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RESEARCH ARTICLE

The fungal opsin gene *nop-1* is negatively-regulated by a component of the blue light sensing pathway and influences conidiation-specific gene expression in *Neurospora crassa*

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Abstract We previously demonstrated that the *nop-1* gene encodes a putative green-light opsin photoreceptor that is highly expressed in cultures that support asexual sporulation (conidiation) in *Neurospora crassa*. In this study, we demonstrate that *nop-1* is a late-stage conidiation gene, through analysis of *nop-1* transcript levels in wild-type strains and mutants blocked at various stages of conidiation. *nop-1* message amounts are similar with constant illumination or darkness during conidiation, consistent with developmental, but not light, regulation of *nop-1* expression. Furthermore, photoinduction experiments using wild type and mutants defective in components of the blue light sensing pathway (*wc-1* and *wc-2*) indicate that *nop-1* mRNA levels are not appreciably affected by brief light exposure during conidiation. Surprisingly, *nop-1* message amounts are greatly elevated in *wc-2* mutants in light or dark, suggesting that the *wc-2* gene product regulates *nop-1* expression in a light-independent manner. Analysis of expression patterns for *al-2*, *con-10* and *con-13*, genes regulated by conidiation and/or blue light, showed that *nop-1* has significant and reproducible effects on all three genes during various stages of conidiation. The results suggest that NOP-1 directly or indirectly modulates carotenogenesis and repression of conidiation-specific gene expression in *N. crassa*.

Keywords Microbial opsins · Conidiation · Blue light · Green light · Photoinduction · White collar genes

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Introduction

In *Neurospora crassa*, macroconidiation (conidiation) is the process of producing multinucleate asexual spores (macroconidia or conidia). This developmental program is initiated by exposure to an air–water interface in cultures with adequate nutrition (rev. in Springer 1993; Ebbole 1998; Borkovich et al. 2004). However, conidiation can be induced in submerged cultures in the presence of limiting nitrogen or carbon or heat stress (That and Turian 1978; Plesofsky-Vig et al. 1983). In the early stages of conidiation, newly-formed aerial hyphae grow away from the surface and change their growth mode from hyphal tip elongation to repeated apical budding, forming hyphal segments with minor constrictions (Springer and Yanofsky 1989). These proconidial aerial chains further mature to form a conidiophore structure late in conidiation that allows for the release of free conidia. Macroconidiation is entrained by the circadian rhythm in *N. crassa* (rev. in Dunlap and Loros 2006), presumably to synchronize spore production and release at optimal times of the day to ensure successful germination (Bell-Pedersen et al. 1996).

Mutants have been identified that are blocked at several different stages of conidiation (Springer and Yanofsky

1989). Early-stage mutants include *fluffyoid* (*fld*), *aconidiate-2* (*acon-2*), *fluffy* (*fl*), and *aconidiate-3* (*acon-3*). These mutants cannot develop past formation of major constrictions. The FL protein is a Gal4p-type C6 zinc cluster transcription factor that is necessary and sufficient to initiate macroconidiation in *N. crassa* (Bailey and Ebbole 1998; Bailey-Shrode and Ebbole 2004; Rerngsamran et al. 2005). Late-stage mutants are *conidial-separation-1* (*csp-1*), *conidial-separation-2* (*csp-2*), and *easily wettable* (*eas*). The *csp-1* and *csp-2* mutants are defective in the release of free conidia. *eas* mutants cannot form the hydrophobic layer found on mature conidia, as they contain a defective hydrophobin gene (Bell-Pedersen et al. 1992; Lauter et al. 1992). The *granular* (*gran*) and *frost* (*fr*) mutants form abnormal conidia. *fr* is homologous to *Saccharomyces cerevisiae CDC1*, and *fr* and *CDC1* have been linked to Mn^{2+} homeostasis in *N. crassa* and *S. cerevisiae*, respectively (Paidhungat and Garrett 1997; Sone and Griffiths 1999).

Conidiation can be synchronized in *N. crassa* mycelial mats freshly-harvested from liquid culture, and this process has been utilized to identify genes whose expression is specific to conidiation (*con* genes: Berlin and Yanofsky 1985a, b). The expression patterns of *con* genes have been monitored throughout the conidial development and individual *con* genes were classified as early or late, depending on the timing of their expression. For example, *con-1*, *con-2*, *con-3*, *con-4* and *con-5* are expressed early in conidiation, while *con-10*, *con-11*, and *con-13* are late-stage *con* genes (Berlin and Yanofsky 1985a).

