Nitrite Reductase Gene Upregulated During Conidiation Is Involved in Macroconidium Formation in *Fusarium oxysporum*

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ABSTRACT

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Fusarium oxysporum produces three kinds of asexual spores, microconidia, macroconidia, and chlamydospores. We previously found that the transcript level of the nitrite reductase gene of *F. oxysporum*, named *FoNIIA*, was markedly upregulated during conidiation compared with during vegetative growth. *FoNIIA* was also found to be positively regulated by *Ren1* that is a transcription regulator controlling development of microconidia and macroconidia. In this study, we analyzed the function of *FoNIIA* in conidiation of *F. oxysporum*. Conidiation cultures showed markedly higher level of accumulation of *FoNiiA* protein as well as *FoNIIA* mRNA than vegetative growth cultures. *FoNIIA* protein was sig-

nificantly decreased in cultures of the *REN1* disruption mutant compared with that of the wild type. These results confirmed that *FoNIIA* expression is upregulated during conidiation and is positively regulated by *REN1*. The *FoNIIA* disruption mutants produced microconidia, macroconidia, and chlamydospores, which were morphologically indistinguishable from those of the wild type. The mutants, however, produced significantly fewer macroconidia than the wild type, although the wild type and mutant strains produced similar numbers of microconidia and chlamydospores. These results demonstrate that nitrite reductase is involved in quantitative control of macroconidium formation as well as nitrate utilization in *F. oxysporum*.

Additional keywords: *F. oxysporum* f. sp. *melonis*, pathogenicity.

Asexual sporulation (conidiation) is a common reproductive mode for fungi (8). In plant diseases, conidia generally act as disseminated propagules, which can be responsible for initiating infection (10). Although the molecular biology of conidiation has been characterized in detail for two experimental fungi, *Aspergillus nidulans* and *Neurospora crassa* (1,23,41), the genetic and molecular mechanisms of conidiation remain largely undefined in plant-pathogenic fungi.

The soilborne fungus *Fusarium oxysporum* is an economically important plant pathogen with worldwide distribution (3,4,12). This fungus contains about 100 intraspecific variants called formae speciales, which cause vascular wilts on different plants (3,4,12). *F. oxysporum* produces three kinds of asexual spores, microconidia, macroconidia, and chlamydospores (Fig. 1) (29). Microconidia are ellipsoidal and have no or one septum (Fig. 1A). Macroconidia are falcate and have three or four septa (Fig. 1B). Globose chlamydospores have thick walls (Fig. 1C). These asexual spores play important roles in the disease cycle of *F. oxysporum*. Microconidia and macroconidia are produced on the stem surface of infected plants and serve as secondary inocula to spread the fungus to the neighboring host plants; chlamydospore is a thick-walled spore that enables a fungus to survive unfavorable conditions and acts as the primary inocula (9,20,35,36).

We previously identified two genes, *REN1* and *FoSTUA*, which encode different types of transcription regulators essential for conidiation of *F. oxysporum* (32,33). *REN1* encodes a protein

with similarity to the *A. nidulans* *MedA*, which has been identified as a developmental regulator of conidiation (6,32). Targeted mutation of *REN1* resulted in defective development of microconidia and macroconidia, and the mutants form rod-shaped, conidium-like cells directly from hyphae. The mutants, however, exhibited normal chlamydospore formation (32). *FoSTUA* encodes a basic helix-loop-helix protein with similarity to the *A. nidulans* *StuA* (13,33). The *FoSTUA* disruption mutants exhibited normal microconidium formation. The mutants, however, lacked conidiophores, resulting in defective development of macroconidia. In contrast, chlamydospore formation was dramatically promoted in the mutants. Thus, *FoSTUA* is a positive and negative regulator for the development of macroconidia and chlamydospores, respectively (33).

We performed expressed sequence tag (EST) analysis during conidiation for discovery of additional genes required for conidiation in *F. oxysporum* (17). We constructed cDNA libraries from two cultures, carboxymethyl cellulose-amended liquid medium (CMCLM) cultures producing microconidia and macroconidia and complete liquid medium (CLM) cultures not producing conidia. A total of 626 and 641 unique genes from CMCLM and CLM libraries, respectively, were identified, and only 130 (~20%) were common in the two libraries. The transcript levels of 496 genes specific for the conidiation library were studied by real-time quantitative polymerase chain reaction (RT-PCR), revealing 42 genes with an over fivefold increase in expression during conidiation conditions (17).

We also compared the transcript levels of 496 conidiation library-specific genes between the wild type and the *REN1* disruption mutant or the *FoSTUA* disruption mutant (18). A total of 35 and three genes were markedly different (over 10-fold increase or decrease) in the transcript levels between the wild type and the *REN1* disruption mutant or the *FoSTUA* disruption mutant, respectively (18). These analyses found that the nitrite reduc-

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*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains a figure showing vegetative growth and pathogenicity of the *FoNIIA* disruption mutants.

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tase gene, named *FoNIIA*, was upregulated during conidiation and positively regulated by *REN1* (17,18).

Utilization of nitrate requires two enzymes, nitrate reductase and nitrite reductase. Nitrate reductase catalyzes the reduction of nitrate to nitrite, and nitrite reductase catalyzes the reduction of nitrite to ammonium (25). We observed that the transcript level of the nitrate reductase gene was not significantly induced in CMCLM cultures and was not regulated by *Ren1* (17,18). These results suggested that nitrite reductase has an important function in conidiation as well as nitrate utilization. In this study, we analyzed the function of nitrite reductase in conidiation of *F. oxysporum*. Conidiation cultures of the wild-type strain showed markedly higher level of accumulation of *FoNiiA* protein as well as *FoNIIA* mRNA than vegetative growth cultures. Targeted mutation of *FoNIIA* resulted in a significant reduction in macroconidia, but not microconidia and chlamydo spores, in conidiation cultures. We propose that nitrite reductase has the function to promote macroconidium formation in *F. oxysporum*.

