

Membrane transporters in self resistance of *Cercospora nicotianae* to the photoactivated toxin cercosporin

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Abstract The goal of this work is to characterize membrane transporter genes in *Cercospora* fungi required for autoresistance to the photoactivated, active-oxygen-generating toxin cercosporin they produce for infection of host plants. Previous studies implicated a role for diverse membrane transporters in cercosporin resistance. In this study, transporters identified in a subtractive cDNA library between a *Cercospora nicotianae* wild type and a cercosporin-sensitive mutant were characterized, including two ABC transporters (CnATR2, CnATR3), an MFS transporter (CnMFS2), a uracil transporter, and a zinc transport protein. Phylogenetic analysis showed that only CnATR3 clustered with transporters previously characterized to be involved in cercosporin resistance. Quantitative RT-PCR analysis of gene expression under conditions of cercosporin toxicity, however, showed that only CnATR2 was upregulated, thus this gene was selected for further characterization. Transformation and expression of CnATR2 in the cercosporin-sensitive fungus *Neurospora crassa* significantly

increased cercosporin resistance. Targeted gene disruption of CnATR2 in the wild type *C. nicotianae*, however, did not decrease resistance. Expression analysis of other transporters in the *cnatr2* mutant under conditions of cercosporin toxicity showed significant upregulation of the cercosporin facilitator protein gene (*CFP*), encoding an MFS transporter previously characterized as playing an important role in cercosporin autoresistance in *Cercospora* species. We conclude that cercosporin autoresistance in *Cercospora* is mediated by multiple genes, and that the fungus compensates for mutations by up-regulation of other resistance genes. CnATR2 may be a useful gene, alone or in addition to other known resistance genes, for engineering *Cercospora* resistance in crop plants.

Keywords Perylenequinone · Photosensitizer resistance · ABC transporter · MFS transporter · Targeted gene disruption · CRG1 · CnATR1 · CnCFP

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Introduction

The purpose of this work is to characterize the role of membrane transporters in auto-resistance of plant pathogenic *Cercospora* fungi to the photoactivated toxin cercosporin they produce for infection of host plants. *Cercospora* species cause devastating leaf-blighting diseases on a wide range of important plant host species worldwide. Much of the reason for their success and broad host range is attributed to their production of the perylenequinone toxin cercosporin (Daub and Chung 2009; Daub et al. 2013). In the presence of light, cercosporin absorbs light energy and reacts with oxygen, generating reactive oxygen species (ROS) such as singlet oxygen (¹O₂) and superoxide (O₂⁻), which kill cells by damaging critical cellular components.

Due to its generation of reactive oxygen species, particularly $^1\text{O}_2$, cercosporin has broad-spectrum toxicity to almost all organisms including mice, bacteria, and many fungi in addition to host and non-host plants. The producing fungi are resistant, however. Thus, the mechanisms used by *Cercospora* fungi to tolerate cercosporin and the ROS it produces are of significant interest, as *Cercospora* auto-resistance genes may be of use in engineering crop plants for resistance to these pathogens. Similar photoactivated perylenequinones are also produced by other plant pathogens including species of *Alternaria*, *Cladosporium*, and *Elsinoë*. Thus, identification of resistance mechanisms against perylenequinones has the potential to provide control strategies against diverse fungal pathogens.

Multiple strategies have been used by our laboratory and others to identify the mechanisms used by *Cercospora* fungi for resistance to cercosporin. Studies on production of $^1\text{O}_2$ quenchers, antioxidants, and antioxidant enzymes have documented an important role in resistance for these compounds, particularly for carotenoids and vitamin B6 (pyridoxal phosphate and its vitamers), which are potent $^1\text{O}_2$ quenchers (Daub et al. 2013; Ehrenshaft et al. 1999). Transformation of tobacco to express *Cercospora* vitamin B6 biosynthetic genes, however, was unsuccessful in elevating levels of cercosporin resistance (Herrero and Daub 2007). Studies with redox-sensitive dyes, reducing agents, and with detection of reduced and oxidized forms of cercosporin (Daub et al. 1992, 2013) also documented the key role of redox potential and the maintenance of the non-photoactive reduced form of cercosporin within fungal hyphae as a core mechanism of cercosporin resistance. In support of these studies, Panagiotis and co-workers demonstrated that overexpression of a yeast FAD pyridine nucleotide reductase gene (*Cpd1*) increased cercosporin resistance in yeast and tobacco (Panagiotis et al. 2007; Ververidis et al. 2001).

Of all possible resistance mechanisms, the role of membrane transporters has been the most studied. Membrane transport proteins efflux a wide array of molecules across membranes (Dahl et al. 2004), and have been implicated in resistance to both endogenously produced and exogenously applied toxic agents. 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 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). Both MFS and

ABC transporters have been shown to provide resistance to cercosporin. These include the MFS transporters CFP (cercosporin facilitator protein) from *C. kikuchii* (Callahan et al. 1999), Bcmfs1 from *Botrytis cinerea* (Hayashi et al. 2002), and MgMfs1 from *Mycosphaerella graminicola* (Roohparvar et al. 2007). ABC transporters shown to play a role in cercosporin resistance include ATR1 from *Cercospora nicotianae* (Amnuaykanjanasin and Daub 2009) and Snq2p from yeast (Ververidis et al. 2001). The precise mechanism(s) by which these transporters impart resistance is not known, but is hypothesized to be due to transport of cercosporin out of the cell.

In our laboratory, studies to identify cercosporin 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 (cercosporin resistance gene 1) (Chung et al. 2003). The *cnrg1*-null mutant (205C3) 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 (WT) and the *cnrg1* null mutant (Herrero et al. 2007). The resulting library contained 185 expressed sequence tags (ESTs) representing genes altered in their regulation between WT and the *cnrg1* mutant, as validated by quantitative RT-PCR (RT-qPCR) analysis. ESTs from the subtraction library were sequenced, classified into functional categories, and characterized as putative resistance genes based on the above-described known mechanisms of cercosporin resistance including reductive detoxification of cercosporin, antioxidants and quenchers of superoxide and singlet oxygen, and efflux out of the cell by membrane transporters. Interestingly, two genes in the *Cercospora* cercosporin biosynthetic cluster (*CTB2*, *CTB5*) were also found in the library. This cluster, called Cercosporin Toxin Biosynthesis (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. 2007a). To date, however, there is no evidence to document a role of the biosynthetic cluster genes in resistance, as a *ctb8* transcription factor null mutant, downregulated for all core cluster genes, is not altered in cercosporin resistance (Chen et al. 2007a).

The goal of this work is to further document the possible role of membrane transporters identified in the *C. nicotianae* subtractive library for their ability to impart cercosporin resistance. In total, eight genes encoding membrane transporters were identified in our subtractive library between *C. nicotianae* WT and the cercosporin-sensitive

cnrg1-null mutant, three of which (the ABC transporter ATR1 and the MFS transporters CFP and CTB4) have previously been characterized (Amnuaykanjanasin and Daub 2009; Callahan et al. 1999; Choquer et al. 2007). Here, we report sequence and phylogenetic characterization of the five remaining predicted transporter genes recovered in the subtractive library: *CnATR2* (ABC Transporter 2), *CnATR3* (ABC Transporter 3), *CnMFS2* (MFS transporter 2), a uracil transporter, and a zinc transport protein. Expression analysis under conditions of cercosporin toxicity indicated a probable role for *CnATR2* in cercosporin resistance, so its role was characterized through expression in the cercosporin-sensitive fungus *Neurospora crassa* as well as by mutant analysis in *C. nicotianae*.

Materials and methods

Fungal strains, culture conditions, and plasmids

All strains of *C. nicotianae* including the 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* ORS-6a (Fungal Genetics Stock Center) was maintained on Vogel's medium (Vogel 1956). *Cercospora nicotianae* mutants used in this work include mutants for the *CnATR1* ABC transporter (Amnuaykanjanasin and Daub 2009), the *CnCRG1* zinc-cluster transcription factor (Chung et al. 2003), and the *CnCTB1* polyketide synthase in the cercosporin biosynthesis cluster (Chen et al. 2007a).

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, USA) or High Fidelity Taq polymerase (Invitrogen) and gene-specific primers (Table 1). The plasmids pGEM-T Easy (Promega, Madison, WI, USA) 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.

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* WT and the *cnrg1* mutant (Herrero et al. 2007). Cloning of *CnATR2* and *CnMFS2* was performed using inverse PCR (Keim et al.

2004). For genes encoding the uracil transporter, the zinc transport protein and *CnATR3*, primers specific to homologs from the closely related maize pathogen *Cercospora zea-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. zea-maydis*, and Genescan (Burge 1998) was used to confirm the locations of introns.

DNA sequencing was performed by Eton Biosciences Inc. (San Diego, CA, USA) and Macrogen USA (Rockville, MD, USA). 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, USA); 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.

Phylogenetic analysis of transporter genes

CnATR2, *CnATR3* and *CnMFS2* protein 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).

Gene expression analysis in *Cercospora*

Differential expression of *CnATR2* between WT and the *cnrg1* mutant (Chung et al. 2003) was assayed as previously described (Amnuaykanjanasin and Daub 2009).