Blue-light has been shown to enhance conidiation and other asexual developmental processes, including the induction of mycelial carotenoid biosynthesis, entrainment of the conidiation circadian rhythm, increased yield of conidia, hyperpolarization of hyphal membranes, and conidiophore phototropism (rev. in Linden et al. 1997; He and Liu 2005). The *N. crassa wc-1* and *wc-2* mutants are defective in all blue-light responses (rev. in Linden et al. 1997; He and Liu 2005) and the respective genes encode zinc-finger transcription factors with PAS domains (Ballario et al. 1996; Linden and Macino 1997). WC-1 and WC-2 together form a white collar complex (WCC) that regulates expression of blue-light inducible genes (Ballario et al. 1996). The WC-1 protein binds a flavin adenine nucleotide cofactor and functions as the actual blue light receptor (Froehlich et al. 2002; He et al. 2002).

The *wc-1* and *wc-2* mutants have been used to characterize the influence of blue light on the expression of conidiation-specific genes. The *al-1* and *al-2* genes, encoding enzymes required for carotenoid synthesis, are developmentally regulated, peaking during the formation of major constrictions and at the separation of free conidia (Li and Schmidhauser 1995). Both *al-1* and *al-2* can be photoinduced in wild type, but not in *wc-1* and *wc-2* strains (Li and Schmidhauser 1995). In contrast, another carotenoid biosynthetic gene, *al-3*, shows WC-dependent photoinducibility only during the early stages of conidiation (Li et al. 1997). The conidiation-specific genes *con-5*, *con-10*, *con-6*, and *con-8* are also subject to photoinduction by blue light during conidiation, but response times vary among the different genes (Lauter and Russo 1991; Lauter and Yanofsky 1993; Carattoli et al. 1995).

We have previously shown that the *N. crassa nop-1* gene encodes a member of the microbial opsin family (Bieszke et al. 1999a; Sharma et al. 2006). Microbial opsins had been well-studied in Archaea (rev. in Brown and Jung 2006; Spudich 2006), but NOP-1 was the first such protein identified in eukaryotic microbes. *nop-1* expression was only detected in cultures grown under conditions that support conidial development (Bieszke et al. 1999a). Analysis of Δ*nop-1* mutant strains revealed subtle defects in the pattern of conidiation in the presence of the mitochondrial ATPase inhibitor oligomycin (Bieszke et al. 1999a). In addition, heterologously-expressed NOP-1 protein bound all-*trans* retinal to form a green-light absorbing pigment with the characteristics of archaeal sensory rhodopsins, suggesting that NOP-1 function may be important for sensory perception in *N. crassa* (Bieszke et al. 1999b). Other work has demonstrated that NOP-1 does not function as a proton pump, in spite of conservation of acidic residues (Asp-131 and Glu-142) at two sites required for proton translocation in bacteriorhodopsin (Brown et al. 2001; Bergo et al. 2002). This contrasts with the observations that an opsin from the filamentous fungus *Leptosphaeria maculans* has proton translocation activity (Waschuk et al. 2005) and mutation of the residue corresponding to Asp-131 in the *L. maculans* opsin leads to a rhodopsin with the characteristics of NOP-1 (Furutani et al. 2006; Fan et al. 2007). The *in vivo* significance of such differences is currently unknown, as a mutant lacking the *L. maculans* rhodopsin has not yet been reported (Idnurm and Howlett 2001).

In this study, we further define the expression pattern and the conditions that regulate *nop-1* expression during conidiation and introduce a possible role for NOP-1 in impacting transcriptional regulation in *N. crassa*. We monitor *nop-1* transcript levels throughout conidiation in wild-type strains. We analyze *nop-1* mRNA amount in several mutants with defects in conidiation and assess the effects of brief light pulses and the *wc* genetic background on *nop-1* expression. Finally, we compare transcript levels for several conidiation and/or light-regulated genes in wild-type and Δ*nop-1* strains. Our results suggest that *nop-1* is a non-photoinducible late-stage conidiation gene that is negatively regulated by the *wc-2* gene product. Our data also support a direct or indirect role for NOP-1 in transcriptional regulation during conidial development and carotenogenesis.

Materials and methods

Strains and growth conditions

The strains used in this study are listed in Table 1. Fluorescent light fixtures with 2-15 Watt F15T8/Natural Sunshine lamps (Philips, USA) were the source of white light for experiments. All tissue samples were immediately quick-frozen in liquid nitrogen after collection. Conidia were propagated on solid Vogel's minimal medium (VM; high nitrogen, with sucrose as carbon source) as described (Davis and Serres 1970) and used to inoculate cultures or directly used for isolation of RNA (see below). Vegetative plate cultures, consisting of hyphae, aerial hyphae and conidiophores, were prepared by inoculation of cellophane-overlaid VM plates in the center with conidia, followed by incubation at room temperature for three days under fluorescent lights. For analysis of gene expression in sexually-differentiated cultures, synthetic crossing medium (SCM; Westergaard and Mitchell 1947) plates (overlaid with cellophane) were inoculated in the center with conidia and incubated under fluorescent lights at room temperature for 6 days. For characterization of the conidiation mutants, cellophane-overlaid VM plates were inoculated in the center using conidia from the various strains. The plates were incubated at room temperature under fluorescent lights for 3 days.

