# *REN1* Is Required for Development of Microconidia and Macroconidia, but Not of Chlamydospores, in the Plant Pathogenic Fungus *Fusarium oxysporum*

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ABSTRACT

The filamentous fungus *Fusarium oxysporum* is a soil-borne facultative parasite that causes economically important losses in a wide variety of crops. *F. oxysporum* exhibits filamentous growth on agar media and undergoes asexual development producing three kinds of spores: microconidia, macroconidia, and chlamydospores. Ellipsoidal microconidia and falcate macroconidia are formed from phialides by basipetal division; globose chlamydospores with thick walls are formed acrogenously from hyphae or by the modification of hyphal cells. Here we describe *rensa*, a conidiation mutant of *F. oxysporum*, obtained by restriction-enzyme-mediated integration mutagenesis. Molecular analysis of *rensa* identified the affected gene, *REN1*, which encodes a protein with similarity to MedA of *Aspergillus nidulans* and Acr1 of *Magnaporthe grisea*. MedA and Acr1 are presumed transcription regulators involved in conidiogenesis in these fungi. The *rensa* mutant and *REN1*-targeted strains lack normal conidiophores and phialides and form rod-shaped, conidium-like cells directly from hyphae by acropetal division. These mutants, however, exhibit normal vegetative growth and chlamydospore formation. Nuclear localization of Ren1 was verified using strains expressing the Ren1-green fluorescent protein fusions. These data strongly suggest that *REN1* encodes a transcription regulator required for the correct differentiation of conidiogenesis cells for development of microconidia and macroconidia in *F. oxysporum*.



ASEXUAL sporulation is a common reproductive mode for a diverse group of agriculturally, industrially, and medically important fungi. Asexual spores of higher fungi are called conidia (Cole 1986). In many plant pathogenic fungi, conidia are the infectious propagules responsible for initiating infection as well as disease dissemination (Dahlberg and Van Etten 1982). Knowledge of the molecular mechanisms controlling conidiation in plant pathogenic fungi will contribute to the search for a target for disease control through reducing primary inocula and spread of the disease. The genetic and molecular mechanisms controlling conidiation have been addressed in detail for two experimental fungi, *Aspergillus nidulans* (Timberlake 1990; Adams *et al.* 1998) and *Neurospora crassa* (Springer 1993). However, the conidiation mechanisms remain largely undefined in plant pathogenic fungi.

*Fusarium oxysporum* is an economically important soil-borne pathogen with worldwide distribution (Armstrong and Armstrong 1981; Beckman 1987). This fungus causes vascular wilts in ~80 botanical species (Armstrong and Armstrong 1981; Beckman 1987). Individual pathogenic strains within the species have a limited host range, and strains with similar or identical host ranges are assigned to intraspecific groups, called formae speciales (Armstrong and Armstrong 1981; Beckman 1987).

*F. oxysporum* is classified in the subdivision Deuteromycotina (fungi imperfecti), because it lacks sexual reproduction. Fusarium species that have sexual stages are classified in Pyrenomycetes of the subdivision Ascomycotina. *F. oxysporum* is unique in its asexual reproduction (Nelson *et al.* 1983); it produces three kinds of asexual spores: microconidia, macroconidia, and chlamydospores (Figure 1). Microconidia are ellipsoidal and have no or one septum (Figure 1A). Macroconidia are falcate and have three or four septa (Figure 1B). Globose chlamydospores have thick walls (Figure 1C). Conidiogenesis of microconidia and macroconidia are phialidic (Nelson *et al.* 1983). Microconidia are formed from phialides in false heads by basipetal division, the developmental mode from the apex toward the base without catenation of cells (Figure 1A). Macroconidia with pronounced foot cells are generally produced from phialides on conidiophores by basipetal division (Figure 1B). Chlamydospores are formed acrogenously from hyphae or by the modification of hyphal cells (Figure 1C). These asexual spores play important roles in the disease cycle of *F. oxysporum*: microconidia and macroco-

Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank Data Libraries under accession nos. AB096070 (*REN1*) and AB113797 (REN1h-1).

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nidia are important as secondary inocula, and chlamydospores are endurance organs in soils and act as inocula (Rowe *et al.* 1977; Couteaudier and Alabouvette 1990; Katan *et al.* 1997; Rekah *et al.* 2000).

We previously described a mutant screen of *F. oxysporum* f. sp. *melonis* using restriction-enzyme-mediated integration (REMI) mutagenesis (Kuspa and Loomis 1992; Lu *et al.* 1994; Inoue *et al.* 2001). This pathogen causes vascular wilt of melon (Leach and Currence 1938). Strain Mel02010 was transformed with the plasmid pSH75, conferring resistance to hygromycin B (Kimura and Tsuge 1993) by the REMI method. We tested 2929 transformants for pathogenicity to melon and selected 43 mutants that showed reduction or complete loss of virulence on susceptible melon cultivars (Inoue *et al.* 2001). Molecular analysis of these mutants allowed us to identify two genes so far, *ARG1* and *FOW1*, essential for virulence of this pathogen (Namiki *et al.* 2001; Inoue *et al.* 2002).

Here we describe the isolation and characterization of *rensa*, a conidiation mutant of *F. oxysporum*. To isolate genes required for conidiation in this fungus, previously isolated REMI transformants (Inoue *et al.* 2001) were tested for conidiation, and the *rensa* mutant was selected. The affected gene, *REN1*, encodes a protein with similarity to MedA of *A. nidulans* (Clutterbuck 1969; Miller *et al.* 1993; Gems and Clutterbuck 1994; Busby *et al.* 1996) and Acr1 of *Magnaporthe grisea* (Lau and Hamer 1998; Nishimura *et al.* 2000). MedA and Acr1 have been identified as developmental regulators of conidiation in these fungi (Clutterbuck 1969; Miller *et al.* 1993; Gems and Clutterbuck 1994; Busby *et al.* 1996; Lau and Hamer 1998; Nishimura *et al.* 2000). A mutation at *REN1* results in a defect in development of microconidia and macroconidia and causes the production of rod-shaped, conidium-like cells in chains by head-to-tail arrays (acropetal division). The mutants, however, exhibit normal vegetative growth and form chlamydospores. Thus, it appears that *REN1* specifically controls the developmental pathway of microconidia and macroconidia in *F. oxysporum*.

## MATERIALS AND METHODS

**Fungal strains, plasmids, and genomic libraries:** Strain Mel02010 (JCM9288) of *F. oxysporum* f. sp. *melonis* (Namiki *et al.* 1994) was used for REMI transformation and identification of *REN1*. The *rensa* mutant (B50-19) was selected from the REMI transformants previously isolated (Inoue *et al.* 2001).

The integrative transformation vector pSH75 (Kimura and Tsuge 1993) was used for transformation of *F. oxysporum*. This vector contains the hygromycin B phosphotransferase gene (*hph*), which is fused to the *A. nidulans trpC* promoter and terminator (Gritz and Davies 1983; Mullaney *et al.* 1985; Kimura and Tsuge 1993).

The β-tubulin gene fragment was amplified from total DNA of Mel02010 by polymerase chain reaction (PCR) with primers C (5′-GAGGAATTCCCAGACCGTATGATG-3′) and D (5′-GCT GGATCCTATTCTTTGGGTCGAACAT-3′; Koenraadt *et al.* 1992). These primers were reported as degenerate primers for PCR amplification of the fungal β-tubulin genes (Koenraadt *et al.* 1992). PCR amplification from total DNA (100 ng) was performed with *Taq* DNA polymerase (Takara, Ohtsu, Japan) according to the manufacturer's instructions. The PCR product of ~0.4 kb was cloned in pGEM-T Easy vector (Promega, Madison, WI) to make pFOTUB1 and sequenced to confirm that it encodes part of β-tubulin. The pFOTUB1 insert was used as a probe for RNA gel blot analysis.

A genomic cosmid library of Mel02010 was constructed with a cosmid vector pMLF2 (An *et al.* 1996) using half-site fill-in reactions (Sambrook *et al.* 1989). Total DNA of Mel02010 was partially digested with *Sau*3AI to generate fragments of ~40 kb and partially filled with dATP and dGTP. The cosmid vector pMLF2 was completely digested with *Xho*I and partially filled with dCTP and dTTP. Partially filled *Sau*3AI genomic DNA fragments were cloned at the partially filled *Xho*I site of pMLF2 to construct a genomic library. Screening of the library by colony hybridization was conducted by the standard method (Sambrook *et al.* 1989).

