Isolation and Molecular Characterization of the
Aspergillus nidulans wA Gene

Maria E. Mayorga and William E. Timberlake
Department of Genetics, University of Georgia, Athens, Georgia 30602
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ABSTRACT

The walls of Aspergillus nidulans conidia contain a green pigment that protects the spores from damage by ultraviolet light. At least two genes, wA and yA, are required for pigment synthesis: wA mutants produce yellow spores, wA mutants produce white spores, and wA mutations are epistatic to yA mutations. We cloned wA by genetic complementation of the wA3 mutation with a cosmid library containing nuclear DNA inserts from the wild-type strain. The wA locus was mapped to an 8.5–10.5-kilobase region by gene disruption analysis. DNA fragments from this region hybridized to a 7500 nucleotide polyadenylated transcript that is absent from hyphae and mature conidia but accumulates during conidiation beginning when pigmented spores first appear. Mutations in the developmental regulatory loci brlA, abaA, wetA and apsA prevent wA mRNA accumulation. By contrast, yA mRNA fails to accumulate only in the brlA- and apsA- mutants. Thus, the level of wA transcript is regulated during conidiophore development and wA activation requires genes within the central pathway regulating conidiation.

CONIDIA of the ascomycetous fungus Aspergillus nidulans contain in their walls a dark green pigment that is not present in other cell types. Pigment is produced as spores mature (Law and Timberlake 1980) and coniders resistance to ultraviolet light (Wright and Pateman 1970; Aramayo, Adams and Timberlake 1989). The production of spore pigment requires expression of at least two genes, yA and wA. yA mutants produce yellow spores, wA mutants produce white spores, and wA, yA double mutants produce white spores (Pontecorvo et al. 1953; Clutterbuck, 1972). The product of yA is a p-diphenol oxidase, or laccase, that converts a yellow pigment precursor to the mature green form (Clutterbuck, 1972; Law and Timberlake 1980; Kurtz and Champe 1982). The product of wA is unknown.

The observations that wA mutants are not deficient in yA-encoded laccase (Clutterbuck 1972) and that wA mutations are epistatic to yA mutations (Pontecorvo et al. 1953) are consistent with the hypothesis that the product of wA catalyzes the synthesis of a yellow pigment intermediate from a colorless precursor (Kurtz and Champe 1982). However, ultrastructural and biochemical studies of A. nidulans conidial walls have shown that wA mutants lack some structural wall components present in wild-type conidia, including melanin and an electron dense outer layer containing α-1,3-glucan (Oliver 1972; Claverie-Martín, Diaz-Torres and Geoghegan 1988). This observation raises the possibility that the product of wA is involved in the synthesis of structural wall components. These components could be required for deposition or localization of the mature green pigment.

We wish to determine the mechanisms regulating expression of wA and yA during conidiophore development. yA has been cloned and its expression shown to be regulated at the level of mRNA accumulation (Yelton, Timberlake and van den Hondeel 1985; O’Hara and Timberlake 1989). In this paper, we describe the physical isolation and preliminary characterization of the wA gene. Our results show that wA codes for a large polyadenylated transcript capable of encoding a protein of up to 250 kDa. The wA transcript is undetectable in vegetative cells, accumulates during conidiation, and is absent from mature spores. Thus, it is likely that wA, like yA, is specifically expressed in the sporogenous phialide cells.

MATERIALS AND METHODS

Aspergillus strains, growth conditions and genetic techniques: A. nidulans strain NK002 (pabaA1, yA2; wA3; veAl, trpC801) was constructed by crossing G324 (yA2; wA3; c12, ywA1, methH2, argB2, galAl; veAl; Glasgow Stock Collection) and FGSC237 (pabaA1, yA2; veAl, trpC801; Fungal Genetics Stock Center) and used as transformation recipient for identification of wA3-complementing clones. Strain NK002 was used to construct diploids with a white-spored strain (TNK15-4) made by transformation of PW1 (bA1; argB2; methG1; veAl; P. Jeglenski, Department of Genetics, University of Warsaw, Poland) with pNK01. Strain TMS003 (pabaA1, yA2; ΔargB::trpCΔB; veAl, trpC801) was constructed by Mary Stringer in our laboratory and used as the transformation recipient for the wA disruption analysis. The white-spored strain TNK22-1 (pabaA1, yA2; wA::argB; ΔargB::trpCΔB; veAl, trpC801) was made by transformation of TMS003 with pNK22 and crossed with
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Figure 1.—Localization of the wA3 complementing activity. (A) A 13.8-kb EcoRI fragment that complemented the wA3 mutation was subcloned from CosNK002 as pNK3 and the indicated restriction sites were mapped. (B) Restriction fragments were subcloned from pNK3 and tested for their ability to complement the wA3 mutation in A. nidulans strain NK002 by cotransformation. Complementation in this white-spored (W) strain gave rise to yellow-spored (Y) colonies because NK002 also carries the ya2 mutation.