In order to evaluate gene expression during a time course of conidiation, strains were grown as described (Berlin and Yanofsky 1985b) with the following modifications. Conidia from various strains were inoculated to a final density of 1×10^6 conidia/ml into silanized 500 ml flasks containing 100 ml liquid VM. The flasks were covered with aluminum foil and shaken at 200 RPM for 20 h at 30°C. The cultures were then filtered under a red safe light onto 5.5 cm Whatman #1 filters and the resulting cell pads placed on top of a monolayer of 0.45 mm glass beads in a 5.5 cm petri dish containing 5 ml of liquid VM. For induction of conidiation under constant conditions, the plates were either incubated under fluorescent white light (fluence rate = 5.6 W/m^2) or in the dark, in a humid atmosphere. For photoinduction experiments, the plates were incubated in the dark for the indicated times, and then transferred to fluorescent lights (fluence rate = 5.6 W/m^2) for 30 min prior to collection.

Northern analysis

All frozen samples were ground to a fine powder, and 200 mg of tissue was used for RNA extraction as described in Sachs and Yanofsky (1991). Northern analysis was performed essentially as described (Tsui et al. 1994), except with Nytran Plus membranes (Schleicher and Schuell). Radiolabeled probes were generated by random priming (Prime A Gene, Promega, Madison, WI, USA) from the following DNA fragments: a 600 bp *Kpn*I-*Bgl*II fragment from pJAB13, a cDNA containing the 5′end of the *nop-1* open reading frame (ORF), a 5.2 Kb *Eco*RV fragment of pTJS542 containing the *al-2* gene (Schmidhauser et al. 1994), and a 500 bp *Bam*HI-*Eco*RI fragment from pBW100 (obtained from D. Ebbole) containing *con-10*. Expression of a rRNA gene was detected by labeling a cosmid containing the gene (obtained from D. Ebbole) or by direct photography of Northern blots. In order to assess *con-13* mRNA levels, a fragment containing most of the coding region (830 bp) in cosmid X11D11 (Orbach et al. 1990) was amplified using PCR and the following primers. The sense primer anneals upstream of the start codon and has the sequence 5′-GCTGGTGTGTGTCAACGCTTAGTG-3′, while the antisense primer anneals after the first intron in the genomic clone and has the sequence 5′-GGAATAGTG

Table 1 *N. crassa* strains used in this study

Strain	Relevant Genotype	Comments	Source
74-OR23-1A	Wild-type, *mat A*	74A	R.L. Weiss, UCLA
39-1	Δ*nop-1*::*hph*$^+$, *mat A*	Δ*nop-1* mutant	Bieszke et al. (1999)
40-10	Δ*nop-1*::*hph*$^+$, *mat a*	Δ*nop-1* mutant	Bieszke et al. (1999)
wc-1	*wc-1*, *mat A*	FGSC#4397, allele ER53	FGSC
wc-2	*wc-2*, *mat A*	FGSC#4407, allele ER33	FGSC
Δ*wc-1*	Δ*wc-1, mat A*	FGSC#11712, allele KO	FGSC
Δ*wc-2*	Δ*wc-2, mat a*	FGSC#11124, allele KO	FGSC
Δ*wc-2*	Δ*wc-2, mat a*	UCR#473, allele KO	Dunlap et al. (2007)
fld	*fld*, *mat A*	FGSC#7022, allele P628	FGSC
fl	*fl*, *mat A*	FGSC#4317, allele flP	FGSC
acon-3	*acon-3*, *mat A*	FGSC#3286, allele RS503	FGSC
csp-2	*csp-2*, *mat A*	FGSC#4085, allele UCLA101	FGSC
fr	*fr*, *mat A*	FGSC#103, allele B110	FGSC
gran	*gr, mat A*	FGSC#794, allele B42	FGSC

FGSC Fungal Genetics Stock Center, Kansas City, KS, USA

GTCTGCGACGACGCAAG-3′. The identity of the PCR product was verified by sequencing, and the fragment was labeled as described above for use as a probe.

mRNA levels were quantitated by performing densitometric analysis of bands on films after exposure of Northern blots using the Alpha Imager TM 2200 Documentation and Analysis System (Alpha Innotech, San Leandro, CA, USA). All the values were standardized to rRNA.