**Fungal transformation:** Protoplast preparation and transformation of *F. oxysporum* were performed by the methods previously described (Inoue *et al.* 2001). Transformants carrying the *hph* gene were selected on regeneration media containing hygromycin B (Wako Pure Chemicals, Osaka, Japan) at 60 μg/ml (Inoue *et al.* 2001). Colonies that appeared 5–10 days after plating on the media were transferred to potato dextrose agar (PDA; Difco, Detroit) containing hygromycin B at 60 μg/ml, and transformants were selected after incubation at 25° for 5 days.

**Test for conidiation:** To induce conidiation of *F. oxysporum* strains, two media were used: carnation leaf agar (CLA; 1.5% agar with sterilized carnation leaf pieces; Fisher *et al.* 1982; Togawa 1992) and carboxymethyl cellulose liquid medium (CMC; 15 g carboxymethyl cellulose, 1 g yeast extract, 1 g $NH_4NO_3$, 1 g $KH_2PO_4$, and 0.5 g/liter $MgSO_4 \cdot 7H_2O$; Cappellini and Peterson 1965).

Strains were grown on PDA at 25° for 5 days. Agar blocks (3 mm in diameter) carrying mycelia were prepared from the resulting colonies and inoculated on CLA, 5 mm apart from carnation leaf pieces. After incubation at 25° for 5–10 days, under continuous BLB (black light blue) light (FL15BLB; Toshiba, Osaka, Japan) (Nirenberg 1990), conidiation was observed with a light microscope (BX50, Olympus, Tokyo). The number of chlamydospores formed in a 5-mm square between agar blocks and leaf pieces was counted with a microscope.

For cryo-scanning electron microscopic observation, strains were grown on CLA at 25° for 5 days, and agar blocks (~2 × 2 mm) containing fungal propagules were cut out from the media. They were attached onto specimen stubs, frozen in liquid nitrogen, and observed with a scanning electron microscope (S-4000, Hitachi, Ibaraki, Japan) after gold coating.

A PDA block (3 mm in diameter) carrying mycelia was inoculated in 50 ml of CMC in a 100-ml Erlenmeyer flask and incubated at 25° for 4 days on an orbital shaker (100 rpm) under continuous fluorescent light (Cappellini and Peterson 1965). The number of conidia was counted with a microscope. The resulting culture was passed through four layers of cheesecloth, and the filtrate was centrifuged at 1600 × *g* for 10 min to collect conidial cells. Cells were fixed in 0.4% *p*-formaldehyde in PME (50 mm PIPES, pH 6.7, 1 mm $MgSO_4$, 20 mm EGTA) for 45 min at room temperature (Borneman *et al.* 2000), washed three times, and mounted in PME containing fluostain I (25 μg/ml; Dojin, Kumamoto, Japan) and Hoechst 33258 (250 μg/ml; Wako Pure Chemicals) to visualize cell walls and nuclei, respectively. Stained cells were observed with a fluorescence microscope (BX50) using a U-MWU filter (Olympus).

For the time course study of conidial development, a PDA block (3 mm in diameter) carrying mycelia was inoculated in 25 ml of complete medium (CM; 10 g $KNO_3$, 5 g $KH_2PO_4$, 2.5 g $MgSO_4 \cdot 7H_2O$, 0.02 g $FeCl_3$, 10 g glucose, 1 g yeast extract, and 1 g/liter peptone; Sanderson and Srb 1965) in a 50-ml Erlenmeyer flask and incubated at 25° for 18 hr on an orbital shaker (100 rpm). The resulting mycelia were collected by centrifugation at 1600 × *g* for 10 min, inoculated in 50 ml of CMC in a 100-ml Erlenmeyer flask, and incubated at 25° for 132 hr on an orbital shaker (100 rpm) under continuous fluorescent light. During incubation, the number of conidia was counted with a microscope at 12-hr intervals. At 0, 12, 24, 36, 48, 60, 72, 96, and 120 hr after inoculation in CMC, fungal tissues were collected from five flasks by centrifugation at 1600 × *g* for 10 min and subjected to RNA isolation as described below.

**Test for vegetative growth and pathogenicity:** To test for vegetative growth of *F. oxysporum* strains, strains were grown on three agar media: PDA, complete medium agar (CMA; CM supplemented with 20 g/liter agar; Sanderson and Srb 1965), and minimal medium agar (MMA; CMA without yeast extract and peptone; Sanderson and Srb 1965). Strains were grown on PDA at 25° for 5 days. PDA blocks (3 mm in diameter) carrying mycelia were inoculated on PDA, MMA, and CMA. After incubation at 25° for 5 days, colony growth and morphology were observed.

Pathogenicity was tested by a root-dip method using the susceptible melon (*Cucumis melo* L.) cultivar Amus as previously described (Inoue *et al.* 2001). Strains were grown in CMC at 25° for 5 days on an orbital shaker (100 rpm) under continuous fluorescent light, and conidial cells were collected as described above. The cell pellet was suspended in sterilized water at $\sim 1 \times 10^7$ conidia/ml, and plant roots were dipped in the suspension for 15 sec. Plants were grown in pots filled with sterilized soil. Ten seedlings, which had a single true leaf, were used for inoculation of each strain, and disease symptoms were assessed 3 weeks after inoculation.

**Nucleic acid isolation and manipulations:** Isolation of total DNA from *F. oxysporum* and DNA gel blot hybridization were performed as previously described (Namiki *et al.* 1994). To isolate total RNA from *F. oxysporum*, strains were grown in 50 ml of CMC, minimal medium (MM; MMA without agar; Sanderson and Srb 1965), and CM (Sanderson and Srb 1965) in 100-ml Erlenmeyer flasks at 25° on an orbital shaker (100 rpm). Fungal tissues were collected by centrifugation at 1600 × *g* for 10 min and ground in liquid nitrogen in a mortar with a pestle. Total RNA was extracted from the powdered mycelia as previously described (Okuda *et al.* 1998). Poly(A)$^+$ RNA was isolated from total RNA with the PolyATtract mRNA isolation systems (Promega) according to the manufacturer's instructions. RNA gel blot hybridization was performed as previously described (Okuda *et al.* 1998).

For analysis of nucleotide sequences, DNA was cloned in pBluescript KS+ (Stratagene, La Jolla, CA) or pGEM-T Easy vector. 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 (Altschul *et al.* 1997). Alignment of nucleotide and amino acid sequences was made with the CLUSTAL W program (Thompson *et al.* 1994).

**Isolation of *REN1*:** Restriction mapping of the pSH75 insertion site in the *rensa* mutant B50-19 revealed the presence of a 7.8-kb *Hin*dIII fragment that contains pSH75 and the flanking genomic DNA (Figure 2). Total DNA of B50-19 was digested with *Hin*dIII, religated, and transformed to *Escherichia coli* DH5α. Ampicillin-resistant transformants were selected, and the rescued plasmid, pRB5019H, was isolated (Figure 2). A 0.4-kb *Bam*HI fragment from pRB5019H was cloned into the *Bam*HI site of pBluescript KS+ to make pBB50-19 (Figure 2). The pBB50-19 insert was used as a probe for screening a genomic library of Mel02010, and a positive clone pcB5019-1 was isolated (Figure 2). A 5.5-kb region containing *REN1* was sequenced as described above. The 1.2-kb *Eco*RV-*Pvu*II fragment internal to *REN1* was cloned into the *Eco*RV site of pBluescript KS+ to make pR1EP (Figure 2) and used as a probe for RNA gel blot analysis.

The *REN1* cDNA was isolated by reverse transcription-PCR (RT-PCR) using the RNA PCR kit Ver. 2.1 (Takara). The cDNA was amplified from total RNA (1 μg) of Mel02010 with primers Rf1 (5′-*ATG*TCTACTGTCAAGTTCCAGT-3′) and Rr1 (5′-*TCA*TGCTCTTGGTGCAGGTTCTGC-3′) according to the manufacturer's instructions (Figure 2). Rf1 and Rr1 contain the *REN1* initiation and termination codons (italics), respectively (Figure 2). RT-PCR products were cloned in pGEM-T Easy vector to determine the sequences.

