FoSTUA, Encoding a Basic Helix-Loop-Helix Protein, Differentially Regulates Development of Three Kinds of Asexual Spores, Macroconidia, Microconidia, and Chlamydospores, in the Fungal Plant Pathogen *Fusarium oxysporum*

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The soil-borne fungus *Fusarium oxysporum* causes vascular wilt of a wide variety of plant species. *F. oxysporum* produces three kinds of asexual spores, macroconidia, microconidia, and chlamydospores. Falcate macroconidia are formed generally from terminal phialides on conidiophores and rarely from intercalary phialides on hyphae. Ellipsoidal microconidia are formed from intercalary phialides on hyphae. Globose chlamydospores with thick walls are developed by the modification of hyphal and conidial cells. Here we describe *FoSTUA* of *F. oxysporum*, which differentially regulates the development of macroconidia, microconidia, and chlamydospores. *FoSTUA* encodes a basic helix-loop-helix protein with similarity to *Aspergillus nidulans* StuA, which has been identified as a transcriptional regulator controlling conidiation. Nuclear localization of FoStuA was verified by using strains expressing FoStuA-green fluorescent protein fusions. The *FoSTUA*-targeted mutants exhibited normal microconidium formation in cultures. However, the mutants lacked conidiophores and produced macroconidia at low frequencies only from intercalary phialides. Thus, *FoSTUA* appears to be necessary to induce conidiophore differentiation. In contrast, chlamydospore formation was dramatically promoted in the mutants. These data demonstrate that FoStuA is a positive regulator and a negative regulator for the development of macroconidia and chlamydospores, respectively, and is dispensable for microconidium formation in cultures. The disease-causing ability of *F. oxysporum* was not affected by mutations in *FoSTUA*. However, the mutants produced markedly fewer macroconidia and microconidia in infected plants than the wild type. These results suggest that *FoSTUA* also has an important role for microconidium formation specifically in infected plants.

Asexual sporulation is a common reproductive mode for fungi. Asexual spores of higher fungi are generally called conidia (16). In many plant-pathogenic fungi, asexual spores are the infectious propagules responsible for initiating infection as well as disease dissemination (18). Knowledge of the molecular mechanisms controlling asexual sporulation in plant-pathogenic fungi will contribute to the search for a target for disease control through reducing primary inocula and spread of disease. However, the mechanisms of asexual sporulation remain largely undefined in plant-pathogenic fungi.

The vascular wilt fungus *Fusarium oxysporum* is an economically important soil-borne pathogen with a worldwide distribution (7, 9). This species includes intraspecific variants, called formae speciales, which cause vascular wilt in ~80 botanical species (7, 9).

F. oxysporum is classified in the subdivision Deuteromycotina (Fungi Imperfecti) because it lacks sexual reproduction. *Fusarium* species that have sexual stages are classified in the subdivision Ascomycotina. *F. oxysporum* is unique in its asexual reproduction: it produces three kinds of asexual spores, macroconidia, microconidia, and chlamydospores (39, 40) (Fig. 1).

Macroconidia are falcate and have three or four septa (Fig. 1A and B). Microconidia are ellipsoidal and have no septa or one septum (Fig. 1C). Globose chlamydospores have thick walls (Fig. 1D). Conidiogenesis of macroconidia and microconidia is phialidic (39, 40). Macroconidia are produced most often from terminal phialides that arise from conidiophores (Fig. 1A) and at low frequencies from intercalary phialides that arise directly from hyphae (Fig. 1B). Microconidia are produced from intercalary phialides in false heads (Fig. 1C). Chlamydospores are generally developed through the modification of hyphal and conidial cells through the condensation of their contents (Fig. 1D). These asexual spores play important roles in the disease cycle: macroconidia and microconidia are produced on the stem surfaces of infected plants and serve as secondary inocula to spread the fungus to neighboring host plants, and chlamydospores are endurance organs in soil and act as primary inocula when suitable host plants are planted in soil (17, 26, 39, 44, 45).

A mutant screen of *F. oxysporum* f. sp. *melonis*, which causes vascular wilt of melon (31), was previously described; this screen was based on restriction enzyme-mediated integration mutagenesis (25, 42). A *rensa* mutant was isolated by screening conidiation mutants; "*rensa*" means catenation in Japanese (42). The affected gene, named *REN1*, which encodes a nuclear protein with similarity to MedA of *Aspergillus nidulans* and Acr1 of *Magnaporthe grisea* (42), was identified. MedA and Acr1 have been reported to act as developmental regulators of

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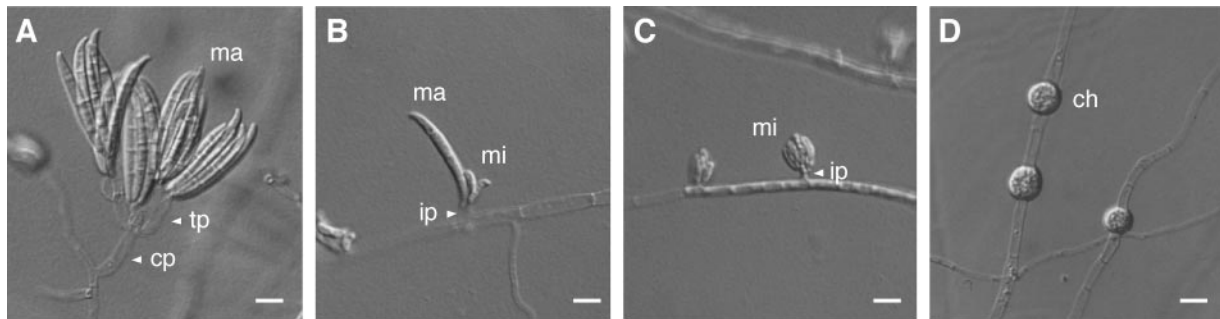
FIG. 1. Conidiation of *F. oxysporum*. Strain Mel02010 was grown on SNA-paper at 25°C for 5 days. (A) Macroconidia (ma) are produced generally from terminal phialides (tp) on conidiophores (cp). (B) Macroconidia are also produced rarely from intercalary phialides (ip) on hyphae. mi, microconidia. (C) Microconidia are produced from intercalary phialides generally in false heads. (D) Chlamydospores (ch) are formed from hyphae. Bars, 10 μm.

conidiation in these fungi (13, 15, 21, 30). Targeted mutation of *REN1* results in the defective development of macroconidia and microconidia, and the mutants form rod-shaped, conidium-like cells directly from hyphae by acropetal division (42). However, the mutants exhibit normal growth and chlamydospore formation (42). Thus, *REN1* is specifically required for the development of macroconidia and microconidia (42).

In *A. nidulans*, at least four regulatory genes (*brlA*, *abaA*, *stuA*, and *medA*) have been reported to control conidial development (1, 5, 12, 13, 15, 32–34). BrlA and AbaA comprise the core pathway required for the transition from vegetative hyphae to conidia, and *brlA* and *abaA* mutant strains are aconidial (1, 5, 12, 49). StuA and MedA are developmental modifiers required for correct cellular differentiation (13, 15, 19, 32, 33, 57); however, *stuA* and *medA* mutant strains produce conidia with normal viability (13, 15, 33).

The *stuA* gene encodes a basic helix-loop-helix (bHLH) transcriptional regulator (19). In *A. nidulans*, conidia are formed through the differentiation of a series of specialized cells: a conidiophore develops as an aerial hyphal branch that swells at its tip to form a globose vesicle, metulae bud from the vesicle, and then phialides bud from the metulae (2, 55). Conidia are formed through interstitial budding of the phialides in chains (2, 55). *stuA* mutants have extremely shortened conidiophores that lack metulae and phialides and produce conidia directly from conidiophore vesicles at low frequencies (15, 33). StuA has been reported to affect conidiation through the spatial and temporal regulation of *brlA* and *abaA* expression (19, 33). *stuA* mutants also show the *stunted* phenotype, resulting from shortened aerial hyphae, and are defective in sexual reproduction (15).