FGSC357 (biA1; wA3) to show linkage of cloned region to wA3. Strains AJC7.1 (biA1; brlAI), GO1 (biA1; abaAI), GO241 (biA1; wetA6), and AJC1.1 (biA1; apsA1) were provided by John Clutterbuck, Department of Genetics, Glasgow University, Scotland. brlAI strains initiate conidiation normally forming conidiophore stalks, but stalks grow indeterminately. abaAI strains form primary sterigmata (metulae) that produce functionally deranged phialides that proliferate instead of forming G1-arrested conidia. wetA6 strains produce normal conidiophores at permissive temperature (30°C) but produce autolytic conidia at restrictive temperature (37°C). apsA1 strains produce sterigmata but nuclei fail to migrate into them, inhibiting phialide and conidium formation. Strain FGSC26 (biA1; veA1) was used for RNA isolations. Strains were grown in appropriately supplemented minimal medium with NO₃ as nitrogen source (Käfer 1977).

For the developmental time course experiment, FGSC26 was inoculated at a density of 3.5 × 10⁵ conidia/ml into supplemented minimal medium containing ampicillin and streptomycin at 25 μg/ml and shaken at 300 rpm at 37°C for 24 hr. Cells (100 ml) were harvested onto 10 cm Whatman No. 1 filter papers by vacuum filtration. Each filter paper was transferred to a Petri dish containing a monolayer of 3-mm glass beads and 18 ml of supplemented minimal medium. Covers were replaced, and the dishes were incubated at 37°C. Samples (four Petri dishes per time point) were taken at 0, 3, 6, 8, 10.5, 12, 15, 20.5, 25, 30, and 40 hr.

Standard A. nidulans genetic (Pontecorvo et al. 1953; Clutterbuck 1974) and transformation (Yelton, Hamer and Timberlake 1984; Timberlake et al. 1985) techniques were used. The wA3-complementing cosmid CosNK002 was isolated from an A. nidulans genomic library in pKBY2 as described by Yelton, Timberlake and Van den Hondel (1986).

Nucleic acid isolation and gel blots: DNA and RNA were isolated as described by Timberlake (1986). DNA was electrophoretically fractionated in formaldehyde-agarose gels and transferred to nylon membranes (Hybond-N, Amersham Corp., Arlington Heights, Illinois). DNA fragments were labeled with 3²P by nick translation and hybridized to filters according to the procedures recommended by the membrane supplier. The transcriptional polarity of wA was determined by using pNK15 as template to make radiolabeled RNA hybridization probes by in vitro transcription with T3 or T7 RNA polymerase.

Plasmid constructions: The following plasmids were constructed by using standard recombinant DNA techniques (Ausubel et al. 1987):

pNK3: A 13.8-kb EcoRI fragment from CosNK002 containing the wA3 complementing activity was inserted into the EcoRI site of pUC13Cm (polynk linker sites: HindIII, PstI, SalI, XhoI, BamHI, SalI, EcorI, provided by Ken Buckley, Department of Genetics, University of Georgia, Athens.)

pNK11: pNK3 was digested with BamHI and religated, deleting the 3.5-kb EcoRI-BamHI fragment and a portion of the vector polynk linker.

pNK12: pNK3 was digested with SalI and religated, deleting the 3.5-kb EcoRI-SalI fragment and a portion of the vector polynk linker.

pNK13: pNK3 was digested with SalI and XhoI and religated, deleting the 10.5-kb EcoRI-XhoI fragment and a portion of the vector polynk linker.

pNK15: A 900-bp SalI-XhoI fragment from pNK12 was ligated into the SalI-XhoI sites of pBluesc ed KS M13* (Stratagene, San Diego, California).