Results

nop-1 is a late-stage conidiation gene

The macroconidiation pathway has been extensively analyzed, and morphological changes have been monitored with respect to time (Springer and Yanofsky 1989). Conidiation begins with the formation of aerial hyphae (1–2 h) that go on to develop minor constrictions (2–4 h). Conidial spores begin to form between each minor constriction, and major constrictions subsequently appear (6–8 h). Septation then occurs between each conidial bud at the major constriction sites (8–10 h), but the conidia remain attached by connectives that form between each spore (10–12 h). These terminally differentiated structures are referred to as conidiophores, and, at the end of their development, mature conidia are released ($\geq$ 16 h).

We previously noted that expression of the *N. crassa* opsin, *nop-1*, is specific to conditions that induce both asexual and sexual spore production (Bieszke et al. 1999a). To further characterize the expression pattern of *nop-1* during conidial development, total RNA was isolated from wild-type cultures incubated to allow synchronized production of conidia in constant light (Berlin and Yanofsky 1985b). RNA was also extracted from purified mature conidia, as well as conidiating vegetative (VM) and sexually differentiated (SCM) plate cultures producing conidia. Northern analysis of these RNA samples revealed that *nop-1* expression is initiated early in conidiation, at 2–4 h, with elevated levels at the later stages, 12–24 h (Fig. 1). The temporal regulation of *nop-1* expression during conidiation is similar in cultures exposed to constant darkness (data not shown). *nop-1* mRNA is also abundant in mature conidia and in conidiating VM and SCM plate cultures (Fig. 1).

Genes whose expression is specific to conidiation (*con* genes) have been identified (Berlin and Yanofsky 1985a). One such *con* gene, *con-10*, is well-characterized in terms of its expression pattern during conidiation. *con-10* is abundantly expressed during the later stages of conidiation (Berlin and Yanofsky 1985a). The expression pattern of *con-10* is remarkably similar to *nop-1* (Fig. 1), including the high transcript levels in purified conidia, and VM and SCM plate cultures. Directly upstream of *con-10* is another *con* gene,

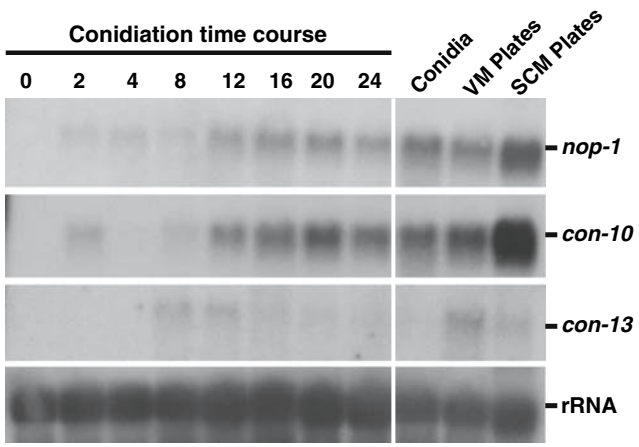

Fig. 1 Expression of *nop-1* and conidiation-specific (*con*) genes during a time course of conidiation. Total RNA was isolated from cultures collected at various times during conidiation (0, 2, 4, 8, 12, 16, 20, and 24 h after transfer to an air–liquid interface), as well as from mature conidia (*conidia*) and vegetative (*VM plates*) and sexually differentiated plate cultures (*SCM plates*) of the wild-type strain 74A. Samples containing 10 μg of total RNA were subjected to Northern analysis, using the *nop-1*, *con-10*, *con-13* and rRNA genes (loading control) as probes. The blot shown is representative of two independent experiments

con-13, whose transcription is independent of *con-10* (Hager and Yanofsky 1990). The *con-13* message is of low abundance and therefore difficult to detect, but this gene is consistently expressed midway through conidiation (Fig. 1). Interestingly, *con-13* message cannot be detected after conidiophore production or in mature conidia (Fig. 1). The message is also weakly expressed in VM and SCM plate cultures, in contrast to *nop-1* and *con-10*. Thus, *con-10* and *nop-1* appear to have similar expression patterns, while that for *con-13* differs.

Mutants that are blocked at different stages of conidiation have been isolated (rev. in Springer and Yanofsky 1989). In order to further characterize the timing of *nop-1* expression during conidiation, we analyzed *nop-1* mRNA levels in vegetative plate cultures from the early conidiation-defective mutants *fluffyoid* (*fld*), *fluffy* (*fl*) and *acondiate-3* (*acon-3*), as well as in the late-stage specific *conidial-separation-2* (*csp-2*) mutant. Previous genetic analysis showed that *acon-2* and *fld* act at the stage of formation of minor constrictions and are upstream of *fl* and *acon-3*, which are both required for development of major constrictions (Springer and Yanofsky 1989). The *csp-2* gene acts later, at the stage of separation of free conidia (Springer and Yanofsky 1989). The *frost* (*fr*) and *granular* (*gran*) mutants were also tested, as they exhibit altered colony morphologies and produce aberrant conidia.