In other fungi, several proteins have been reported to contain segments highly similar to the bHLH region of StuA and have been termed APSES proteins (Asm-1, Phd1, StuA, Efg1, and Sok2) (6, 22, 53, 56). In *Neurospora crassa*, Asm-1 is involved in several processes, including spore germination, vegetative growth, and sexual sporulation (6). Phd1 and Sok2 of *Saccharomyces cerevisiae* and Efg1 of *Candida albicans* regulate pseudohyphal growth and morphology (22, 50, 53, 56).

Here we report the cloning and functional analysis of *FoSTUA*, the *F. oxysporum* homologue of *stuA*. FoStuA contains a segment highly similar to the bHLH motif of APSES proteins and localizes in nuclei. Targeted mutation of *FoSTUA*

resulted in a lack of conidiophores, from which macroconidia are differentiated, and in increased chlamydospore formation. The mutants produced microconidia normally in cultures but not in infected plants. Thus, it appears that FoStuA differentially controls the developmental pathways for macroconidia, microconidia, and chlamydospores in *F. oxysporum*.

MATERIALS AND METHODS

Fungal strains, plasmids, and genomic library. Strain Mel02010 (JCM9288) of *F. oxysporum* f. sp. *melonis* (38) was used in this study. Mel02010 and its transformants were maintained routinely on potato dextrose agar (PDA) (Difco).

The integrative transformation vectors pSH75 (28) and pII99 (37) were used for the transformation of *F. oxysporum*. These vectors carry *hph* (23) and *nptII* (8), respectively, fused to the *A. nidulans trpC* promoter and terminator (36).

A genomic cosmid library of Mel02010 constructed in cosmid vector pMLF2 (4) was described previously (42). Screening of the library by colony hybridization was conducted by the standard method (46).

Fungal transformation. Protoplast preparation and transformation of *F. oxysporum* were performed as previously described (24, 25). Transformants carrying *hph* or *nptII* were selected on regeneration medium containing hygromycin B (Wako Pure Chemicals) at 60 μg/ml or Geneticin (Gibco) at 180 μg/ml, respectively (24, 25).

Test for conidiation. To induce conidiation in *F. oxysporum* strains, two media were used: synthetic low-nutrient agar medium (SNA), containing (in weight/volume) 0.1% KH_2PO_4, 0.1% KNO_3, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.02% glucose, 0.02% sucrose, and 2% agar (41), and liquid carboxymethyl cellulose medium (CMC), containing (in weight/volume) 1.5% carboxymethyl cellulose, 0.1% yeast extract, 0.1% NH_4NO_3, 0.1% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ (14). Sterilized filter paper (1 by 2 cm) was placed on the SNA surface before the inoculation of fungal strains (41).

Strains were grown on PDA at 25°C for 5 days. Agar blocks (3 mm in diameter) carrying mycelia were prepared from the resulting colonies and inoculated onto SNA 5 mm away from the filter paper pieces. After incubation at 25°C for 5 days under continuous black blue light (Toshiba FL15BLB), conidiation was observed with a light microscope (Olympus BX50). The number of chlamydospores formed in a 5-mm square between the agar block and the filter paper was counted.

A PDA block carrying mycelium was inoculated into 50 ml of CMC in a 100-ml Erlenmeyer flask and incubated at 25°C for 4 days on an orbital shaker (100 rpm) under continuous fluorescent light (14). The numbers of macroconidia and microconidia were counted with a microscope. Conidia were fixed in 0.4% *p*-formaldehyde and stained with Fluostain I (25 μg/ml; Dojin) and Hoechst 33258 (250 μg/ml; Wako) to visualize cell walls and nuclei, respectively, as previously described (42). Stained cells were observed with a BX50 fluorescence microscope and a U-MWU filter (Olympus).

For the time course study of conidial development, a PDA block carrying mycelium was inoculated into 25 ml of liquid complete medium (CM) (47) in a 50-ml Erlenmeyer flask and incubated at 25°C for 18 h on an orbital shaker (100 rpm). The resulting mycelium was collected by centrifugation at 1,600 × *g* for 10 min, inoculated into 50 ml of CMC in a 100-ml Erlenmeyer flask, and incubated

A

B
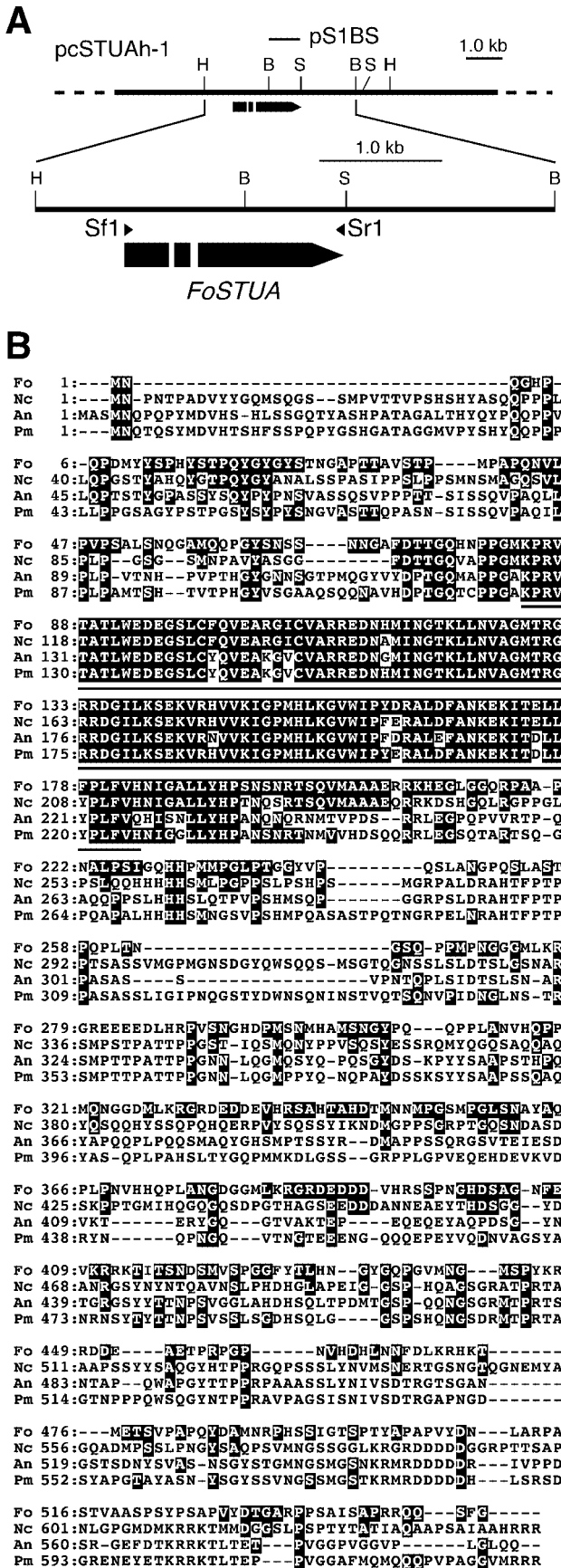
as described above. During incubation, the numbers of macroconidia and microconidia were counted with a microscope at 12-h intervals from 0 to 132 h. At 0, 12, 24, 36, 48, 60, 72, 96, and 120 h after inoculation into CMC, fungal tissues were collected from five flasks by centrifugation and subjected to RNA isolation as described below.

