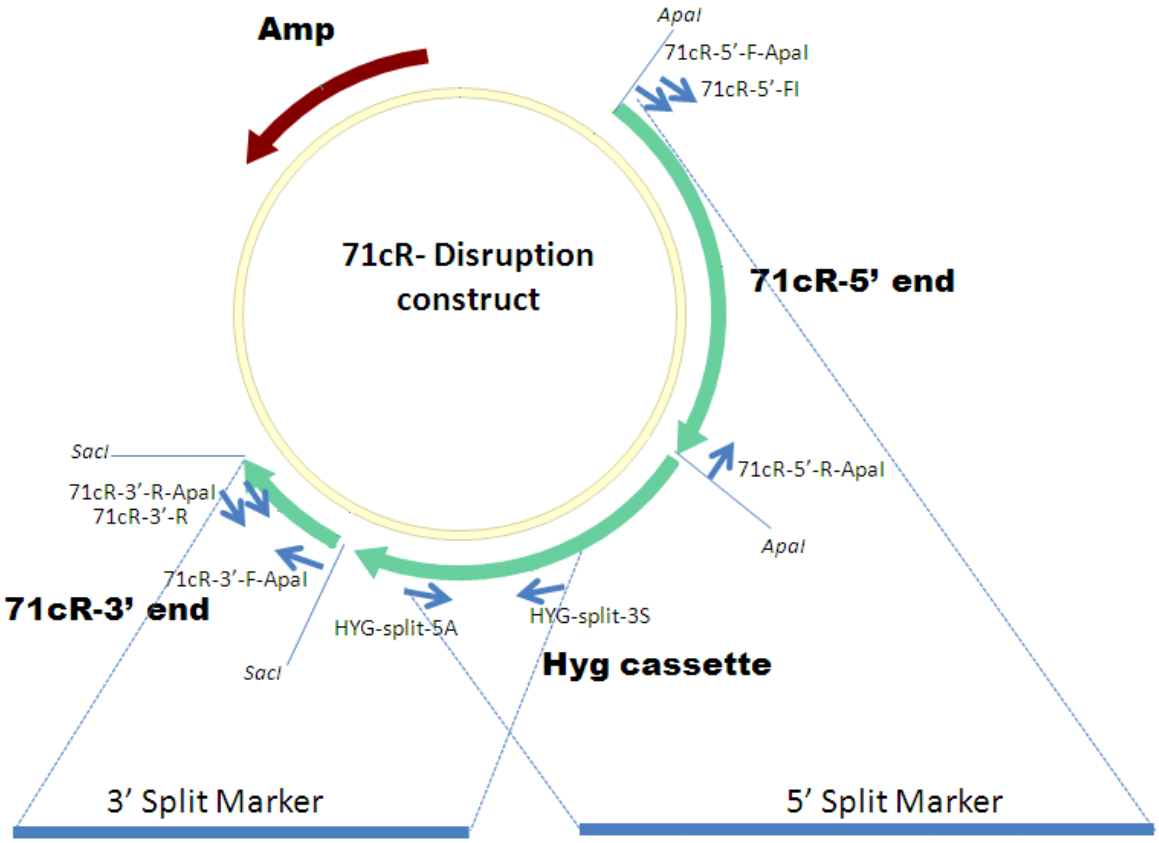

Figure 2. 71cR Disruption construct and split markers. The 5' and 3' ends of the 71cR sequence were ligated onto each side of hyg casette of pCB1636 to construct the 71cR-disruption construct. The split markers were amplified from this disruption construct by using 71cF-5'-F and Hyg-split5A (for 5' split marker) and 71cf-3'-R and Hyg-split3S (for 3' split marker).

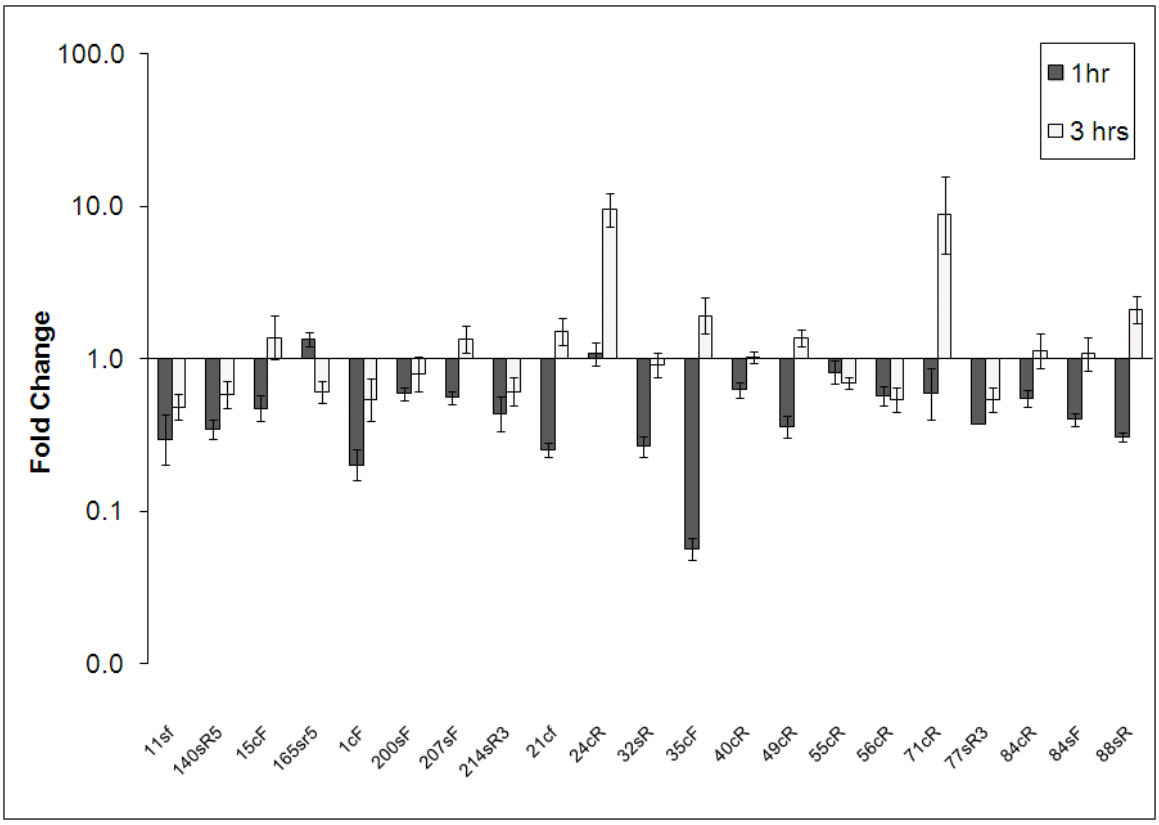

Figure 3. Quantitative RT-PCR analysis of gene expression of hypothetical protein genes in the *C. nicotianae atr1* mutant treated with cercosporin in the light. Each sample was normalized against the actin control, and fold-change relative to no-cercosporin was calculated according to the $2^{(-\Delta\Delta C(T))}$ method (Livak and Schmittgen, 2001). Data represent the mean of two independent experiments. Error bars represent 95% confidence intervals.

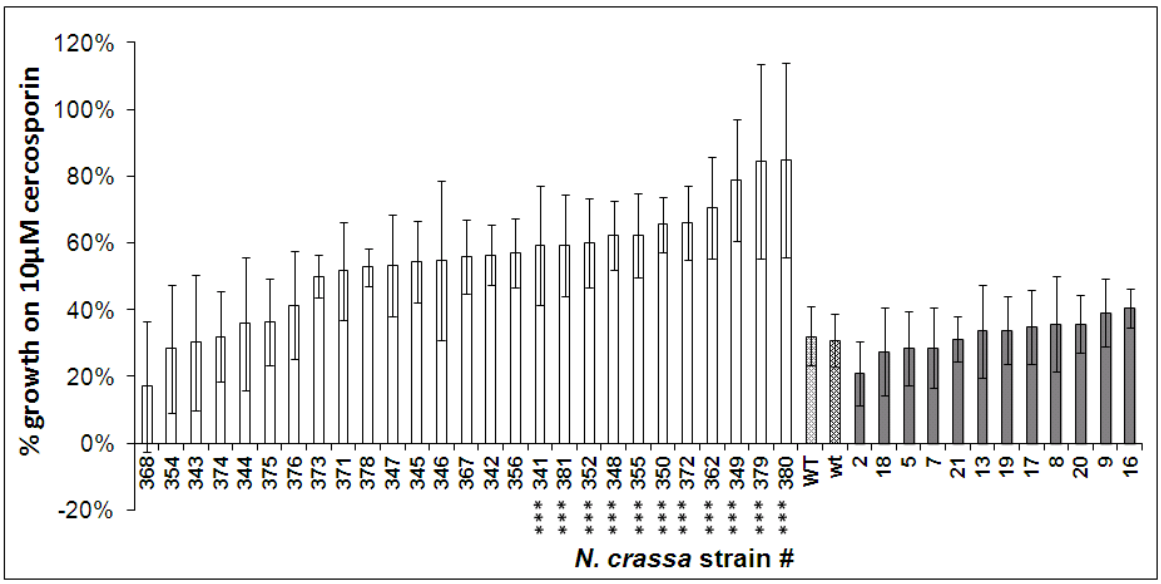

Figure 4. Cercosporin resistance of *Neurospora crassa* 71cR-transformed strains (white bars), wild type *N. crassa* (patterned bars; WT = 71CR control; wt = 24cF control), and 24cF-transformed strains (grey bars). Data are the result of two independent experiments with 5 replications each. Strains marked with *** have significantly greater resistance than wild type ($P < 0.05$). Error bars represent 95% confidence intervals.