Assays for expression under conditions of cercosporin toxicity utilized disruption mutants for *CnCTB1* (*cnctb1*), *CnATR1* (*cnatr1*) (Amnuaykanjanasin and Daub 2009), and for *CnATR2* (*cnatr2*). 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 10 μ M cercosporin or with 0.5 % acetone (used

Table 1 Primer sequences

CnATR2 disruption construct cloning	
ATR2-5'-F1w/ApaI	ATTATA <u>GGGCCCT</u> CCTGGGCATTGTTTCGATCCTCTAC
ATR2-5'-R1w/ApaI	ATTATA <u>GGGCCCC</u> ATGAGAACAGTCGTGATGTATGCGAGAAC
ATR2-3'-F1w/EcoRI	ATTATA <u>GAATTC</u> TCCACTCTACCAAGGCTCTGTTCG
ATR2-3'-R1w/SacI	ATTATA <u>GAGCTC</u> ACCTGGTCGGCAGTGTTCTCATC
Amplification of split markers	
ATR2-5'-F1	TCCTGGGCATTGTTTCGATCCTCTAC
ATR2-3'-R1	ACCTGGTCGGCAGTGTTCTCATC
HYG-split3S	CGTTGCAAGACCTGCCTGAA
HYG-split5A	GGATGCCTCCGCTCGAAGTA
Screening <i>atr2</i>-disruption strains	
ATR2-5' F2	TGCTTGGTGGCGTCGCTAG
ATR2-3'-R2	ATCCACTTCAGGGTCGAGCATG
Hyg-F2	TGAACCATCTTGTCAAACGACAC
HYG-R	TGTCGGGCGTACACAAATCG
14c-2700R	GATCGTTTGCTTGAGCTTCGAG
ATR2-F1	ACAGGAAGTCACCCATCGTTACC
14c-5 ORFa-F	CTGGAGGTCTGAAGGATTACTG
CnATR2-expressing construct	
14c-start2 w/Xba	<u>GCTCTAGA</u> ACCTCGACATGGGTGACTACA
14c-3end	GCTTTCTGAAGTACTGCGACTGC
Screening the presence of CnATR2 in <i>N. crassa</i> transformants	
ATR2-F1	ACAGGAAGTCACCCATCGTTACC
ATR2-R1	AACGACGAGACCGAGCTTCC
Amplification of cDNA for RT-qPCR	
ATR1-F	GTCGCTTGCACCGTCTACTG
ATR1-R	CATGGTAGCCTTCTGCTTCTCA
ATR2-F	CTAGCAAGCTCTCCTCGATTCC
ATR2-R	GATCGTTTGCTTGAGCTTCGAG
MFS2-F	AGCCGGCAATGTCACACTTAC
MFS2-R	TCAAGGCGCAGTATGGATCTT
CFP-F	ACGCCGGAAGTGTCTTATGC
CFP-R	TGCGGTGTGTCGAGTGTGA
ATR3-F	TGTCATTACCGGCTCCATGTT
ATR3-R	TGCTGCTGCACATAGCCTGT
Uracil-F	GGAGCCAAATATCACCTGAGCTT
Uracil-R	CGCCCCAAATCCCAAAG
Zinc-F	CCAGCCCTCAGGTGGAGA
Zinc-R	TGAGTGCTCGCCTTTACCAC
ATR2-F	CTAGCAAGCTCTCCTCGATTCC
ATR2-R	GATCGTTTGCTTGAGCTTCGAG
NC-Tubulin 2F	CCC CGGTCTCAAGATGT
NC-Tubulin 2R	CGCTTGAAGAGCTCCTGGAT
CerActin-F	TGACGATGCGCCACGAGCTGT
CerActin-R	TTGATTGGAGCCTCGGTGAGC
Cer18S-F	TGGTGGAGTGATTGTCTGCTT
Cer18S-R	TCCCCAGCACGACAGAGTTT

Bold and underlined sequences represent restriction enzymes targets

to solubilize cercosporin). After cercosporin or acetone treatment, cultures were incubated under high light intensities (55–65 $\mu\text{E m}^2/\text{s}$) before harvesting mycelia for RNA extraction and RT-qPCR analysis.

Total RNA was extracted from lyophilized and homogenized mycelia with TRI-REAGENT (Sigma-Aldrich, St Louis, MO, USA) 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, an LiCl and ethanol precipitation steps were performed, after which samples were treated with DNase Turbo DNA-free™ Kit (Ambion, Austin, TX) according to the manufacturer's recommendations.

Protocols for cDNA synthesis and qPCR were same as used in Beseli et al. (2015). Each sample was normalized against the following *C. nicotianae*-specific internal controls: 18S rRNA for *cnctb1* and actin for *cnrg1*, *cnatr1*, *cnatr2*. Primers used to amplify each gene are shown in Table 1. Fold changes were calculated according to the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen 2001; Schmittgen et al. 2000; Winer 1999). Two independent experiments with three replications each were performed.

Transformation of *N. crassa* with *CnATR2*

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 *XbaI*) and 14c-3end (Table 1). This PCR fragment was digested with *XbaI*, and ligated to the pTxA-1 that was digested with *XbaI* and *SmaI*. 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. Protoplasts were generated as previously described (Beseli et al. 2015).

For transformation, a total of 2×10^7 protoplasts were incubated with 10 μg of plasmid DNA and regenerated in the presence of 100 $\mu\text{g}/\text{ml}$ hygromycin. Single hygromycin (hyg)-resistant colonies that formed after 2–3 days were transferred to new plates and were continuously maintained under selection (100 $\mu\text{g}/\text{ml}$ hygromycin) for subsequent manipulations. Transformation and presence of *CnATR2* were 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.

Screening of *N. crassa* *CnATR2* transformants for cercosporin and photosensitizer resistance

The *CnATR2* transformants confirmed by PCR analysis were tested for cercosporin resistance on split petri plates as previously described (Beseli et al. 2015). Radial growth of colonies was measured at 16 h after inoculation, and % growth on cercosporin was calculated relative to the acetone control on the same plate. Each transformant was replicated five times, and the experiment was repeated two times. The mean value of all 10 samples for each strain and the corresponding standard errors were calculated and significant differences between WT and each transformant were calculated using Dunnett's *t* Tests (Dunnett 1955).

Strains of *N. crassa* *CnATR2* transformants that demonstrated resistance to cercosporin were further tested for resistance to other photosensitizers. Assays for resistance were conducted as above using 10 μM of hypericin (Alfa Aesar, Ward Hill, MA, USA) and 10 μM of rose bengal (Sigma-Aldrich, St. Louis, MO, USA). Hypocrellin A (Abcam, Cambridge, MA, USA) was used at 5 μM due to its high toxicity. Hypocrellin A and hypericin were solubilized in acetone, and control medium contained the same amount of acetone (0.5 %). Rose bengal was dissolved in water. Each transformant and the WT *N. crassa* were replicated five times and the experiment was repeated twice.

For gene expression analysis, total RNA was extracted from lyophilized and homogenized mycelia of five randomly chosen transformants, and RT-qPCR analysis was conducted as previously described (Beseli et al. 2015). Each sample was normalized against the *N. crassa*-specific tubulin control.

Targeted gene disruption in *C. nicotianae*

For targeted disruption of the *CnATR2* gene in WT *C. nicotianae*, a split-marker recombination technique was modified as previously described (You et al. 2009). The 5' [from 200 base pairs (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/*ApaI*, ATR2-5'-R1 w/*ApaI* and ATR2-3'-F1 w/*EcoRI*, ATR2-3'-R1 w/*SacI*. The PCR products were digested with the appropriate restriction enzymes (ATR2-5' digested with *ApaI*; ATR2-3' digested with *EcoRI* and *SacI*) for cloning into the receptor plasmid pCB1636 (Sweigard et al. 1997). Plasmid pCB1636 was first digested with *ApaI* and ligated with *ApaI* digested ATR2-5'. Recombinant plasmids isolated from this step were selected based on the insert's correct orientation, and

subsequently digested with *EcoRI* and *SacI* and ligated with *ATR2-3'* to obtain a *CnATR2* disruption construct (Supplemental Fig. 1). Using this construct as a template, 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) except that cultures were grown in PDB. For transformation, 1×10^7 protoplasts were incubated with 10 μg of each of the two split marker PCR fragments in the presence of spermidine (3 mM) and heparin (0.5 mg/ml). Transformants were originally selected in medium containing 125 $\mu\text{g}/\text{ml}$ of hygromycin. Hyg-resistant colonies were transferred to PDA amended with 125 $\mu\text{g}/\text{ml}$ hygromycin, and were transferred a minimum of five 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 $\mu\text{g}/\text{ml}$ hygromycin using the E.Z.N.A. Fungal DNA Mini kit (Omega Bio-tek, Norcross, GA, USA).