Test for vegetative growth and pathogenicity. To test for the vegetative growth of *F. oxysporum* strains, the strains were grown on three agar media: PDA, complete medium agar (CMA) (47), and minimal medium agar (MMA) (47). PDA blocks (3 mm in diameter) carrying mycelia were inoculated onto PDA, MMA, and CMA. After incubation at 25°C for 5 days, colony growth and morphology were observed.

Pathogenicity was tested by a root dip method with susceptible melon (*Cucumis melo* L.) cultivar Amus and conidial suspensions (~10^7 conidia/ml in sterilized water) as previously described (25). Disease symptoms were assessed 3 weeks after inoculation.

Nucleic acid analysis. Isolation of total DNA from *F. oxysporum* and DNA gel blot hybridization were performed as previously described (25, 38). Isolation of total RNA from *F. oxysporum*, preparation of poly(A)$^+$ RNA, and RNA gel blot hybridization were performed as previously described (42, 43).

For analysis of nucleotide sequences, DNA was cloned in pBluescript KS(+) (Stratagene) or pGEM-T Easy (Promega). DNA sequences were determined by using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and an automated DNA sequencer (model 373A; Applied Biosystems). DNA sequences were analyzed with BLAST (3). Nucleotide and amino acid sequences were aligned with the CLUSTAL W program (54).

Isolation of *FoSTUA*. The *FoSTUA* fragment was amplified from total DNA of strain Mel02010 by PCR with the primers stuA-1f (AATGGCATGATCAACGGCAC) and stuA-1r (AGATGCATCGGGCCAATCTT) and with *Taq* DNA polymerase (Takara). These primers were designed on the basis of the conserved bHLH-encoding regions of *A. nidulans stuA* (19, 33) and *N. crassa asm-1* (6). The PCR products were cloned in vector pGEM-T Easy. Several clones were sequenced, and one clone was found to contain a PCR product with the expected size of ~130 bp and to encode a peptide with strong similarity to corresponding regions of APSES proteins. This PCR product was used as a probe for screening of a genomic cosmid library of Mel02010, and a positive clone, named pcSTUAh-1, was isolated (Fig. 2A). A 6.0-kb region containing the *stuA* homologue was sequenced, and a putative open reading frame (ORF) for *FoSTUA* was identified.

FoSTUA cDNA was isolated by reverse transcription (RT)-PCR by using an RNA PCR kit, version 2.1 (Takara). The cDNA was amplified from total RNA (1 μg) of strain Mel02010 with the primers Sf1 (*ATG*AACCAAGGCCATCCCCA) and Sr1 (*CTA*ACCGAAAGATTGTTGTC) (Fig. 2A) according to the manufacturer's instructions; Sf1 and Sr1 contain the *FoSTUA* initiation and termination codons (italic type), respectively. The RT-PCR products were cloned in vector pGEM-T Easy to determine the sequences.

Construction of *FoSTUA*-GFP gene fusion vectors. The green fluorescent protein (GFP) expression vectors pYTGFP-N and pYTGFP-C were used to make *FoSTUA*-GFP gene fusion vectors. These vectors contain the GFP (enhanced GFP [EGFP]) ORF fused to the *A. nidulans trpC* promoter and terminator (24, 27, 36). Vectors pYTGFP-N and pYTGFP-C were made for N-terminal and C-terminal fusions of GFP, respectively, to target proteins (24). As a control, we used plasmid pYTGFPc, which carries only the GFP ORF under the control of the *trpC* promoter and terminator (24).

FoSTUA cDNA was amplified from poly(A)$^+$ RNA of strain Mel02010 by

FIG. 2. *F. oxysporum FoSTUA* gene. (A) Map of the *FoSTUA* locus. The bars pointing to the right indicate *FoSTUA* with introns (white segments). The 0.8-kb BglII-SalI fragment of *FoSTUA* was cloned in pBluescript KS(+) to make pS1BS, which was used as a probe for RNA gel blot analysis. Arrowheads above *FoSTUA* denote the orientations and locations of the oligonucleotide primers (Sf1 and Sr1) used in RT-PCR experiments. B, BglII; H, HindIII; S, SalI. (B) Similarity of FoStuA to other proteins. The amino acid sequence of FoStuA (Fo) was aligned with those of Asm-1 of *N. crassa* (Nc; Genbank accession no. CAD70882), StuA of *A. nidulans* (An; accession no. AAA33325), and StuA of *P. marneffei* (Pm; accession no. AAM27919). Amino acids that are conserved between FoStuA and any of the others are indicated as white letters on a black background. The most conserved bHLH motifs among the four sequences are underlined (amino acids 84 to 184 of FoStuA).

RT-PCR with the primers FoSTUA1N-f (AAA<u>GGATCC</u>TG*ATG*AACCAAG GCCATCCCC) and FoSTUAN-r (CCC<u>CTGCAG</u>*CTA*ACCGAAAGATTGTT GTCT); FoSTUAN-f has a BamHI site (underlined) with the initiation codon (italic type), and FoSTUAN-r has a PstI site (underlined) with the termination codon (italic type). The amplified DNA was digested with BamHI and PstI and cloned into the BamHI-PstI site of pYTGFP-N to make pGFP-FoSTUA, resulting in an N-terminal fusion of GFP to FoStuA.

FoSTUA cDNA was also prepared from poly(A)$^+$ RNA of strain Mel02010 by RT-PCR with the primers FoSTUAC-f (ACA<u>TCTAGA</u>TGAACCAAGGCCA TCCCCAGC) and FoSTUAC-r (CTC<u>CTGCAG</u>TGCTCTTGGTGCAGGTTC TGC); FoSTUAC-f contains an XbaI site (underlined) with the initiation codon (italic type), and FoSTUAC-r contains a PstI site (underlined) fused to the last codon of *FoSTUA*. The amplified DNA was digested with XbaI and PstI and cloned into the XbaI-PstI site of pYTGFP-C to make pFoSTUA-GFP, resulting in a C-terminal fusion of GFP to FoStuA.

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

Nucleotide sequence accession number. The *FoSTUA* sequence has been deposited in the DDBJ/EMBL/GenBank databases under accession number AB180746.

RESULTS

Isolation of *FoSTUA*. To isolate the APSES gene homologue from *F. oxysporum*, part of the homologue was amplified from DNA of strain Mel02010 by PCR. The PCR product was used as a probe to screen a cosmid genomic library of Mel02010. A positive clone, pcSTUAh-1, was isolated and partially restriction mapped (Fig. 2A). Sequencing of a 6.0-kb region in pcFoSTUA-1 revealed a putative ORF encoding a protein with similarity to APSES proteins.

cDNA was prepared from total RNA of strain Mel02010 by RT-PCR with primers Sf1 and Sr1 (Fig. 2A). RT-PCR amplification produced a 1.7-kb fragment of DNA. Comparison of the genomic sequence with that of the RT-PCR product indicated that the gene has two introns (52 and 65 bp) and three exons (340, 133, and 1177 bp) and potentially encodes a 550-amino-acid protein.

A BLAST database search revealed that the deduced amino acid sequence has significant similarity to those of APSES proteins, such as *N. crassa* Asm-1 (6), *Penicillium marneffei* StuA (11), and *A. nidulans* StuA (33)—32.1, 28.1, and 27.1% identical, respectively (Fig. 2B). Thus, the gene containing the ORF of interest was named *FoSTUA*. The similarity was most evident in amino acids 84 to 184 of *F. oxysporum*, which correspond to the bHLH region (6, 11, 19, 33) (Fig. 2B).