Northern analysis revealed that *nop-1* mRNA can not be detected in the vegetative mutants *fr* and *gran*, or in the early-stage developmental mutants *fld* and *fl* (Fig. 2). In contrast, *nop-1* is weakly-expressed in the *acon-3* and *csp-2*

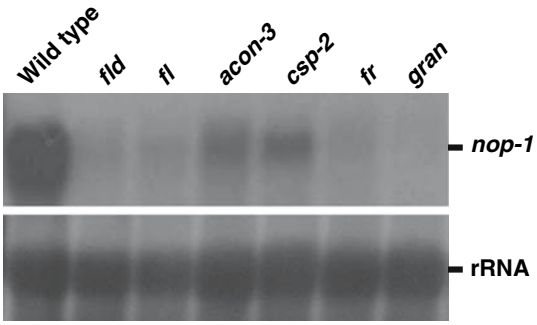

Fig. 2 *nop-1* expression in developmental mutants. Samples containing 10 µg of total RNA from the indicated strains (Table 1) were used to prepare Northern blots. Membranes were probed with the *nop-1* and rRNA genes, as shown. Wildtype is strain 74A. Blot shown is representative of two independent experiments

mutants (Fig. 2). The difference in *nop-1* expression noted in the *fl* and *acon-3* mutants, which appear to be blocked at the same stage of conidiation, suggests a difference in temporal regulation of gene expression by *fl* or *acon-3* that is not apparent at the gross morphological level. The lack of *nop-1* mRNA in the *fr* and *gran* mutants, which develop aberrant conidia, suggests that the defects in these mutants may occur early in the conidiation timeline. The absence of *nop-1* mRNA in mutants blocked at early stages of conidiation and the weak transcription of *nop-1* observed in the *csp-2* mutant blocked in the later stages of conidiation are consistent with the observation that *nop-1* expression is specific to late conidial development.

Expression of *con-10* is induced by heat shock temperatures in wild-type conidiating cultures (Lee and Ebbole 1998). To determine if elevated temperature can enhance *nop-1* expression, levels of *nop-1* mRNA in conidiating cultures at room temperature were compared to those in identical cultures shifted to 45°C (heat shock conditions) for the last hour of incubation. The results showed that *nop-1* levels were not elevated by heat (data not shown). Thus, despite their similar expression pattern during conidiation, *con-10* and *nop-1* do not share heat shock-inducibility.

nop-1 is not photoinducible and is negatively regulated by WC-2

Blue light has been shown to enhance production of conidia (Lauter et al. 1997) and to induce expression of a number of genes associated with conidiation and asexual development (rev. in Linden et al. 1997). WC-1 and WC-2 are transcription factors required for the blue-light signaling pathway in *N. crassa* (Ballario et al. 1996; Linden and Macino 1997). Increased expression of blue light-regulated genes is blocked in the *wc-1* or *wc-2* genetic background (rev. in Linden et al. 1997), He and Liu (2005)]. Examples are the carotenoid biosynthetic genes, *al-1* and *al-2* (Li and Schmidhauser 1995) and the *con* genes, *con-5*, *con-6*, *con-8* and *con-10* (Lauter and Russo 1991; Lauter and Yanofsky 1993; Carattoli et al. 1995).

Based on the proposed role of NOP-1 as a photoreceptor, we were interested to determine whether *nop-1* is regulated by the blue-light sensing pathway. We first evaluated *nop-1* mRNA levels in the *wc-1* and *wc-2* mutant backgrounds (alleles ER53 and ER33, respectively; see Table 1) under constitutive light and darkness. The pattern of *nop-1* expression in the white-collar mutants was similar to that of wild type under both conditions (data not shown). However, since *N. crassa* possesses a strong photoadaptation pathway in response to long-term light exposure (rev. in He and Liu 2005), we also tested the effect of short (30 min) light pulses on *nop-1* expression. We included the photoinducible *con-10* gene as a control. For these experiments, we also took advantage of strains carrying deletions of the *wc-1* and *wc-2* ORFs (Δ*wc-1* and Δ*wc-2*; see Table 1). Northern analysis revealed that *nop-1* could not be photoinduced in any genetic background (Fig. 3). In contrast, *con-10* mRNA was abundant in light-exposed wild-type cultures and was absent from Δ*wc-1* and Δ*wc-2* mutants (Fig. 3). Surprisingly, *nop-1* levels were significantly elevated in the *wc-2* background with and without photoinduction (Fig. 3). We obtained the same result with another Δ*wc-2* strain (UCR#473; data not shown). Although not as robust of a response, we also often observed slightly higher amounts of *nop-1* message in Δ*wc-1* mutants (Fig. 3; data not shown). These results suggest that *nop-1* is not photoinduced but instead is negatively regulated in a constitutive manner by components of the major blue-light sensing pathway in *N. crassa*, most notably by the PAS-domain transcription factor WC-2.