MATERIALS AND METHODS

Fungal strains and culture conditions. Strains Mel02010 (JCM9288) and DR1 (*REN1* disruption mutant) of *F. oxysporum* f. sp. *melonis* (28,32) were used in this study. DR1 was generated from Mel02010 by transformation-mediated disruption of *REN1* (32). Strains were maintained routinely on potato dextrose agar (PDA; Difco, Detroit, MI).

To compare the transcript and protein levels of *FoNIIA* between conidiation and vegetative growth cultures of *F. oxysporum*, strains were grown in CMCLM (15 g of carboxymethyl cellulose, 1 g of $(\text{NH}_4)_2\text{SO}_4$, 1 g of KH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1 g of yeast extract per liter) (7) and CLM (1 g of $\text{Ca}(\text{NO}_3)_2$, 0.2 g of KH_2PO_4 , 0.25 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.15 g of NaCl, 0.02 g of FeCl_3 , 10 g of glucose, 1 g of yeast extract, and 1 g of peptone per liter) (39) at 25°C for 5 days on an orbital shaker (100 rpm). To analyze effects of nitrogen sources on the transcript and protein levels of *FoNIIA* during vegetative growth, strains were grown in 50 ml of minimal liquid medium (MLM; 1 g of ammonium tartrate, 0.2 g of KH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.15 g of NaCl, 0.02 g of FeCl_3 , and 10 g of glucose per liter) (39) at 25°C for 3 days, and the resulting mycelia were transferred to 50 ml of MLM supplemented with 0.1% ammonium tartrate or 0.1% NaNO_2 as a sole nitrogen source in 100-ml Erlenmeyer flasks and grown at 25°C for 24 h on an orbital shaker (100 rpm). These cultures were subjected to isolation of RNA and proteins.

Nucleic acid isolation and manipulation. Isolation of total DNA and RNA from *F. oxysporum* was performed as previously described (18,28,37). Poly(A)⁺ RNA was isolated from total RNA with the Oligo dT30 mRNA Purification Kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Plasmid DNA and bacterial artificial chromosome (BAC) DNA were extracted by the standard methods (38).

Restriction enzyme digestion of fungal genomic DNA and fractionation in agarose gels were performed by the standard methods (38). Fractionated DNA was transferred to Hybond N⁺ nylon membranes (GE Healthcare Bio-Sciences, Piscataway, NJ) and subjected to hybridization and detection using Gene Images Random-Prime Labeling and Detection Reagents (GE Healthcare Bio-Sciences) according to the manufacturer's instruction. PCR was performed using *Taq* DNA polymerase (Takara Bio).

DNA sequences were determined with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK) and an automated fluorescent DNA sequencer (Model 373A, Applied Biosystems) according to the manufacturer's instructions. DNA sequences were analyzed with BLAST program (2).

Real-time quantitative PCR was performed in a LightCycler Quick System 350S (Roche Diagnostic, Indianapolis, IN) using the One Step SYBR RT-PCR Kit (Takara Bio) as previously described (17,18). The PCR primers 2F02-f (5'-GTC AGA AGC GAC CAG CTT TC-3') and 2F02-r (5'-GAT ACC GTT TGG CAA CTC GT-3') were designed to amplify an *FoNIIA* cDNA fragment of 120-bp using the primer design software in DNAsis Pro Ver. 2.2 (Hitachi Software Engineering, Yokohama, Japan). Previously designed and optimized primers FOMeF (5'-ACT TAC CCC GCC ACT TGA G-3') and FOMeR (5'-CCA GGC GTA CTT GAA GGA AC-3') for the elongation factor 1- α gene (*EF1 α*) as a constitutively expressed endogenous control (17,18) were used. The transcript level of *FoNIIA* was measured four times with independently isolated RNA. To verify that the efficiencies of the target (*FoNIIA*) and endogenous control reactions were approximately equal, reactions were performed with serial dilutions of RNA as templates. The threshold cycle was calculated with a LightCycler Software version 3.5 (Roche Diagnostic) using the second-derivative-maximum method (LightCycler Operator's Manual version 3.5, Roche Diagnostic), and the transcript levels in samples were calculated from the gene-specific standard curve (17,18). The transcript level of *FoNIIA* was normalized to that of *EF1 α* (value = 1).

Genomic library. A genomic BAC library of Mel02010 was constructed with a BAC vector pcc1BAC (Epicentre, Madison, WI). Chromosome-sized DNA molecules in agarose plugs were partially digested with *Sau3AI* (Takara Bio) and fractionated in a 1.0% certified low melt agarose (Bio-Rad Laboratories, Richmond, CA) gel on a contour-clamped homogeneous electric field (CHEF) apparatus (CHEF-DRII; Bio-Rad Laboratories) as previously described (16). A piece of agarose containing DNA fragments of about 150 kb was excised and treated with β -agarase (New England BioLabs, Ipswich, MA) according to the manufacturer's instructions. Recovered DNA was ligated with *Bam*HI-digested, dephosphorylated pcc1BAC using T4 DNA ligase (Takara Bio). Ligation products were transformed by electroporation into *Escherichia coli* ElectroMAX DH10B (Invitrogen, San Diego, CA) using an electroporation apparatus GenePulser (Bio-Rad Labora-

Fig. 1. Conidiation of *Fusarium oxysporum*. The wild-type strain Mel02010 was grown on a synthetic low-nutrient agar-paper medium at 25°C for 5 days. **A**, Microconidia (mi) are produced from intercalary phialides (ip) in false heads. **B**, Macroconidia (ma) are produced from terminal phialides (tp) on conidiophores (cp). **C**, Chlamydo spores (ch) are formed from hyphae. Bar markers represent 10 μm .

tories), and a total of 3,072 colonies were obtained. The average insert size of 12 clones analyzed was 114.3 kb, and 3,072 clones were expected to contain a total of 351 Mb, that is, about 5.9-fold of the *F. oxysporum* genome size (about 59.9 Mb).