Expression of *FoSTUA*. *Fusarium* species are known to produce conidia when they are grown in CMC (14). Ohara et al. previously observed that *F. oxysporum* also produces macroconidia and microconidia in CMC but not in CM or liquid minimal medium (MM) (42).

The timing of conidial development and *FoSTUA* expression was observed in CMC. Strain Mel02010 was grown in CM at 25°C for 18 h. The resulting mycelia were inoculated into CMC and incubated at 25°C on an orbital shaker. Microconidia and macroconidia first appeared at 24 and 48 h, respectively, after inoculation into CMC, and their numbers increased up to 120 h (Fig. 3A). Poly(A)$^+$ RNA was prepared from fungal tissues, and the gel blot was probed with pS1BS containing the *FoSTUA* fragment (Fig. 2A). The probe hybridized to 2.7-kb bands in RNAs from inoculated mycelia which had been grown in CM and from fungal tissues grown in CMC with similar signal intensities (Fig. 3B). This result suggested that *FoSTUA*
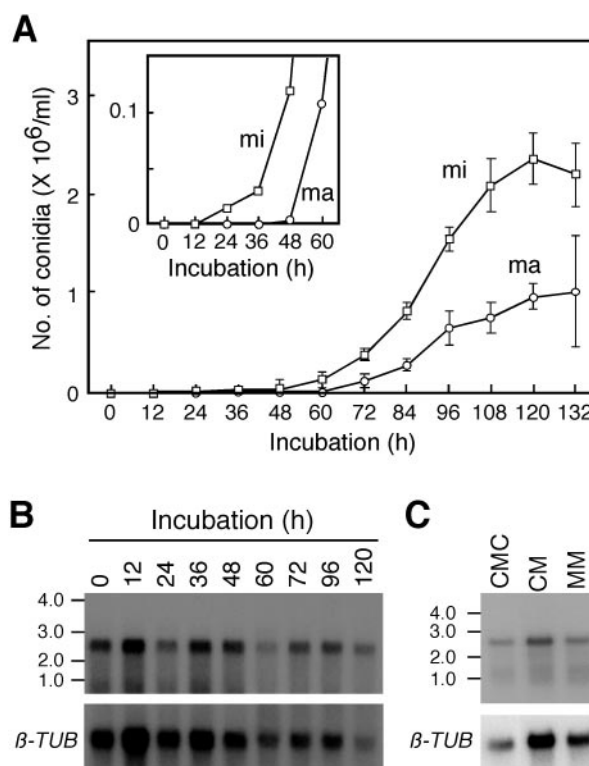

FIG. 3. Time course of conidial development and *FoSTUA* expression. (A) Time course of conidial development. Mycelia of strain Mel02010 grown in CM were inoculated into CMC and incubated at 25°C for 132 h. The numbers of macroconidia (ma) and microconidia (mi) were counted at 12-h intervals with a microscope; data represent the means and standard deviations of five replications. The inset shows the time course of conidial development during the initial 60 h of incubation on an expanded scale. (B) Time course of *FoSTUA* expression during conidiation. Fungal tissues of strain Mel02010 grown for the indicated periods were collected, and poly(A)$^+$ RNA was prepared from the tissues. Poly(A)$^+$ RNA (~5 μg/lane) was electrophoresed in a 1.5% agarose gel containing 2.2 M formaldehyde. The blot was probed with pS1BS containing the *FoSTUA* fragment (Fig. 2A). 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*) (42). (C) *FoSTUA* expression during conidiation and vegetative growth. Strain Mel02010 was grown in CMC, CM, and MM at 25°C for 4 days, and poly(A)$^+$ RNA was prepared from the cultures. The RNA gel blot was probed with pS1BS and pFOTUB1.

is constitutively expressed during conidiation as well as during vegetative growth.

To verify the expression of *FoSTUA* during vegetative growth, strain Mel02010 was grown in CM and MM, in which conidiation is not induced, at 25°C for 4 days, and poly(A)$^+$ RNA was prepared from the cultures. The RNA gel blot was probed with pS1BS (Fig. 2A). The probe hybridized to 2.7-kb bands in RNAs from fungal tissues grown in CMC, CM, and MM (Fig. 3C). Thus, *FoSTUA* is expressed by *F. oxysporum* not only during conidiation but also during vegetative growth.

Intracellular localization of GFP-tagged FoStuA. FoStuA contains a bHLH structure, and its amino acid sequence is significantly similar to those of APSES proteins (Fig. 2B), which have been considered to be transcriptional regulators (6, 11, 19, 22, 53, 56). To test the intracellular localization of
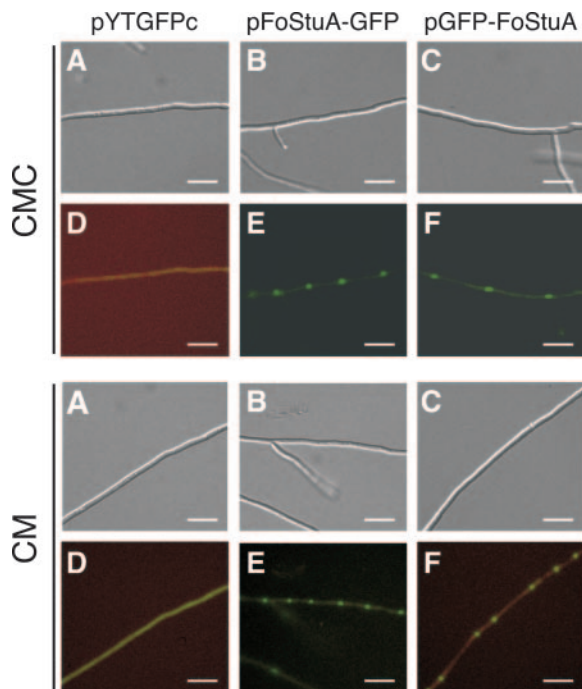
FIG. 4. Intracellular localization of GFP-tagged FoStuA. Transformants with pYTGFPc (A and D), pFoStuA-GFP (B and E), and pGFP-FoStuA (C and F) were grown in CMC and CM at 25°C for 4 days. The resulting mycelia were observed with a fluorescence microscope and a U-MWIG filter (Olympus) for GFP fluorescence. Bars, 20 μm. (A to C) Differential interference contrast images. (D to F) GFP fluorescence images.

FoStuA, we made strains expressing FoStuA-GFP and GFP-FoStuA fusions and observed the intracellular distribution of the GFP fluorescence in the strains by fluorescence microscopy. We constructed *FoSTUA*-GFP and GFP-*FoSTUA* gene fusions under the control of the *A. nidulans trpC* promoter as pFoStuA-GFP and pGFP-FoStuA, respectively. These constructs were introduced into strain Mel02010 by cotransformation with plasmid pSH75, conferring hygromycin B resistance. As a control, Mel02010 was transformed with plasmid pYTGFPc, which carries the GFP gene under the control of the *trpC* promoter.

Transformants were grown in CMC and CM, and their mycelia were observed with a fluorescence microscope. 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 (Fig. 4). Of 10 pFoStuA-GFP transformants, 6 emitted GFP fluorescence; of 13 pGFP-FoStuA transformants, 6 emitted GFP fluorescence. In all of the transformants expressing FoStuA-GFP or GFP-FoStuA fusions, GFP fluorescence was targeted to nuclei in mycelial cells grown in CMC and CM (Fig. 4), suggesting that FoStuA localizes in nuclei. These results were consistent with the prediction that FoStuA acts as a transcriptional regulator.