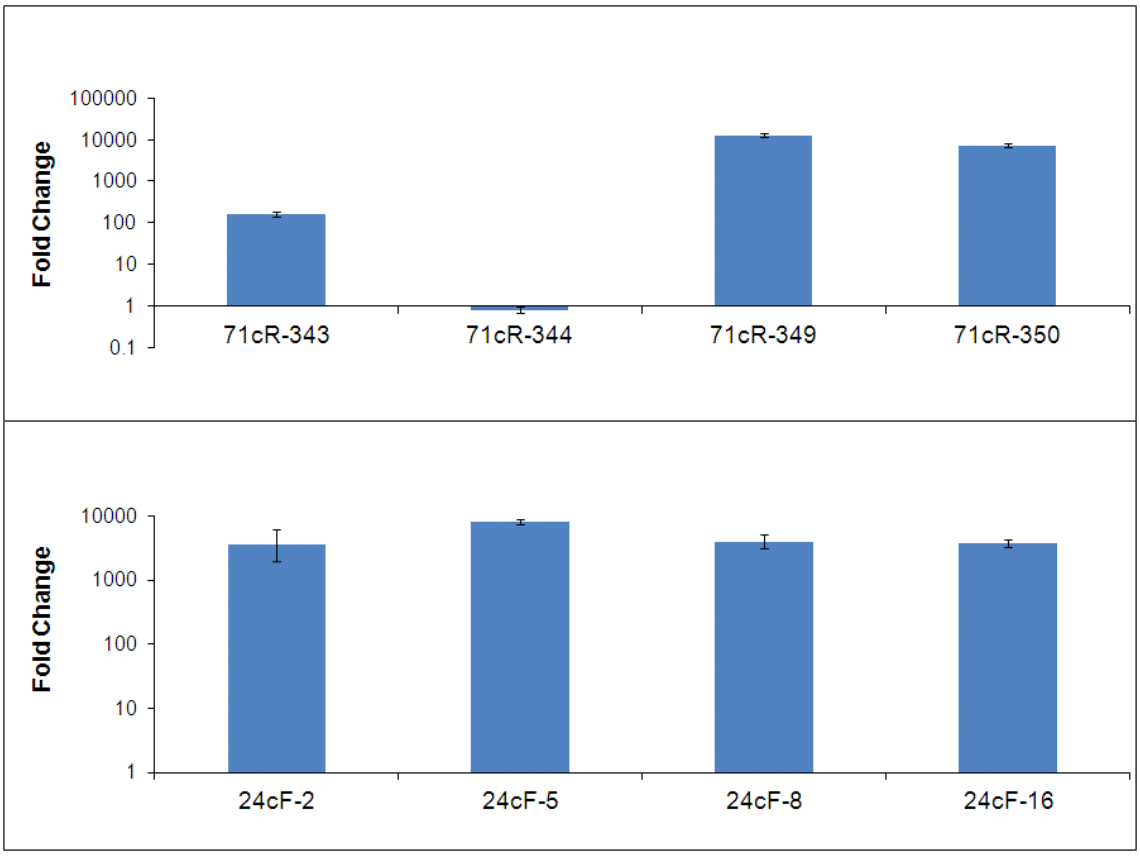

Figure 5. Quantitative RT-PCR analysis of gene expression of *71cR* and *24cF* genes in selected *Neurospora crassa* transformants. Each sample was normalized against the tubulin control, and fold-change relative to wild type *Neurospora crassa* control (no transgene) was calculated according to the $2^{(-\Delta\Delta C(T))}$ method. Error bars represent 95% confidence intervals.

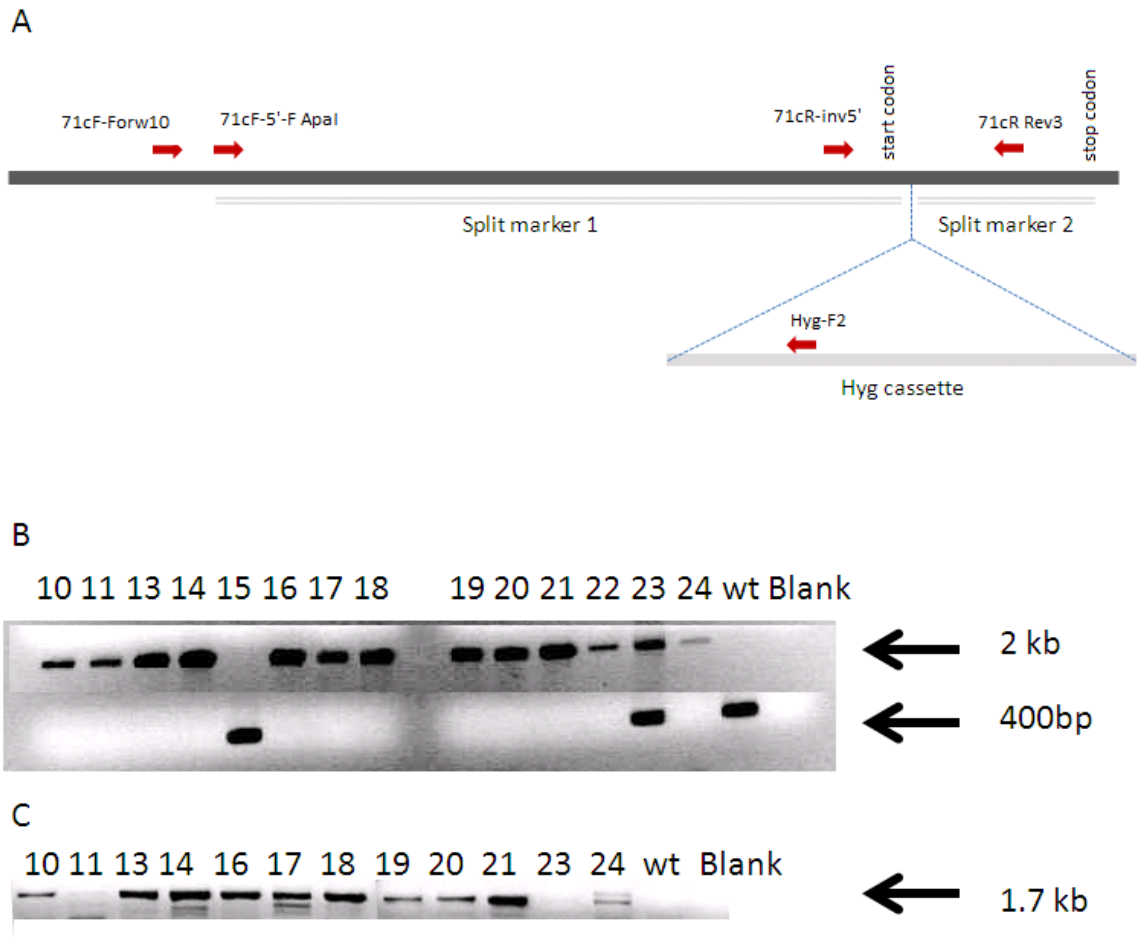

Figure 6. Disruption of the 71cR. **A**. The gDNA of wt *C. nicotianae* is shown as black bar with the start and stop codons of 71cR indicated. In disrupted lines the hyg cassette is predicted to integrate in the location specified by dotted lines. The homologous regions of each split marker are represented as double lines. Location of primers used in PCR screening is also shown. **B**. Gel image of screening of putative 71cR disruptants with primers that spans the hyg cassette integration site (71cR-Rev3 and 71cR-inv5'). Integration results in a 2 kb band (top); wt band is 400bp (indication of non-disruptant genotype). Wild type *C. nicotianae* and transformant #15 showed only the wild type band; transformant #23 has both bands indicating an ectopic integration of the split markers. **C.** Bands represent the 1.7 kb amplification of the 5' integration site by using 71cR locus-specific forward primer outside the split marker 1 sequence (71cR-Forw 10) and a reverse primer specific to the hyg cassette sequence (Hyg-F2).

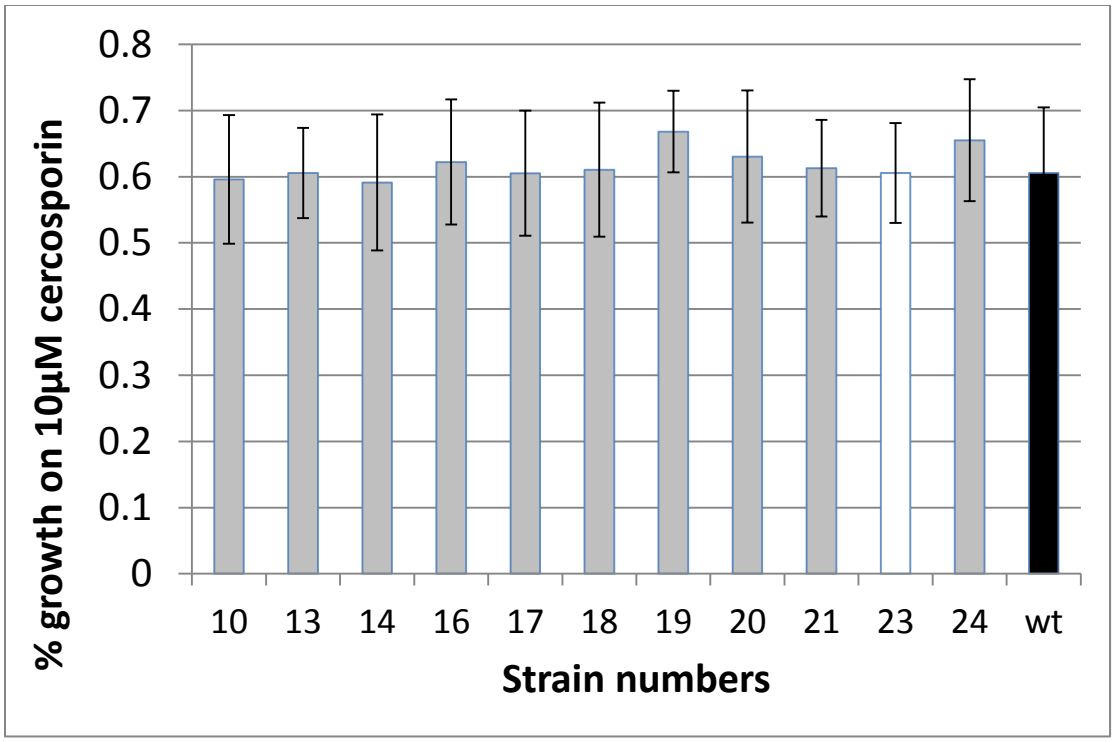

Figure 7. Cercosporin resistance of *Cercospora nicotianae* wild type (black bar), 71cR-disruptant strains (grey bars) and 71cR-non-disruptant strain (transformed with the split markers but 71cR gene was not disrupted (white bar). Data are the results of two independent experiments with 12 replications each. Error bars represent standard error corresponding the average of 24 samples.