Colony blotting to Hybond N+ nylon membranes was performed by the standard method (38), and Gene Images Random-Prime Labeling and Detection Reagents were used for colony hybridization.

Isolation of nitrite reductase gene. The insert of EST clone WCMC-2_F02 encoding putative nitrite reductase (17) was used as a probe for screening a genomic BAC library of Mel02010, and a positive clone BACFoNIIA was isolated. An 8.0-kb region containing WCMC-2_F02 sequence was sequenced, and a putative open reading frame (ORF) of *FoNIIA* was identified.

The *FoNIIA* cDNA was isolated by reverse transcription-PCR (RT-PCR) using the RNA PCR Kit version 2.1 (Takara Bio). The cDNA was amplified from total RNA (1 µg) of Mel02010 with primers NIRf (5'-ATG ACT GCA ACG ACA CAA A-3') and NIRr (5'-CTA CCA TTC CGG CGC AC-3') (Fig. 2A) according to the manufacturer's instructions. NIRf and NIRr contain the *FoNIIA* initiation and termination codons (italics), respectively. RT-PCR products were cloned into the plasmid pCR2.1 (Invitrogen) to determine the sequences.

The sequence of *FoNIIA* has been deposited in DDBJ/EMBL/GenBank databases under accession no. AB274526.

Preparation of protein extracts and immunoblotting. Fungal tissues (500 mg) were suspended in 300 µl of protein extraction buffer (10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 2% sodium dodecyl sulfate (SDS), 5 mM EDTA, and complete protease inhibitor cocktail set (Roche) 1 tablet per 50 ml) and broken by agitation with zirconia beads (3.0 mm) at 4°C for 3 min using Micro Smash MS-100R (TOMY, Tokyo, Japan). After centrifugation at 1,600 × g for 10 min, the supernatants of the lysates were collected. All protein concentration was determined by the Bradford method (5) using Bio-Rad Protein Assay Kit (Bio-Rad) with bovine serum albumin as a standard.

Total protein samples were separated by SDS-polyacrylamide gel electrophoresis using NuPAGE 4 to 12% Bis-Tris Gel (Invitrogen) and blotted to Hybond-ECL (GE Healthcare Bio-Sciences) by using an electroblotting apparatus Trans-Blot Semi-Dry (Bio-Rad Laboratories). FoNIIA protein was detected using the antibody raised against an epitopic peptide (CTRNM-ELEDDRQKQR) of FoNIIA conjugated to keyhole limpet hemocyanin in rabbit (prepared by Takara Bio). Antibody binding was visualized using enhanced chemiluminescence (ECL) western blotting detection system (GE Healthcare Bio-Sciences) after binding a horseradish peroxidase-conjugated secondary antibody.

Fungal transformation. The *FoNIIA* disruption vector pGDN1 was constructed by cloning the 1.6-kb internal fragment of *FoNIIA* into the integrative transformation vector pSH75 (21), which carries hygromycin B resistance gene (*hph*). The 1.6-kb *FoNIIA* fragment was amplified by PCR with primers Dnf (5'-TCG TGG GGT TGG GCA TGG TT-3') and DNr (5'-CCT GCT TCT TGG CAC CAA AC-3') (Fig. 2A). The amplified DNA was cloned into the *EcoRV* site of pSH75 to make pGDN1.

The complementation vector pCON1 was constructed by cloning the entire *FoNIIA* with the In-Fusion Dry-Down PCR Cloning Kit (Clontech, Mountain View, CA). The 7.4-kb fragment, which includes the *FoNIIA* exon and intron region and its 2.0-kb upstream and 2.0-kb downstream regions, was amplified from BACFoNIIA DNA by PCR using primers CNf (5'-CGA AGT TAT CAG TCG ACG CAT GTG CCT TAT GTA GAG-3') and CNr (5'-AAT GGT CTA GAA AGC TCT ACC ATT CCG GCG CAC CGC-3') (Fig. 2A). These primers have 16 bases (underlined) identical to sequences at the ends of the linearized plasmid pDNR-CMV (Clontech). The In-Fusion cloning reaction and transformation in *E. coli* Fusion-Blue Competent Cells

(Clontech) yielded the *FoNIIA* complementation vector pCON1. The plasmid pCON1 was introduced into the *FoNIIA* disruption mutant by co-transformation with the transformation vector pIH99 (27), which carries geneticin resistance gene (*nptII*).

Protoplast preparation and transformation of *F. oxysporum* were performed as previously described (19). Transformants carrying *hph* or *nptII* were selected on regeneration medium containing hygromycin B (Wako Pure Chemicals, Osaka, Japan) at 100 µg/ml or geneticin (Gibco BRL, Life Technology, Gaithersburg, MD) at 150 µg/ml, respectively (19,27). To assess nitrite utilization of transformants, transformants were grown on minimal agar medium (MAM; MLM supplemented with 2% agar) (39) containing 0.1% ammonia tartrate or 0.1% NaNO₂ as a sole nitrogen source.

Test for conidiation. To induce conidiation of *F. oxysporum* strains, two media were used: CMCLM for formation of microconidia and macroconidia and synthetic low nutrient agar medium (SNA; 1.0 g of KH₂PO₄, 0.5 g of (NH₄)₂SO₄, 0.5 g of MgSO₄·7H₂O, 0.5 g of KCl, 0.2 g of glucose, 0.2 g of sucrose, and 20 g of agar per liter) (31) for formation of microconidia, macroconidia, and chlamydospores. Sterilized filter paper (1 × 2 cm) was placed on SNA surface before inoculation of fungal strains (31).