**Construction of the *REN1-EGFP* fusion vectors:** The green fluorescent protein (GFP)-expression vectors pYTGFP-N and pYTGFP-C were used to make the *REN1-EGFP* fusion vectors. These vectors contain the *GFP* (*EGFP*) open reading frame (ORF), which is fused to the *A. nidulans trpC* promoter and terminator (Mullaney *et al.* 1985; Inoue *et al.* 2002). In pYTGFP-N, the last codon of the *GFP* ORF is fused to 15 nucleotides encoding a glycine linker followed by *Bam*HI, *Sma*I, *Pst*I, and *Eco*RI sites (Kimura *et al.* 2001; Inoue *et al.* 2002). In pYTGFP-C, the *trp*C promoter is followed by *Xba*I, *Bam*HI, *Sma*I, and *Pst*I sites and 15 nucleotides encoding a glycine linker fused to the initiation codon of the *GFP* ORF (Inoue *et al.* 2002). For a control, we used the plasmid pYTGFPc, which carries only *GFP* under the control of the *trpC* promoter and terminator (Inoue *et al.* 2002).

The *REN1* cDNA was amplified from poly(A)$^+$ RNA of Mel02010 by RT-PCR with primers REN1N-f (5′-GAGTGAT C*ATG*TCTACTGTCAAGTTCCAGT-3′) and REN1N-r (5′-TTT CTGCAG*TCA*TGCTCTTGGTGCAGGTTC-3′). REN1N-f has a *Bcl*I site (underlined) with the initiation codon (italics); REN1N-r has a *Pst*I site (underlined) with the termination codon (italics). The amplified DNA was digested with *Bcl*I and *Pst*I and cloned into the *Bam*HI-*Pst*I site of pYTEGFP-N to make pGFP-REN1, resulting in a N-terminal fusion of GFP to Ren1.

The *REN1* cDNA was amplified from poly(A)$^+$ RNA of Mel02010 by RT-PCR with primers REN1C-f (5′-GAGTCTA G*ATG*TCTACTGTCAAGTTCCAGT-3′) and REN1C-r (5′-CTC CTGCAGTGCTCTTGGTGCAGGTTCTGC-3′). REN1C-f contains a *Xba*I site (underlined) with the initiation codon (italics); REN1C-r contains a *Pst*I site (underlined) fused to the last codon of *REN1*. The amplified DNA was digested with *Xba*I and *Pst*I and cloned into the *Xba*I-*Pst*I site of pYTGFP-C to make pREN1-GFP, resulting in a C-terminal fusion of GFP to Ren1.

All the PCR products cloned in the vectors were sequenced to confirm the fact that no nucleotide substitution had occurred during amplification.

**Observation of intracellular localization of the GFP-tagged Ren1:** Transformants of the wild-type strain with pYTGFPc, pREN1-GFP, and pGFP-REN1 were grown in CMC and CM at 25° for 4 days. The resulting mycelia were observed under a fluorescence microscope (BX50) using U-MWIG filter (Olympus) for GFP fluorescence.

## RESULTS

**The *rensa* mutant:** REMI transformants previously isolated from the wild-type strain Mel02010 (Inoue *et al.*

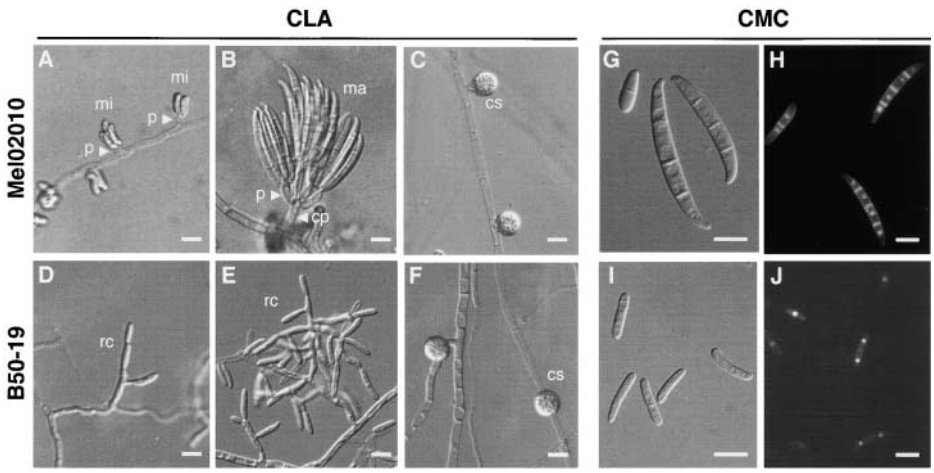



FIGURE 1.—Conidiation of the *rensa* mutant. (A–F) The wild-type strain Mel02010 and the *rensa* mutant B50-19 were grown on CLA at 25° for 5 days (A–D and F) or 10 days (E). Bars, 10 μm. mi, microconidium; p, phialide; ma, macroconidium; cp, conidiophore; cs, chlamydospore; rc, rod-shaped, conidium-like cell. (G–J) Mel02010 and B50-19 were grown in CMC at 25° for 4 days on an orbital shaker (at 100 rpm). The resulting cells were observed with a differential phase contrast microscope (G and I). The cells were stained with fluostain I and Hoechst 33258 to visualize cell walls and nuclei, respectively, and observed with a fluorescence microscope (H and J). Bars, 10 μm.

2001) were grown on the conidiation medium CLA. *F. oxysporum* produces microconidia, macroconidia, and chlamydospores on this medium (FISHER *et al.* 1982; TOGAWA 1992). Of 1792 REMI transformants tested, we identified one strain, B50-19, with a defect in development of microconidia and macroconidia. Although Mel02010 formed microconidia and macroconidia from phialides by basipetal division (Figure 1, A and B), B50-19 lacked normal conidiophores and phialides and formed rod-shaped, conidium-like cells directly from hyphae by acropetal division (Figure 1D). The rod-shaped cells had no or one septum, and their cell width was slenderer than that of microconidia and macroconidia of the wild type (Figure 1D). By prolonged incubation, B50-19 frequently formed rod-shaped cells in branching chains (Figure 1E). We called this mutation *rensa*, which means "catenation" in Japanese. The *rensa* mutant produced chlamydospores acrogenously from hyphae or by the modification of hyphal cells, as did the wild type (Figure 1, C and F). The number of chlamydospores formed on the media between inocula and carnation leaf pieces was not significantly different between Mel02010 and B50-19: $3.70 \pm 1.24$ chlamydospores/mm$^2$ and $3.11 \pm 0.31$ chlamydospores/mm$^2$, respectively, in the average of three cultures.

Fusarium species are known to produce microconidia and macroconidia when they are grown in CMC on an orbital shaker (CAPPELLINI and PETERSON 1965). The wild-type and *rensa* mutant strains were grown in CMC at 25° for 4 days. The wild type formed a number of microconidia and macroconidia (Figure 1G): $3.7 \times 10^6$ microconidia/ml and $1.8 \times 10^6$ macroconidia/ml in the averages of three cultures. The *rensa* mutant, however, formed a smaller number of rod-shaped, slender cells (Figure 1I): $0.4 \times 10^6$ cells/ml in the average of three cultures. The rod-shaped cells had no or one septum and were similar in size and morphology to conidium-like cells formed on CLA (Figure 1, D and I), suggesting that these cells are aberrant conidia.

Conidial cells were stained with fluostain I and Hoechst 33258 to visualize cell walls and nuclei, respectively. Microconidia and macroconidia from the wild type contain a single nucleus in each cell (Figure 1H). Rod-shaped cells from the mutant also contain a single nucleus in each cell (Figure 1J). This result suggests that the *rensa* mutation does not affect the coupling of cell and nuclear division during conidiation.

**The tagged locus in the *rensa* mutant:** The *rensa* mutant B50-19 was generated by REMI transformation of Mel02010 with the plasmid pSH75 in the presence of *Bam*HI. DNA gel blot analysis using the pSH75 probe demonstrated that this mutant had two copies of pSH75 as a tandem array (Figure 2). About half of one of the copies was deleted, and the *Bam*HI site in the junction of deleted plasmid and chromosomal DNA had been lost (Figure 2). The analysis identified a 7.8-kb *Hin*dIII fragment, which included the complete pSH75 and the flanking genomic DNA from one side of the inserted plasmid (Figure 2). The 7.8-kb *Hin*dIII fragment was recovered by plasmid rescue as pRB5019H (Figure 2).