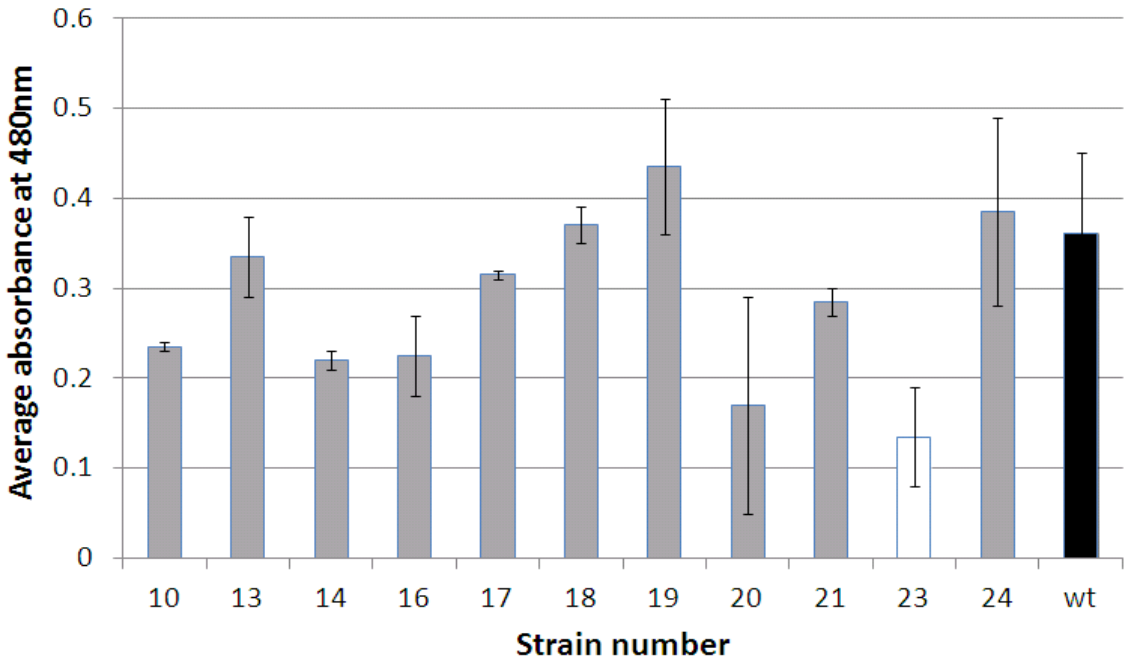

Figure 8. Cercosporin production assay of *Cercospora nicotianae* wild type (black bar), 71cR-disruptant strains (grey bars) and 71cR-non-disruptant strain (transformed with the split markers but 71cR gene was not disrupted) (white bar). Data are the results of two independent experiments with 2 replications each. Error bars represent standard error corresponding the average of 4 samples.

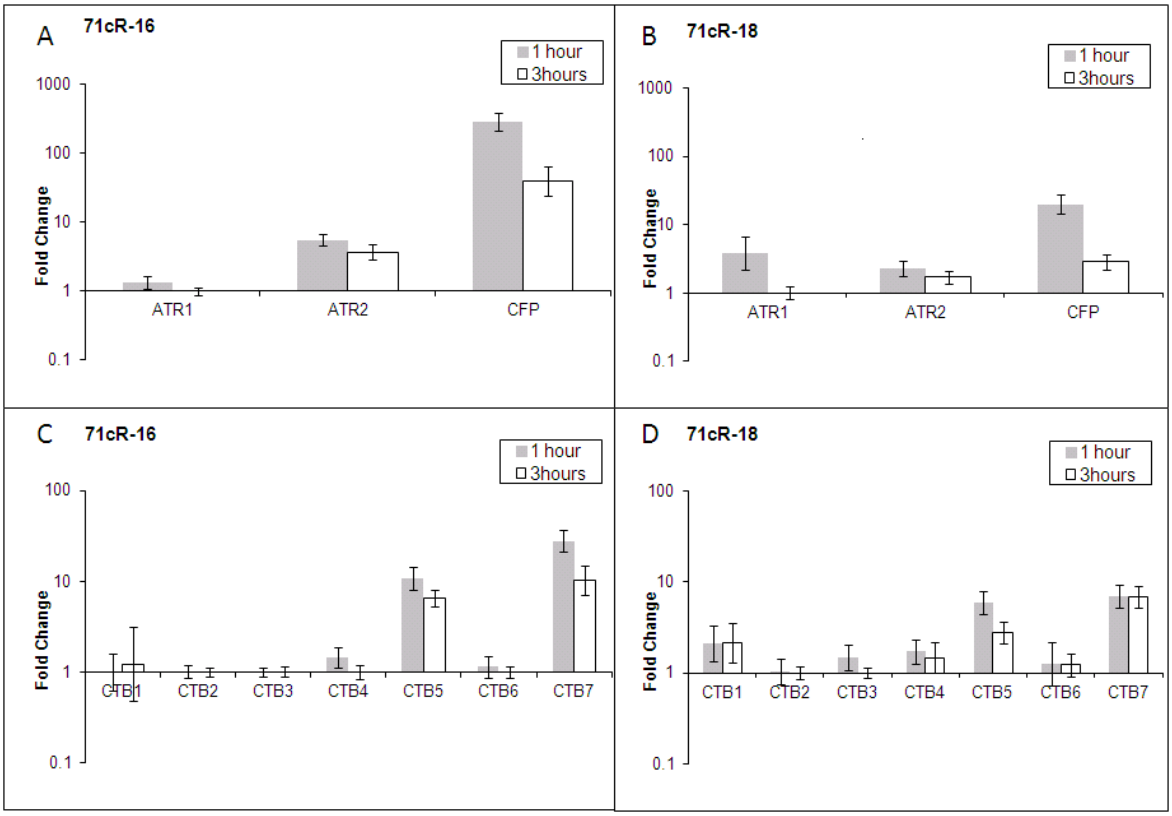

Figure 9. Quantitative RT-PCR analysis of gene expression of genes in the *C. nicotianae 71cR* mutant treated with cercosporin in the light. Each sample was normalized against the actin control, and fold-change relative to no cercosporin control was calculated according to the $2^{(-\Delta\Delta C(T))}$ method. Data represent the mean of two independent experiments. Error bars represent 95% confidence intervals. **A.** Expression of 3 transporters in 71cR disruptant #16. **B.** Expression of 3 transporters in 71cR disruptant #18. **C.** Expression of CTB genes in 71cR disruptant #16. **D.** Expression of CTB genes in 71cR disruptant #18.

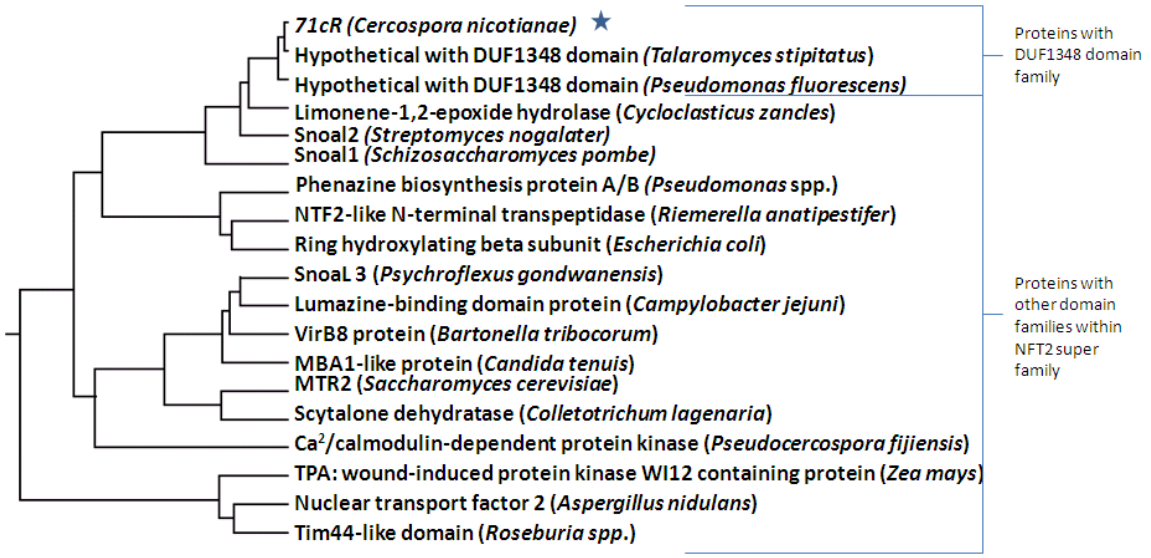

Figure 10. Phylogenetic tree of proteins homologous to 71cR protein in *C. nicotianae* generated by ClustelW2. Tree shows 71cR amino acid sequence, a hypothetical protein from *Talaromyces stipitatus* (XP_002479782.1) (the closest homolog to 71cR), and a *Pseudomonas fluorescens* protein (Protein Pfl_3262) in the same domain family (DUF1348), along with 16 proteins from different organisms with known domains representing each of the characterized domains within the NTF2-like super family. The accession numbers with the genus names where these proteins were found were included in Supplemental Table S1. 71cR protein from *C. nicotianae* (denoted with star) forms a unique branch with the *Talaromyces stipitatus* protein and these two proteins cluster with the *Pseudomonas fluorescens* protein with the DUF1348 family domain. These proteins are then grouped with a protein with limonene-1,2-epoxide hydrolase domain family and then to SnoaL2 and SnoaL1 domain family.