Fig. 2. The nitrite reductase gene *FoNIIA* of *Fusarium oxysporum*. **A**, Map of the *FoNIIA* locus. The arrowed bar indicates *FoNIIA* with introns (white segments). Arrowheads denote the location of oligonucleotide primers used in polymerase chain reaction (PCR) and reverse transcription-PCR experiments. **B**, BamHI; Bg, BglII; P, PvuII; X, XbaI. **B**, Amino acid sequence of FoNIIA. I to IV, NADH/FAD-binding domain, ferredoxin-like binding domain, nitrite and sulphite reductase domain, and Rieske domain, respectively.

The wild type and mutant strains were grown on PDA at 25°C for 5 days. Agar blocks (3-mm diameter) carrying mycelia were prepared from the resulting colonies and inoculated in 50 ml of CMCLM in a 100-ml Erlenmeyer flask. Each strain was inoculated in two sets of five flasks, and each set was incubated at 25°C for 5 days on an orbital shaker (100 rpm) under continuous fluorescent light or dark condition (7). For each flask, the numbers of microconidia and macroconidia were counted twice using a microscope.

A PDA block carrying mycelium was inoculated on SNA-paper medium, 5 mm away from filter paper pieces. Each strain was inoculated on two sets of five SNA-paper plates, and each set was incubated at 25°C for 5 days under continuous fluorescent light or dark condition. For each plate, numbers of chlamydospores formed in 5-mm squares between agar blocks and filter paper were counted using a microscope.

Test for vegetative growth and pathogenicity. To test for vegetative growth of *F. oxysporum* strains, they were grown on three agar media: PDA, complete agar medium (CAM; CLM supplemented with 2% agar) (39), and MAM. CAM and MAM were supplemented with 0.1% ammonium tartrate as a sole nitrogen source. PDA blocks (3 mm in diameter) carrying mycelia were inoculated on PDA, CAM, and MAM. After incubation at 25°C for 5 days, colony growth and morphology were recorded.

Pathogenicity was tested by a root-dip inoculation of susceptible melon (*Cucumis melo* L.) cultivar Amus with bud cell suspensions ($\approx 1 \times 10^6$ cells/ml in sterilized water) as previously described (28). Fifteen seedlings with a single true leaf were inoculated with bud cell suspension of each strain. Pathogenicity was also tested by planting 15 seedlings with a single true leaf in soil infested with bud cells ($\approx 1 \times 10^5$ cells/g of soil) of each strain. Disease symptoms were observed 3 weeks after inoculation.

RESULTS AND DISCUSSION

Isolation of *FoNIIA*. A BAC clone, named BACFoNIIA, was isolated by screening of a BAC genomic library of Mel02010 using the insert of EST clone WCMC-2_F02 carrying the partial cDNA of putative nitrite reductase gene as a probe. Sequencing of the 8.0-kb region of BACFoNIIA detected a putative ORF encoding a protein with strong similarity to nitrite reductases from fungi (Fig. 2A).

The cDNA of 3.3 kb was prepared from total RNA of Mel02010 by RT-PCR with primers NIRf and NIRr (Fig. 2A). Comparison of the genomic sequence with that of RT-PCR product indicated that the gene has three introns (53, 50, and 51 bp) and four exons (65, 151, 1,973, and 1,079 bp) and potentially encodes a 1,089-amino acid protein.

A database search using BLAST algorithm revealed that the deduced amino acid sequence has strong similarity to those of fungal nitrite reductases, such as *A. nidulans* NiiA (34) and *N. crassa* Nit-6 (14) with 62.3 and 61.6% identity, respectively. The cloned gene encodes four domains typical of nitrite reductases: NADH/FAD-binding domain, ferredoxin-like binding domain, conserved domain in nitrite and sulphite reductases, and Rieske domain containing iron-sulfur clusters (Fig. 2B) (14,40). Thus, this gene was named *FoNIIA*.

Expression of *FoNIIA*. *F. oxysporum* produces microconidia and macroconidia in CMCLM, but not in CLM, and exhibits vigorous vegetative growth in CLM (17,32,33). The transcript and protein levels of *FoNIIA* in the wild-type strain Mel02010 were tested in CMCLM (conidiation) cultures and CLM (vegetative growth) cultures by real-time quantitative PCR and immunoblot analyses, respectively. *FoNIIA* showed 63-fold higher transcript level in CMCLM cultures than in CLM cultures (Fig. 3A). *FoNiiA* protein was markedly abundant in CMCLM cultures compared to CLM cultures (Fig. 3A). These results indicate that the transcript level of *FoNIIA* is upregulated during conidiation, resulting in increase of *FoNiiA* protein.

Nitrite reductase catalyzes the reduction of nitrite to ammonium (25). We also tested the transcript and protein levels of *FoNIIA* in Mel02010 grown in MLM containing NaNO_2 or ammonium tartrate as a sole nitrogen source. The transcript level of *FoNIIA* was 12-fold higher in NaNO_2 cultures than in ammonium tartrate cultures, and *FoNiiA* protein was more abundant in NaNO_2 cultures than in ammonium tartrate cultures (Fig. 3B), indicating that *FoNIIA* expression is induced in nitrite medium.

We previously found that *FoNIIA* is positively regulated by the transcription regulator Ren1, essential for conidiation of *F. oxysporum* (32). The wild-type strain Mel02010 and the *REN1* disruption mutant DR1 were grown on MAM containing ammonium tartrate or NaNO_2 as a sole nitrogen source at 25°C for 6 days, and colony diameters were measured. The wild type and mutant strains showed similar growth on ammonium tartrate medium (Fig. 4A). The *REN1* disruption mutant, however, showed poor growth on NaNO_2 medium compared with the wild type (Fig. 4A). Although the wild type and mutant strains did not differ in growth on NaNO_2 medium during the initial 2 days of incubation, colony growth of the mutant was restricted after that (Fig. 4A and B). Such poor growth of the *REN1* disruption mutant on NaNO_2 medium was probably due to the lower expression level of *FoNIIA*. The expression level of *FoNIIA* was compared between the wild type and the mutant grown in MLM supplemented with NaNO_2 . The transcript level of *FoNIIA* was 11-fold lower in the mutant than in the wild type, and accumulation of *FoNiiA* protein was less in the mutant than in the wild type (Fig. 3C). These results confirmed that *FoNIIA* is positively regulated by Ren1.