Targeted deletion of *FoSTUA*. To determine the function of *FoSTUA* in developmental processes in *F. oxysporum*, homologous recombination was used to replace *FoSTUA* with plasmid pGDS1, which contains the *nptII* cassette flanked by 5′ and 3′ sequences from *FoSTUA* (Fig. 5A). Strain Mel02010 was transformed with pGDS1, and 36 transformants were isolated. Transformants were grown on SNA-paper. All of the transformants produced macroconidia, microconidia, and chlamydospores, which were similar in morphology to those of the wild type (Fig. 1). However, 29 transformants were distinguishable from the wild type in the quantities of macroconidia and chlamydospores produced: the numbers of macroconidia and chlamydospores were markedly decreased and increased, respectively, in these transformants. Microscopic observation showed that these transformants completely lacked conidiophores and formed macroconidia only from intercalary phialides that arose directly from hyphae.

The mode of integration of pGDS1 in four transformants (DS1 to DS4) lacking conidiophores was analyzed by DNA gel blot analysis. As controls, four transformants (CT1 to CT4) showing wild-type conidiation were also used. Total DNAs of strain Mel02010 and transformants were digested with EcoRV, and the blot was probed with the 0.8-kb BglII-SalI fragment integrated in pGDS1 (Fig. 5A). The probe hybridized to ~3.6-kb EcoRV fragments in Mel02010 and the CT transformants (Fig. 5B). However, all of the DS transformants lacked 3.6-kb bands (Fig. 5B). DS1, DS3, and DS4 had ~6.3-kb bands resulting from homologous recombination, and DS2 had an ~6.4-kb band (Fig. 5B). The homologous integration of pGDS1 in DS2 was probably accompanied by a deletion of the flanking region.

The expression of *FoSTUA* in the *FoSTUA* deletion (Δ*FoSTUA*) strains was determined by RNA gel blot analysis. Poly(A)$^+$ RNAs were prepared from fungal tissues of strains Mel02010, DS1, DS2, DS3, and CT1 grown in CMC. The RNA gel blot was probed with pS1BS (Fig. 2A). The probe hybridized to 2.7-kb bands in Mel02010 and CT1 but not to any bands in DS1, DS2, and DS3 (Fig. 5C). DNA and RNA gel blot analyses clearly showed that targeted mutation of *FoSTUA* caused a deficiency in conidiophore development and promoted chlamydospore formation in DS transformants.

Macroconidia, microconidia, and chlamydospores of Δ*FoSTUA* mutants. To further analyze the role of *FoSTUA* in the conidiation of *F. oxysporum*, strain Mel02010, Δ*FoSTUA* mutants (DS1 to DS3), and an ectopic transformant (CT1) were grown in CMC, and their macroconidia and microconidia were qualitatively and quantitatively observed. The Δ*FoSTUA* mutants produced macroconidia and microconidia which were not different in morphology from those of Mel02010 and CT1 (Fig. 6A). Conidia were stained with Fluostain I and Hoechst 33258 to visualize cell walls and nuclei, respectively. Macroconidia and microconidia from Mel02010 and the mutants contained a single nucleus in each cell (Fig. 6A), suggesting that mutation of *FoSTUA* does not affect the coupling of cell division and nuclear division during conidiation.

The formation of macroconidia and the formation of microconidia were quantitatively compared between wild-type and mutant strains. The numbers of macroconidia were clearly reduced in the Δ*FoSTUA* mutants (Fig. 6B). Observation of fungal tissues grown in CMC showed that strains Mel02010 and CT1 produced many conidiophores. The mutants, however, produced no conidiophore-like structures (data not shown), suggesting that the mutants produced macroconidia only from intercalary phialides on hyphae. In contrast, the
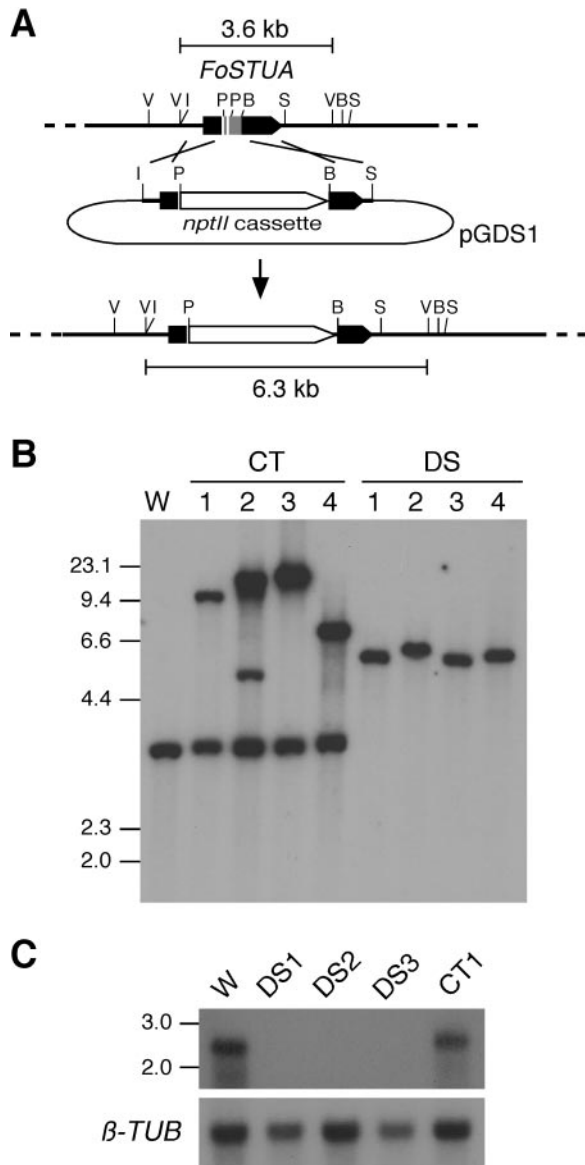
FIG. 5. Transformation-mediated targeting of *FoSTUA*. (A) Structure of the *FoSTUA* locus before and after homologous integration of the *FoSTUA*-targeting vector pGDS1. To make pGDS1, a 0.9-kb EcoRI-PvuII fragment and a 0.8-kb BglII-SalI fragment containing the 5′ and 3′ regions of *FoSTUA*, respectively, were cloned into transformation vector pII99. B, BglII; I, EcoRI; V, EcoRV; P, PvuII; S, SalI. (B) DNA gel blot analysis of pGDS1 transformants. Total DNA (~2 μg/lane) from the wild-type strain and the pGDS1 transformants was digested with EcoRV and fractionated in a 0.8% agarose gel. The blot was probed with the 0.8-kb BglII-SalI fragment from pGDS1. Sizes (in kilobases) of marker DNA fragments (HindIII-digested λ DNA) are indicated on the left. W, wild-type strain Mel02010; CT1 to CT4, transformants showing normal conidiation; DS1 to DS4, transformants lacking conidiophores. (C) RNA gel blot analysis of pGDS1 transformants. The wild-type strain and the pGDS1 transformants were grown in CMC at 25°C for 4 days, and poly(A)$^+$ RNA was prepared from the cultures. Poly(A)$^+$ RNA (~5 μg/lane) was electrophoresed in a 1.5% agarose gel containing 2.2 M formaldehyde. The blot was probed with pS1BS containing the *FoSTUA* fragment (Fig. 2A). Sizes (in kilobases) of marker RNA fragments are indicated on the left. The blot was also probed with pFOTUB1 containing the β-tubulin gene fragment (β-*TUB*) (42).
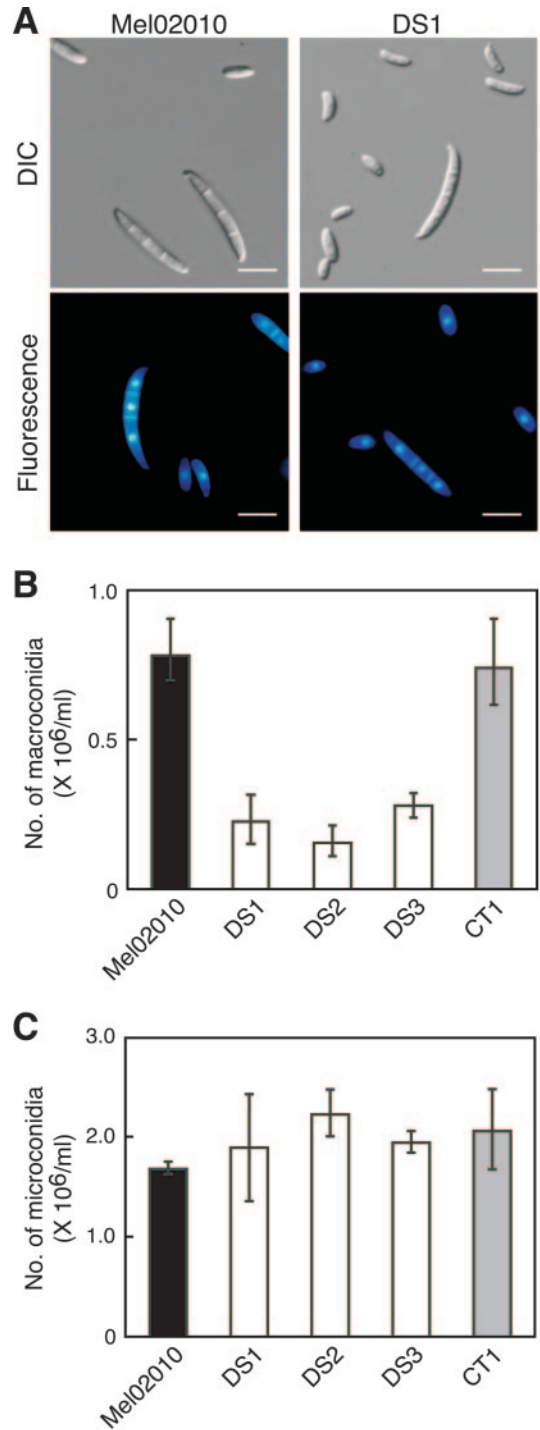