pNK19: A 4-kb XhoI fragment from pNK12 was ligated into the XhoI site of pIC19-H (Marsh, Erfle and Wykes 1984).
A. nidulans wA Gene

FIGURE 2.—RNA blot analysis of the wA region. (A) RNA was isolated from conidiating cultures (D), hyphae (H), or abaA-induced hyphae (A), fractionated in a denaturing agarose gel, and a blot was hybridized with radiolabeled pNK3 DNA. Locations of molecular weight standards and A. nidulans rRNAs were determined by ethidium bromide staining of the gel. (B) Restriction map of the pNK3 insert showing the extent and direction (arrow) of wA transcription.

RESULTS

Complementation of the wA3 mutation: DNA from a pKBY2 cosmid library containing wild-type A. nidulans inserts (YELTON, TIMBERLAKE and VAN DEN HONDEL 1985) was used to transform strain NK002 (pabaAI, yA2; wA3; veAI; trpC80I) to tryptophan-independence. Complementation of the wA3 mutation in this strain was expected to lead to formation of yellow conidia because of the yA2 mutation. One of 2950 trpC+ transformants produced yellow spores. This strain was colony purified. DNA was isolated, subjected to in vitro lambda packaging, and used to transduce Escherichia coli HB101 to ampicillin resistance. Three colonies grew and cosmid DNA was isolated from them. No differences were found between the electrophoretic patterns of digests of the three cosmids with four restriction endonucleases.

Cosmid DNA from each of the three E. coli transductants was used to transform NK002 to tryptophan-independence. With each, >50% of the transformants produced yellow conidia.

Localization of the wA3 complementing activity: The wA3-complementing activity was localized to a 13.8-kb EcoRI fragment (Figure 1A) by using individual, gel-isolated fragments from CosNK002 to complement the mutation as described by TIMBERLAKE et al. (1985). Subclones of this fragment were tested for their ability to complement wA3 by cotransformation of NK002 with pTA11, containing the A. nidulans trpC gene. Figure 1B shows that an XhoI fragment from coordinate positions 6.5–10.5 complemented the mutation. Two other fragments containing this XhoI fragment also complemented, whereas flanking fragments did not.

Transcription mapping of the wA region: To investigate transcription from the putative wA region, pNK3 (Figure 2A), 11, 12, 13, 15 and 19 were used to probe blots of gel-fractionated RNA from conidiating cultures (which contain hyphae, conidiophores and conidia), hyphae, or vegetative cells in which development had been artificially induced by forced expression of abaA (MIRABITO, ADAMS and TIMBERLAKE 1989). With the exception of pNK13, the clones
hybridized to a 7.5K nucleotide (nt) RNA (Figure 2A) that is absent from hyphae but present in conidiating cultures and in abaA-induced cells. Except for clones pNK15 and pNK19, these clones also hybridized to a 2.5K nt RNA that was present in all lanes just below the position of 25S rRNA. A band just above this one and a second band just below the position of 18S rRNA were visible in many blots, but appeared to be artifactual, because they were also present in blots hybridized with unrelated probes and were not present in blots from gels containing poly(A)* RNA (Figure 5C). These results, together with the complementation data, suggest that the region from the BamHI site at coordinate position 3.7 to the XhoI site at coordinate position 10.5 codes for wA mRNA as depicted in Figure 2B. The direction of wA transcription was determined by blot hybridization with strand-specific RNA probes and is also indicated in Figure 2B. The region from the XhoI (10.5) site to the EcoRI (13.8) site codes for a 2.5K nt RNA.

**Demonstration of wA identity:** To determine if this transcription unit corresponds to wA, we cotransformed A. nidulans PW1 (wA+; argB-) with pNK15, containing a 900-bp SalI–XhoI fragment from coordinate positions 5.5–6.4 (Figure 3), and pSalargB, containing the argB gene. A white-spored, arginine-independent strain (TNK15-4) was selected and colony purified. Southern blot analysis of DNA from PW1 and TNK15-4 showed that pNK15 had integrated by the single homologous recombination event depicted in Figure 3, hence disrupting the putative wA transcription unit. Diploids were constructed between TNK15-4 and NK002, and all were white-spored. Southern blot analysis of DNA from the component haploids and several diploids confirmed that the diploids contained wA regions from both parents (Figure 3C). In addition we crossed a white-spored disruptant, TNK22-1 (pabaA1, ya2; wA::argB; ΔargB::trpCΔB; veA1, trpC801) with FGSC357 (biA1; wA3). Of 15,000 progeny scored from recombinant cleistothecia 13 were green-spored and 20 were yellow-spored giving a recombination frequency of 0.22%. Thus, the insertional mutation is tightly linked to the wA3 mutation. These results, in conjunction with the complementation and transcription mapping data, confirm that the cloned region contains wA.