In addition of nitrite reductase, nitrate reductase is also required for nitrate utilization: this enzyme catalyzes the reduction of nitrate to nitrite, the first step for nitrate assimilation (25). However, we previously observed that the nitrate reductase gene

Fig. 3. Transcript (upper column) and protein (lower column) levels of *FoNIIA*. Transcript level of *FoNIIA* in each culture was quantified by quantitative real-time polymerase chain reaction (PCR) analysis and normalized to that of *EF1α* (value = 1). Each value represents the mean and standard deviations of four experiments with independently isolated RNA. *FoNiiA* protein was detected by immunoblot analysis with anti-*FoNiiA* antibody. Total proteins (10 µg) from each culture were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted on nitrocellulose membranes. The blots were treated with anti-*FoNiiA* antibody, and antibody binding was visualized using enhanced chemiluminescence (ECL) western blotting detection system after binding a horseradish peroxidase-conjugated secondary antibody. **A**, The wild-type strain Mel02010 was grown in carboxymethyl cellulose-amended liquid medium (CMCLM; conidiation phase) and complete liquid medium (CLM; vegetative growth phase) at 25°C for 5 days. **B**, Mel02010 was grown in minimal liquid medium (MLM) supplemented with NaNO_2 (NO_2^-) or ammonium tartrate (NH_4^+) as a sole nitrogen source. **C**, Mel02010 (WT) and the *REN1* disruption mutant (DR1) were grown in MLM supplemented with NaNO_2 (NO_2^-).

was not significantly induced in CMCLM cultures and was not regulated by Ren1 (17,18). Deng et al. (11) found using cDNA array with 3,372 tentative unique transcripts that the nitrite reductase gene was highly expressed in conidia and germinated conidia, but not in vegetative mycelia, of *F. oxysporum*. All these data suggested that nitrite reductase has an important function in conidiogenesis and conidial germination as well as nitrate utilization.

Targeted disruption of *FoNIIA*. To examine the function of *FoNIIA* in nitrite utilization and conidiation in *F. oxysporum*, homologous recombination was employed to disrupt *FoNIIA* with the plasmid pGDN1, which contains a 1.6-kb internal fragment of *FoNIIA* and the *hph* cassette (Fig. 5A). Strain Mel02010 was transformed with pGDN1, and 19 transformants were isolated. When these transformants were grown on MAM containing ammonium tartrate or NaNO₂, 12 transformants exhibited normal growth on both media. The remaining seven transformants (DN1 to DN7) exhibited normal growth on ammonium medium, but exhibited very poor growth on nitrite medium (Fig. 5B), suggesting that these transformants were deficient in nitrite utilization due to disruption of *FoNIIA*.

Fig. 4. Colony growth of the *REN1* disruption mutant on ammonium and nitrite media. The wild-type strain Mel02010 and the *REN1* disruption mutant DR1 were grown on minimal agar medium supplemented with ammonium tartrate (NH₄⁺) or NaNO₂ (NO₂⁻) as a sole nitrogen source at 25°C, and colony diameters were measured at 24-h intervals. **A**, Data represent the means and standard deviations of three replications. **B**, Photographs were taken after 4 days of incubation.

Fig. 5. Transformation-mediated disruption of *FoNIIA*. **A**, Structure of the *FoNIIA* locus before and after homologous integration of the *FoNIIA* disruption vector pGDN1. To make pGDN1, the 1.6-kb internal fragment of *FoNIIA* was cloned into the transformation vector pSH75. **B**, *Bam*HI. **B**, Growth of the *FoNIIA* disruption mutant on ammonium and nitrite media. Strains were grown on minimal agar medium supplemented with ammonium tartrate (NH₄⁺) or NaNO₂ (NO₂⁻) as a sole nitrogen source at 25°C for 4 days. WT, wild-type strain Mel02010; DN1, *FoNIIA* disruption mutant; CN1, *FoNIIA*-complemented transformant made from DN1. **C**, DNA gel blot analysis of pGDN1 transformants. Total DNA (0.5 µg/lane) from Mel02010 and the pGDN1 transformants was digested with *Bam*HI and fractionated in a 0.8% agarose gel. The blot was probed with the 1.6-kb *FoNIIA* fragment from pGDN1. Sizes (in kilobases) of marker DNA fragments (*Hind*III-digested λDNA) are indicated on the left. DN1 to DN4, *FoNIIA* disruption mutants; EC1 to EC4, ectopic transformants. **D**, Polymerase chain reaction (PCR) analysis of genetic complementation of *FoNIIA* disruption mutant. Total DNA from each strain was used as templates for PCR with primer pair CNf and CNr. CN1 to CN3, *FoNIIA*-complemented transformants made from DN1. **E**, Microconidia and macroconidia produced by Mel02010 and DN1. Strains were grown in carboxymethyl cellulose-amended liquid medium at 25°C for 4 days under continuous fluorescent light. Bar markers represent 10 µm.

The integration mode of pGDN1 in four transformants (DN1 to DN4) showing poor growth on nitrite medium was analyzed by DNA gel blot analysis. As controls, four transformants (EC1 to EC4) showing normal growth on nitrite medium were also used. Total DNA of Mel02010 and transformants was digested with *Bam*HI, and the blot was probed with the 1.6-kb *FoNIIA* fragment cloned in pGDN1 (Fig. 5A). The probe hybridized to ≈ 9.8 -kb bands in the wild-type strain and EC transformants (Fig. 5A and C). We expected that the probe would hybridize to ≈ 6.7 - and ≈ 11.2 -kb bands, instead of ≈ 9.8 -kb band, in DN transformants, if *FoNIIA* was disrupted by homologous integration of pGDN1 into the *FoNIIA* locus (Fig. 5A). The hybridization patterns of DN transformants were consistent with expected ones (Fig. 5C).