FIG. 6. Conidiation of Δ*FoSTUA* mutants. (A) Macroconidia and microconidia of wild-type and Δ*FoSTUA* mutant strains. Conidia were stained with Fluostain I and Hoechst 33258 to visualize cell walls and nuclei, respectively, and were observed with differential interference contrast (DIC) and fluorescence microscopes. Bars, 10 μm. (B and C) Numbers of macroconidia and microconidia produced by wild-type and Δ*FoSTUA* mutant strains. Strains were grown in CMC at 25°C for 4 days. The resulting macroconidia and microconidia were counted; data represent the means and standard deviations of three replications. Mel02010, wild type; DS1 to DS3, Δ*FoSTUA* mutants; CT1, ectopic transformant.

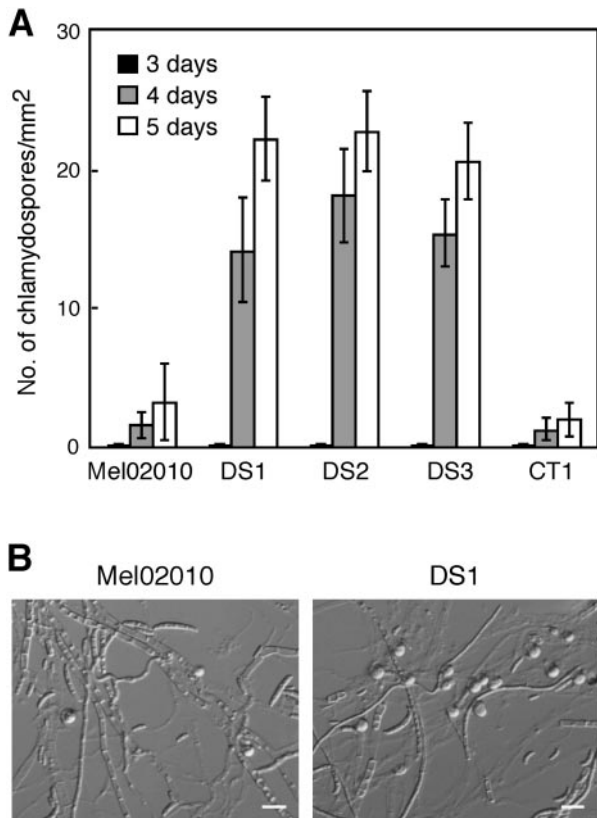FIG. 7. Chlamydospore formation by Δ*FoSTUA* mutants. (A) Numbers of chlamydospores produced by wild-type and Δ*FoSTUA* mutant strains. Strains were grown on SNA-paper at 25°C, and the numbers of chlamydospores produced were counted at 3, 4, and 5 days of incubation. Data represent the means and standard deviations of three replications. Mel02010, wild type; DS1 to DS3, Δ*FoSTUA* mutants; CT1, ectopic transformant. (B) Microscopic photographs of strains Mel02010 and DS1 grown on SNA-paper. Photographs were taken at 5 days of incubation. Globose organs are chlamydospores. Bars, 30 μm.
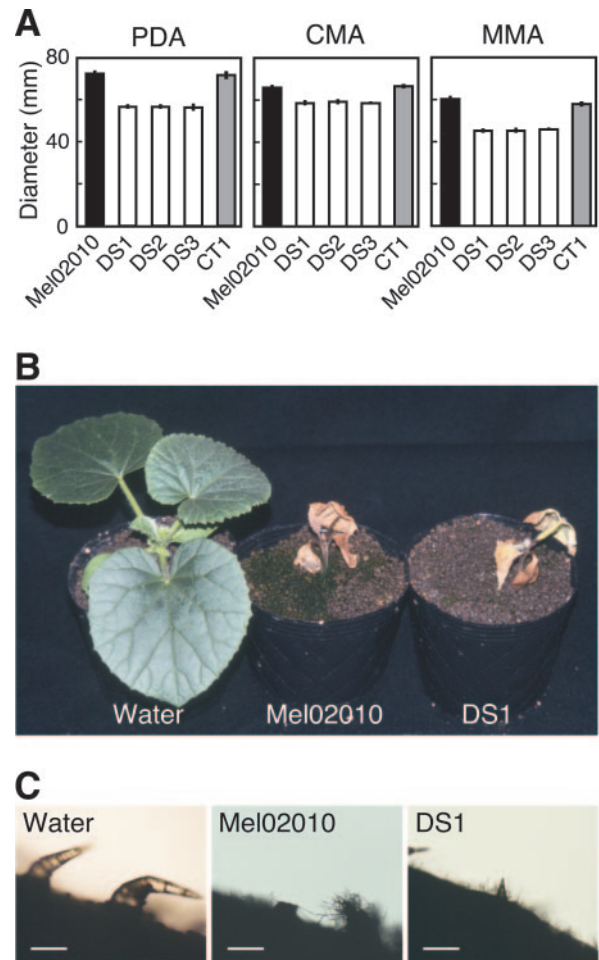

FIG. 8. Vegetative growth and pathogenicity of Δ*FoSTUA* mutants. (A) Colony growth of wild-type and Δ*FoSTUA* mutant strains. Strains were grown on PDA, CMA, and MMA at 25°C for 5 days. Colony diameters were measured; data represent the means and standard deviations of three replications. Mel02010, wild type; DS1 to DS3, Δ*FoSTUA* mutants; CT1, ectopic transformant. (B and C) Pathogenicity of and colonization by wild-type and Δ*FoSTUA* mutant strains. Seedlings of cultivar Amus with a single true leaf were inoculated with conidial suspensions (~10^7 conidia/ml) of wild-type strain Mel02010 and Δ*FoSTUA* mutant DS1 by a root dip method. Control plants were immersed in water. Photographs of plants were taken 3 weeks after inoculation (B). Stem surfaces immediately above crowns were observed with a microscope (C). Bars, 100 μm.

numbers of microconidia did not differ significantly between wild-type and mutant strains (Fig. 6C). These results indicate that *FoSTUA* is essential for the development of conidiophores, from which macroconidia arise, and is dispensable for microconidium formation in cultures.