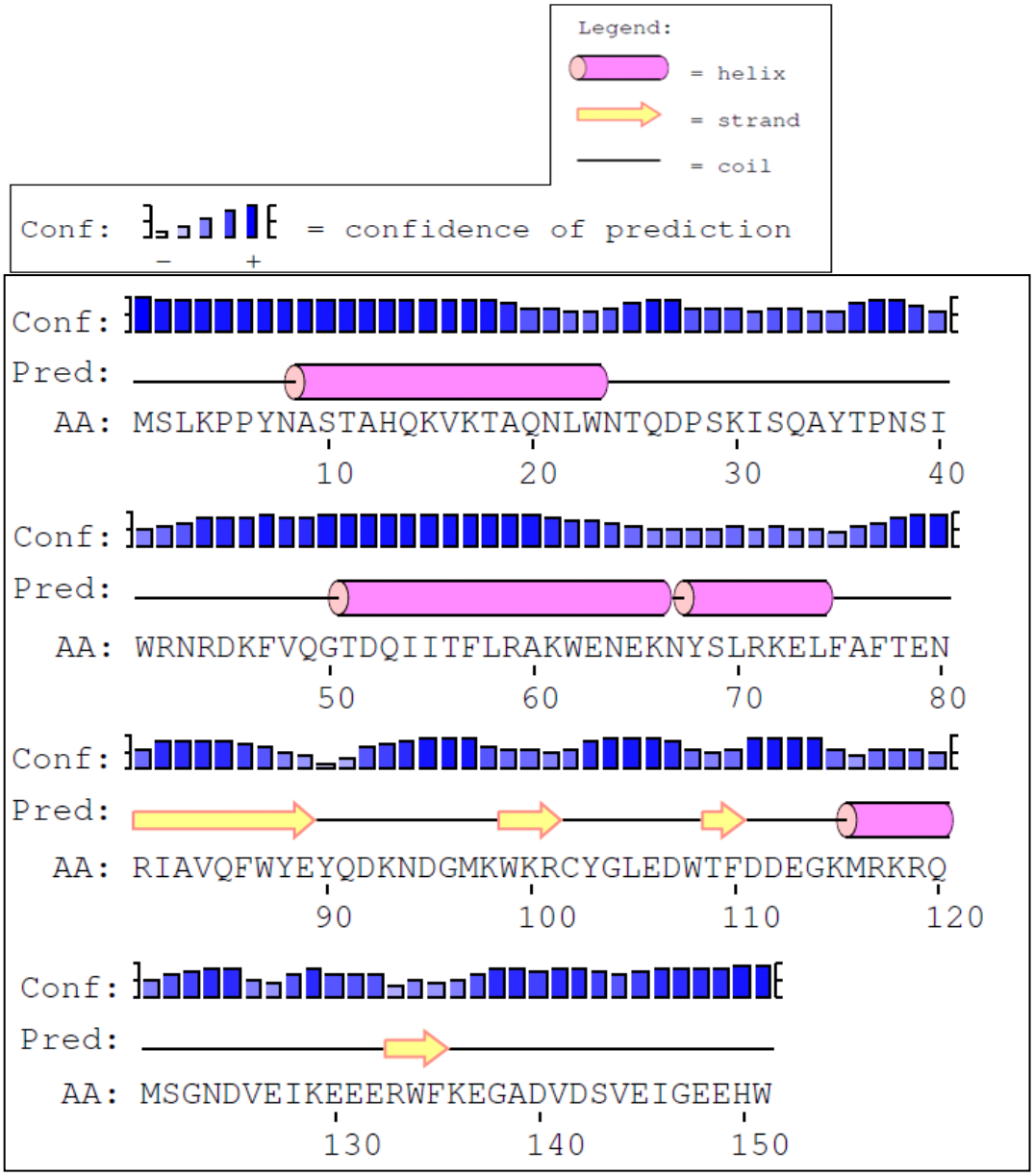

Figure 11. 71cR secondary structure predictions from PSIPRED server (Buchan et. al., 2013). There are 4 alpha helixes and 4 beta sheets. The sequence of amino acids relative to each structure is shown (AA). The confidence of prediction is included as bars.

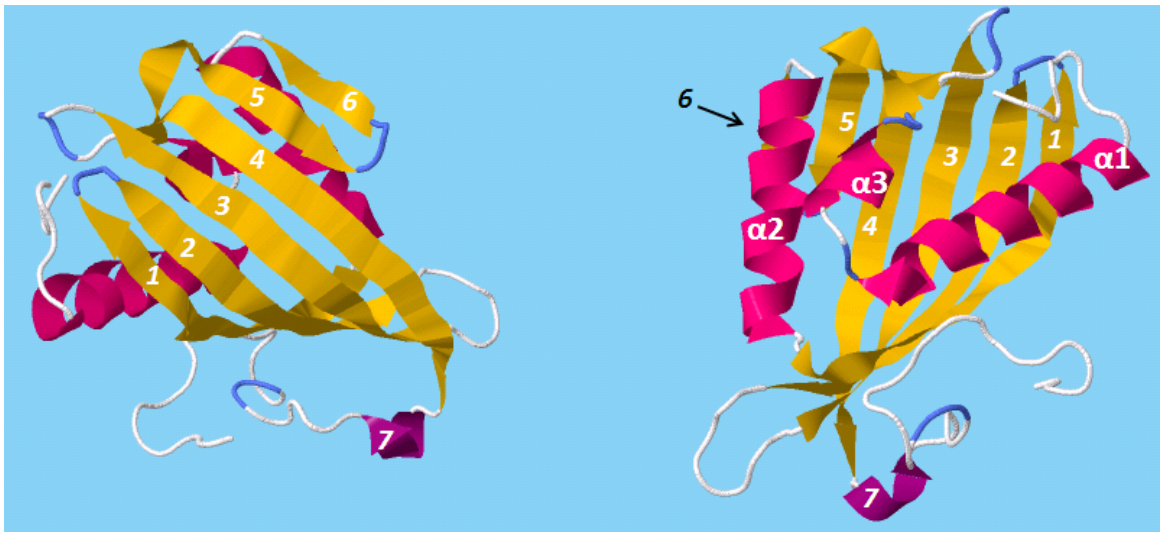

Figure 12. The tertiary structure of 71cR amino acid sequence from Raptor (Källberg et. al., 2012). The two figures represent the same structure from different angles. The beta sheets are numbered from 1-7 and alpha helixes are numbered from α1-α3. The structure of 71cR protein is a half barrel structure with 5 beta sheets enclosing the α1. The two other alpha helices are located at the tip of the structure. The predicted modal states that this protein forms a dimer to complete the barrel structure.

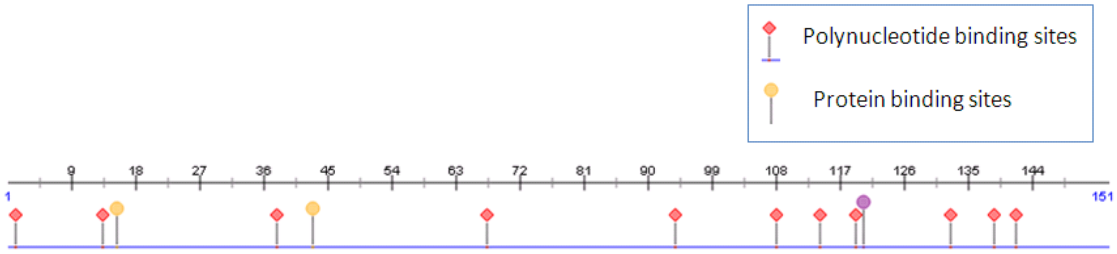

Figure 13. The Solvent Accessibility prediction for 71cR using PredictProtein (Rost et. al., 2004). There are 3 possible polynucleotide binding regions and 11 protein binding regions (Ofran et. al., 2007). The relative positions of each binding sited are indicated relative to amino acid number shown along the top. Lines with circles represent possible polynucleotide binding sites and lines with a diamond shape represent protein binding regions.

Table 1: Primer sequences[12]

Table 1-A. Primers used for q-RTPCR analysis

	Forward Primer	Reverse Primer
11sf	AGTGGCTAATCTGCTCTGG	CACTGCTATTCCTGTTGG
140sR5	ATTGGTTCGTGAGCTTCAGC	TGGCTTGATGCTTTCGTAGC
15cF	ACATGCCGCACTCTGTTCATTTGG	GAAGCCCACAACCTGCAACCATTT
165sr5	CCATGGATTAGGTGAATGC	ATCATCGCGCAACTCG
1cF	CCGTTCCTGCAGAGCTCAAG	GCCATTGACCATGTTGAGCATGTC
200sF	TGCTGATCCGCTAAATGCTAAAAC	GCCGATGGACAAGGGTATAAGATC
207sF	ATGCTCGATCCTCCGAACCA	CGGCAGCTTTGAGCGTCTTT
214sR3	ATCAGAAGAGACAGCATAAAGC	AAGTGGTGGCTCAGCGTGG
21cf	CATTGGACACTGCTGGTGC	GAGGAAATGATCGAGAACAGC
24cF	TGCTTTCACCTTCAAGTTCGAC	TGCCCTTGCCAAAGCTAGG
32sR	TGTTAGAGAGTAAAACGGCGTGG	AAGTCAAGCACCACATTGAGTTGC
35cF	ATGTTTCAGATCCTCCTGC	GACAGAGCAGCAGGGTGG
40cR	TTGGTCCATCCCTGATCCTGTTGT	TCAGACTCAGCGAGCGAAGGATTT
49cR	AACAAGACTGGTGCATCTTCC	TGATAGCACTGGGAGGTGC
55cR	GGAGACAGCCAAGCAAGAAGTC	GGGAAGAAGGCGATTGAGGA
56cR	ATCGTTCAAGACCGAGAGGCTCAA	ATGCCGAGAGATCAATGTCCCGAT
71cR	TCAAGCCACCCTACAATGCCTCAA	TTATTTGGTCGGTGCCTTGGACGA
77sR3	TGAGTGGTCGCTTGATTCG	ATCTGGACCCGAAATCGTGC
84cR	ATCTTCCTGATCGCCTCGG	AGCCCAAAGCGCATAGTGG
84sF	AGTGGGAGTGGGAGTCTTGG	AACACTGGAGAACGAATCAACG
88sR	ATCTGCCGACCTCATCTCACTG	AAGACACGACAAATCTGGACAGTTC

Table 1-B. Primers used for cloning the genes in pToxA plasmid

71cR-F-EcoRI	TTTAAT**GAATTC**AGTGAACACGAACGACTAGGATG
71cR-R-HindIII	TTTAAT**AAGCTT**TCCACGATACGAACTAATGCTCACC
24cF-F-EcoRI	TTTAAT**GAATTC**TCGTAACATCGTTGGGTCAG
24cF-R-HindIII	TTTAAT**AAGCTT**TCGTAACATCGTTGGGTCAG

Table 1-C. PCR screening of putative *N. crassa* 71cR transformants

71cR-F	GAGGAGGAGAGGTGGTTCAAGGA
24cF-F	TTCCAATCTACGTTTTCGACCCTG
HYG-R	TGTCGGGCGTACACAAATCG
ToxA-Rev1	ATAAAGGGCTAAGGTGTCCGTCC

Table 1 continued

71cR-R	TTATTTGGTCGGTGCCTTGGACGA
24cF-R	ATCAACTGGTAGGCGACTGTGAC

Table 1-D. Primers used for cloning 71cR disruption construct and amplification of split markers

71cF-5'-F ApaI	ATTAAT**GGGCCC**TGCTCGTCATCTTGCTCATCG
71cF-5'-R ApaI	ATTAAT**GGGCCC**AGAGACATTTTGAGATGGAATTCG
Hyg-split3S	CGTTGCAAGACCTGCCTGAA
Hyg-split5A	GGATGCCTCCGCTCGAAGTA
71cf-3'-R Sac1	ATTAAT**GAGCTC**CAAGTCAAATCCAC
71cF-3'-F Sac1	ATTAAT**GAGCTC**GCCACCCTACAATGCCTCAAC
71cF-5'-F	TGCTCGTCATCTTGCTCATCG
71cf-3'-R	GAGCTCCAAGTCAAATCCAC

Table 1-E. Primers used for screening for putative *C. nicotianae* 71cR disruptants

71cR-Rev3	TCTTCCACTTCATCCCATCATTCTTA
71cR-inv5'	CCTAGCATCTCAATCTCACCAACTAAC
71cR-Forw10	CTAGATGAGACGACGCCTGATC
Hyg-F2	TGAACCATCTTGTCAAACGACAC