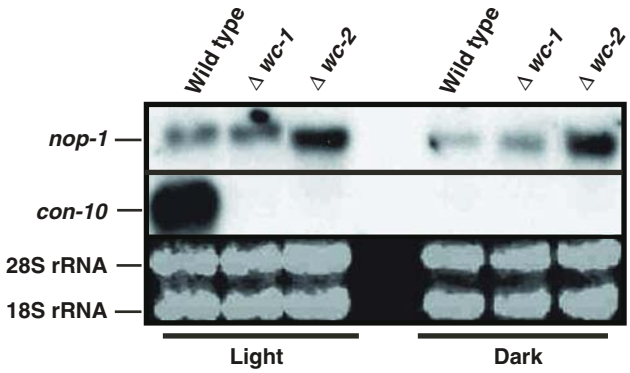

Fig. 3 Photoinduction of *nop-1* transcript levels in wild type and the blue-light sensing mutants *wc-1* and *wc-2*. Cultures of wild-type (74A), Δ*wc-1* (FGSC#11712) and Δ*wc-2* (FGSC#11124) strains were allowed to conidiate for 8 h in constant darkness, then either kept in the dark or exposed to light for 30 min prior to collection and RNA extraction. Northern blots were prepared and probed using *nop-1* or *con-10*. The rRNAs were detected by photography of the ethidium bromide-stained blots. Blot shown is representative of three independent experiments

nop-1 influences the levels of several genes that are expressed during conidiation

nop-1 expression is increased during the later stages of conidiation. This pattern is consistent with NOP-1 functioning during the formation of conidiophores and/or the release of mature conidia. Thus, it is possible that a NOP-1 pathway may ultimately regulate the expression of other genes specific to conidiation. NOP-1 binds retinal and forms a light-absorbing pigment characteristic of archaeal sensory rhodopsins in vitro (Bieszke et al. 1999a, b) and has been speculated to have similar light-absorbing capabilities in vivo. Therefore, a preliminary analysis of *nop-1* as a potential regulator of light and/or conidiation-specific gene expression was initiated by evaluating mRNA levels of *al-2*, *con-10* and *con-13* in wild-type and Δ*nop-1* strains. *al-2* is a blue-light regulated gene that encodes phytoene synthase, an important enzyme for carotenoid biosynthesis in *N. crassa* (Schmidhauser et al. 1994). As mentioned above, *con-10* expression is also blue-light regulated, but the function of its gene product is unknown (Corrochano et al. 1995). *con-13* is another *con* gene of unknown function whose expression is not blue-light regulated (Hager and Yanofsky 1990; Lauter and Russo 1991). Therefore, comparison of levels of the *al-2*, *con-10* and *con-13* transcripts in the Δ*nop-1* and wild-type background would reveal whether NOP-1 function was specific to light-regulation (depicted by photoinduced changes in *al-2* and *con-10* expression) or directed to conidiation (differences in *con-10* and *con-13* expression levels). mRNA levels were assessed in three independent experiments and a representative Northern blot is shown in Fig. 4. Densitometry was used to quantitate mRNA levels for the three genes at 8, 12, 16, and 24 h into conidiation (Table 2; some values not available due to low mRNA levels).

The results show that *nop-1* exerts a subtle, but reproducible effect on levels of all three transcripts during at least two time points during conidiation (Fig. 4; Table 2). *nop-1* affects *al-2* expression at two time points in light-exposed cultures; levels of *al-2* mRNA are reduced twofold