The 0.4-kb *Bam*HI fragment (pBB50-19) from pRB5019H (Figure 2) was used as a probe to screen a cosmid genomic library of Mel02010. A positive clone, pcB5019-1, was isolated and partially restriction mapped (Figure 2). Restriction sites downstream of the tagged site in pRB5019H were identical to those of pcB5019-1 (Figure 2). However, restriction sites upstream of the tagged site were different (Figure 2), indicating that the plasmid integration had caused a deletion of genomic DNA in B50-19.

**A putative open reading frame at the tagged locus:** Sequencing of the 5.5-kb region in pcB5019-1 found a putative ORF1 from the tagged site in B50-19 (Figure 2). ORF1 consists of three exons (45, 187, and 1940 bp) divided by two introns (184 and 139 bp) and potentially encodes a 724-amino-acid protein. The introns were initially deduced on the basis of consensus sequences for 5′ and 3′ splice signals typical of fungal genes (BRU-

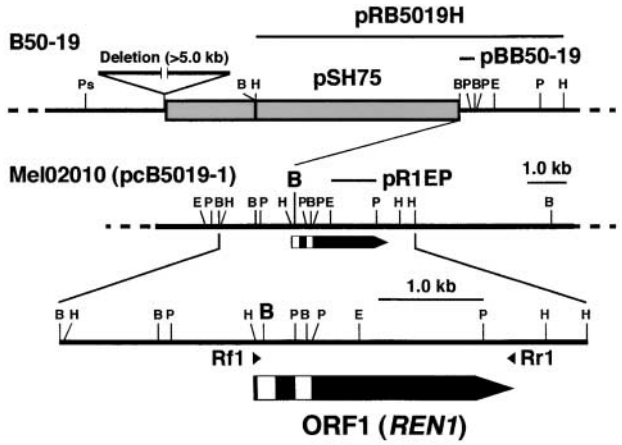



FIGURE 2.—The tagged locus in the *rensa* mutant B50-19. The 7.8-kb *Hin*dIII fragment was recovered from total DNA of B50-19 by plasmid rescue as pRB5019H. Restriction sites of the tagged locus in B50-19 were deduced by restriction mapping of pRB5019H and DNA gel blot analysis of B50-19 with the pSH75 probe. The pBB50-19 insert was used as a probe for screening a genomic cosmid library of the wild-type strain Mel02010, and the clone pcB5019-1 was isolated. The arrowed bar indicates ORF1 (*REN1*) with introns (open segments). The sequence analysis of pRB5019H and pcB5019-1 showed that the insertion of the transformation vector pSH75 in B50-19 had occurred at the *Bam*HI site in the first intron accompanied by deletion of the upstream region (>5.0 kb). The pR1EP insert was used as a probe for RNA gel blot analysis. Arrowheads above ORF1 denote orientation and location of oligonucleotide primers (Rf1 and Rr1) used in RT-PCR experiments. B, *Bam*HI; E, *Eco*RV; H, *Hin*dIII; P, *Pvu*II; Ps, *Pst*I.

CHEZ *et al.* 1993). To confirm the introns, the cDNA was prepared from total RNA of Mel02010 by RT-PCR with primers Rf1 and Rr1 (Figure 2). The RT-PCR amplification produced a 2.2-kb fragment of DNA. Comparison of the genomic sequence with that of the RT-PCR product confirmed that the two introns are spliced. The sequence analysis of pRB5019H and pcB5019-1 showed that the insertion of the transformation vector pSH75 in B50-19 had occurred at the *Bam*HI site in the first intron accompanied by deletion of the upstream region (Figure 2). Preliminary analysis found that >5.0 kb genomic DNA was deleted in B50-19.

The deduced amino acid sequence encoded by ORF1 reveals similarity to those of MedA of *A. nidulans* (GenBank accession no. AF080599), Acr1 of *M. grisea* (GenBank accession no. AB096705), and EAA33615 of *N. crassa* (GenBank accession no. AABX01000175): 28.6, 32.9, and 40.8% identical to MedA, Acr1, and EAA33615, respectively (Figure 3). MedA and Acr1 are presumed transcription regulators involved in the conidial development in these fungi (MILLER *et al.* 1993; BUSBY *et al.* 1996; LAU and HAMER 1998; NISHIMURA *et al.* 2000). Although EAA33615 was estimated as a hypothetical protein of *N. crassa*, its function has not been identified (GALAGAN *et al.* 2003). The similarity was most evident in amino acids 395–554 in the *F. oxysporum* ORF1 (Figure 3). The BLAST searches with available genome sequence databases of filamentous fungi and yeasts found no other proteins containing this conserved region. These data suggest that this region is a highly conserved, unique motif in a novel class of presumed transcription regulators involved in conidial development of filamentous fungi. LAU and HAMER (1998) identified a glutamine-rich region in Acr1 (amino acids 281–330 in Figure 3), which shares similarity to several transcription factors including GBF of *Dictyostelium discoideum* (SCHNITZLER *et al.* 1994) and SIN3 of *Saccharomyces cerevisiae* (WANG *et al.* 1990). However, the ORF1 product, MedA, and EAA33615 lack such regions (Figure 3). The ORF1 product also shows weak similarity to proline- and hydroxyproline-rich proteins from higher plants, which constitute cell wall architecture (STIEFEL *et al.* 1988; CAELLES *et al.* 1992).

***REN1* is essential for conidiation:** We identified ORF1, which encodes a protein homologous to MedA and Acr1, in the tagged site in the *rensa* mutant. However, it appeared that the plasmid integration had been accompanied with deletion of chromosomal DNA in the mutant. To assess whether mutation of ORF1 is responsible for the *rensa* phenotype, homologous recombination was employed to replace ORF1 with the plasmid pGDR1, which contains the *hph* gene flanked by the 5′ and 3′ sequences from ORF1 (Figure 4A). The 0.5-kb *Bam*HI-*Eco*RV fragment and the 1.2-kb *Cla*I-*Hin*dIII fragment containing the 5′ and 3′ regions of ORF1, respectively, were cloned into the transformation vector pSH75 to make the ORF1-targeting vector pGDR1 (Figure 4A).

The wild-type strain Mel02010 was transformed with pGDR1, and 42 transformants were isolated. Each transformant was tested for conidiation on CLA. All the transformants normally formed chlamydospores. Of 42 transformants, 28 produced microconidia and macroconidia, as did the wild type. However, the remaining 14 transformants exhibited the *rensa* phenotype: they produced rod-shaped, slender cells by acropetal division.

The integration mode of pGDR1 in four transformants (DR1–DR4) showing the *rensa* phenotype was analyzed by DNA gel blot analysis. As controls, four transformants (WT1–WT4) showing normal conidiation were also used. Total DNA of Mel02010 and transformants was digested with *Bam*HI, and the blot was probed with the 1.2-kb *Cla*I-*Hin*dIII fragment integrated in pGDR1 (Figure 4A). The restriction map of pcB5019-1 predicts that the probe would hybridize to a 5.8-kb *Bam*HI fragment in the wild type (Figure 4A); however, four bands of ~2.5, 5.8, 7.0, and 23.0 kb hybridized (Figure 4B). The 5.8-kb signal was intense, and the others were weak. Thus, the wild type has the ORF1 homologs. All WT transformants contained these four bands and additional bands, resulting from ectopic integration of pGDR1 (Figure 4B). All DR transformants, however, lacked 5.8-kb bands but had 8.8-kb bands, as expected when the targeting vector