[1] Bold and underlined sequences represent restriction enzymes targets

[2]The primer sets used for screening each hypothetical genes from a *C. nicotianae atr1* disruptant for their induction with cercosporin toxicity by using q-RTPCR

Table 2: Conserved domains of the *C. zeae maydis* homologs of hypothetical proteins in the subtractive library

	Domains of the homologs from *C. zea maydis*
11sf	Binding peptidoglycan in bacteria and chitin in eukaryotes
140sR5	Heme-binding cytochrome domain of fungal cellobiose dehydrogenases
15cF	Nucleotide-sugar transporter
165sr5	Hypothetical protein-no domains
1cF	Pyridoxal phosphate (PLP)-dependent enzyme
200sF	Lipid binding and membrane curvature sensing modules
207sF	G protein-coupled receptor common in photoreceptor cells
214sR3	R3H domain is predicted to be binding ssDNA
21cf	Glutathione-dependent formaldehyde-activating enzyme
24cR	Phospholipid methyltransferase
32sR	S mat+
35cF	Retrotransposons
40cR	Hypothetical protein
49cR	Putative cell wall mannosidase from *Candida albicans* glycosyl hydrolase
55cR	Cytochrome P450
56cR	S mat+ , Retinal pigment epithelial membrane protein
71cR	Small polyketide cyclases--> catalyse ring closure steps in the biosynthesis of Polyketide antibiotics
77sR3	NADB_Rossmann super family-NAD(P)(+)-binding proteins
84cR	Phospholipid methyltransferase
84sF	Pyruvate carboxyltransferase-catalyzes the cleavage of HMG-CoA
88sR	Pyrrolidone carboxyl peptidase

Supplemental Table S1. Proteins used in phylogenetic analysis and their GenBank accession numbers.

Protein	**Accession number**
71cR (Cercospora nicotianae)	AHN52028
Hypothetical with DUF1348 domain (*Talaromyces stipitatus*)	EED19348
Hypothetical with DUF1348 domain *(Pseudomonas fluorescens)*	2IMJ_D
Limonene-1,2-epoxide hydrolase (*Cycloclasticus zancles*)	AGS39004
Snoal2 *(Streptomyces nogalater)*	2GEX_A
Snoal1 *(Schizosaccharomyces pombe)*	NP_592801
Phenazine biosynthesis protein A/B *(Pseudomonas* spp.)	EJM14221
NTF2-like N-terminal transpeptidase (*Riemerella anatipestifer*)	ADZ12144
Ring hydroxylating beta subunit (*Escherichia coli*)	KDA89273
SnoaL 3 (*Psychroflexus gondwanensis*)	WP_003443406
Lumazine-binding domain protein (*Campylobacter jejuni*)	AGV55990
VirB8 protein (*Bartonella tribocorum*)	YP_001610004
MBA1-like protein (*Candida tenuis*)	XP_006684488
MTR2 (*Saccharomyces cerevisiae*)	CAA82029
Scytalone dehydratase (*Colletotrichum lagenaria*)	BAA13009
Ca^2/calmodulin-dependent protein kinase (*Pseudocercospora fijiensis*)	XP_007931892
TPA: wound-induced protein kinase WI12 containing protein (*Zea mays*)	DAA64234
Nuclear transport factor 2 (*Aspergillus nidulans*)	AAK71467
Tim44-like domain (*Roseburia spp*.)	WP_022243554

CHAPTER 4: The role of *Cercospora zeae maydis* homologs of *Rhodobacter sphaeroides* $^1O_2$ resistance genes in resistance to the photoactivated toxin cercosporin

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Additional key words: *Cercospora*, *Rhodobacter*, singlet oxygen, cercosporin, aldehyde dehydrogenase; aldo/keto reductase; succinyl-CoA ligase; O-Acetylhomoserine (thiol) lyase; peptide methionine sulphoxide reductase; glutathione S-transferase.

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Abstract

The photosynthetic bacterium *Rhodobacter sphaeroides* and plant pathogenic fungus *Cercospora nicotianae* have been used as models for understanding resistance to singlet oxygen ($^1O_2$), a highly-toxic reactive oxygen species. In *Rhodobacter* and *Cercospora* $^1O_2$ is derived, respectively, from photosynthesis and from an $^1O_2$-generating toxin cercosporin which the fungus produces to parasitize plants. We identified common genes recovered in transcriptome studies of putative $^1O_2$ resistance genes in these two systems, suggesting common $^1O_2$ resistance mechanisms. To determine if the *Cercospora* homologs of *R. sphaeroides* $^1O_2$ resistance genes are involved in cercosporin resistance, we expressed the genes in the cercosporin-sensitive fungus *Neurospora crassa* and assayed for increases in cercosporin resistance. *N. crassa* transformants expressing genes encoding aldo/keto reductase, succinyl-CoA ligase, O-acetylhomoserine (thiol) lyase, peptide methionine sulphoxide reductase, and glutathione S-transferase did not have elevated levels of cercosporin resistance. Several transformants expressing aldehyde dehydrogenase were significantly more resistant to cercosporin. Expression of the transgene and enzyme activity did not correlate with resistance, however. We conclude that although the genes tested in this study are important in $^1O_2$ resistance in *R. sphaeroides*, their *C. zeae maydis* homologs are not involved in cercosporin resistance.

1. Introduction

Due to its unique chemistry, oxygen is both an essential component of most organisms as well as a potential toxicant. Oxygen in its ground state has two unpaired electrons with a parallel spin, restricting its reactivity. Excitation of oxygen causes changes in unpaired electron number (such as in superoxide and peroxide) or spin direction (such as in singlet oxygen [$^1O_2$]), relieving the spin restriction and leading to high reactivity (Halliwell 2007).

Among reactive oxygen species (ROS), $^1O_2$ is one of the most damaging to cells partly because $^1O_2$ is not common in cells outside of photosynthetic organisms, where $^1O_2$ is generated from pigments of the photosystems as a byproduct of photosynthesis (Daub et al., 2013). $^1O_2$ can react with many macromolecules in cells including proteins, lipids, and nucleic acids, and cause the production of more ROS including organic peroxides and sulfoxides. Several mechanisms have been found in photosynthetic organisms that protect them from $^1O_2$ damage (Glaeser et al., 2011). These include quenchers such as carotenoids, catalases, glutathione-dependent and independent detoxification of oxidation products, and increasing protein turn-over for the replacement of the $^1O_2$ damaged proteins. Outside of photosynthetic organisms, organisms that synthesize photosensitizers also encounter $^1O_2$. Photosensitizers are compounds that are activated by light, and then react with oxygen to generate ROS. Fungi that produce photoactivated perylenequinone compounds, for example, use these compounds to parasitize host plants, and must tolerate the $^1O_2$ produced. Studies on perylenequinone producers have documented similar $^1O_2$ resistance mechanisms to those

found in photosynthetic organisms including quenchers and catalases as well as other mechanisms such as membrane transporters and reductases that act on perylenequinones (Daub et al., 2013).

Damage due to reactions of $^1O_2$ with cellular macromolecules disturbs cellular function in many organisms, and studies to characterize cellular resistance mechanisms are of significant interest. Much of the work on understanding cellular $^1O_2$ resistance has utilized two model systems, *Rhodobacter* spp. and *Cercospora* spp. *Rhodobacter sphaeroides* is a photosynthetic, Gram-negative bacterium. In *R. sphaeroides*, the two sigma transcription factors, RpoE and RpoHII, were found to regulate some of the genes involved in $^1O_2$ resistance (Nuss et al., 2009). Beside these two transcription factors, the RNA chaperone (Hfq) plays a role in $^1O_2$ response by binding to small regulatory RNAs and regulating the activity of several mRNAs. A proteomic analysis between a wild-type strain and an *hfq* deletion mutant in the presence and absence of $^1O_2$ identified 79 differentially expressed proteins (Berghoff et al., 2011). Many of these proteins were same as those identified to be regulated by RpoE and RpoHII. The putative $^1O_2$-resistance proteins regulated by these regulatory elements include proteins involved in processes such as metabolic enzymes, protein folding, redox reactions, membrane transport, oxidative phosphorylation, protein turnover, amino acid metabolism, DNA repair, and $^1O_2$ scavenging (Berghoff et al., 2011; Nuss et al., 2009).

We have been studying resistance mechanisms utilized by the plant pathogenic fungus *Cercospora* to the photoactivated perylenequinone toxin cercosporin, which it

produces in order to parasitize plants (Daub et al., 2013). Cercosporin generates $^1O_2$ in the presence of light, causing peroxidation of the membrane lipids of plant hosts and allowing for cell death and colonization by the fungus. *Cercospora* species are also resistant to a broad range of structurally diverse $^1O_2$-generating photosensitizers, making it a model for studies of cellular $^1O_2$ resistance. *Cercospora* species defend against $^1O_2$ through variety of methods including quenchers and membrane transporters as well as maintaining the toxin inside the cell in a non-toxic, reduced form (Daub et al., 1992). As in *R. sphaeroides*, a transcription factor, CRG1, was identified in *C. nicotianae* to be involved in resistance to cercosporin (Chung et al., 2003). A subtractive hybridization strategy identified 185 genes that are differentially regulated between wild-type (wt) and a *crg1* mutant (Herrero et al., 2007). We hypothesize that some of these genes encode cercosporin resistance.

A comparison of genes in the cercosporin resistance library with genes in *Rhodobacter* regulated by RpoE, RpoHII, and Hfq identified several genes encoding enzymes that were common between the two systems (Daub et al., 2013), including ones involved in generating reducing power, protein turn-over and repair, or involved in antioxidant activity. The purpose of this work was to determine if the *Cercospora* homologs of *R. sphaeroides* $^1O_2$ resistance genes are involved in cercosporin resistance. For this work we expressed the genes in the cercosporin-sensitive fungus *Neurospora crassa* and assayed for increases in cercosporin resistance, an assay previously shown to be an effective approach to characterizing cercosporin-resistance genes (Herrero and Daub 2007).

2. Materials and Methods

2.1 Cloning C. zeae-maydis genes into expression vector

The putative cercosporin resistance genes to be characterized in this work were selected from the *C. nicotianae* EST sequences found in the subtractive library between a wt *C. nicotianae* strain and the *crg1* mutant (Herrero et al., 2007). Sequences were cloned from the closely related species *C. zeae maydis,* which has recently been sequenced (http://www.jgi.doe.gov). The genes studied are as follows, named according to their putative function and the library gene names assigned in *C. nicotianae* (Herrero et al., 2007): Aldehyde dehydrogenase (48cR); Aldo/keto reductase (152sR); Succinyl-CoA ligase (10sF); O-Acetylhomoserine (thiol) lyase (59sF); Peptide methionine sulphoxide reductase (108sR); Glutathione S-transferase (65cR).