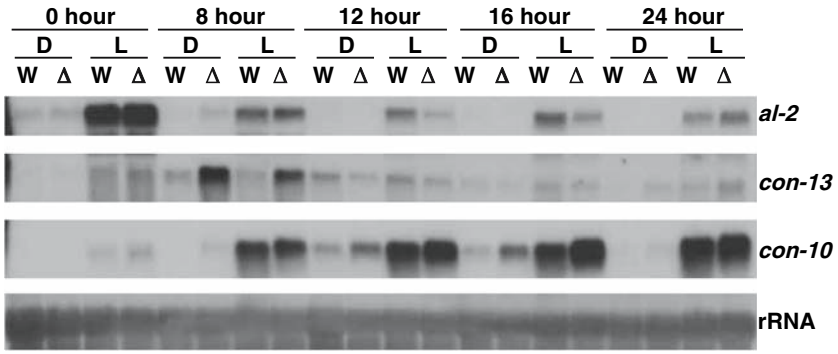

Fig. 4 Photoinduced gene expression during conidiation in Δ*nop-1* and wild-type strains. Cultures were grown in the dark for the indicated times and then either kept in the dark (D) or transferred to light for 30 min (L) before collection and subsequent RNA extraction. Samples containing 10 μg of total RNA from wild-type strain 74A (W) and Δ*nop-1* strain 39-1 (*triangle*) were used to prepare Northern blots. Membranes were probed with the *al-2*, *con-13*, *con-10* and rRNA genes, as indicated. Blot shown is representative of three independent experiments

Table 2 Relative levels of *al-2*, *con-13* and *con-10* transcripts in Δ*nop-1* vs. wild-type strains

Probe	RNA levels at various times during conidiation (% wild type)[a]							
	8 h		12 h		16 h		24 h	
	Dark[b]	Light[c]	Dark	Light	Dark	Light	Dark	Light
al-2	134 ± 21	150 ± 25	NQ[d]	46 ± 06	NQ	200 ± 22	NQ	124 ± 12
con-13	347 ± 45	473 ± 200	198 ± 68	97 ± 03	NQ	NQ	NQ	NQ
con-10	89 ± 30	91 ± 19	221 ± 53	128 ± 08	245 ± 65	191 ± 09	123 ± 15	135 ± 4

[a] Northern analysis was performed with the indicated probe using blots from three independent experiments, comparing wild-type strain 74A to a Δ*nop-1* mutant (strain 40-10 or 39-1). Densitometric measurements of band intensity were normalized using rRNA as a loading control. The values from two representative experiments were compared to the wild-type strain (100%). Errors were calculated as the standard error of the mean

[b] Dark, samples were kept in complete darkness

[c] Light, samples were incubated in dark for the time specified, and then transferred to light for 30 min

[d] NQ, not quantitated (mRNA levels too low)

at 12 h, but are twice those of wild type at 16 h (Fig. 4; Table 2). Levels of *con-13* message are elevated in Δ*nop-1* mutants relative to wild type at 8 h into conidiation (light or dark; Fig. 4; Table 2) and at 12 h in dark-grown cultures (Table 2; Δ*nop-1* lane in Fig. 4 is underloaded). We also noted minor apparent photosuppression of *con-13* transcript in both Δ*nop-1* and wild-type strains at 8 h into conidiation (Fig. 4). The *con-10* transcript is present at higher levels in Δ*nop-1* strains relative to wild type at 12 and 16 h in dark-grown cultures; photoinduced levels are also elevated in Δ*nop-1* mutants at 16 h (Fig. 4; Table 2). Thus, NOP-1 appears to play a minor role in influencing both light and conidiation-specific gene expression in *N. crassa*.

Discussion

Conidiation is subject to complex regulation in *N. crassa*, as it is influenced by light, desiccation, CO_2 levels, nutrient-availability, and can be entrained by the circadian rhythm (rev. in Davis 2000). Light and developmental regulation of *nop-1* mRNA levels during conidiation was investigated to further probe possible functions for NOP-1. *nop-1* is highly expressed in the later stages of conidiation independent of light, consistent with a developmentally-regulated gene. Furthermore, the pattern of weak or absent *nop-1* transcription in the developmental mutants supports conidiation-specific expression of *nop-1*. Interestingly, *nop-1* expression parallels the formation of conidiophores and release of mature conidia, suggesting that NOP-1 may function during the later stages of conidiation.

The pattern of *nop-1* expression is similar to that of *con-10* during conidiation. The function of *con-10* is unknown, but the encoded protein is similar to a *Bacillus subtilis* protein that is expressed under conditions of starvation and stress (Mueller et al. 1992; Ebbole 1996). Although *nop-1* expression is not enhanced by elevated temperature, the NOP-1 protein may play a role during heat-shock or other stress responses. NOP-1 is related to a fungal opsin-related protein (ORP) group that includes *N. crassa* ORP-1 (Bieszke et al. 1999a) and several members that are putative heat shock/stress response proteins in yeast (Seymour and Piper 1999; Hahn et al. 2004). One ORP, *Saccharomyces cerevisiae* HSP30, negatively regulates the plasma membrane H^+-ATPase during heat shock (Piper et al. 1997). Δ*nop-1* strains were tested in the presence of plasma, vacuolar, and mitochondrial H^+-ATPase inhibitors (Bieszke et al. 1999a). Δ*nop-1* mutants exhibited defects in vegetative growth in the presence of the mitochondrial H^+-ATPase inhibitor, oligomycin (Bieszke et al. 1999a), suggesting a possible function for NOP-1 in the response to stress. The observed links between NOP-1, CON-10, heat shock proteins and stress-inducing factors suggest that the late-stage conidial gene products NOP-1 and CON-10 may function in the same regulatory pathway under certain stress conditions.