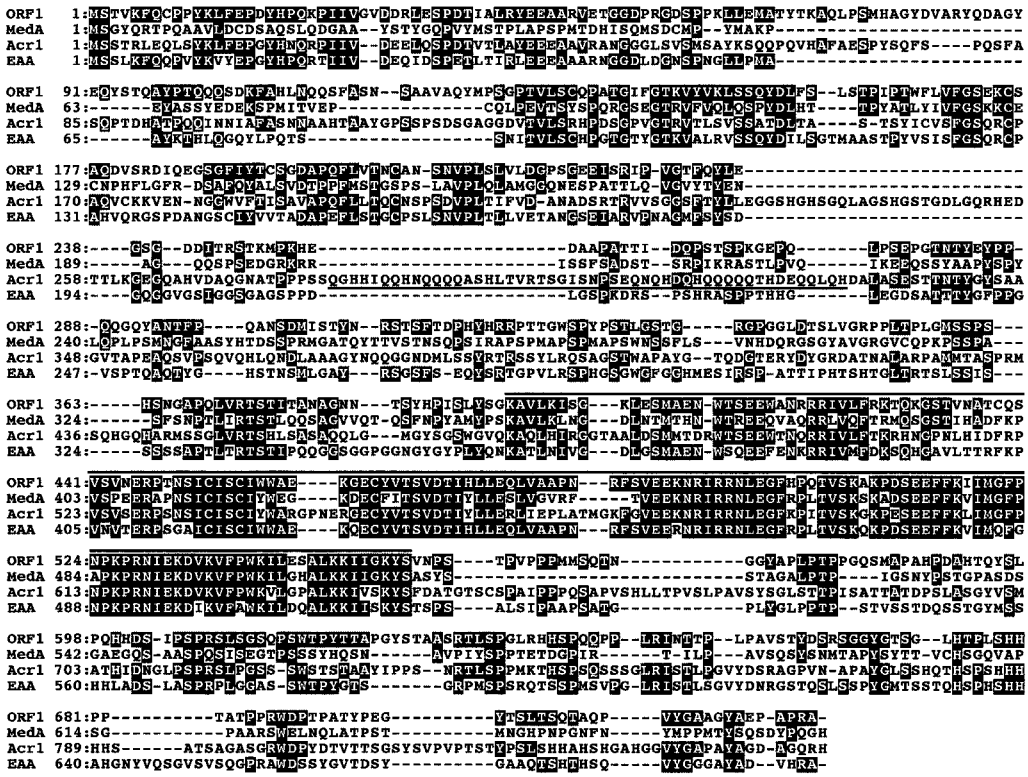


FIGURE 3.—Similarity of the ORF1-encoded protein to other proteins. Amino acid sequence encoded by ORF1 was aligned with those of MedA of *A. nidulans* (GenBank accession no. AF-080599), Acr1 of *M. grisea* (GenBank accession no. AB-096705), and EAA33615 (EAA) of *N. crassa* (GenBank accession no. AABX01000 175). Amino acids that are conserved between the ORF1 product and any of the others are indicated as white letters on a black background. The most conserved region among four sequences is indicated by a line above the sequences (amino acids 395–554 of ORF1). The glutamine-rich region in Acr1 is underlined (amino acids 281–330 of Acr1).

pGDR1 was integrated into chromosomal DNA by homologous recombination (Figure 4, A and B). DR transformants preserved 2.5-, 7.0-, and 23.0-kb bands (Figure 4B). These results clearly showed that the *rensa* phenotype of DR transformants resulted from disruption of ORF1. Thus, the gene encoding ORF1 was designated *REN1*.

We observed conidiogenesis of the REMI mutant B50-19 and the *REN1*-targeted mutant DR1 with a cryo-scanning electron microscope (Figure 4C). These mutants lacked normal conidiophores and phialides and formed rod-shaped, catenated cells directly from hyphae (Figure 4C). Formation pattern and morphology of rod-shaped cells were similar in B50-19 and DR1 (Figure 4C). Although the first cells from hyphae looked like conidiophores, they were slenderer than conidiophores of the wild type.

**Expression of *REN1*:** Expression of *REN1* was determined by RNA gel blot analysis. Mel02010 was grown in CMC, CM, and MM liquid media at 25° for 4 days on an orbital shaker. Mel02010 formed microconidia and macroconidia in CMC, but not in CM and MM. Poly(A)$^+$ RNA was prepared from the cultures. The RNA gel blot was probed with pR1EP containing the *REN1* fragment (Figure 2). Unexpectedly, the probe hybridized to three bands of ~2.6, 3.4, and 4.8 kb in RNA from fungal tissues grown in CMC (Figure 5A). The 3.4-kb signal was intense, and the others were weak. This result indicates that the *REN1* region is transcribed in a complex manner. These three bands were also detected in RNA from mycelia grown in CM and MM (Figure 5A). Thus, *F. oxysporum* expresses *REN1* during both conidiation and vegetative growth.

Expression of *REN1* in the *REN1*-targeted mutants was determined by RNA gel blot analysis. Poly(A)$^+$ RNA was prepared from fungal tissue of Mel02010, B50-19, and DR1 grown in CMC at 25° for 4 days. The RNA gel blot was probed with pR1EP (Figure 2). The probe hybridized to three bands of ~2.6, 3.4, and 4.8 kb in Mel02010, but to no bands in B50-19 and DR1 (Figure 5B). This result indicates that these three bands are all related to transcripts from the *REN1* locus.

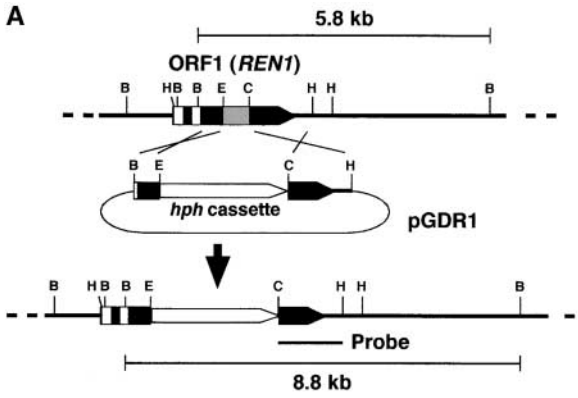
The timing of conidial development and *REN1* expression was observed in CMC. Mel02010 and DR1 were grown in CM at 25° for 18 hr. The resulting mycelia were inoculated in CMC and incubated at 25° for 132 hr. In Mel02010, microconidia and macroconidia first appeared at 12 and 48 hr, respectively, after inoculation in CMC, and their numbers apparently increased to 120 hr (Figure 6A). Poly(A)$^+$ RNA was prepared from fungal tissue, and the gel blot was probed with pR1EP (Figure 2). The probe weakly hybridized to only a 3.4-kb band in RNA of inoculated mycelia grown in CM for 18 hr (Figure 6B). After inoculation of the mycelia in CMC, two RNA bands of ~3.4 and 4.8 kb were detected at 12–60 hr, and three RNA bands of ~2.6, 3.4, and 4.8 kb were detected at 72–120 hr (Figure 6B). This result suggests that at least 3.4- and 4.8-kb transcripts from *REN1* are essential for development of microconidia and macroconidia.

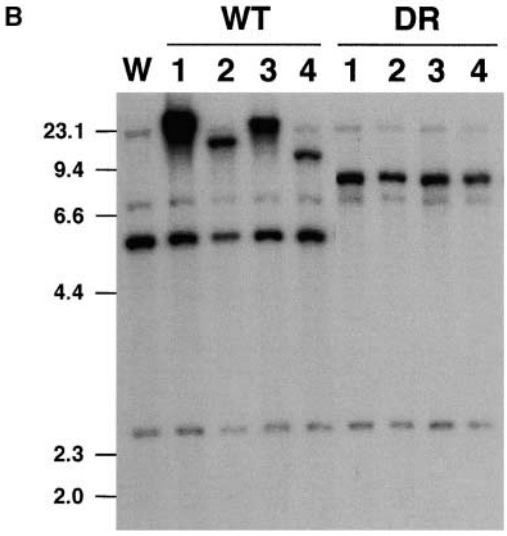
In DR1, conidium-like cells first occurred at 60 hr after inoculation in CMC, and the number gradually

increased to 108 hr (Figure 6A). Thus, it appears that conidiation of the *REN1* mutant is delayed.

**Vegetative growth and pathogenicity of the *REN1*-targeted mutants:** Vegetative growth of the wild-type and *REN1*-targeted strains was evaluated by measuring colony diameters grown on PDA, CMA, and MMA at 25° for 5 days. Colony diameters of the REMI mutant B50-19 and the *REN1*-targeted mutants (DR1–DR3) were not significantly different from those of the wild type (Figure 7A). To the unaided eye there was no apparent difference in colony morphology between the wild-type and mutant strains (Figure 7B). These results indicate that *REN1* is dispensable for vegetative growth.