Screening of *cnatr2* 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 $\mu\text{E m}^2/\text{s}$) at room temperature. Cercosporin was extracted with 5 N KOH and concentrations were measured as previously described (Jenns et al. 1989). For sporulation, cultures were grown on V8 agar (30 % V8 juice, 0.045 M CaCO_3 , 2 % agar) at 18 °C. To induce conidiation, the cultures were brushed with a sterile paint brush after 4 days and incubated under the same conditions for four additional days. To recover spores, plates were flooded with water and brushed gently. Spores were quantified using a hemocytometer. For inoculations, spores were diluted to a final concentration of 3.3×10^4 cells/ml, and 50 ml of spore solution was atomized onto the upper leaves of 3-month-old 'Burley 21' tobacco plants (3 plants per strain). Plants were covered with plastic bags for 4 days to provide high humidity conditions. Disease ratings were taken 7 weeks after inoculation.

Results

Sequence analysis of transporters

Five genes encoding transporters recovered in a subtractive library between the *C. nicotianae* WT and the cercosporin-sensitive *cnrg1* transcription factor mutant were analyzed. RT-qPCR analysis showed that EST 103cF (*CnMFS2*) was strongly downregulated in *cnrg1*, whereas 86cR (*CnATR3*) and 53cR (encoding the uracil transporter) were both upregulated in *cnrg1* (Herrero et al. 2007). Preliminary microarray analysis (unpublished data) of the expression of 184sR5 (encoding the zinc transport protein) and 14c (*CnATR2*) showed little difference in expression of either gene between the WT and the *cnrg1* mutant.

Full-length sequences were obtained for the five transporter genes. *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), similar to most eukaryote transporters (Lamping et al. 2010). *CnATR3* has a 4717 bp genomic sequence, 4404 bp ORF and six introns (GenBank accession# KC959476). As with *CnATR2*, the protein consists of two similar halves with transmembrane and nucleotide-binding domains. *CnMFS2* (GenBank accession # GU646037) has 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. The zinc transport protein gene has an 1865 bp genomic 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-Bauer et al. 2011). The uracil transporter gene has a 1176 bp genomic sequence and lacks introns (GenBank accession# AF306523.1). The gene includes solute binding domains found in Solute Carrier (SLC) families five and six 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^- dependent plasma membrane transporters (neurotransmitters) (Kristensen et al. 2011). NCS1 transporters are essential secondary active transporters for nucleobases, hydantoins, and vitamins and work 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*

Table 2 Homologs of transporters included in this study

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

cerevisiae (Pantazopoulou and Diallinas 2007). Homologs of each of the transporter proteins identified are shown in Table 2.

Phylogenetic analysis of CnATR2, CnATR3, and CnMFS2

As noted above, ABC and MFS transporters have been identified in previous studies to have roles in cercosporin production and resistance, thus we compared CnATR2, CnATR3, and CnMFS2 to these and other ABC and MFS transporters using phylogenetic analysis (Figs. 1, 2; Supplemental Table S1). 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. 1). 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. 1). 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. 1a). 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. 1a).

For the MRP-like (ABC-C) group, the ABC1 and ABC2 domains of fungal transporters were clustered with considerably lower bootstrap support (Fig. 1b). 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. 2, Supplemental Table S1) including the *S. cerevisiae* Dtr1p that has been experimentally verified for transport of bisformyldityrosine into the surface of ascospores (Felder et al. 2002). Filamentous fungal and yeast dityrosine transporters were clearly separated

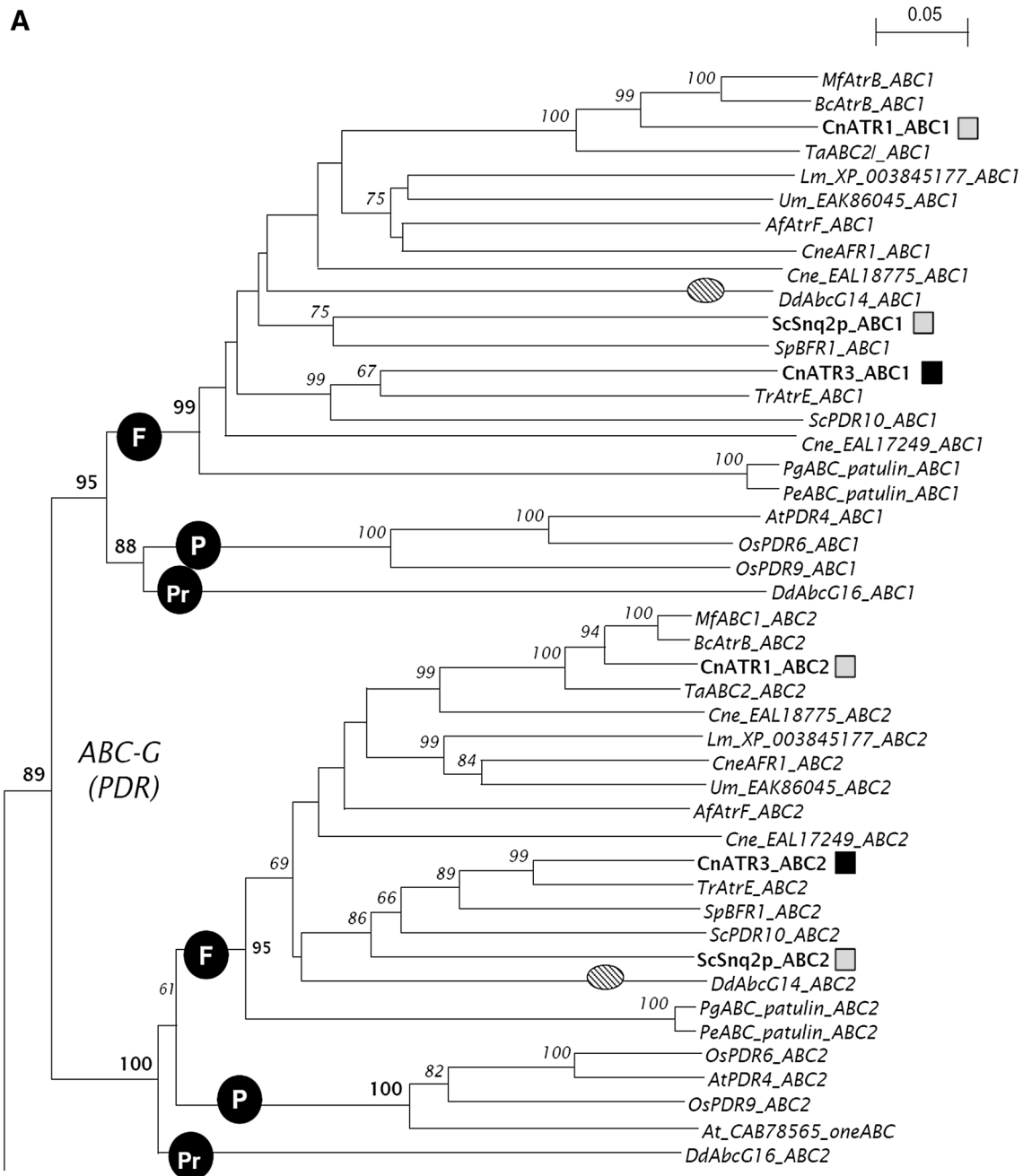


Fig. 1 Phylogenetic analysis of ABC transporters. Fungal, plant, protist, and animal branches are noted in *black circles* as F, P, Pr, and A, respectively. Transporters from other taxonomic groups within those branches (e.g. *Dictyostelium* transporter clustering within the fungal group) are noted by *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 ScSsq2p are marked by *gray squares*. CnATR1, CnATR3, and ScSsq2p grouped with the subfamily ABC-G (PDR) transporters (*panel a*). CnATR2 grouped with ABC-C (MRP) transporters (*panel b*). Bootstrap analysis was performed with 1000 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 S1

in the tree with strong bootstrap support (Fig. 2). Two MFS transporters previously shown to play a role in cercosporin resistance, CFP from *Cercospora kikuchii* and *C. nicotianae* (Amnuaykanjanasin and Daub 2009; Callahan et al. 1999)

and Bcmfs1 from *Botrytis cinerea* (Hayashi et al. 2002), were clustered together in a distinct group with 100 % bootstrap support. CnCTB4, the MFS transporter found within the cercosporin biosynthetic cluster, is also shown.