Both early and late responses to blue-light exposure have been documented in *N. crassa* (rev. in Linden et al. 1997). Blue-light initiates early responses that are important for entraining the circadian rhythm of conidiation, controlling membrane potential during hyphal elongation, and inducing carotenogenesis in mycelial cultures. The late blue-light effects include the phototrophic responses and enhancement of spore production during both asexual and sexual reproduction. Both early and late responses are regulated by two blue-light dependent transcription factors, WC-1 and WC-2. We did not observe a significant increase in *nop-1* mRNA levels after a brief light pulse at any point during conidiation in wild type or the *wc* mutants. However, *nop-1* expression levels are elevated in *wc-2* mutants in light or dark conditions. These results suggest that *nop-1* expression is negatively regulated by *wc-2* in a light-independent manner. These observations also support WC-1-independent regulation of gene expression by WC-2; this is of interest, as WC-2 is a PAS-domain containing transcription factor that, in contrast to WC-1, does not bind flavin or serve directly as the blue-light photoreceptor (Froehlich et al. 2002; He et al. 2002).

NOP-1 may have a sensory role in the later-stages of conidiation, as NOP-1 was shown to form a green-light absorbing pigment upon binding the all-*trans* retinal chromophore and to possess spectral properties similar to those of the archaeal sensory rhodopsins (Bieszke et al. 1999b). Green light effects are not well characterized in fungi, but green light has been shown to affect spore production in the plant-pathogens *Trichometasphaeria turcica* and *Alternaria solani*, either alone or in a synergistic interaction with blue light (rev. in Klein 1992). In *N. crassa*, conidiophore phototropism and production of mature conidia, both late-stage conidiation events, are subject to regulation by blue light (Siegel et al. 1968; Lauter 1996). Thus, NOP-1 acting as a green-light receptor could potentially regulate these or other conidial developmental processes as an adjunct to the blue-light signaling pathway.

Carotenogenesis is induced by blue-light in basal hyphae, but constitutive during conidiation in *N. crassa* (Harding and Turner 1981). Carotenoids are thought to play a protective function in fungi, serving as scavengers for reactive oxygen species (Schroeder and Johnson 1995; Michan et al. 2003; Iigusa et al. 2005). The *al-2* gene is required for carotenogenesis and serves as a reporter gene for this process. The *al-2* transcript is photoinduced during conidiation, in a WC-1- and WC-2-dependent manner (Li and Schmidhauser 1995). Relative to wild type, *al-2* transcript levels are reduced at 12 h, but elevated at 16 h in light-exposed Δ*nop-1* conidiating cultures. We cannot determine whether the differences seen in the Δ*nop-1*

photoinduced samples are light-dependent, as the *al-2* transcript could not be detected in 12-h wild-type and Δ*nop-1* dark-grown cultures. However, this result may point to a sensory role for NOP-1, possibly as a green-light receptor serving as an adjunct to the blue-light pathway. A link between opsins/ORPs and carotenogenesis has also been revealed in the filamentous fungus *Fusarium fujikurori* (Prado et al. 2004). The ORP-encoding gene *carO* is found in a carotenoid gene cluster. Expression of *carO* is deregulated by overproduction of carotenoids and is induced by light and by heat shock. However, similar to other fungal opsin and *orp* genes, targeted deletion of *carO* did not reveal any phenotypes.

The effect of *nop-1* on expression of the conidiation-specific genes *con-10* and *con-13* is light-independent, as their expression is significantly elevated in the Δ*nop-1* mutants compared to wild type with and without light exposure. This suggests a role for NOP-1 in negative regulation of these two *con* genes during development. Interestingly, *nop-1* expression is highest throughout the formative stages of conidiation and remains elevated even into the time points after conidial release. Since Δ*nop-1* strains do not exhibit obvious defects in conidiation (Bieszke et al. 1999a), any contribution of NOP-1 to a negative-regulatory process during conidiation must be functionally redundant to another pathway and/or operate under different environmental conditions.

Taken together, our results suggest that NOP-1 may play an auxiliary role during late-stage conidial events and/or stress responses. It is possible that NOP-1 may act in concert or share overlapping functions with other proteins, namely *N. crassa* ORP-1. Future studies will focus on genome-wide transcriptional profiling to identify genes regulated by green light and *nop-1*, and on genetic approaches to help discern the role of *nop-1* and *orp-1* in the diverse photobiology of *N. crassa*.

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