The *REN1*-targeted mutants were tested for pathogenicity to melon plants. The wild-type and mutant strains were grown in CMC, and the resulting conidial cells were used as inocula. Ten seedlings of susceptible cultivar Amus with a single true leaf were inoculated with cell suspension of each strain by the root-dip method (Inoue *et al.* 2001), and disease symptoms were observed 3 weeks after inoculation. The wild-type and mutant strains caused typical wilt symptoms on all seedlings (Figure 7C). Thus, it appears that the rod-shaped cells of the *REN1*-targeted mutants preserve the abilities to



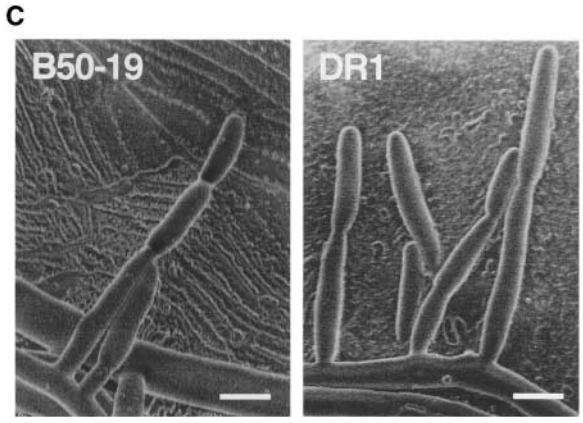




Figure 4.—Transformation-mediated targeting of ORF1. (A) Structure of ORF1 before and after homologous integration of the ORF1-targeting vector pGDR1. The targeting vector pGDR1 contains *hph* flanked by the 5′ sequence of 0.5 kb and the 3′ sequence of 1.2 kb from ORF1 in pSH75. B, *Bam*HI; C, *Cla*I; E, *Eco*RV; H, *Hin*dIII. (B) DNA gel blot analysis of pGDR1 transformants. Total DNA (2 μg/lane) from the wild-type strain and transformants was digested with *Bam*HI and fractionated in a 0.8% agarose gel. The blot was probed with the 1.2-kb *Cla*I-*Hin*dIII fragment from pGDR1. Sizes (in kilobases) of marker DNA fragments (*Hin*dIII-digested λDNA) are indicated on the left. W, wild-type strain Mel02010; WT1–WT4, transformants showing normal conidiation; DR1–DR4, transformants showing *rensa* phenotype. (C) Scanning electron micrograph of conidium-like cells of the REMI mutant B50-19 and the *REN1*-targeted mutant DR1. Strains were grown on CLA at 25° for 5 days and observed with a scanning electron microscope. Bars, 10 μm.

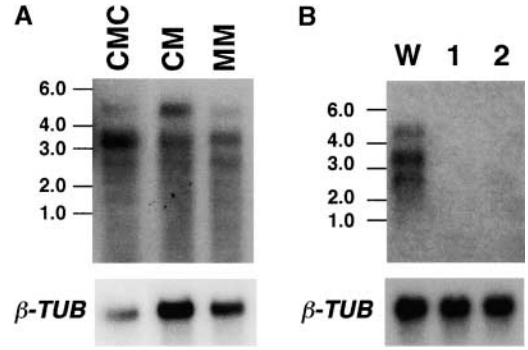



Figure 5.—RNA gel blot analysis of *REN1*. The wild-type strain Mel02010 was grown in CMC, CM, and MM at 25° for 4 days (A). The wild-type strain (W), the REMI mutant B50-19 (lane 1), and the *REN1*-targeted mutant DR1 (lane 2) were grown in CMC at 25° for 4 days (B). Poly(A)$^+$ RNA was prepared from the cultures. RNA (5 μg/lane) was electrophoresed in a 1.5% agarose gel containing 2.2 M formaldehyde. The blots were probed with pR1EP containing the *REN1* fragment (Figure 2). Sizes (in kilobases) of marker RNA fragments (Novagen, Madison, WI) are indicated on the left. The blots were also probed with pFOTUB1 containing the β-tubulin gene fragment (β-*TUB*).

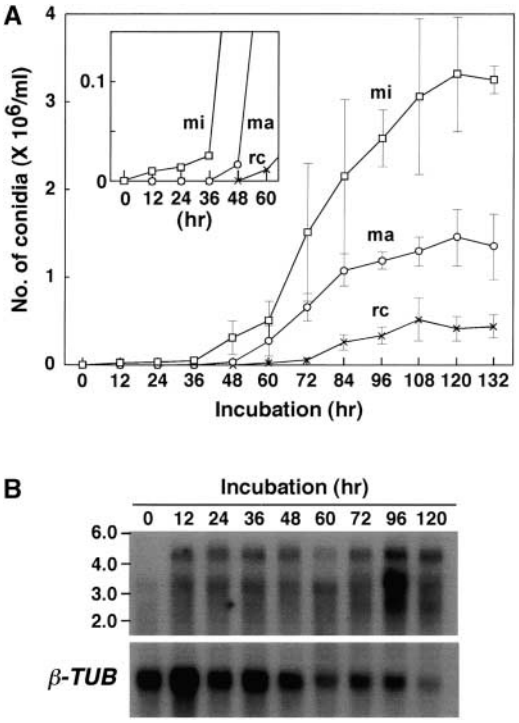



FIGURE 6.—Time course of conidial development of the wild-type strain Mel02010 and the *REN1*-targeted mutant DR1. (A) Mycelia of each strain grown in CM was inoculated in CMC and incubated at 25° for 132 hr. Numbers of microconidia (mi) and macroconidia (ma) of Mel02010 and of rod-shaped, conidium-like cells (rc) of DR1 were counted at 12-hr intervals with a microscope, and data represent means ±SD of five replications. (B) Fungal tissue of Mel02010 grown for indicated periods was collected, and poly(A)$^+$ RNA was prepared from the tissue. RNA (5 μg/lane) was electrophoresed in a 1.5% agarose gel containing 2.2 M formaldehyde. The blots were probed with pR1EP containing the *REN1* fragment (Figure 2). Sizes (in kilobases) of marker RNA fragments (Novagen) are indicated on the left. The blot was also probed with pFOTUB1 containing the β-tubulin gene fragment (β-*TUB*).

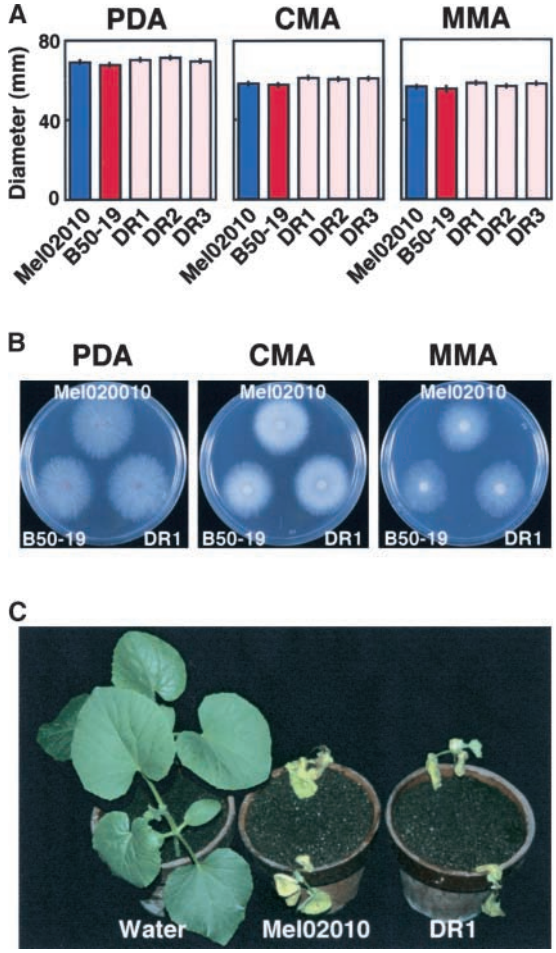



FIGURE 7.—Vegetative growth and pathogenicity of *REN1* mutants. (A and B) Colony diameter (A) and morphology (B) of *REN1* mutants. The wild-type strain Mel02010, the REMI mutant B50-19, and the *REN1*-targeted mutants (DR1–DR3) were grown on PDA, CMA, and MMA at 25° for 5 days. Colony diameter was measured, and data represent means ±SD of three replications. (C) Pathogenicity of *REN1* mutant. Mel02010 and DR1 were grown in CMC at 25° for 5 days, and the resulting conidial cells were collected. Seedlings of the cultivar Amus with a single true leaf were inoculated with cell suspension (~$1 \times 10^7$ conidia/ml) of each strain by the root-dip method. Control plants were immersed in water. Photograph was taken 3 weeks after inoculation.

infect host plant tissue and to cause disease symptoms under the conditions tested.