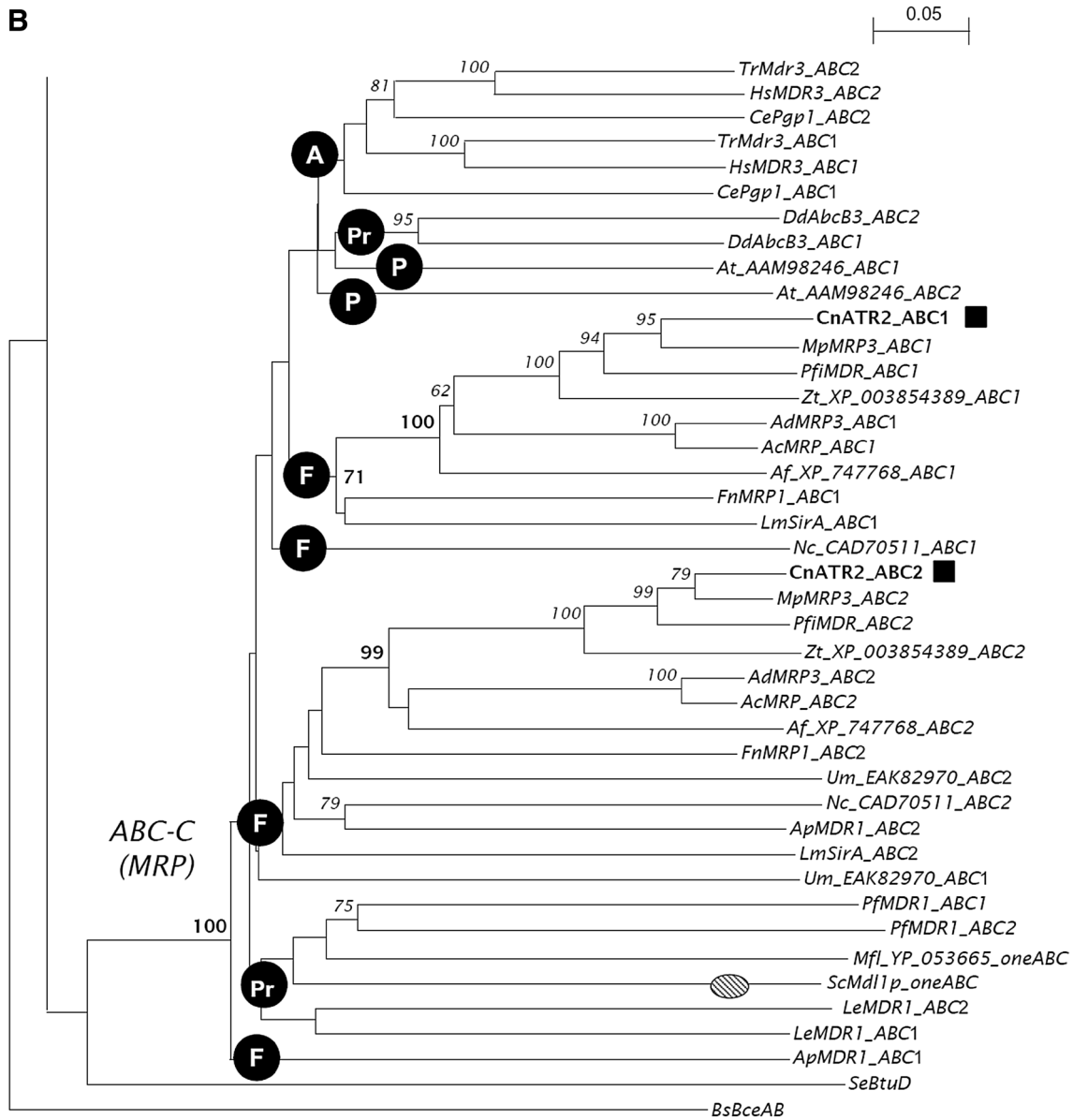


Fig. 1 continued

Expression of transporters under conditions of cercosporin toxicity

To test for a possible role in cercosporin resistance, the expression of the five transporter genes along with the previously characterized *CnCFP* was assayed under conditions of cercosporin toxicity by treating the cercosporin-sensitive *cnatr1* mutant (Amnuaykanjanasin and Daub 2009) with cercosporin under high light conditions. Both *CnCFP* and *CnATR2* were strongly induced when cercosporin toxicity was induced (Fig. 3). *CnCFP* was induced more than 50-fold after both 1 and 3 h of treatment, and *CnATR2* was upregulated 9–16-fold. By contrast, there was no induction

of *CnMFS2*, *CnATR3*, or the uracil transporter or the zinc transport protein genes. Based on these gene expression results, *CnATR2* was chosen for further characterization.

***CnATR2* characterization**

CnATR2 was one of six of the 185 ESTs recovered in the subtractive library that was found in both the forward (downregulated in *cnarg1* relative to WT) and reverse (up-regulated in *cnarg1* relative to WT) libraries (Herrero et al. 2007). As noted earlier, preliminary macroarray data (unpublished) demonstrated little difference in *CnATR2* expression between WT and the *cnarg1* mutant. We tested

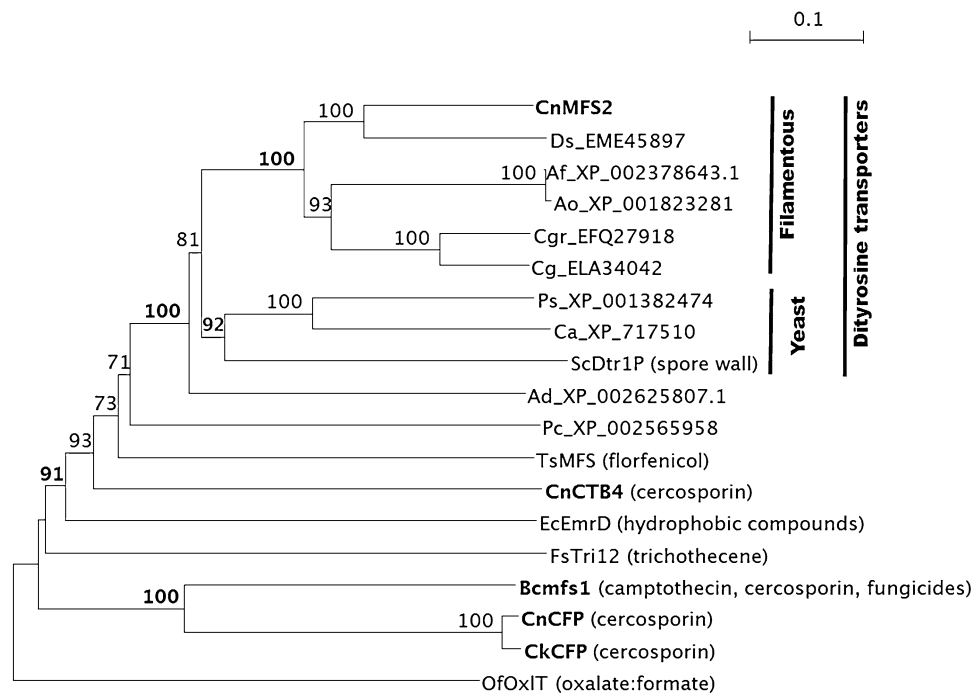
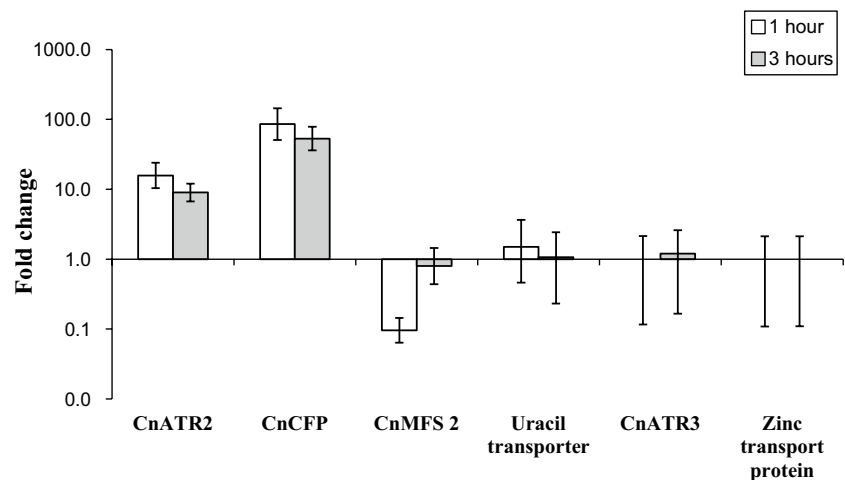


Fig. 2 Phylogenetic analysis of MFS transporters. The phylogenetic tree includes known fungal MFS transporters, with target compounds, when known, indicated in *parenthesis*. Transporters in this study as well as those previously shown to be involved in cercosporin production or resistance are shown 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 1000 replicates, and values of >60 % are given at the nodes. A bacterial MFS transporter, *Oxalobacter formigenes* *OxIT*, was used as the outgroup in this tree. Protein names and accession numbers for each sequence are shown in supplemental Table S1

Fig. 3 RT-qPCR analysis of gene expression of transporter genes in the *C. nicotianae cnatr1* 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 $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Data represent the mean of two independent experiments each with three technical replications. Error bars represent 95 % confidence intervals



the expression of *CnATR2* in WT and the *cnarg1* mutant using qRT-PCR (Fig. 4a). As medium composition was previously shown to affect relative gene expression between *C. nicotianae* WT and the *cnarg1* mutant in other transporters (*CnCFP*, *CnCTB4*, *CnATR1*) (Amnuaykanjanasin and Daub 2009), we tested cultures grown in both PDB and CM liquid medium. The *C. nicotianae* WT and the *cnarg1* mutant both synthesize cercosporin when grown in CM medium,

however, only WT synthesizes cercosporin in PDB medium. *CnATR2* gene expression was downregulated in the *cnarg1* mutant relative to WT when grown in PDB medium, but upregulated in CM medium. This pattern is the same as the expression pattern of the previously characterized *CnATR1* gene that is involved in cercosporin resistance (Amnuaykanjanasin and Daub 2009). To test directly if *CnATR2* expression is regulated by the presence of cercosporin, we assayed

