Forkhead-associated Domains of the Tobacco NtFHA1 Transcription Activator and the Yeast Fhl1 Forkhead Transcription Factor Are Functionally Conserved*

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NtFHA1 encodes a novel protein containing the forkhead-associated (FHA) domain and the acidic domain in Nicotiana tabacum. NtFHA1 functions as a transactivator and is targeted to the nucleus. The sequence of the FHA domain of NtFHA1 is significantly homologous to that of the Fhl1 forkhead transcription factor of yeast. FHL1 was previously identified as a suppressor of RNA polymerase III mutations, and the fhl1 deletion mutant exhibited severe growth defects and impaired rRNA processing. Ectopic expression of the FHA domain of NtFHA1 (but not its mutant form) resulted in severe growth retardation in yeast. Similarly, expression of Fhl1, its FHA domain, or chimeric Fhl1 containing the NtFHA1 FHA domain also inhibited yeast growth. Yeast cells overexpressing the FHA domains of NtFHA1 and Fhl1 contained lower levels of mature rRNAs and exhibited rRNA-processing defects, similar to the fhl1 null mutant. Chimeric Fhl1 (but not the mutant form with a small deletion in its FHA domain) fully complemented the growth and rRNA-processing defects of the fhl1 null mutant, demonstrating that the FHA domain of NtFHA1 can functionally substitute for the FHA domain of Fhl1. These results demonstrate that the FHA domains of NtFHA1 and Fhl1 are conserved in their structure and function and that the FHA domain of Fhl1 is critically involved in regulation of rRNA processing in yeast. NtFHA1 function in plants may be analogous to Fhl1 function in yeast.

Eukaryotic transcription factors have been divided into many classes depending on their characteristic DNA-binding domains. The forkhead domain is a highly conserved 110-amino acid DNA-binding region found in a class of transcription factors (1). This domain was named after the Drosophila forkhead protein that is required for the proper formation of the terminal structure of the Drosophila embryos (2). In Saccharomyces cerevisiae, several forkhead transcription factors (Fhl1, Hcm1, Fkh1, and Fkh2) have been identified (3–6). Fhl1 has been implicated in RNA polymerase III function, the mutation of which leads to aberrant rRNA processing (3), whereas the Fkh1 and Fkh2 proteins, which share homology with each other in their forkhead domain and N-terminal region, regulate the cell cycle and pseudohyphal growth (5, 6).

In addition to the forkhead DNA-binding domain, some forkhead transcription factors also possess a forkhead-associated (FHA) domain. FHA domains are highly diverse protein-protein interaction modules characterized by a 55–75-residue motif with only 7 residues that are >65% conserved among 120 family members (7). Not being restricted to a group of forkhead transcription factors, the FHA domain has been found in a wide variety of proteins from both prokaryotes and eukaryotes (8). Rad53, a protein kinase involved in DNA damage response and cell cycle arrest in Saccharomyces cerevisiae, contains two FHA domains separated by a central Ser/Thr kinase domain (9). Yeast Yhr5 and FraH of Arabidopsis contain zinc finger motifs beside the FHA domain (7). Another group of the FHA domain-containing proteins are protein kinases such as Dun1, Spk1, and Mek1 of yeast, all of which have been reported to act in the nucleus and to respond to signals related to DNA replication and repair (10). In plants, the FHA domain was identified in Arabidopsis kinase-associated protein phosphatase (KAPP) through interaction cloning using an Arabidopsis receptor-like kinase as bait (11). Further study of the interaction between the KAPP and receptor-like kinase (RLK) proteins provided the evidence for involvement of FHA domains in phosphorylation-dependent protein-protein interactions (12). KAPP is involved in plant RLK signaling pathways on the inner face of the plasma membrane, unlike other FHA domain-containing proteins that are localized in the nucleus. The tertiary structure of the FHA domain has been solved in FHA2 (13) and FHA1 (14) of Rad53. Although the primary sequences of these two FHA domains are divergent, their tertiary structures are remarkably similar. The tertiary structure consists of a β-sandwich containing two twisted antiparallel β-sheets and a short α-helix at the C terminus.