**Disruption analysis of the wA region:** The limits of the wA genetic locus were determined by testing

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**Figure 3.**—Disruption of the wA gene. pNK15 was linearized with SalI, mixed with pSalargB, and the mixture was used to transform the green-spored A. nidulans strain PW1 to arginine-independence. Transformants were scored for the production of white spores. Genomic integration of pNK15 by the single crossover event shown in panel A is expected to give rise to a duplication of the hatched SalI–XhoI fragment and to the two novel EcoRV fragments shown in panel B. (C) DNA from white-spored transformant TNK15-4, PW1, NK002, and three white-spored diploids (1-3) derived from a TNK15-4/NK002 heterokaryon was digested with EcoRV and subjected to Southern blot analysis with the 900-bp SalI–XhoI fragment (coordinate positions 5.5–6.4) from pNK15 as probe.
A. nidulans \( wA \) Gene

![Diagram of restriction enzyme digests and conidial color](image)

**A. nidulans**

**Gene 77**

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**CONIDIAL COLOR**

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- W
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- W
- W
- W
- W

**wA Region**

**Figure 4.** Disruption analysis of the \( wA \) region. The restriction fragments shown were subcloned into pDC1, containing the \( \text{argB} \) gene. Plasmids were linearized by digestion with restriction enzymes that cut at the junction of pDC1 and the subcloned fragment and used to transform *A. nidulans* strain TMS003 (\( pabaAl, yA2; \Delta \text{argB:trpCAB}; wA1, \text{trpC801} \)) to arginine-independence. Transformants were allowed to conidiate and scored for production of white (W) spores. "Y" indicates that no white-spored colonies were observed in >500 transformants. The inferred position of \( wA \) is indicated at the bottom of the figure.

the ability of cloned fragments from pNK3 to disrupt gene function by integration events of the type illustrated in Figure 3A. Figure 4 shows that fragments from coordinate positions 1.8–10.5 were capable of disrupting \( wA \) function and are, therefore, presumably completely contained within the locus. A \( \text{SmaI} \) fragment from coordinate positions 0.5–2.3 produced no white-spored colonies, nor did an \( \text{XhoI-ClaI} \) fragment from coordinate positions 10.5–11.1, thereby establishing the outer limits of \( wA \).

**Developmental regulation of \( wA \):** To determine the pattern of accumulation of \( wA \) transcript during conidiophore development, RNA was isolated at various times after inducing development and a gel blot was hybridized with \( wA \) and \( yA \) probes. Figure 5 shows that the 7.5k nt \( wA \) transcript appeared at 15 hr, a time when the first pigmented conidia were being formed, whereas the 2.2K nt \( yA \) transcript appeared at 10.5 hr, at the time immature conidia were being formed. The \( wA \) transcript was not detected in poly(A)+ RNA from developmentally abnormal mutant strains carrying the \( \text{brlA1, abaAl, wetA6 or apsA1} \) alleles (Figure 5C; see MATERIALS AND METHODS), nor in RNA from purified spores (data not shown). As expected (O'HARA and TIMBERLAKE 1989), the \( yA \) transcript was not detected in RNA from \( \text{brlZAl} \) or \( \text{apsAl} \) strains (Figure 5C).