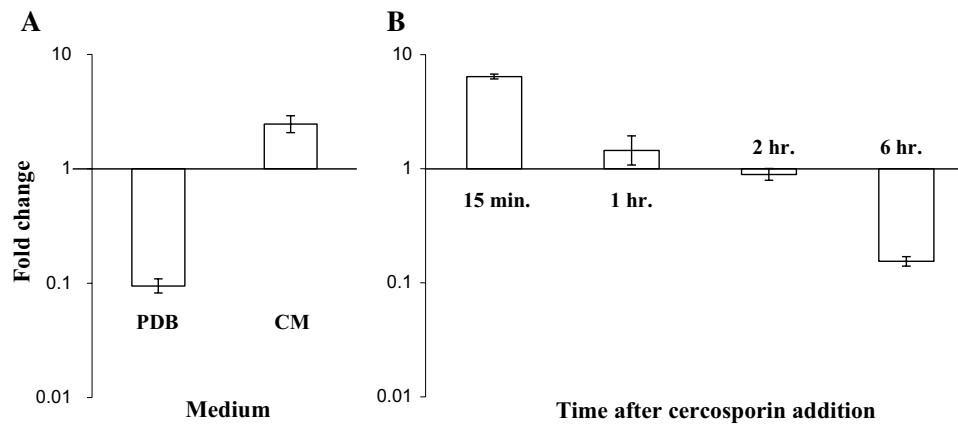


Fig. 4 Expression analysis of *CnATR2*. **a** Differential expression of *CnATR2* in the cercosporin-sensitive *C. nicotianae* *cnrg1* mutant, deficient for the CRG1 transcription factor. Cultures were grown for 6 days in two different culture media: potato dextrose broth (PDB) and CM medium. Gene expression was quantified using RT-qPCR, with each sample normalized against the actin control. **b** Expression of *CnATR2* in response to cercosporin in a non-cercosporin-producing *C. nicotianae* mutant deficient for the CTB1 polyketide synthase

in the cercosporin biosynthetic pathway. Gene expression was quantified using RT-qPCR normalized against the 18S rRNA control. Cultures were treated with cercosporin or 0.5 % acetone, and RNA was extracted at 15 min, and 1, 2 and 6 h after addition of cercosporin. Expression in response to cercosporin is shown relative to the acetone control. Data for both analyses were calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Error bars represent 95 % confidence intervals

CnATR2 expression in a *C. nicotianae* strain mutant for the CnCTB1 polyketide synthase in the cercosporin biosynthetic pathway and thus defective in cercosporin production (Choquer et al. 2005). *CnATR2* expression was strongly upregulated within 15 min of the addition of cercosporin, and then decreased (Fig. 4b).

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*, an assay previously used to identify putative cercosporin resistance genes (Beseli et al. 2015). 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 μ M inhibits radial growth of *N. crassa*, resulting in approximately 30 % of the radial growth on medium lacking cercosporin (Fig. 5a). Of the 23 PCR-positive transformants tested, six were found to be significantly more resistant to cercosporin than WT ($P < 0.05$). Mean resistance of the remaining transformants varied from 40 to 60 %.

Five randomly chosen *CnATR2* transformants screened for resistance were selected to analyze the expression of *CnATR2* (Fig. 5b). RT-qPCR analysis of *CnATR2* expression in all five of the *CnATR2* transformants showed high levels of expression (tubulin-normalized) relative to the WT control. Transformants # 8 and # 21 that were not

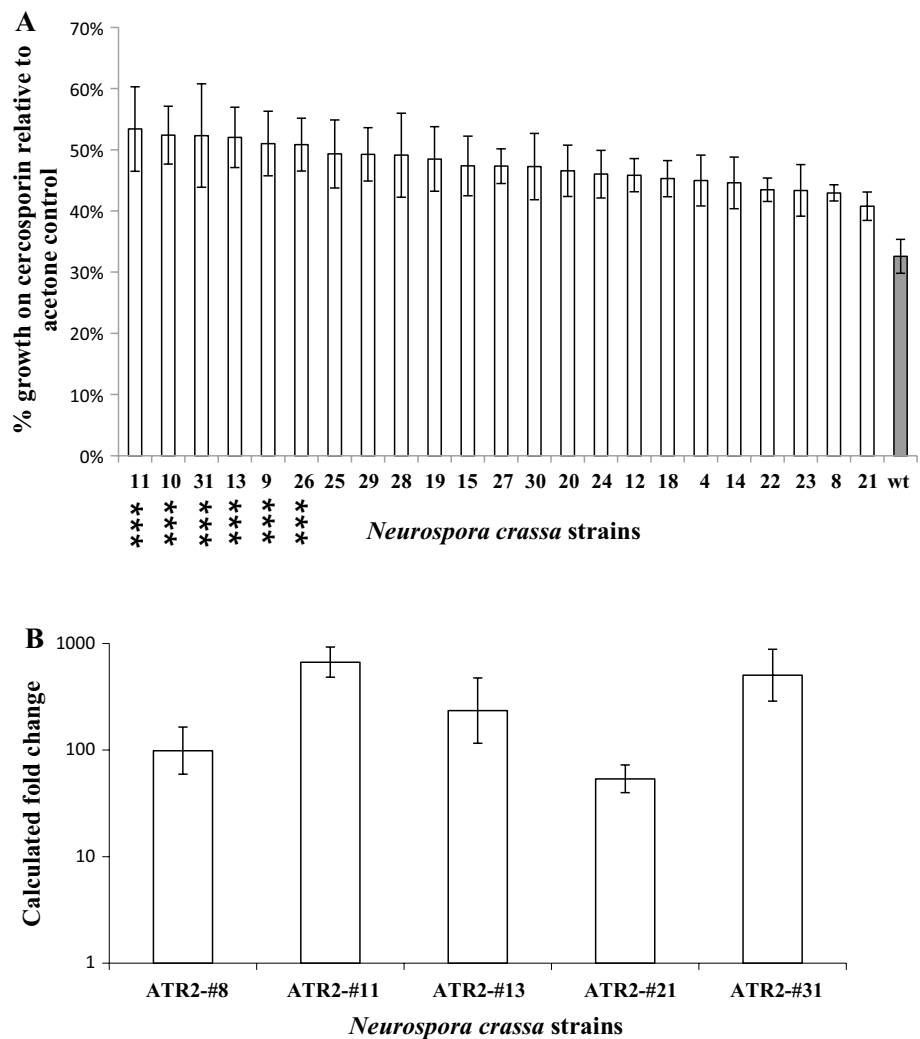
statistically more resistant than WT had the lowest expression (99-fold and 54-fold, respectively). The cercosporin-resistant transformants (# 11, 13, 31) had the highest expression (669-fold, 235-fold, and 505-fold, respectively). Thus, *CnATR2* expression correlated with resistance in the transformants tested.

To test if expression of *CnATR2* could impart resistance to other 1O_2 -generating photosensitizers, we tested response of the cercosporin-resistant *N. crassa* transformants to other photosensitizers: hypocrellin A (a perylenequinone analog from the fungus *Hypocrella bambusae*); hypericin [an extended quinone from the plant *Hypericum perforatum* (St. John's Wort)], and the structurally unrelated photosensitizer rose bengal. Assay of toxicity to the *N. crassa* WT in the dark showed a lack of dark toxicity by hypericin and hypocrellin A, similar to cercosporin; however, rose bengal suppressed *N. crassa* growth in the dark by approximately 50 % (data not shown). Results of the toxicity assays in the light with the WT *N. crassa* and the cercosporin-resistant *CnATR2* transformants are shown in Fig. 6. Hypocrellin A was the most toxic, and was used at a lower concentration (5 μ M) due to its high toxicity. Hypericin showed similar levels of toxicity as does cercosporin, and rose bengal was the least toxic. For all compounds, the *CnATR2* transformants were not significantly more resistant than WT *N. crassa*.

atr2 disruption mutants of *C. nicotianae*

A split marker strategy was used to generate *C. nicotianae* disruption mutants by homologous recombination. A total

Fig. 5 Transformation of the cercosporin-sensitive fungus *Neurospora crassa* with *CnATR2*. **a** Cercosporin resistance of *N. crassa* wild type (gray bar) and *CnATR2*-transformed strains (white bars). Data are radial growth on 10 μ M cercosporin relative to the acetone control. Data are the results of two independent experiments with five replications each. Strains marked with three asterisks have significantly greater resistance than wild type ($P < 0.05$). Error bars represent standard error. **b** RT-qPCR analysis of gene expression of *CnATR2* transformants #8, 11, 13, 21 and 31. Each sample was normalized against the tubulin control, and fold-change relative to the non-transformed wild type *Neurospora crassa* control was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Error bars represent 95 % confidence intervals



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 *cnatr2*-disruption mutants were tested for cercosporin sensitivity by growing them on cercosporin-containing medium in the light. WT 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 WT, the non-disrupted transformants, or the *cnatr2* disruption mutants in radial growth on cercosporin.