To observe chlamydospore formation, strain Mel02010, three mutants (DS1 to DS3), and strain CT1 were grown on SNA-paper. Wild-type and mutant strains produced chlamydospores from 3 days after inoculation, and the numbers increased by 4 and 5 days (Fig. 7A). Chlamydospores of the mutants were similar in morphology to those of the wild type (Fig. 7B). However, the mutants produced many more chlamydospores than the wild type—6.7 to 7.5 times more than Mel02010 at 5 days of incubation (Fig. 7). These results indicate that mutation of *FoSTUA* leads to marked promotion of chlamydospore formation. It is likely that FoStuA has contrary functions in the developmental pathways of macroconidia and chlamydospores.

Vegetative growth and pathogenicity of Δ*FoSTUA* mutants. Vegetative growth of Δ*FoSTUA* mutants (DS1 to DS3) was evaluated by measuring the diameters of colonies grown on

three agar media, PDA, CMA, and MMA, at 25°C for 5 days. On all of the media, the colonies of the mutants were slightly smaller than those of strains Mel02010 and CT1 (Fig. 8A). Although the wild-type strain produced abundant aerial hyphae on all media, the mutants produced fewer aerial hyphae, especially on MMA (data not shown). These results indicate that *FoSTUA* is involved in but is not essential for vegetative growth.

Δ*FoSTUA* mutants (DS1 to DS3) were tested for pathogenicity for melon plants. Fifteen seedlings of susceptible cultivar Amus with a single true leaf were inoculated with a conidial suspension of each strain by the root dip method (25). The mutants caused typical wilt symptoms on all seedlings, as did the wild type. Figure 8B shows disease symptoms caused by

TABLE 1. In planta conidiation of Δ*FoSTUA* mutants[a]

Inoculum[b]	Mean ± SD no. (10^4/cm of stem) of:	
	Macroconidia	Microconidia
Water	None	None
Mel02010	62.6 ± 21.7	5.0 ± 1.2
DS1	0.4 ± 0.6	0.2 ± 0.5
DS2	0.2 ± 0.5	0.2 ± 0.5
DS3	0.4 ± 0.6	0.4 ± 0.6
CT1	50.6 ± 17.8	4.0 ± 0.7

[a] Seedlings of cultivar Amus were inoculated with a conidial suspension (~10^7 conidia/ml) of each strain by a root dip method. Control plants were immersed in water. Stem segments (~1.0 cm) immediately above crowns were cut out from inoculated plants 3 weeks after inoculation. The segments were soaked in sterilized water (1.0 ml) and vigorously shaken to suspend conidia. The numbers of macroconidia and microconidia in the suspensions were counted with a microscope; data are from five replications.
[b] Mel02010, wild type; DS1 to DS3, Δ*FoSTUA* mutants; CT1, ectopic transformant.

strains Mel02010 and DS1 as examples. The timing of symptom appearance and the severity of symptoms were almost the same on plants inoculated with wild-type and mutant strains, indicating that the Δ*FoSTUA* mutants retained the abilities to infect host plant tissues and to cause disease symptoms under the conditions tested.

F. oxysporum invades from roots of host plants, colonizes roots and stems, and produces conspicuous masses of macroconidia and microconidia on stem surfaces at the late stage of symptom development (26, 44, 45). We observed mycelia of wild-type and mutant strains growing on stem surfaces 3 weeks after inoculation. The mutants colonized stems and produced mycelia on the surfaces, as did the wild type (Fig. 8C), suggesting that *FoSTUA* is dispensable for colonization of host plants.

We also observed conidiation of wild-type and mutant strains in infected plants. Stems (~1.0 cm) immediately above crowns were cut out from diseased plants and soaked in sterilized water for suspending conidia. The numbers of macroconidia and microconidia in the suspensions were counted under a microscope. Although the wild-type strain produced large number of macroconidia in infected plants, the mutants produced very few macroconidia (Table 1). Although the mutants showed normal microconidium formation in CMC (Fig. 6C), they produced 10- to 20-fold fewer microconidia than the wild type in infected plants (Table 1). This result suggests that *FoSTUA* may have more critical roles in conidiation in host plants than in cultures.

DISCUSSION

***FoSTUA* is essential for conidiophore development.** In this study, we isolated *FoSTUA*, which differentially regulates the development of three kinds of asexual spores, macroconidia, microconidia, and chlamydospores, in *F. oxysporum*. FoStuA belongs to a group of APSES proteins which contain highly conserved bHLH motifs of ~100 amino acids (6, 19, 22, 33, 53, 56). The APSES proteins have been identified to regulate developmental processes in fungi, such as the formation of metulae and phialides in *A. nidulans* and *P. marneffei* (11, 19, 33), sexual reproduction in *N. crassa* (6), pseudohypal growth
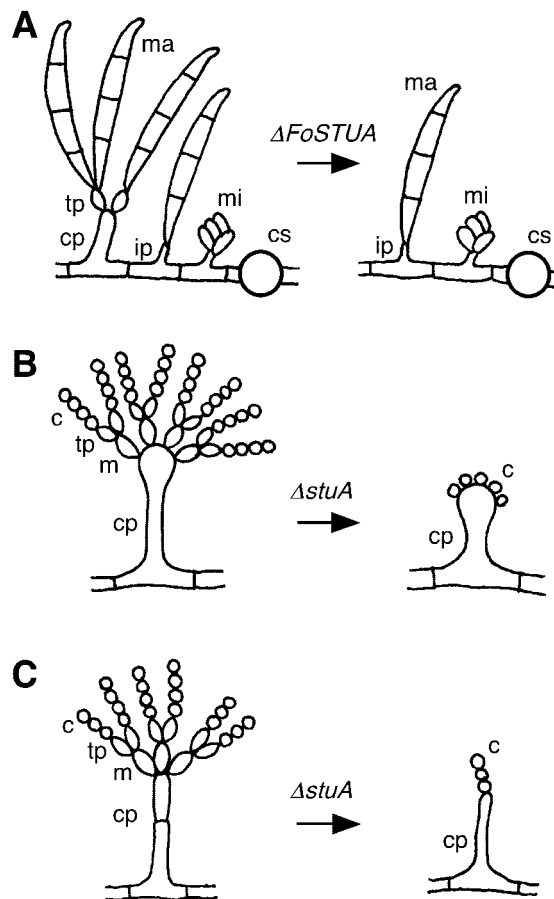

FIG. 9. Schematic representation of conidiogenesis of Δ*FoSTUA*, Δ*stuA*, and Δ*stuA* mutants of *F. oxysporum* (A), *A. nidulans* (B), and *P. marneffei* (C), respectively. ma, macroconidium; mi, microconidium; tp, terminal phialide; ip, intercalary phialide; cp, conidiophore; cs, chlamydospore; m, metula; c, conidium.

in *S. cerevisiae* (22, 50, 56), and dimorphic switching and chlamydospore formation in *C. albicans* (51, 53).