**DISCUSSION**

The results presented in this paper show that we have cloned the *A. nidulans* \( wA \) gene, because (1) CosNK002 complements the \( wA3 \) mutation at high frequencies, (2) fragments from within the CosNK002 insert also complement the mutation, (3) disruption of the putative \( wA \) transcription unit through homologous recombination between pNK15 and the genome produces colonies displaying the white-spored phenotype, (4) diploids formed between one such white-spored disruptant strain and a \( wA3 \) mutant strain displayed the \( wA^- \) mutant phenotype, and (5) a
**Figure 5.**—Developmental regulation of wA. (A) RNA was isolated from strain FGSC26 at intervals after inducing development. Phialides were first observed at 8 hr postinduction and pigmented conidia were first observed at 1.5 hr. Gel blots were hybridized with a radiolabeled 900-bp SalI-XhoI wA internal fragment from pNK15 and a plasmid containing a 1.5-kbp BamHI yA internal fragment. (B) An ethidium bromide-stained gel run in parallel with the gel used in panel A. (C) Poly(A)＋ RNA, isolated from strain FGSC26 at 0 and 24 hr after inducing development and from strains carrying mutations in the morphogenetic loci brlA, abaA, wetA or apsA (see MATERIALS AND METHODS) was hybridized with wA and yA probes as in blot from panel A.

**wA** insertional mutation was tightly linked to the **wA3** mutation.

The results further show that the level of **wA** transcript is developmentally regulated. **wA** mRNA was not detected in spores or hyphae, but accumulated in conidial cultures beginning at the time when conidia first appeared. It also accumulated during artificially induced development in the **alcA** by **abaA** strain TPM1 (Figure 2A; **MIRABITO, ADAMS and TIMBERLAKE 1989**). Like **yA** mRNA (O’**HARA and TIMBERLAKE 1989**), **wA** mRNA was not detected in developmentally abnormal, aphaidial strains carrying either the brlA or apsA mutations. In contrast to **yA** mRNA, **wA** mRNA was also not detected in **abaA** or **wetA6** mutants, both of which produce phialides. Neither **wA** nor **yA** transcripts were detected in mature conidia. Thus, two phialidic strains that either produce no conidia (**abaA**) or unpigmented conidia that autolyze (**wetA6**) fail to accumulate **wA** mRNA. As **wA** is required for the production of normal conidia and its transcript is absent from spores, it must be expressed in phialides. Thus, our results imply that **abaA** and **wetA** mutations interfere with expression of some phialide-specific genes (e.g., **wA**) without interfering with expression of others (e.g., **yA**) or completely inhibiting phialide formation.

The results of the disruption analyses reported here have some interesting implications concerning the efficiency of plasmid integration by homologous recombination in **A. nidulans**. The recipient strain for the transformation experiments used to map **wA**, TMS003, was deleted for the **argB** locus, therefore precluding integration of the **argB**-bearing transformation plasmids at the corresponding locus by homologous recombination. When the transforming plasmids were linearized with restriction enzymes that cut at the junction of **A. nidulans** DNA and vector sequences, integration at **wA** occurred in >50% of the transformants, whereas with circular plasmids homologous integration at **wA** occurred in <5% of the transformants. Transformations with **argB**-bearing plasmids were also done in **A. nidulans** strain **PW1** (**argB**). Homologous integration at **wA** was also more efficient with linearized plasmids but less frequent (~5%) than with the **argB** deletion strain. These results indicate that integration of plasmids in **A. nidulans** can be directed to specific chromosomal locations by introducing double-strand breaks in the DNA molecules used for transformation, as in *Saccharomyces cerevisiae* (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981).

**wA** codes for an unusually large polyadenylated transcript (7.5K nt) that, unexpectedly, accumulates later during development than does the **yA** transcript. **wA** transcript accumulation requires brlA, abaA and wetA activities, whereas **yA** requires only brlA activity. These differences suggest that even though both genes appear to have related functions and their transcripts may be expressed in the same cell type (phialides), they could be regulated by different mechanisms. The epistatic relationship between **wA** and **yA**, and the observation that **yA** encodes a p-diphenol oxidase present in spore walls, has led to the hypothesis that **wA** encodes an enzyme responsible for synthesis of a yellow pigment intermediate that is converted to the mature green form by the **yA** product (PONTECORVO et al. 1953; CLUTTERBUCK 1972; LAW and TIMBERLAKE 1980; KURTZ and CHAMPE 1982). The fact that cell walls of **wA**-mutant conidia lack some wall components (OLIVER 1972; CLAVERIE-MARTIN, DIAZ-TORRES and GOGHEGAN 1988) suggests a more complex function for **wA** than pigment intermediate synthesis. DNA sequence analysis and in situ localization of the **wA** product will help in elucidating
the function of wA in spore differentiation.

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LITERATURE CITED


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