As other transporters (ATR1, CFP) have shown a dual role in both cercosporin resistance and production, eight of the *cnatr2* disruption mutants (# 17, # 62, # 65, # 75, # 78, # 151, # 173 and # 181), two non-disrupted transformants (# 58 and # 59) and WT *C. nicotianae* were selected for assay of

cercosporin production (Fig. 9a). There was no significant difference between cercosporin production between WT and the *cnatr2* transformants suggesting that *CnATR2* is not involved in cercosporin production. All eight *cnatr2* disruption mutants, two non-disrupted transformants and WT *C. nicotianae* were also tested for changes in sporulation (Fig. 9b). Average production of spores ranged from 0.8 to 2.6×10^5 spores/plate, and there was no significant difference in the number of spores produced by the strains. All eight disruption mutants, two non-disrupted transformants and WT *C. nicotianae* were then used for pathogenicity assays on tobacco. Disease severity was assayed by counting the number of coalesced lesions on inoculated leaves; there were no significant differences between the disruption mutants and WT *C. nicotianae* (Fig. 9c).

Expression of other transporters and cercosporin biosynthetic genes in *cnatr2* disruption mutants

Expression of *CnATR2* in *N. crassa* demonstrated that *CnATR2* can provide cercosporin resistance; however, *atr2*

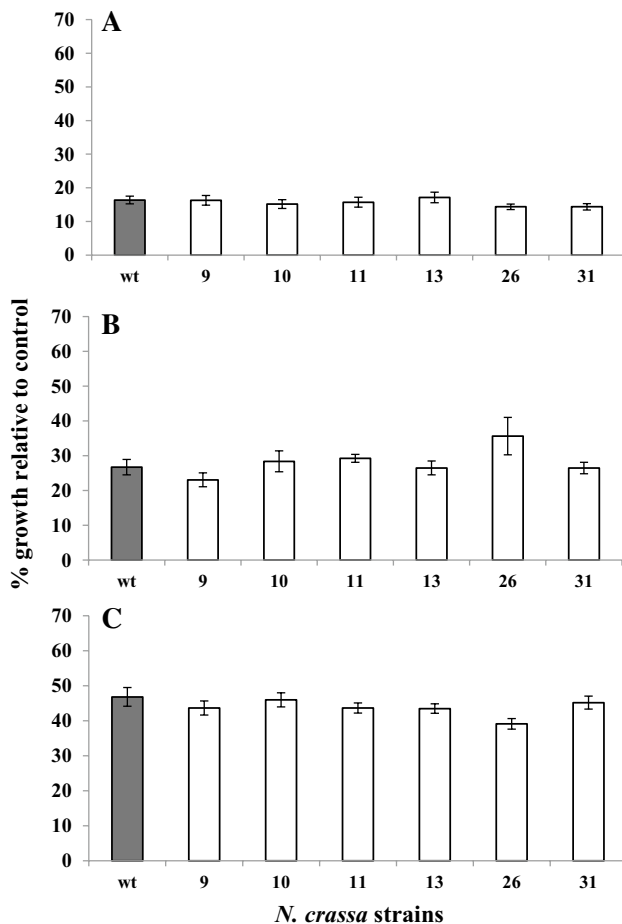


Fig. 6 Growth of *Neurospora crassa* *CnATR2* WT (gray bar) and cercosporin-resistant transformants (white bars) on medium containing $^1\text{O}_2$ -generating photosensitizers: **a** 5 μM hypocrellin A, **b** 10 μM hypericin and **c** 10 μM rose bengal. Data shown are relative to growth on the acetone control (hypocrellin A, hypericin) or water control (rose bengal). Data are the results of two independent experiments with five replications each. Each data point is reported as mean (\pm SE)

mutants of *C. nicotianae* were not more sensitive to cercosporin than WT. 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 upregulated in the *cnatr2* 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 h and over 170-fold at 3 h. These results suggest that the lack of sensitivity of the *cnatr2* mutant may be due to compensation by overexpression of *CnCFP*.

The subtractive library of putative cercosporin resistance genes also includes two genes from the cercosporin biosynthetic pathway: *CnCTB2*, encoding an *O*-methyltransferase, and *CnCTB5*, 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 *cnatr2* mutant showed significant upregulation of *CnCTB5* as well as *CnCTB7*, encoding a second FAD/FMN-dependent oxidoreductase (Fig. 10b); expression of *CnCTB2* and the other biosynthetic genes including the *CnCTB4* transporter was not altered.

Discussion

Cercospora fungi are highly successful plant pathogens due to their production of the photosensitizing toxin cercosporin. Cercosporin shows almost universal toxicity to cells because it produces reactive oxygen species including the highly toxic $^1\text{O}_2$. For many years, our work has been focused on understanding the genes and mechanisms involved in cercosporin autoresistance (Daub et al. 2013), as these genes may have utility in engineering crop plants for resistance. In this work, we focused on the study of membrane transporters in *Cercospora nicotianae*. Transporters have previously been shown to provide cercosporin resistance in several fungal species. Also, genes encoding membrane transporters were recovered in a subtractive library between the *C. nicotianae* WT strain and a cercosporin-sensitive mutant deficient for the CRG1 transcription factor required for resistance (Herrero et al. 2007).

Membrane transporters shown to play a role in resistance to cercosporin are diverse. In *Cercospora*, both the ABC transporter *ATR1* (Amnuaykanjanasin and Daub 2009) and the MFS transporter *CFP* (Callahan et al. 1999) were found to play a role in resistance; both of these were also recovered in the subtractive library. In addition, studies in other fungal systems 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 *Campylothecha acuminata* (Hayashi et al. 2002, Roohparvar et al. 2007). The role of transporters has also been shown in medical studies using the structurally similar photosensitizer hypericin, where increased expression of ABC transporters in cancer cells reduced utility of hypericin as an anti-cancer agent (Jendzelovsky et al. 2009).

For this study, we cloned and conducted bioinformatics analysis to identify the remaining transporter genes in our subtractive library. We 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