To confirm disruption of *FoNIIA* in DN transformants, genetic complementation of the *FoNIIA* disruption mutant DN1 with the wild-type *FoNIIA* was performed. The plasmid pCON1, which contains the entire *FoNIIA* exon and intron regions with the upstream and downstream regulatory sequences, was introduced into DN1 by co-transformation with the plasmid pII99, conferring resistance to geneticin (27). When geneticin-resistant transformants were tested for growth on MAM containing ammonium tartrate or NaNO₂, three transformants (CN1 to CN3) normally grew on NaNO₂ medium as well as ammonium tartrate medium (Fig. 5B). PCR analysis of these transformants with primers CNf and CNr verified the presence of the introduced 7.4-kb fragment containing *FoNIIA* (Fig. 5A and D). These results confirmed that *FoNIIA* encodes nitrite reductase.

Conidiation of *FoNIIA* disruption mutants. To assess the role of *FoNIIA* in conidiation of *F. oxysporum*, formation of

microconidia, macroconidia, and chlamydozoospores of the *FoNIIA* disruption mutants (DN1 to DN3) was compared with that of the wild-type strain Mel02010, the ectopic pGDN1 transformant EC1, and the *FoNIIA*-complemented transformant CN1. Strains were grown in CMCLM containing (NH₄)₂SO₄ as a sole nitrogen source under continuous fluorescent light or dark condition, and formation of microconidia and macroconidia was observed. The *FoNIIA* disruption mutants produced microconidia and macroconidia with similar morphology to those produced by the wild type (Fig. 5E), indicating that *FoNIIA* is not essential for morphogenesis of these conidia.

The expression of *FoNIIA* is positively regulated by Ren1 (18). *REN1* controls the early step of development of microconidia and macroconidia, and the *REN1* disruption mutants lack conidiophores and phialides and form rod-shaped, conidium-like cells, instead of microconidia and macroconidia, directly from hypha (32). Thus, we supposed that mutation of *FoNIIA* might cause morphological abnormality of these conidia. The *FoNIIA* disruption mutants, however, produced microconidia and macroconidia with normal morphology. These results suggest that Ren1 controls a number of genes including *FoNIIA*, which function in different steps of the conidiation process, and that *FoNIIA* may not contribute at the early step of conidiogenesis.

Number of microconidia and macroconidia produced by each strain was counted after incubation in CMCLM for 5 days. Number of microconidia produced by the *FoNIIA* disruption mutants and the other strains was not significantly different (Fig. 6A). However, number of macroconidia produced by the *FoNIIA* disruption mutants was significantly fewer than those produced by

Fig. 6. Conidiation of the *FoNIIA* disruption mutants. **A**, Strains were grown in carboxymethyl cellulose-amended liquid medium at 25°C for 4 days under continuous fluorescent light (FL) or dark condition, and the resulting microconidia and macroconidia were counted. **B**, Strains were grown on synthetic low nutrient agar-paper medium at 25°C for 5 days under continuous FL or dark condition, and chlamydozoospores were counted. Data represent the means and standard deviations of five replications. Asterisk indicates statistically significant difference between the wild-type and each transformant according to *t* test ($P < 0.05$). WT, wild-type strain Mel02010; DN1 to DN3, *FoNIIA* disruption mutants; EC1, ectopic transformant; CN1, *FoNIIA*-complemented transformant made from DN1.

the other strains: ≈ 50 and 20% of the wild-type strain Mel02010 under continuous fluorescent light and dark conditions, respectively (Fig. 6A). The *FoNIIA*-complemented transformants produced macroconidia as did the wild type (Fig. 6A).

To observe chlamydospore formation, strains were grown on SNA-paper medium under continuous fluorescent light or dark conditions. The *FoNIIA* disruption mutants formed chlamydospores acrogenously from hyphae or by the modification of hyphal cells, as did the wild type. The number and morphology of chlamydospores were not significantly different between wild type and mutants under both light and dark conditions (Fig. 6B). Although it was difficult to accurately count numbers of microconidia and macroconidia formed on SNA-paper medium, the mutants produced apparently fewer macroconidia than the wild type under both light and dark conditions. The wild type and mutant strains seemed to produce similar number of microconidia. These results indicate that nitrite reductase is required for wild-type macroconidium formation but not for microconidium and chlamydospore formation.

There were no significant differences in colony growth on PDA, CAM, and MAM between the wild type and the *FoNIIA* disruption mutants. The *FoNIIA* disruption mutants caused typical wilt symptoms on melon seedlings, when seedlings were inoculated with bud cell suspension by a root-dip method and were planted in soil infested with bud cells. Timing of symptom appearance and severity of symptoms were indistinguishable on plants inoculated with the wild type and the *FoNIIA* disruption mutants, indicating that the mutants preserve the ability to infect host plant tissue and to cause disease symptoms under the conditions tested.

The mutation of *FoNIIA* affected macroconidium formation, but not formation of microconidia and chlamydospores, vegetative growth on ammonium medium, and virulence in *F. oxysporum*. The genetic role of nitrite reductase in quantitative control of macroconidium formation is uncertain. The effect of mutation of nitrite reductase genes on conidiation has not been reported in any fungi, including *A. nidulans* and *N. crassa*. Because we used CMCLM containing $(\text{NH}_4)_2\text{SO}_4$ as a sole nitrogen source in test for formation of microconidia and macroconidia, nitrite reductase activity was not required for nutritional nitrogen supply in both the wild type and the *FoNIIA* disruption mutants. Thus, different function of nitrite reductase is likely to have a role in macroconidium formation. Nitrite reductase is also involved in denitrification process, in which this enzyme generates nitric oxide (NO) from nitrite (44). Nitric oxide is known as a potent signaling molecule with multiple biological functions in animal and plants (26,43). In fungi, nitric oxide was reported to be involved in sporangioophore development of *Phycomyces blakesleeana* (24), photo-conidiation of *N. crassa* (30), conidiation of *Coniothyrium minitans* (15), and conidial germination of *Aspergillus fumigatus* (22) and *Colletotrichum coccodes* (42). Nitric oxide production in these fungi was identified to be mediated by nitric oxide synthases (NOS), but not nitrite reductases (15,24,30,42). Therefore, further studies to detect nitric oxide generation during conidiation in the wild type and the *FoNIIA* disruption mutant should be performed.