Targeted mutation of *FoSTUA* in *F. oxysporum* causes a lack of production of conidiophores, from which terminal phialides differentiate, followed by macroconidia that arise from phialides by basipetal division (Fig. 9A). Δ*FoSTUA* mutants, however, retain the abilities to differentiate intercalary phialides and to form macroconidia from intercalary phialides at low frequencies (Fig. 9A). Thus, the mutants produce macroconidia at lower frequencies than the wild type. In contrast, the mutants normally produce microconidia, because microconidia are generated from intercalary phialides. These results indicate that *FoSTUA* is required specifically for the initiation of conidiophore differentiation from hyphae in macroconidium development.

Of the fungal APSES proteins, the StuA proteins of *A. nidulans* and *P. marneffei* have been identified to be required for asexual spore development (11, 19, 33, 57). The modes of conidiogenesis in these two fungi are similar and consist of the sequential development of conidiophores, metulae, phialides, and conidia (Fig. 9B and C). In addition to *stuA*, the *abaA* homologue of *P. marneffei* has been isolated and characterized

as a conidiation regulator (10). Δ*stuA* mutants of these fungi can form conidiophores but not metulae and phialides (11, 19, 33, 57) (Fig. 9B and C). The mutants, however, produce conidia directly from conidiophores (11, 19, 33, 57) (Fig. 9B and C). Thus, the StuA proteins of these fungi are essential for the differentiation of two types of sterigmata (metulae and phialides). In contrast, FoStuA of *F. oxysporum* is required primarily for the development of conidiophores but not phialides. These data suggest that FoStuA of *F. oxysporum* has a role in conidiation that is different from the roles of the StuA proteins of *A. nidulans* and *P. marneffei*.

Ohara et al. isolated *REN1*, which encodes a putative transcriptional regulator, from a restriction enzyme-mediated integration transformant showing abnormal conidiation (42). Ren1 shows significant similarity to *A. nidulans* MedA (13). Mutation of *medA* results in aberrant conidiophores with branching chains of metulae, delayed differentiation of conidia, and frequent reinitiation of secondary conidiophores (13, 15, 21). Conidiation of the *medA* mutant is not completely blocked but is delayed (13, 15, 21). However, mutation of *REN1* in *F. oxysporum* leads to a lack of normal conidiophores and phialides and to the production of chains of rod-shaped, conidium-like cells directly from hyphae (42). The conidium-like cells also branch, unlike cells and conidia during normal conidiogenesis. Thus, Ren1 appears to be a core component of the conidiogenesis pathway in *F. oxysporum*, while its homologue MedA acts only as a modifier of this pathway in *A. nidulans* (13, 15, 21). Our data concerning the functions of FoStuA and Ren1 suggest that the molecular machinery underlying conidiation in *F. oxysporum* differs significantly from that in *A. nidulans*.

Recently, the genome sequence of *F. graminearum* became available (http://www-genome.wi.edu/annotation/fungi/fusarium/). On the website, we searched for the *F. graminearum* homologues of four genes (*brlA*, *abaA*, *stuA*, and *medA*) of *A. nidulans* that control conidiogenesis and found two putative genes that encode proteins significantly similar to StuA and MedA. These genes have exon and intron organizations similar to those of *FoSTUA* and *REN1* and encode proteins strongly similar to FoStuA and Ren1 (data not shown). However, no *brlA* or *abaA* homologues were found in the *F. graminearum* genome, although BrlA and AbaA are core components of the conidiogenesis pathway in *A. nidulans* (1, 5, 12, 49). These results suggest that conidiation regulators and their functions differ between *Fusarium* species and *A. nidulans*. Together with the fact that FoStuA and Ren1 localize in nuclei, we propose that Ren1 and FoStuA are transcriptional regulators in the core pathway for conidiation in *F. oxysporum*. Identification of the target genes of FoStuA and Ren1 is needed to assess the precise functions of these regulators in *F. oxysporum* conidiation. Analysis of the *N. crassa* genome sequence showed that *N. crassa* also possesses no *brlA* or *abaA* homologues (20).

***FoSTUA* acts as a repressor of chlamydospore formation.** *FoSTUA* is expressed in CMC and also in CM and MM, in which conidiation is not induced. GFP-tagged FoStuA is localized in nuclei in mycelial cells grown in CMC and CM. These features of *FoSTUA* and its product seemed to be responsible for the phenotypes of the Δ*FoSTUA* mutants. The colony growth of the mutants was slightly slower on solid media, and the mutants produced fewer aerial hyphae. This growth phe-

notype resembles that of the Δ*stuA* mutant of *A. nidulans* (15). In *A. nidulans*, *stuA* is expressed in both conidiating and vegetative hyphae (32). However, in *P. marneffei*, *stuA* is not expressed in vegetative hyphae, and deletion of *stuA* causes no abnormalities in growth or morphology of vegetative hyphae (11).

F. oxysporum has the ability to produce thick-walled cells, termed chlamydospores, in hyphae and conidia under certain environmental conditions (17, 39, 48, 52). Chlamydospores of *Fusarium* species provide the principal means of long-time survival during unfavorable periods in soil and play an important role as the primary inocula infecting plants (17, 39, 48, 52). Ohara et al. observed that a mutation of *REN1* has no effect on chlamydospore formation (42). Thus, we concluded that the developmental pathway of chlamydospores is genetically independent of that of macroconidia or microconidia. However, mutation of *FoSTUA* leads to the marked promotion of chlamydospore formation. FoStuA appears to be involved not only in conidiophore development but also in chlamydospore formation, although it controls these two developmental processes in opposite manners.

Among fungi from which APSES proteins have been characterized, *C. albicans* also produces chlamydospores. Chlamydospores of *F. oxysporum* and *C. albicans* are formed under similar conditions: chlamydospore formation is induced by aging and by unfavorable conditions of growth, such as low temperatures and carbon starvation (29, 35, 39, 48, 51, 52). In *C. albicans*, deletion of *EGF1*, encoding an APSES protein, results in a complete deficiency in chlamydospore formation (51). Thus, FoStuA and Egf1 have opposite functions in chlamydospore formation. Egf1 of *C. albicans* has a dual role in hyphal development and chlamydospore formation, in which Egf1 acts as an activator. In contrast, FoStuA functions as a repressor of chlamydospore formation but also as an activator of the development of conidiophores and macroconidia. The molecular mechanisms for chlamydospore formation in these fungi are unexplored; identification of target genes of FoStuA may be useful for understanding the molecular mechanisms of the chlamydospore formation that is an important trait of *F. oxysporum*.

***FoSTUA* is dispensable for disease-causing ability but important for in planta conidiation.** Δ*FoSTUA* mutants of *F. oxysporum* caused wilt symptoms in susceptible melon plants, as does the wild type, under the conditions tested. The mutants also grew invasively in stems at the late stage of infection. These results strongly suggest that *FoSTUA* is not involved in invading root tissues, colonizing roots and stems, and causing symptoms.

After symptom development, *F. oxysporum* pathogens produce conspicuous masses of macroconidia and microconidia on the surfaces of infected plants (39, 48, 52). Macroconidia are formed predominantly on plant surfaces, although microconidia are generally predominant in artificial culture media. Macroconidia and microconidia serve to spread the fungus within the plant as well as outside the plant (39, 48, 52). In the final stages of wilt disease development, chlamydospores arise on macroconidia, microconidia, and mycelia (39, 48, 52). Conidiation in infected plants is an essential step for the disease cycle. Macroconidium formation by Δ*FoSTUA* mutants in infected plants was very poor. Although the mutants showed

normal microconidium formation in cultures, they produced markedly fewer microconidia in infected plants than the wild type. These results suggest that *FoSTUA* has an important role in microconidium formation, specifically in infected plants, and that *FoSTUA* is essential for the life cycle of *F. oxysporum*.

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