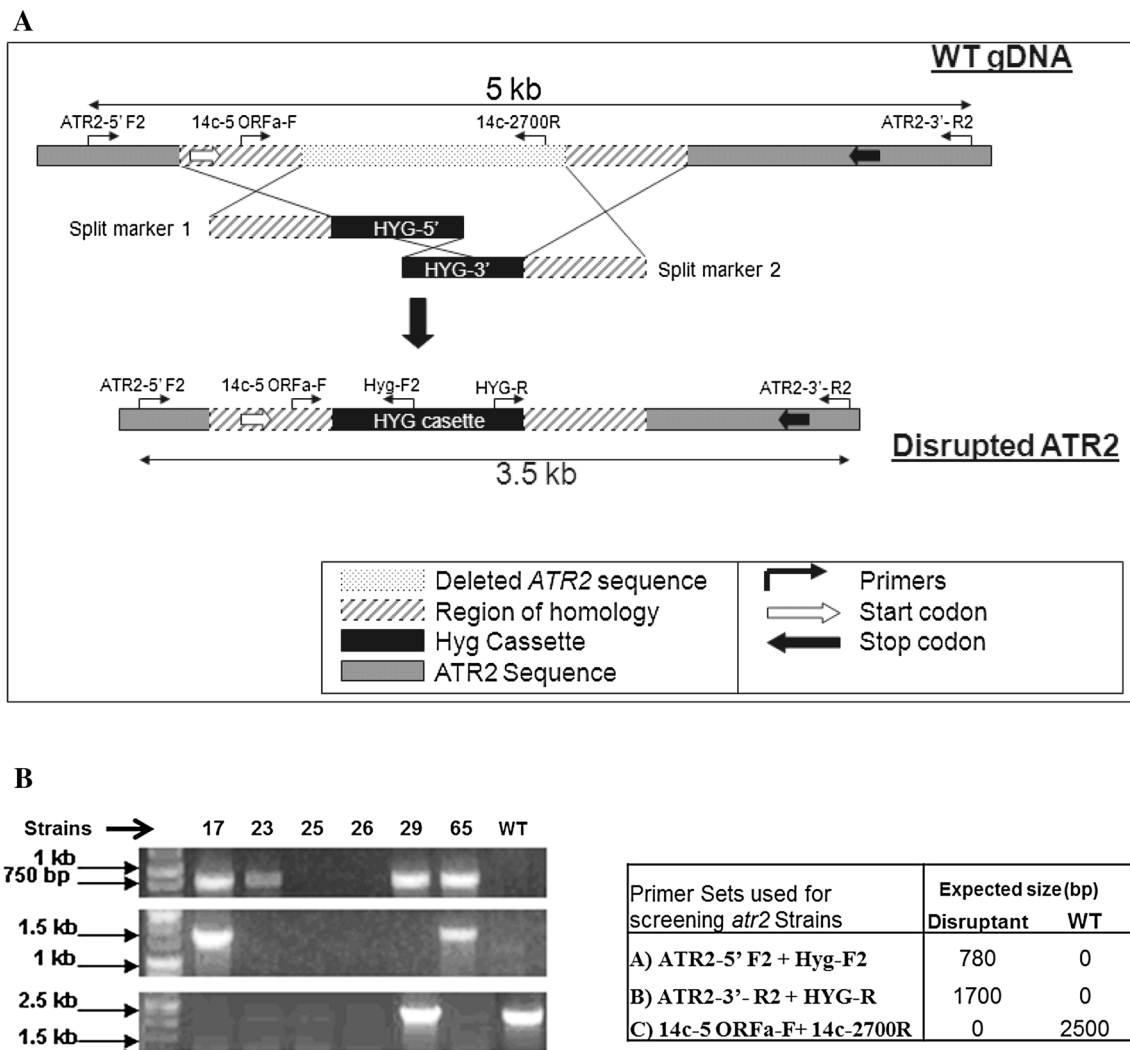


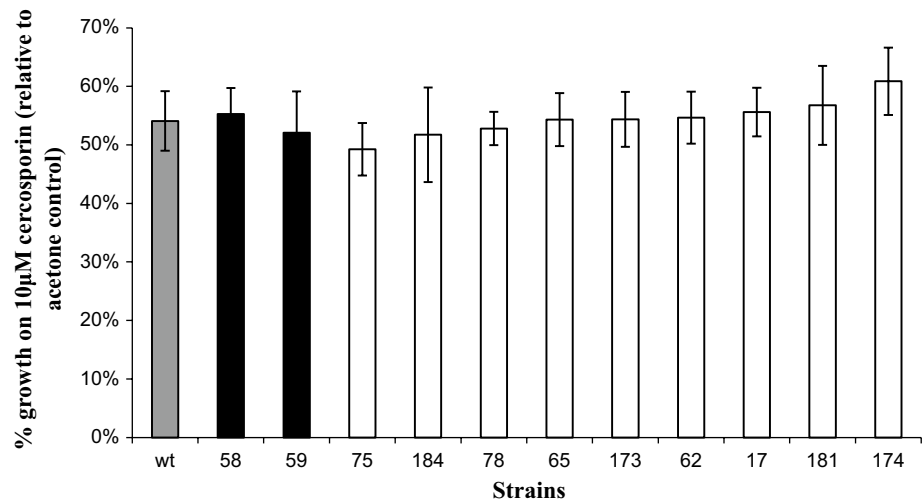
Fig. 7 Targeted gene disruption of *CnATR2* in *C. nicotianae* wild type (WT) using a split-marker recombination method (You et al. 2009). **a** Diagrams of *C. nicotianae ATR2* genomic DNA, split marker PCR fragments, and resulting disruption sequence. *Hatched bars* show regions of homology between 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 # 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 (# 29) 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

been implicated in cercosporin resistance and production, we conducted a phylogenetic analysis of the ABC and MFS transporters along with other transporters previously implicated in cercosporin resistance or production. ABC transporters previously characterized to play a role in cercosporin resistance (the *C. nicotianae ATR1* and *S. cerevisiae Snq2p*) were found in the PDR (pleiotropic drug resistance) subfamily (Fig. 1A). Our phylogenetic analysis showed that *CnATR3* clusters in this group. 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* 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 to be involved in resistance, but rather in maintenance of plasma membrane asymmetry of budding yeast (Rockwell et al. 2009). The two ABC domains of the previously characterized *CnATR1* clustered within their own group in the PDR subfamily, along with

Fig. 8 Cercosporin resistance of *C. nicotianae* wild type (gray bar), *CnATR2* non-disruption transformants (black bars) and disruption mutants (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-disruption hyg-resistant transformants and *cnatr2* disruptants



transporters in the Leotiomycetes *Monilinia fructicola* and *B. cinerea*. 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). *CnATR1*'s role in cercosporin resistance makes this group of ABC transporters strong candidates for toxin resistance. In our study, however, *CnATR3* expression was unchanged under conditions of cercosporin toxicity; thus, it is not known at this time what role *CnATR3* plays in *C. nicotianae*.

CnATR2 clustered with the MRP (multidrug resistance-associated protein-like) transporters (Fig. 1b). The ABC domains of *CnATR2* cluster with those of *Mycosphaerella populorum* MRP3, *Mycosphaerella fijiensis* MDR, and *Zymoseptoria tritici* ABC. 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 demonstrate that *CnATR2* can impart cercosporin resistance to *N. crassa*, suggesting that the homologous *Mycosphaerella* and *Zymoseptoria* transporters would be candidates for investigating possible roles in resistance to perylenequinones.

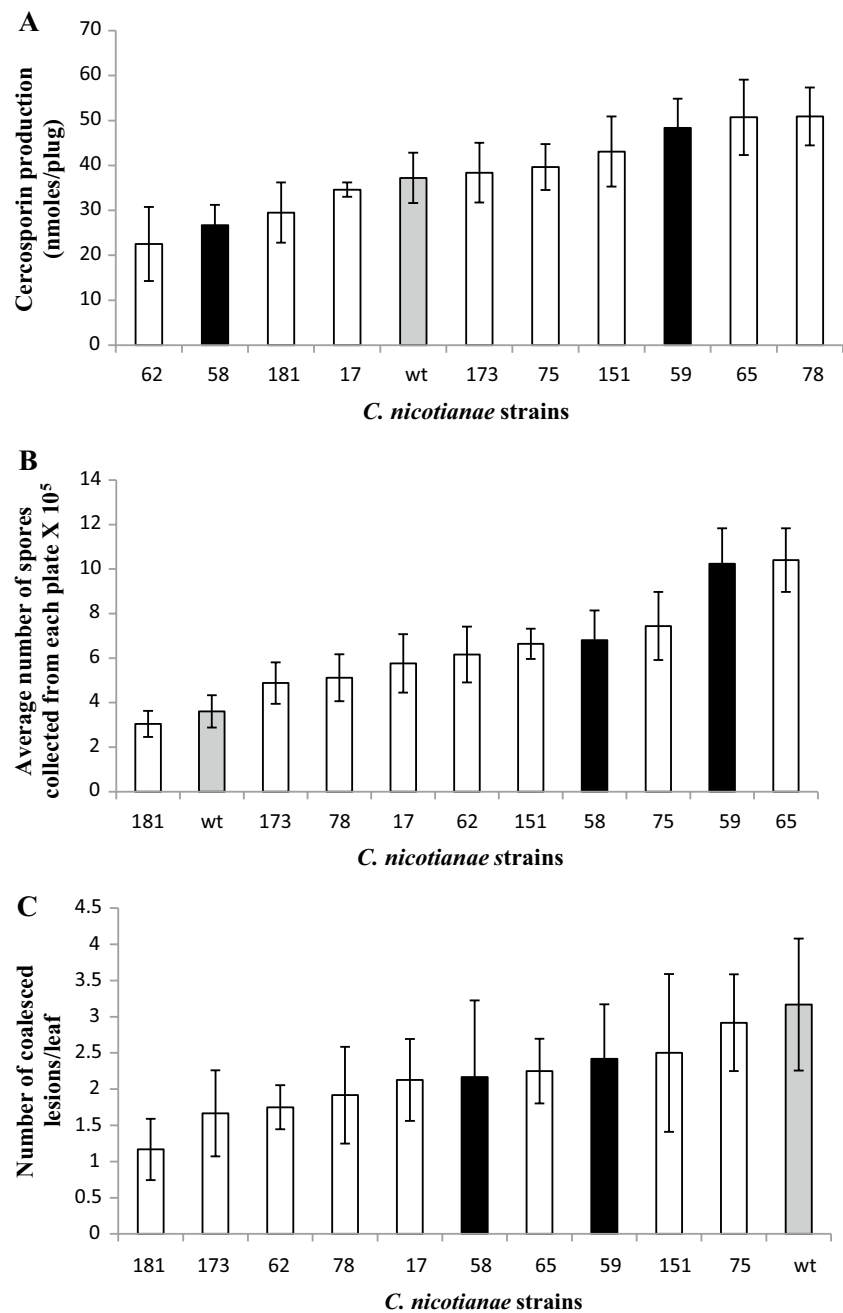
Two of the transporters previously shown to have a role in cercosporin resistance are MFS transporters, *Bcmf1* from *B. cinerea* (Hayashi et al. 2002) and *CFP* from *C. nicotianae* and *C. kikuchii* (Callahan et al. 1999). *CnMFS2*, investigated in this study, did not cluster with these transporters, nor did any of these cluster with *CnCTB4*, the MFS transporter in the cercosporin biosynthetic cluster. *CnCTB4* has been shown to be regulated by *CRG1* (Daub et al. 2013). However, in this study we found that *CnCTB4* was not induced under conditions of cercosporin toxicity, a result consistent with previous studies documenting no role for *CnCTB4* in cercosporin resistance

(Choquer et al. 2007; Daub et al. 2013). *CnMFS2* clusters with a transporter protein from *Dothistroma septosporum* (teleomorph = *Mycosphaerella pini*), a fungus also in the Dothidiomycetes which produces the photoactivated toxin dothistromin (De Wit et al. 2012; Stoessl et al. 1990). The *Dothistroma* transporters 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) and with no known role in protection against toxic compounds. We found that *CnMFS2* was not upregulated under conditions of cercosporin toxicity.

To test for a role of these transporters in cercosporin resistance, we assayed for changes in expression when the cercosporin-sensitive *cnatr1* transporter mutant was exposed to cercosporin toxicity. Only two of the transporter genes were upregulated. The previously characterized *CnCFP* (Callahan et al. 1999) was induced almost 100-fold at both time points tested (1 and 3 h). *CnATR2* was also induced approximately 10-fold at both time points. As indicated above, there was no induction of *CnATR3*, *CnMFS2*, or genes encoding the uracil transporter or the zinc transport protein.