We previously found 35 genes, which were markedly different (>10 -fold increase or decrease) in the transcript levels between the wild type and the *REN1* disruption mutant (18). Of 35 genes, 26 including *FoNIIA* correspond to upregulated genes in CMCLM cultures of the wild-type strain, which were identified by the EST analysis of CMCLM and CLM cultures (17). Of the 26 genes, 16 are of unknown function. The remaining 10 genes possibly encode proteins with significant similarity to known proteins, such as membrane transporters, transcription factors, V-SNARE involved in vacuolar transport, small monomeric GTPase, and iron transferase (17,18). These genes provide candidates for further studies directed at understanding fungal conidiogenesis and its

molecular regulation. Gene-disruption studies are in progress to determine whether any of these genes are required for conidial development of *F. oxysporum*. This approach will provide a unique and novel insight into conidiogenesis of *F. oxysporum* that provides the infectious propagules playing important roles in the disease cycle of this pathogen.

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LITERATURE CITED

- Adams, T. H., Wieser, J. K., and Yu, J. H. 1998. Asexual sporulation in *Aspergillus nidulans*. Microbiol. Mol. Biol. R. 62:35-54.
- Altschul, S. F., Madden, T. L., Schäfer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402.
- Armstrong, G. M., and Armstrong, J. K. 1981. Formae speciales and races of *Fusarium oxysporum* causing wilt diseases. Pages 391-399 in: *Fusarium Disease, Biology and Taxonomy*. P. E. Nelson, T. A. Toussoum, and R. J. Cook, eds. The Pennsylvania State University Press, University Park, PA.
- Beckman, C. H. 1987. The Nature of Wilt Diseases of Plants. The American Phytopathological Society Press, St. Paul, MN.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.
- Busty, T. M., Miller, K. Y., and Miller, B. L. 1996. Suppression and enhancement of the *Aspergillus nidulans medusa* mutation by altered dosage of the *bristle* and *stunted* genes. Genetics 143:155-163.
- Cappellini, R. A., and Peterson, J. L. 1965. Macroconidium formation in submerged cultures by a nonsporulating strain of *Gibberella zeae*. Mycologia 57:962-966.
- Cole, G. T. 1986. Models of cell differentiation in conidial fungi. Microbiol. Rev. 50:95-132.
- Couteaudier, Y., and Alabouvette, C. 1990. Survival and inoculum potential of conidia and chlamydospores of *Fusarium oxysporum* f. sp. *lini* in soil. Can. J. Microbiol. 36:551-556.
- Dahlberg, K. R., and Van Etten, J. L. 1982. Physiology and biochemistry of fungal sporulation. Annu. Rev. Phytopathol. 20:218-301.
- Deng, Y., Dong, H., Jin, Q., Dai, C., Fang, Y., Liang, S., Wang, K., Shao, J., Lou, Y., Shi, W., Vakalounakis, D. J., and Li, D. 2006. Analysis of expressed sequence tag data and gene expression profiles involved in conidial germination of *Fusarium oxysporum*. Appl. Environ. Microbiol. 72:1667-1671.
- Di Pietro, A., Madrid, M. P., Caracul, Z., Delgado-Jarana, J., and Roncero, M. I. G. 2003. *Fusarium oxysporum*: Exploring the molecular arsenal of a vascular wilt fungus. Mol. Plant Pathol. 4:315-325.
- Dutton, J. R., Johns, S., and Miller, B. L. 1997. StuAp is a sequence-specific transcription factor that regulates developmental complexity in *Aspergillus nidulans*. EMBO J. 16:5710-5721.
- Exley, G. E., Colandene, J. D., and Garrett, R. H. 1993. Molecular cloning, characterization, and nucleotide sequence of *nit-6*, the structural gene for nitrite reductase in *Neurospora crassa*. J. Bacteriol. 175:2379-2392.
- Gong, X., Fu, Y., Jiang, D., Li, G., Yi, X., and Peng, Y. 2007. L-arginine is essential for conidiation in the filamentous fungus *Coniothyrium minitans*. Fungal Genet. Biol. 44:1368-1379.
- Hatta, R., Shinjo, A., Ruswandi, S., Kitani, K., Yamamoto, M., Akimitsu, K., and Tsuge, T. 2006. DNA transposon fossils present on the conditionally dispensable chromosome controlling AF-toxin biosynthesis and pathogenicity of *Alternaria alternata*. J. Gen. Plant Pathol. 72:210-219.
- Iida, Y., Ohara, T., and Tsuge, T. 2006. Identification of genes up-regulated during conidiation of *Fusarium oxysporum* through expressed sequence tag analysis. Fungal Genet. Biol. 43:179-189.
- Iida, Y., Ohara, T., and Tsuge, T. 2007. Identification of genes with changes in transcript levels caused by mutations in conidiation regulator genes *REN1* and *FoSTUA* in *Fusarium oxysporum*. J. Gen. Plant Pathol. 73:158-167.