To test their function in cercosporin resistance, each of the genes were cloned into the expression vector pTxA-1 (Amnuaykanjanasin and Daub 2009) (Fig. 1) and transformed into the cercosporin-sensitive fungus *Neurospora crassa*. Genes were amplified from *C. zeae-maydis* using gene-specific primers with restriction sites shown in Table 1-A. The PCR fragments were digested with the appropriate restriction enzymes and ligated to the pTxA-1 digested with the same restriction enzymes (Fig. 1).

2.2 N. crassa protoplast generation and transformation

Each plasmid was transformed into *N. crassa* protoplasts using polyethylene glycol (PEG)-mediated transformation. *N. crassa* protoplast generation and transformation was

carried out as previously described (Vollmer and Yanofsky 1986). The cell-wall lysing mix consisted of 1.5% (m/v) of Sigma-Aldrich's lysing enzymes and 1.5% (v/v) of β-glucuronidase (Sigma-Aldrich, St. Louis, MO) in 100ml of 1M sorbitol. A total of 2 X 10^7 protoplasts in 100µl were incubated with 10 µg of plasmid DNA and regenerated in the presence of 100 µg/ml hygromycin (hyg). Single hyg-resistant colonies were transferred to new plates with 100 µg/ml hyg at least 4 times before confirming the presence of the transgene by PCR screening using gene-specific primers (Table 1-B). An NaOH DNA extraction method described previously (Wang et al., 1993), was used to extract gDNA for PCR screening analysis.

2.3 Screening of N. crassa transformants for cercosporin resistance

PCR-positive transformants were assayed for resistance to cercosporin. Split petri plates were prepared with one half containing Vogel's medium (Vogel 1956) supplemented with 10 µM cercosporin and the other half supplemented with 0.5% acetone (used to solubilize the cercosporin). Mycelial plugs (6 mm) of the transformants or wt *N. crassa* were inoculated onto each side of the split plates, and incubated at room temperature under continuous fluorescent light (55-65 $\mu E.m^{-2}sec^{-1}$). Radial growth of colonies was measured 21 hours after inoculation, and % growth on cercosporin was calculated relative to acetone controls on the same plate. Each transformant was replicated five times, and the experiment was repeated two times.

2.4 Gene expression analysis of transformants

For each gene, total RNA was extracted from four randomly chosen *N. crassa* transformants using TRI-REAGENT (Sigma-Aldrich, St Louis, MO) following the manufacturer's protocol. Samples were treated with DNAse (Turbo DNA-free^{TM} Kit, Ambion, Austin, TX). Synthesis of cDNA was carried out using Taqman Reverse Transcription Reagents (Applied Biosystems, Somerville, MA) according to the manufacturer's recommendations. SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used for q-RTPCR reactions. The primers used to amplify each gene are shown in Table 1-C, and the cycling parameters for cDNA synthesis and q-RTPCR reactions were those previously used by Herrero et al (Herrero et al., 2007). Single amplicons were identified using a melting point analysis protocol (60–90 ^{0}C every 0.5 0C for 1 second). The q-RTPCR reactions were performed in triplicate, and negative controls included: i) the use of RNA as a template to check for gDNA contamination in samples, and ii) a water control. Each sample was normalized against a tubulin control, and fold-change relative to wt *N. crassa* (no transgene) was calculated according to the $2^{-\Delta\Delta}C_T$ method (Livak and Schmittgen 2001); (Schmittgen et al., 2000; Winer 1999).

2.5 Aldehyde dehydrogenase enzyme assay

For testing aldehyde dehydrogenase enzyme activity, Aldehyde Dehydrogenase Activity Colorimetric Assay Kit (BioVision, Inc. Milpitas, CA) was used according to manufacturer's protocol. Five randomly selected *N. crassa* aldehyde dehydrogenase

transformants (#63, 82, 84, 86, 87), wt *N. crassa* and wt *C. nicotianae* were grown on cellomembrane on Vogel's medium (*N. crassa*) or potato dextrose agar (PDA; Difco) (*Cercospora*) for 4 days. For each, 100 mg of mycelium was homogenized for 30 seconds in 4 ml of 0.1M sodium pyrophosphate buffer. The samples were centrifuged for 5 minutes at 13000 rpm at 4 ^{0}C. 10 µl of the sample was used for each 50 µl reaction. Boiled samples were used as controls. The samples were read in a Synergy HT microplate reader (BioTek Instruments, Inc. Winooski, VT).

3. Results

3.1 Transformation of N. crassa and cercosporin sensitivity assay

Homologs to the *Rhodobacter* $^1O_2$ resistance genes were identified from *C. nicotianae* EST sequences in a subtractive library between the wt *C. nicotianae* and the cercosporin sensitive *crg1* mutant. As a genome sequence for *C. nicotianae* is not available, we cloned the genes from the closely related *C. zeae maydis* whose genome has been sequenced. Six genes were cloned and tested for the ability to impart cercosporin resistance: aldehyde dehydrogenase (48cR), aldo/keto reductase (152sR), succinyl-CoA ligase (10sF), O-acetylhomoserine (thiol) lyase (59sf), peptide methionine sulfoxide reductase (108sR) and glutathione S transferase (65cR).

To test the ability of the *Cercospora* genes to impart resistance to cercosporin, we transformed them into the cercosporin-sensitive fungus *N. crassa* and assayed for increased resistance to cercosporin. After each transformation event, colonies were selected on

Vogel's medium amended with hyg, and hyg-resistant transformants were screened for the presence of the target gene by PCR screening. PCR-positive transformants were then assayed for cercosporin resistance. Results of cercosporin resistance assays for each transformant are shown in Fig. 2. Growth of wt *N. crassa* is inhibited about 70% when grown on medium amended with 10 μM cercosporin and grown in the light. None of the *N. crassa* transformants transformed with genes encoding aldo/keto reductase, succinyl-CoA ligase, O-acetylhomoserine (thiol) lyase, peptide methionine sulfoxide reductase and glutathione S transferase showed any increase in resistance to cercosporin (Fig. 2). By contrast, 2 out of 13 transformants transformed with aldehyde dehydrogenase were significantly more resistant to cercosporin than wt *N. crassa* ($P < 0.05$) (Fig. 2).

3.2 Transgene expression of selected N. crassa transformants

For each gene, selected transformants were assayed by q-RTPCR to quantify expression of the transgene. Expression of transgenes was normalized to a tubulin control and shown relative to wt *N. crassa* (Fig. 3). Transgenes were shown to be expressed in all transformants tested. Thus the lack of resistance in the aldo/keto reductase, succinyl-CoA ligase, O-acetylhomoserine (thiol) lyase, peptide methionine sulfoxide reductase and glutathione S transferase transformants is not due to lack of expression. Rather we conclude that these genes are unable to impart elevated resistance to cercosporin.

For the aldehyde dehydrogenase transformants, we tested relative expression of transformants with significant increases in resistance (# 84, 86) as compared to those with no

increase in resistance (# 63, 82, 87) (Fig. 3). Expression was detected in all transformants with the resistant aldehyde dehydrogenase transformant, #86 having less than the expression of other aldehyde dehydrogenase transformants.

3.3 Aldehyde dehydrogenase enzyme activity

Gene expression results of the aldehyde dehydrogenase transformants did not correlate with resistance data. We thus assayed for aldehyde dehydrogenase enzyme activity in the *N. crassa* aldehyde dehydrogenase transformants compared to the wt control. Activity in wt *C. zeae-maydis*, the source of the transgene, was also tested. The assay utilized a commercial kit for aldehyde dehydrogenase activity that measures the amount of NADH produced from the oxidation of acetaldehyde by aldehyde dehydrogenase. Results are shown in Fig. 4. We hypothesized that if this enzyme is important in cercosporin resistance (through generating reducing power due to the production of NADH), we would expect to see higher activity of the enzyme in *C. zeae-maydis*. However, the highest activity was found in wt *N. crassa* and in transformants (#87 and 63, Fig. 2) that did not have increased resistance. The lowest activity was in *C. zeae-maydis.* The two transformants that had significantly greater resistance to cercosporin had the lowest aldehyde dehydrogenase activity other than the wt *C. nicotianae*. Thus aldehyde dehydrogenase activity, at least as assayed through this assay, does not correlate with cercosporin resistance.

4. Discussion

Cercospora species have been used as a model for understanding $^1O_2$ and oxidative stress resistance in non-photosynthetic organisms due to their production of and resistance to the $^1O_2$- generating toxin cercosporin (Daub et al., 2013). The transcription factor, CRG1, was shown to be required for cercosporin resistance (Chung et al., 2003), and was used to identify putative cercosporin resistance genes. A subtractive library of genes differentially regulated between wt *C. nicotianae* and a *crg1* mutant (Herrero et al., 2007) identified 185 genes putatively involved in cercosporin resistance. In this study, we chose to definitively characterize the role in cercosporin resistance of genes from the subtractive library that are homologs to $^1O_2$ resistance genes in the photosynthetic bacterium *Rhodobacter sphaeroides.* Evidence for a role of homologs of the *Rhodobacter* $^1O_2$ resistance genes in cercosporin resistance would document common $^1O_2$ resistance mechanisms in two evolutionarily distinct organisms (*Rhodobacter* spp. and *Cercospora* spp.), and strengthen the evidence for their essential role in $^1O_2$ resistance.

Genes tested in this study encode proteins predicted to impart $^1O_2$ and cercosporin resistance. Three of the genes tested encode enzymes (aldehyde dehydrogenase, aldo/keto reductase and succinyl-CoA ligase) that generate reducing power by producing NADH. In *Cercospora*, the ability of the fungus to maintain cercosporin in a reduced and thus non-photoactive state, has been shown to be a major resistance mechanism (Daub et al., 1992). All three of these genes are induced in wt *R. sphaeroides* with the addition of $^1O_2$. As they are involved in primary metabolic functions such as pentose or TCA cycles, homologs are

present in many organisms including *N. crassa*. Evidence that the *Cercospora* homologs could impart cercosporin resistance would document the need for further characterization of differences between these enzymes in *Cercospora* and *Rhodobacter* as compared to those in $^1O_2$-sensitive organisms.