**Intracellular localization of the GFP-tagged Ren1:** The predicted amino acid sequence of Ren1 is significantly similar to those of MedA of *A. nidulans* and Acr1 of *M. grisea* (Figure 3). Although *medA* and *ACR1* have been suggested to encode transcription regulators (MILLER *et al.* 1993; BUSBY *et al.* 1996; LAU and HAMER 1998; NISHIMURA *et al.* 2000), their nuclear localization has not been verified. Ren1 also shows weak similarity to proline- and hydroxyproline-rich proteins, cell wall proteins of higher plants. To test intracellular localization of Ren1, we made strains expressing Ren1-GFP and GFP-Ren1 fusion proteins and observed intracellular distribution of the GFP fluorescence in the strains under fluorescence microscopy. We constructed the *REN1-GFP* and *GFP-REN1* gene fusions under the control of the *A. nidulans trpC* promoter (MULLANEY *et al.* 1985) as pREN1-GFP and pGFP-REN1, respectively. These constructs were introduced into Mel02010 by cotransformation with the plasmid pSH75 conferring hygromycin B resistance. For a control, Mel02010 was transformed with the plasmid pYTGFPc, which carries *GFP* under the control of the *trpC* promoter.

Transformants were grown in CMC and CM, and their mycelia were observed under fluorescence microscopy. Of 10 pYTGFPc transformants, 7 expressed GFP. In these transformants, GFP fluorescence did not localize in any cell components throughout mycelia grown in CMC and CM (Figure 8). Of 13 pREN1-GFP transformants, 9 emitted GFP fluorescence; of 14 pGFP-REN1 transformants, 3 emitted GFP fluorescence. In all the transformants expressing Ren1-GFP and GFP-Ren1 fusions,

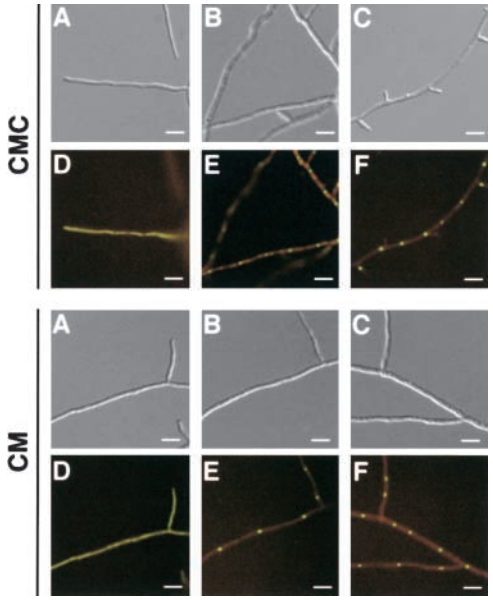



FIGURE 8.—Intracellular localization of the GFP-tagged Ren1. Transformants with pYTGFPc (A and D), pREN1-GFP (B and E), and pGFP-REN1 (C and F) were grown in CMC and CM at 25° for 4 days. (A–C) Differential interference contrast images. (D–F) GFP fluorescence images. Bars, 20 μm.

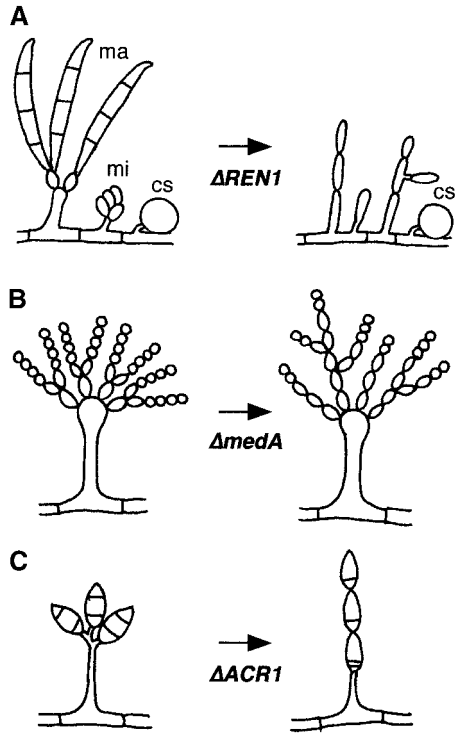



FIGURE 9.—Schematic of conidiogenesis of *REN1*, *medA*, and *ACR1* mutants of *F. oxysporum* (A), *A. nidulans* (B), and *M. grisea* (C), respectively. ma, macroconidium; mi, microconidium; cs, chlamydospore.

GFP fluorescence was targeted in nuclei in mycelial cells grown in CMC and CM (Figure 8), suggesting that Ren1 localizes in nuclei and acts as a transcription regulator.

**The *REN1* homologs:** DNA gel blot analysis showed the presence of the *REN1* homologs in the wild-type strain Mel02010. As shown in Figure 4B, the 5.8-kb *Bam*HI fragment contains *REN1*, and the 2.5-, 7.0-, and 23.0-kb fragments contain the homologs. To isolate the homologs, a cosmid genomic library of Mel02010 was screened with a probe of the 1.2-kb *Cla*I-*Hin*dIII fragment used in Figure 4B. Positive clones were digested with *Bam*HI, and pcREN1h-1, pcREN1h-2, and pcREN1h-3, which contained the 2.5-, 7.0-, and 23.0-kb *Bam*HI fragments, respectively, were isolated. The *REN1* homolog (REN1h-1) in pcREN1h-1 was sequenced. Comparison of sequences of *REN1* and REN1h-1 detected a highly conserved region of ~0.6 kb (91.4% identity) within the third exon of *REN1*. However, REN1h-1 has a termination codon in this region. Their 5′ and 3′ regions flanking to the conserved region have lower identity (~50%), and REN1h-1 contains many termination codons in these regions (data not shown). Partial sequencing of the *REN1* homologs in pcREN1h-2 and pcREN1h-3 revealed that the homologs show strong similarity to REN1h-1 and also have many termination codons (data not shown). These results strongly suggest that the *REN1* homologs are pseudogenes.

## DISCUSSION

Molecular analysis of the REMI mutant B50-19 of the plant pathogenic fungus *F. oxysporum* identified the *REN1* gene, essential for development of microconidia and macroconidia. In *F. oxysporum*, microconidia are formed from phialides in false heads by basipetal division; macroconidia are formed from phialides on conidiophores by basipetal division. The *REN1* mutants lack normal conidiophores and phialides and form rod-shaped, slender cells directly from hyphae by acropetal division. Thus, *REN1* is required for the correct differentiation of conidiophores and phialides. The mutants produce rod-shaped cells in a single chain at the early stage of development and frequently form the cells in branching chains at the later stage of development. Since the wild-type strain never forms microconidia and macroconidia in a chain, cell division switches from acropetal to basipetal mode at the phialide stage. *REN1* also may be essential for the switch from acropetal to basipetal division during conidiogenesis.

The *REN1* mutants form normal chlamydospores. The number of chlamydospores of the mutants was also similar to that of the wild type. These results indicate that *REN1* is not required for chlamydospore formation. Chlamydospores are formed acrogenously from hyphae or by the modification of hyphal cells. Thus, it appears that the developmental pathway of chlamydospores is genetically independent from those of microconidia and macroconidia.