19. Inoue, I., Ohara, T., Namiki, F., and Tsuge, T. 2001. Isolation of pathogenicity mutants of *Fusarium oxysporum* f. sp. *melonis* by insertional mutagenesis. *J. Gen. Plant Pathol.* 67:191-199.
20. Katan, T., Shlevin, E., and Katan, J. 1997. Sporulation of *Fusarium oxysporum* f. sp. *lycopersici* on stem surface of tomato plants and aerial dissemination of inoculum. *Phytopathology* 87:712-719.
21. Kimura, N., and Tsuge, T. 1993. Gene cluster involved in melanin biosynthesis of the filamentous fungus *Alternaria alternata*. *J. Bacteriol.* 175:4427-4435.
22. Kunert, J. 1995. Effect of nitric oxide donors on survival of conidia, germination and growth of *Aspergillus fumigatus* in vitro. *Folia Microbiol.* 40:238-244.
23. Lengeler, K. B., Davidson, R. C., D'souza, C., Harashima, T., Shen, W. C., Wang, P., Pan, X., Waugh, M., and Heitman, J. 2000. Signal transduction cascades regulating fungal development and virulence. *Microbiol. Mol. Biol. R.* 64:746-785.
24. Maier, J., Hecker, R., Rocke, P., and Ninnemann, H. 2001. Role of nitric oxide synthase in the light-induced development of sporangiophores in *Phycomyces blakesleeana*. *Plant Physiol.* 126:1323-1330.
25. Marzluf, G. A. 1997. Genetic regulation of nitrogen metabolism in the fungi. *Microbiol. Mol. Biol. R.* 61:17-32.
26. Mur, L. A. J., Carver, T. L. W., and Prats, E. 2006. NO way to live; the various roles of nitric oxide in plant-pathogen interactions. *J. Exp. Bot.* 57:489-505.
27. Namiki, F., Matsunaga, M., Okuda, M., Inoue, I., Nishi, K., Fujita, Y., and Tsuge, T. 2001. Mutation of an arginine biosynthesis gene causes reduced pathogenicity in *Fusarium oxysporum* f. sp. *melonis*. *Mol. Plant-Microbe Interact.* 14:580-584.
28. Namiki, F., Shiomi, T., Kayamura, T., and Tsuge, T. 1994. Characterization of the formae speciales of *Fusarium oxysporum* causing wilts of cucurbits by DNA fingerprinting with the nuclear repetitive DNA sequences. *Appl. Environ. Microbiol.* 60:2684-2691.
29. Nelson, P. E. 1981. Life cycle and epidemiology of *Fusarium oxysporum*. Pages 51-80 in: *Fusarium Wilt Diseases of Plants*. M. E. Mace, A. A. Bell, and C. H. Beckman, eds. Academic Press, New York.
30. Ninnemann, H., and Maier, J. 1996. Indications for the occurrence of nitric oxide synthases in fungi and plants and the involvement in photoconidiation of *Neurospora crassa*. *Photochem. Photobiol.* 64:393-398.
31. Nirenberg, H. I. 1990. Recent advances in the taxonomy of *Fusarium*. *Stud. Mycol.* 32:91-101.
32. Ohara, T., Inoue, I., Namiki, F., Kunoh, H., and Tsuge, T. 2004. *REN1* is required for development of microconidia and macroconidia, but not of chlamydospores, in the plant pathogenic fungus *Fusarium oxysporum*. *Genetics* 166:113-124.
33. Ohara, T., and Tsuge, T. 2004. *FoSTUA*, encoding a basic helix-loop-helix protein, differentially regulates development of three kinds of asexual spores, macroconidia, microconidia, and chlamydospore, in the fungal plant pathogen *Fusarium oxysporum*. *Eukaryot. Cell* 3:1412-1422.
34. Pain, A., Woodward, J., Quail, M. A., Anderson, M. J., Clark, R., Collins, M., Fosker, N., Fraser, A., Harris, D., Larke, N., Murphy, L., Humphray, S., O'Neil, S., Pertea, M., Price, C., Rabinowitsch, E., Rajandream, M. A., Salzberg, S., Saunders, D., Seeger, K., Sharp, S., Warren, T., Denning, D. W., Barrell, B., and Hall, N. 2004. Insight into the genome of *Aspergillus fumigatus*: Analysis of a 922 kb region encompassing the nitrate assimilation gene cluster. *Fungal Genet. Biol.* 41:443-453.
35. Rekah, Y., Shtienberg, D., and Katan, J. 2000. Disease development following infection of tomato and basil foliage by airborne conidia of the soilborne pathogens *Fusarium oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* f. sp. *basilici*. *Phytopathology* 90:1322-1329.
36. Rowe, R. C., Farely, J. D., and Coplin, D. C. 1977. Airborne spore dispersal and recolonization of steamed soil by *Fusarium oxysporum* in greenhouses. *Phytopathology* 67:1513-1517.
37. Saitoh, K., Togashi, K., Arie, T., and Teraoka, T. 2006. A simple method for a mini-preparation of fungal DNA. *J. Gen. Plant Pathol.* 72:348-350.
38. Sambrook, J., and Russell, D. W. 2001. *Molecular Cloning: A Laboratory Manual*. 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
39. Sanderson, K. E., and Srb, A. M. 1965. Heterokaryosis and parasexuality in the fungus *Ascochyta imperfecta*. *Am. J. Bot.* 42:72-81.
40. Schneider, D., and Schmidt, C. L. 2005. Multiple Rieske proteins in prokaryotes: Where and why? *Biochim. Biophys. Acta* 1710:1-12.
41. Springer, M. L. 1993. Genetic control of fungal differentiation: The three pathways of *Neurospora crassa*. *Bioessays* 15:365-374.
42. Wang, J., and Higgins, V. J. 2005. Nitric oxide has a regulatory effect in the germination of conidia of *Colletotrichum coccodes*. *Fungal Genet. Biol.* 42:284-292.
43. Wendehenne, D., Pugin, A., Klessing, D. F., and Durner, J. 2001. Nitric oxide: Comparative synthesis and signaling in animal and plant cells. *Trends Plant Sci.* 6:177-183.
44. Zumft, W. G. 1997. Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. R.* 61:533-616.