Two of these genes characterized in this work encode O-Acetylhomoserine (thiol) lyase and peptide methionine sulphoxide reductase, both involved in amino acid metabolism. In *Cercospora* spp., a mechanism involved in repairing amino acids damaged by $^1O_2$ has not been documented. However, in *Rhodobacter* spp., amino acid turnover is a major documented resistance mechanism against $^1O_2$. One of the easiest oxidized amino acid by $^1O_2$ is methionine. Peptide methionine sulfoxide reductase catalyzes the reduction of the oxidized form of methionine, methionine sulfoxide, back to methionine in proteins (Weissbach et al., 2002). O-Acetylhomoserine (thiol) lyase is also involved in methionine biosynthesis and might have a role in recycling the damaged methionine by $^1O_2$ (Yamagata 1989).

The last gene characterized in this work encodes a glutathione S-transferase (GST). This group of proteins are known to involve in detoxification reactions by catalyzing the conjugation of the reduced form of glutathione (GSH), an important antioxidant, to toxic substrates such as phenols, aflatoxins and ROS (Sheehan et al., 2001). These enzymes also have peroxidase and isomerase activities. GST genes are present in wide variety of organisms from animals to plants to bacteria to fungi.

In spite of the known role of the 6 *Rhodobacter* homologs in $^1O_2$ resistance, we found that only one, aldehyde dehydrogenase, was able to impart cercosporin resistance when transformed into *N. crassa*. *N. crassa* transformants with the other 5 genes did not show any elevated levels of cercosporin resistance, even though we confirmed strong expression of the genes. Thus we conclude that these enzymes are not involved in resistance to cercosporin.

Although we recovered aldehyde dehydrogenase transformants with elevated cercosporin resistance, only two transformants out of 13 were found to be significantly more resistant than wt *N. crassa* (Fig. 2). To confirm our resistance assay results, we assayed transgene expression in selected transformants that were or were not resistant to cercosporin. The results showed that the transgene was expressed in each of the transformants assayed (Fig. 3), and there was no correlation between the increase in cercosporin resistance in aldehyde dehydrogenase transformants and the level of expression. We then assayed aldehyde dehydrogenase enzyme assays to measure the generation of NADH (Fig. 4); we hypothesized that cercosporin resistance should correlate with NADH production given the importance of reducing power in cercosporin resistance. Our results, however, showed an inverse relationship between NADH production and cercosporin resistance. The aldehyde dehydrogenase activity in *C. zeae maydis* (greatest resistance) resulted in the lowest levels of NADH, followed by the two resistant *N. crassa* transformants. The wt *N. crassa* and the three transformants that did not impart resistance were the highest NADH producers.

In summary, the photosynthetic bacterium *Rhodobacter* and the photosensitizer-generating fungus *Cercospora* have both been used as models for understanding $^1O_2$

resistance. Studies of transcription factors associated with resistance in these organisms identified putative $^1O_2$ resistance genes common to both species. Our studies, however, failed to document a role for these genes in resistance to the toxin cercosporin. We conclude that although the homologs of the *C. zeae maydis* genes that are tested in this study are important in $^1O_2$ resistance in *R. sphaeroides*, they are not involved in cercosporin resistance in *Cercospora* species.

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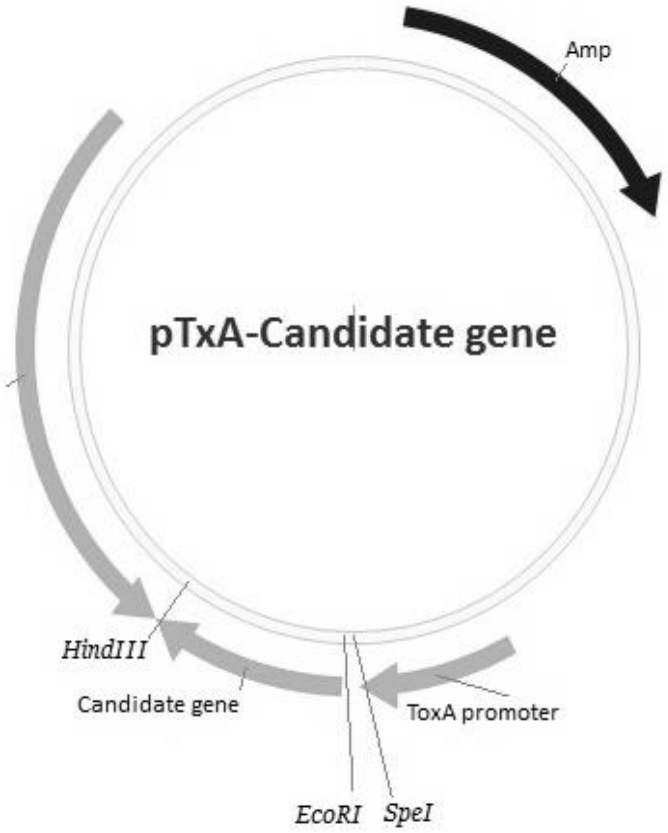

Figure 1 . Expression vector for cloning the genes for *N. crassa* transformation. The vector was modified from pTxa-1 (Amnuaykanjanasin et al., 2009), which has a Tox A promoter, a constitutive promoter from *Pyrenophora tritici-repentis*, the Hyg cassette and the Amp cassette for selection in fungi and *Escherichia coli*, respectively. For each of the genes transformed to *N. crassa*, *C. zeae maydis* gDNA was amplified using the sequence specific primers with incorporated restriction enzyme sites (Table 1). These PCR fragments were digested with the appropriate restriction enzymes and ligated to the pTxA-1 in between ToxA promoter and Hyg cassette that was digested with the same restriction enzymes. The locations of the restriction enzymes, HindIII, EcoRI and SpeI, are specified in the figure.

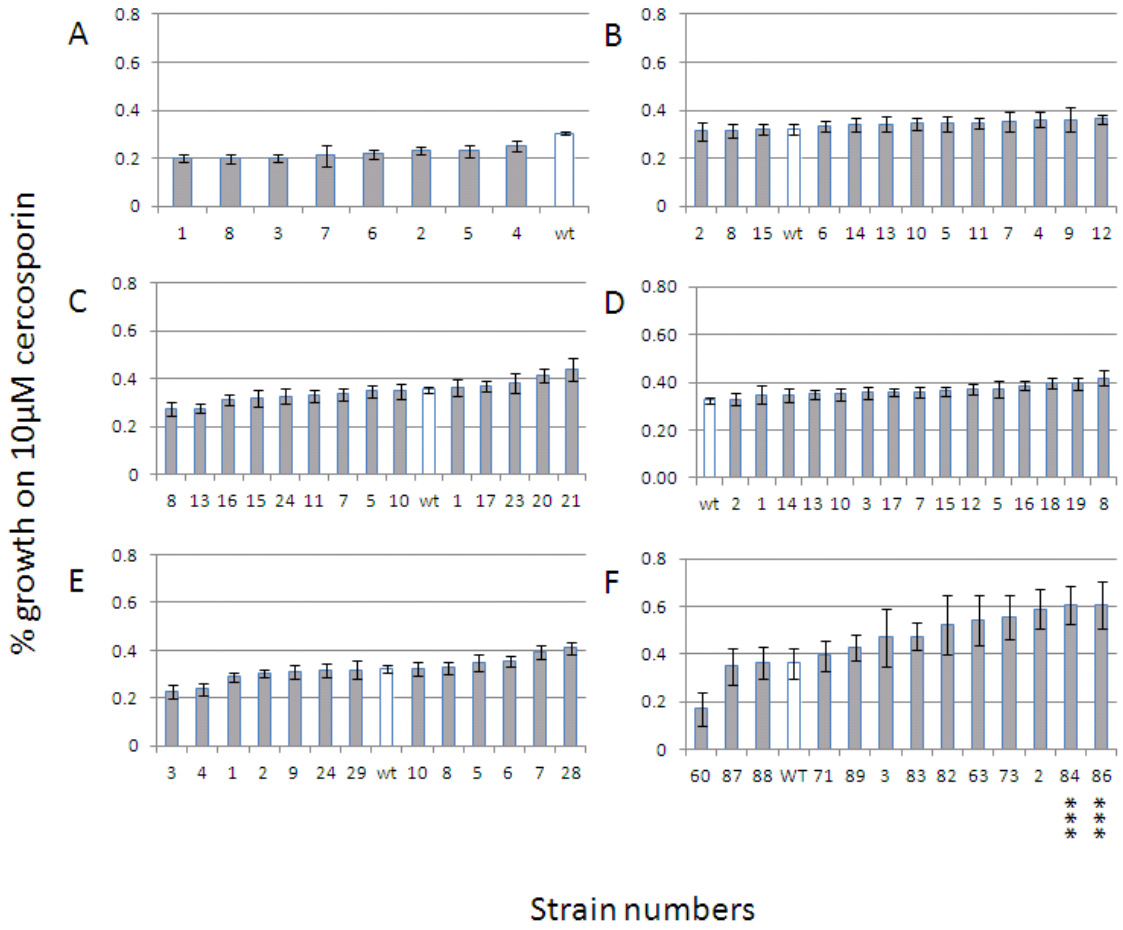

Figure 2 . Cercosporin resistance of *N. crassa* wild type (white bars) and strains transformed with *C. zeae-maydis* resistance genes (grey bars). Strain numbers are given on the x axis and the % growth on 10µM cercosporin (relative to no cercosporin control) on the y axis. Each graph represents the results of resistance assay of *N. crassa* transformants transformed with following genes: Fig. 1-A Peptide methionine sulphoxide reductase, 1-B Aldo/ Keto reductase, 1-C Gutathione S transferase, 1-D O-Acetylhomoserine lyase, 1-E Succinyl-CoA ligase, 1-F Aldehyde dehydrogenase. For each graph, data are the results of two independent experiments with 5 replications each. Strains marked with *** have significantly greater resistance than wild type ($P < 0.05$). Error bars represent 95% confidence intervals.