Upregulation of *CnATR2* in response to cercosporin toxicity led us to further characterize its role in resistance. We showed that *CnATR2* is transiently upregulated in response to cercosporin, and that its regulation in the *cnarg1* transcription factor mutant was similar to that of *CnATR1*, previously shown to play a role in both cercosporin resistance and production. We then tested the ability of *CnATR2* to increase cercosporin resistance in the cercosporin-sensitive fungus *N. crassa*. We used this assay successfully in a previous study to characterize library genes that are homologs of $^1\text{O}_2$ -resistance genes in the photosynthetic bacterium *Rhodobacter sphaeroides* (Beseli et al. 2015). Under the conditions of the assay, growth of WT *N. crassa* is

Fig. 9 Cercosporin production, spore production, and pathogenicity of *Cercospora nicotianae* *atr2* disruption mutants (*white bars*), wild type (*gray bars*), and non-disruption transformants (*black bars*). **a** Cercosporin production by *cnatr2* disruption mutants. 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 ($P < 0.05$). **b** Sporulation assay. *Bars* represent mean spore number per petri dish. Data are the average of 7 plates. Each data point is reported as mean (\pm SE). **c** Pathogenicity assay. *Bars* represent mean number of coalesced lesions on four infected leaves per plant from three plants. Each data point is reported as mean (\pm SE). Although variation was seen between strains in all three assays, there were no statistically significant differences between wild type and the *cnatr2* disruption mutants for cercosporin production, spore production, or pathogenicity

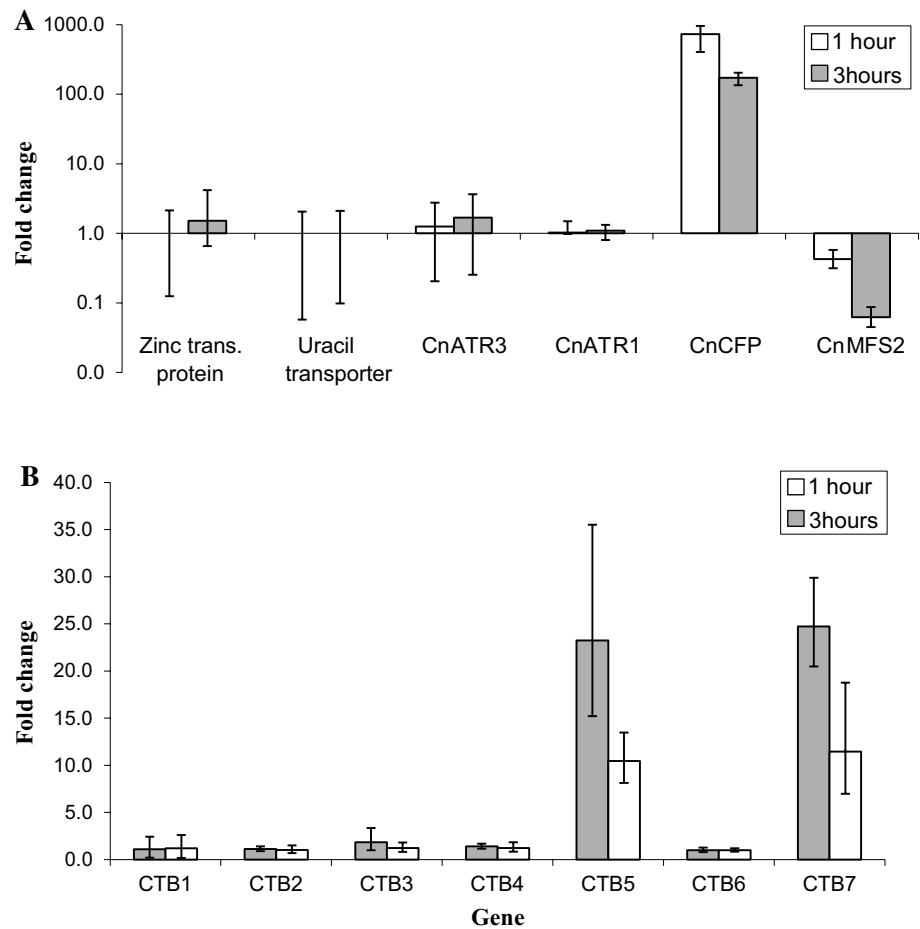


inhibited by about 70 % whereas growth of the resistant *C. nicotianae* is inhibited by about 40 %. Assay of *N. crassa* CnATR2 transformants identified six that had significantly more resistance to cercosporin than WT, confirming the ability of CnATR2 to impart cercosporin resistance. Not all of the transformants 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 CnATR2 in selected *N. crassa* transformants was analyzed, and a correlation was found between expression of the transgene

and resistance of the transformant. We thus hypothesize that ATR2 imparts resistance, but that the effect is small and high levels of expression are required. Assays with the other photosensitizers also suggest that resistance is specific to cercosporin.

It is important to note that *N. crassa* has 17 ABC transporters that function in efflux (Toone 2011). The primers used for amplification of CnATR2 in the *N. crassa* transformants were selected based on lack of similarity to sequences in WT *N. crassa*. Specificity of the primers used to screen for CnATR2 were tested by PCR, and lack of amplification in WT *N. crassa* was confirmed. The *N. crassa* ABC

Fig. 10 RT-qPCR analysis of gene expression in *cnatr2* 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 $2^{-\Delta\Delta CT}$ 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



transporter with the closest homology to CnATR2 has only 49 % identity; thus, no close homologs are present. *N. crassa* has some resistance to cercosporin, but we have no evidence that this is due to any of the ABC transporters.

The role of CnATR2 in cercosporin resistance was further characterized by creating disruption mutants. Previous studies with *cnatr1* and *ckcfp* transporter mutants in *C. nicotianae* and *C. kikuchii* 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* *ckcfp* disruption mutants, resistance was decreased 40–45 % (Callahan et al. 1999). Production of cercosporin was also significantly decreased in both *ckcfp* and *cnatr1* disruption mutants, and *ckcfp* disruption mutants 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, there was no difference between the *cnatr2* disruptants and WT *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* *cnatr2* mutants led us to test if induction of other transporters compensates for the *cnatr2* mutation, as is seen in the *cnatr1* mutant where both *CnCFP* and *CnATR2* are induced under conditions of cercosporin toxicity. We tested the expression of *CnATR1*, *CnATR3*, *CnCFP*, *CnMFS2*, and the uracil transporter and zinc transfer protein genes in the *cnatr2* mutant under conditions of cercosporin toxicity. Of these genes, only *CnCFP* was induced, but it was strongly induced (200–700-fold). Given the documented role of CFP in cercosporin resistance, we hypothesize that upregulation of *CnCFP* may compensate for mutations in both *CnATR1* and *CnATR2*. Further research with double mutants of *CnCFP* with *CnATR1* or *CnATR2* will be needed to investigate this hypothesis.

In addition to induction of transporters in the *cnatr2* mutant, we also tested induction of the *CTB* genes in the cercosporin biosynthetic pathway. We have hypothesized that resistance genes may be found within the biosynthetic cluster, but gene disruption experiments in *C. nicotianae*

have not shown decreases in resistance in *cnctb* mutants (Chen et al. 2007b). Based on our hypothesis in this study that upregulation of other resistance genes compensates for mutations, we tested whether the *cnctb* genes were upregulated in the *cnatr1* and *cnatr2* mutants under conditions of cercosporin toxicity. In the *cnatr1* mutant, all genes in the biosynthetic cluster (*CTB1-8*) were upregulated (data not shown), a result that was unexpected due to the cercosporin deficiency caused by the loss of CnATR1. Cercosporin production is not affected in the *cnatr2* mutant, but we found that two of the genes, *CnCTB5* and *CnCTB7*, were upregulated. These two genes encode putative FAD/FMN- or NADPH-dependent oxidoreductases (Chen et al. 2007b), and reduction of cercosporin is a major mechanism of cercosporin resistance (Daub et al. 1992, 2013). It is possible that CnCTB5 and CnCTB7 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 investigated transporters identified in a subtractive library of genes putatively involved in *C. nicotianae* resistance to the photoactivated toxin cercosporin. Phylogenetic analysis did not show close homologies with other transporters previously shown to play a role in cercosporin resistance; however, analysis of gene expression in response to cercosporin and under conditions of cercosporin toxicity identified *CnATR2* as a putative resistance gene, a result confirmed by the ability of this gene to impart resistance to *N. crassa*. Disruption mutants were not altered in resistance, but other known and putative resistance genes were upregulated in the mutants and may compensate for the *cnatr2* mutation. We hypothesize that cercosporin resistance in *Cercospora* is mediated by multiple mechanisms that are carefully regulated and redundant to ensure resistance. This work adds to previous studies in *Cercospora* and in other fungi showing the importance of membrane transporters in resistance to cercosporin. Current work in the laboratory is focused on transforming tobacco with the *CnATR1* and *CnCFP* genes to impart *Cercospora* disease resistance. Based on our results, *CnATR2* is a good candidate to be included in our plant transformation studies.

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