Ren1 shows significant similarity to MedA of *A. nidulans* and Acr1 of *M. grisea*. Modes of conidiogenesis are

highly complex in conidial ascomycete fungi, and at least 20 modes of conidial ontogeny have been described (Cole 1986). Conidial ontogeny in *F. oxysporum* and *A. nidulans* is phialidic, and that in *M. grisea* is sympodial (Figure 9). In *A. nidulans*, the conidiophore vesicle bears two types of sterigmata (metulae and phialides), and chains of conidia are produced from phialides (Figure 9B). A mutation at *medA* results in aberrant conidiophores with branching chains of metulae, delayed conidial differentiation, and frequent reinitiation of the secondary conidiophore (Figure 9B; Clutterbuck 1969; Gems and Clutterbuck 1994; Busby *et al.* 1996). In *M. grisea*, conidiogenesis involves the production of three-celled conidia, borne sympodially on an aerial conidiophore (Figure 9C). A mutation at *ACR1* results in the production of head-to-tail (acropetal) arrays of conidia (Figure 9C; Lau and Hamer 1998; Nishimura *et al.* 2000). These observations demonstrate that mutations at *REN1*, *medA*, and *ACR1* in these fungi result in a similar phenotype, in which conidiogenesis cells are reiterated in chains (Figure 9).

Although MedA and Acr1 have been suggested to be transcription regulators involved in conidiation of these fungi (Clutterbuck 1969; Miller *et al.* 1993; Gems and Clutterbuck 1994; Busby *et al.* 1996; Lau and Hamer 1998; Nishimura *et al.* 2000), their nuclear localization has not been verified. We verified nuclear import of Ren1 by using strains expressing the GFP-tagged Ren1. Both the Ren1-GFP and GFP-Ren1 fusion proteins were targeted to nuclei. The fact that the nuclear localization of Ren1 is not affected by either N-terminal or C-terminal fusions of GFP suggests that Ren1 has the nuclear import signal in the internal region. The structural similarity among Ren1, MedA, and Acr1 and the nuclear localization of Ren1 strongly suggest that these proteins represent members of a novel class of conserved regulators controlling conidial pattern formation. Recently, the genome sequence of *F. graminearum* has become available on the website of the Whitehead Institute (http://www-genome.wi.mit.edu/annotation/fungi/fusarium/). We searched the *REN1* homolog of *F. graminearum* on the website and found a putative gene, which has the exon/intron organization similar to *REN1* and encodes a protein strongly similar to Ren1.

In *A. nidulans*, five transcription regulator genes (*brlA*, *abaA*, *wetA*, *medA*, and *stuA*) have been identified as controlling conidial development (Clutterbuck 1969; Boylan *et al.* 1987; Adams and Timberlake 1988; Mirabito *et al.* 1989; Miller *et al.* 1992; Busby *et al.* 1996; Adams *et al.* 1998). BrlA, AbaA, and WetA comprise the core pathway required for the transition from vegetative hyphae to conidia (Boylan *et al.* 1987; Sewall *et al.* 1990; Marshall and Timberlake 1991; Adams *et al.* 1998). BrlA is required to initiate conidiation (Han *et al.* 1993; Prade and Timberlake 1993). AbaA is required for phialide differentiation, and WetA is essential for maturation of conidia (Sewall *et al.* 1990; Marshall and Timberlake 1991). Some target genes have been identified for BrlA and AbaA (Aramayo and Timberlake 1993; Chang and Timberlake 1993; Andrianopoulos and Timberlake 1994).

StuA and MedA represent components of a developmental modifier pathway required for correct cellular differentiation (Clutterbuck 1969; Miller *et al.* 1992; Busby *et al.* 1996; Wu and Miller 1997). StuA modulates expression of the core regulatory genes *brlA* and *abaA* (Miller *et al.* 1992). MedA represses premature expression of *brlA* during early development and downregulates *brlA* during later stages (Busby *et al.* 1996). MedA is also required as a coactivator of the *abaA* expression (Busby *et al.* 1996). Conidiation of the *medA* mutant is not completely blocked, but is delayed (Clutterbuck 1969; Gems and Clutterbuck 1994). However, a mutation at *REN1* in *F. oxysporum* leads to a lack of normal conidiophores and phialides and to production of aberrant conidia. The aberrant conidia also branch, unlike normal conidiogenesis cells and conidia. We propose that *REN1* comprises the core pathway for the development of microconidia and macroconidia in *F. oxysporum*. Identification of the target genes of Ren1 is needed to assess the precise function of Ren1 in conidial development of *F. oxysporum*.

The *REN1* locus was found to have at least three mRNAs of ~2.6, 3.4, and 4.8 kb. When vegetative mycelia grown in CM were inoculated in CMC to induce conidiation, microconidia and macroconidia occurred from 12 and 48 hr, respectively, after inoculation, and their numbers increased to 120 hr. During incubation in CMC, two mRNA of ~3.4 and 4.8 kb accumulated in fungal tissue at the early stage (12–60 hr), and an additional ~2.6-kb mRNA also accumulated at the later stage (72–120 hr). Thus, at least 3.4- and 4.8-kb mRNAs seem to be necessary for development of microconidia and macroconidia. These three mRNAs were also detected in mycelia grown in the nonconidiation media CM and MM for 96 hr, although *REN1* is dispensable for vegetative growth. The Ren1-GFP fusion proteins localized in nuclei in mycelial cells grown in CMC and also in CM. Thus, the *REN1* expression and nuclear localization of Ren1 are essential, but not enough, for conidial development.

The *A. nidulans medA* locus has two transcription start sites that give rise to two mRNAs (Miller *et al.* 1993). Both mRNAs have long reader sequences that contain multiple mini-ORFs, although the roles of two transcripts and mini-ORFs have not been characterized (Miller *et al.* 1993). Although transcription start sites of three mRNAs of *REN1* have not been identified, three mini-ORFs (225, 210, and 270 bp) were found within the 1.5-kb region upstream of the *REN1* ORF (data not shown). These mini-ORFs are probably not exons of *REN1*, because we could not find introns with consensus sequences for 5′ and 3′ splice signals to join mini-ORFs and *REN1* ORF as a single ORF. The *brlA* and *stuA* loci

in *A. nidulans* also have two overlapping transcription units (*brlA*α and *brlA*β in *brlA* and *stuA*α and *stuA*β in *stuA*), which are different in transcription start sites (Miller *et al.* 1992; Han *et al.* 1993; Prade and Timberlake 1993; Wu and Miller 1997). Both transcription units in *brlA* and *stuA* are essential for normal development of conidia (Miller *et al.* 1992; Han *et al.* 1993; Prade and Timberlake 1993; Wu and Miller 1997). Complexity in transcriptional and translational regulation is probably a common feature in the developmentally regulatory genes for conidiation in these fungi.

The *REN1* mutants of *F. oxysporum* could cause wilt symptoms in susceptible melon plants, as did the wild type, under the conditions tested. In contrast, a mutation at *ACR1* in *M. grisea* results in a drastic reduction of virulence in rice plants (Lau and Hamer 1998). The lifestyles and infection mechanisms of the soil-borne pathogen *F. oxysporum* and the foliar pathogen *M. grisea* are highly divergent. *F. oxysporum* infects host plants in a manner typical of soil-borne pathogens: conidia germinate in response to root exudates, produce penetration hyphae that attach to root surface and penetrate it directly, and grow invasively in host plant tissue (Rodríguez-Gálvez and Mendgen 1995). Conversely, conidia of *M. grisea* attach to the leaf, germinate, and differentiate dome-shaped, melanized appressoria, able to generate sufficient turgor pressure to penetrate the plant cuticlar surface (Talbot 1995). Reduced virulence phenotype of the *ACR1* mutants is due mainly to inability to form appressoria (Lau and Hamer 1998). A variety of conidiation mutants of *M. grisea* also fail to produce normal appressoria, resulting in nonpathogenic or reduced virulent phenotypes (Hamer *et al.* 1989; Shi and Leung 1995; Shi *et al.* 1998). These observations demonstrate that conidiation and appressorium formation in *M. grisea* may involve an overlapping set of gene products (Hamer *et al.* 1989; Lau and Hamer 1998; Shi *et al.* 1998). *F. oxysporum* directly penetrate root surface without formation of typical appressoria (Rodríguez-Gálvez and Mendgen 1995). Thus, Ren1 probably is not essential for penetration of root surface or for colonization in the plant tissue.

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