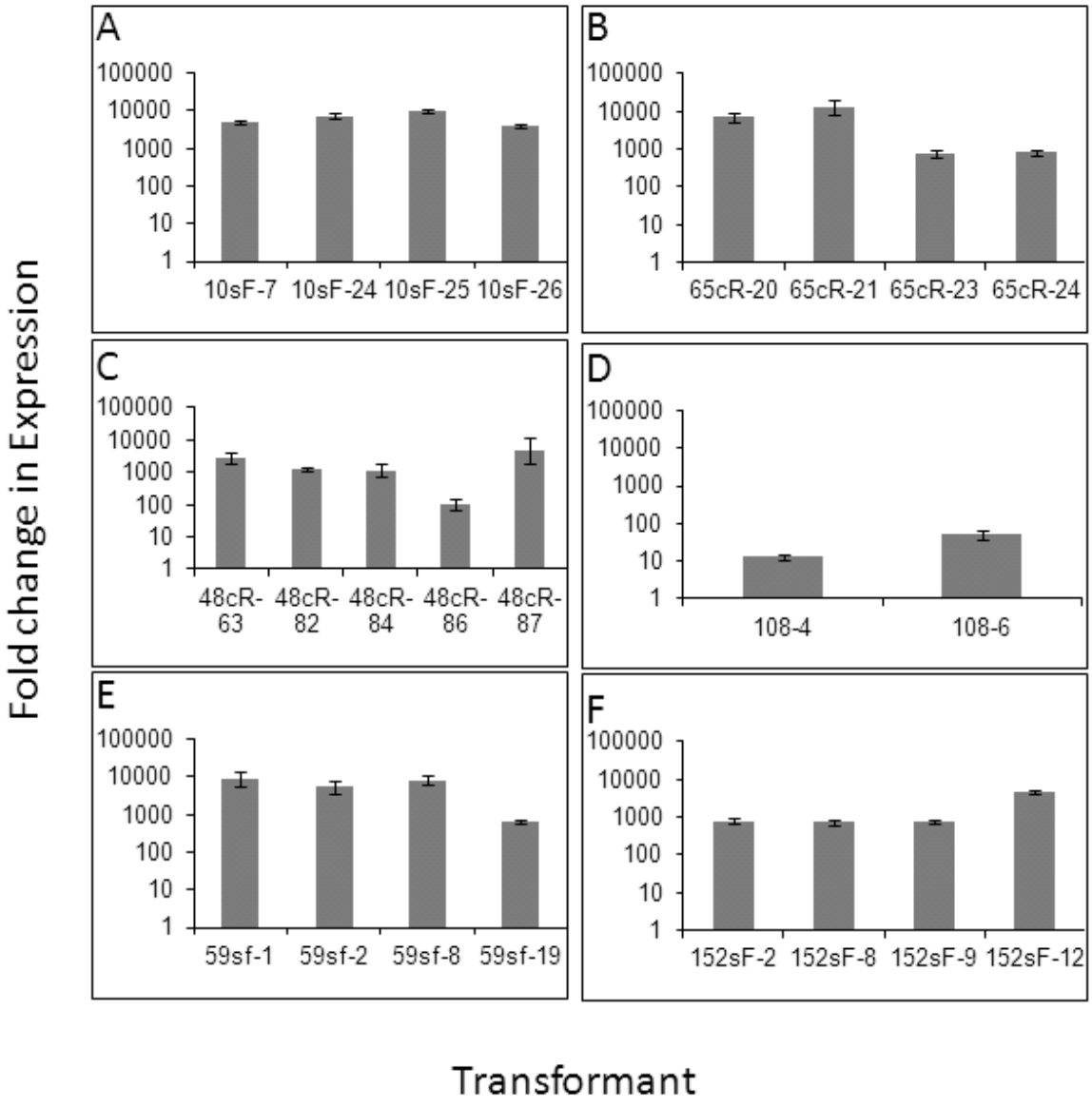

Figure 3. Quantitative RT-PCR analysis of expression of *C. zeae-maydis* genes in selected *N. crassa* transformants. Each sample was normalized against the tubulin control, and fold-change relative to wild type *Neurospora crassa* control (no transgene) was calculated according to the $2^{(-\Delta\Delta C(T))}$ method (Livak and Schmittgen, 2001). Error bars represent 95% confidence intervals. 1-A Succinyl-CoA ligase, 1-B Gutathione S transferase, 1-C Aldehyde dehydrogenase, 1-D Peptide methionine sulphoxide reductase, 1-E O-Acetylhomoserine lyase, 1-F Aldo/ Keto reductase. The y axis represents the fold change and the x axis represents the transformant (from Fig. 2).

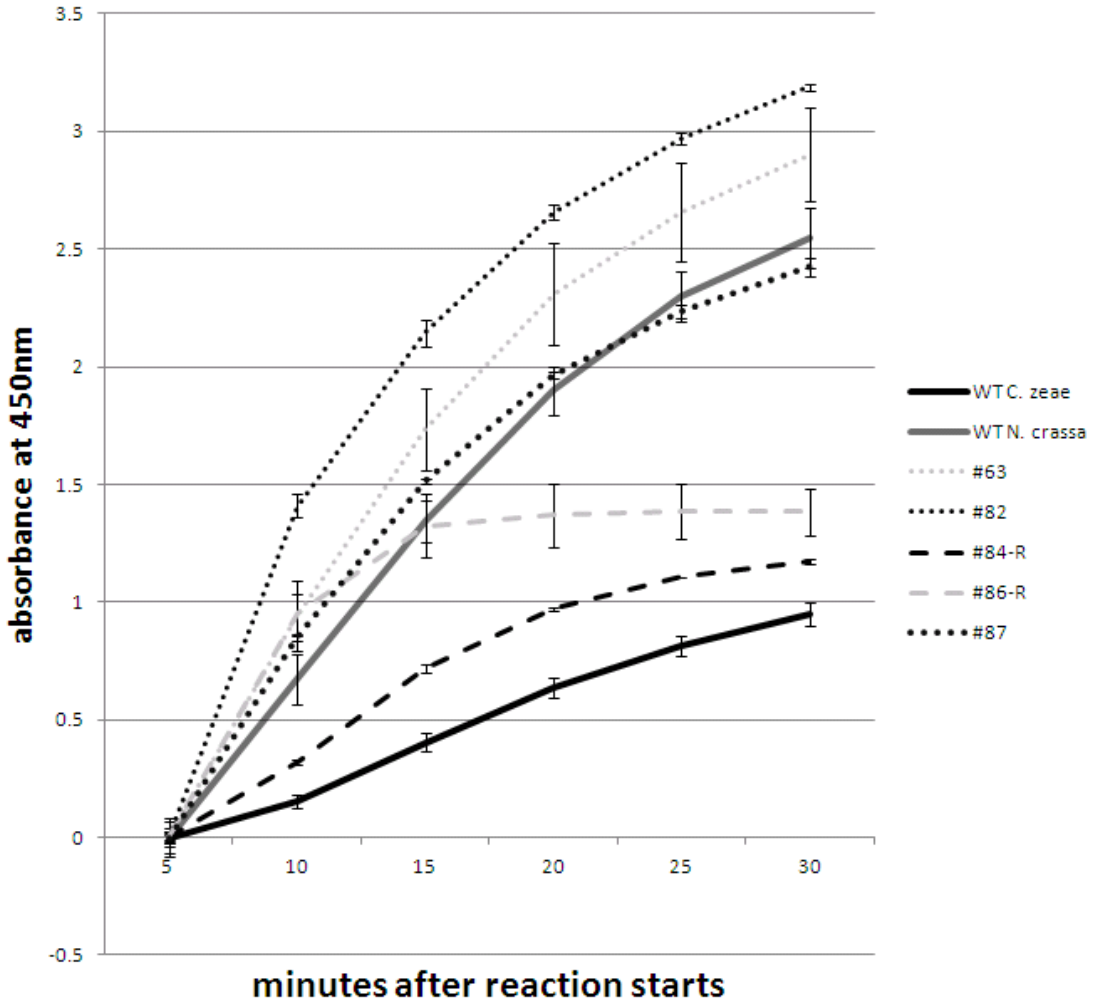

Figure 4. Aldehyde dehydrogenase enzyme assay. Acetaldehyde oxidation by aldehyde dehydrogenase in homogenates from wt and *N. crassa* transformants. Assay measures NADH production, assayed by reduction of acetaldehyde, detected by absorbance at 450 nm. Data shown are values minus readings from boiled homogenate controls. Each line represents the average of two independent experiments. Error bars represent standard error. The enzyme activity in the cercosporin-resistant *N. crassa* transformants, #84 and 86, are shown as dashed lines; activity in non-resistant transformants are shown with dotted lines. Aldehyde dehydrogenase activities of wt *N. crassa and C. zeae maydis* are shown as solid lines.

Table 1: Primer sequences[12]

A. Primers used for cloning genes in pToxA expression vector[1]	
10sF-F-HindIII	ATTATA**AAGCTT**TGACCTCTCCTCTTCCATTCC
10sF-R-HindIII	ATTATA**AAGCTT**TGCAATGAGATGCTTCATGC
48cR-F-EcoRI	ATTATA**GAATTC**CGTCCATCTGACCAGCACTG
48cR-R	GCAGAGATATGTGAGAGGGAGATATG
108sR-F-SpeI	ATTATA**ACTAGT**ATACTCCTCATCATAGCAGGAATTGC
108sR-R-EcoRI	ATTATA**GAATTC**TTCGCCGTCTGCCCTTC
152sF-F-EcoRI	ATTATA**GAATTC**TGTCCGACACCTTTCAACTCG
152sf-R-HindIII	ATTATA**AAGCTT**TAGACAACTGAATCGTTCGGAGG
59sF-F-EcoRI	ATTATA**GAATTC**CTTCAGTTATTCACGGAACAGTTATCG
59sF-R-HindIII	ATTATA**AAGCTT**TTCCAGACCAACATCCTGCG
65cR-F-HindIII	ATTATA**AAGCTT**TTCTCTGCCACTGCACTCCG
65cR-R-EcoRI	ATTATA**GAATTC**GTATGTGCTGTCGATGTTTGGC
B. PCR screening of transformants for the presence of the transgene [2]	
ToxA-F	ATAAAGGGCTAAGGTGTCCGTCC
10sF-R	TGGAAACCGATTCGACAAAACC
48cR -R2	AGTAGCCCTTGTCTCCCTTGCG
108sF-R1	GTGGGGTCGAAGACGATTTGG
152sR-R2	ACTTTGCCCTCGTTGACG
59sF-R1	TCGTGGCGTAAATGGGAAC
65cR-R1	ATGTCGAGGACTCCGTAGAGG
C. Expression analysis	
10sF-F	CATTGAGGCGGAGATCCC
10sF-R	TGGAAACCGATTCGACAAAACC
48cR- F	CGTCGTCGGTGACCCATTCG
48cR -R2	AGTAGCCCTTGTCTCCCTTGCG
108sF-F1	TACATTGGAGGCGACACGCAG
108sF-R1	GTGGGGTCGAAGACGATTTGG
152sR-F2	CTGAGTTACATTGACCTGTATCTACTGC
152sR-R2	ACTTTGCCCTCGTTGACG
59sF-F1	CCGAGATTCGAGACCTTGCAG
59sF-R1	TCGTGGCGTAAATGGGAAC
65cR-F1	AGCTGTCCCAGTGTGAGCAG
65cR-R1	ATGTCGAGGACTCCGTAGAGG

[1] Bold and underlined sequences represent restriction enzymes targets

[2] Screening for each transgene was with ToxA-F and the corresponding reverse primer