In this study, we report a novel FHA domain-containing transcription activator in plants, designated NtFHA1. The FHA domains of NtFHA1 and the yeast Fhl1 forkhead transcription factor have conserved functions in regulation of cell growth and rRNA processing in yeast. Furthermore, the NtFHA1 FHA domain is fully functional within Fhl1 to rescue the phenotypes of slow growth and defective rRNA processing in the yeast fhl1 null mutant. These results indicate that Nt-
FHA1 in plant cells may perform a function analogous to that of FHL1 in yeast, probably by associating with other transcription factors. This is the first functional analysis of the FHA domain-containing transcription factors in plants.

**EXPERIMENTAL PROCEDURES**

**FHA Domain Constructs**—FHA domain constructs and site-directed cFhl1 mutants were generated by PCR and recombinant PCR (15) using synthetic oligonucleotides, and the mutations were confirmed by DNA sequencing. cFhl1Δ33 was generated by digestion of the cFhl1 construct (Fhl1 with its FHA domain replaced with the NtFHA1 FHA domain) with NcoI and HphI and subsequent self-ligation. This leads to internal deletion of 33 amino acids that include 4 conserved residues of the FHA domain without causing reading frame shift.

**Yeasts Strains and Culture**—Experiments were carried out in the EGY48 (MATα, his3, trpl, ura3, LexAop-LEU2) and W303-1A (MATα, ade2-1, his3-110, leu2-3, trpl-1, ura3-1) genetic backgrounds as indicated. For complementation assay, the FHA domain replaced with the NtFHA1 FHA domain, with NcoI and HphI and subsequent self-ligation. This leads to internal deletion of 33 amino acids that include 4 conserved residues of the FHA domain without causing reading frame shift.

**Synchronization of Tobacco BY2 Cells and Analysis of NtFHA1 Gene Expression**—Cultured cells were achieved by a 24-h subculture of stationary phase cells (7 days old) in medium containing aminophosphitocin (5 mg/liter, Sigma), followed by extensive washes as described (16). Mitotic index was measured as described (16). Northern blot analysis was carried out with 50 μg of total RNA for each lane as described under “DNA and RNA Gel Blot Analyses.”

**Flow Cytometry**—For cell cycle analysis, 1-ml aliquots were removed from yeast cultures and fixed in 70% ethanol. 300 μl of cells (2 × 10⁶ cells) were washed with 50 μl sodium citrate (pH 7.5) and incubated for 3 h with 1 ml of ethanol and 50 μl RNase A in 50 μl sodium citrate at 37 °C. Cells were treated with 4 μg/ml propidium iodide, sonicated, and analyzed using a BD Biosciences FACScan and CellQuest software.

**Transcription Activation Assays**—For effector constructs, various NtFHA1 cDNA fragments were cloned into a PhoHis vector (17) using a BamHI site to generate LexAop-nfg and XhoI sites, generating in-frame fusions of the LexA DNA-binding domain and the full-length or truncated open reading frames of NtFHA1. As a control, the DNA fragment containing the B42 activation domain was PCR-amplified from the pB42AD plasmid (CLONTECH) and cloned into pLexA using EcoRI and XhoI sites. To measure the transcription activation, the effector constructs were transformed into the BY2 strain, and the transformants were tested for growth in Leu− and for β-galactosidase activity. Three separate colonies were assayed for each construct, and β-galactosidase activity was determined as A₄₂₀₅₄₆/n.h.

**Subcellular Localization of the NtFHA1-GFP Protein**—The NtFHA1 cDNA corresponding to amino acid residues 1–209 was PCR-amplified and cloned into the 2.26-GFP vector (17) using a BamHI site to generate an NtFHA1-GFP in-frame fusion under the control of the cauliflower mosaic virus 35 S promoter. As a control for nuclear localization, the pNL5-RFP construct encoding a red fluorescent protein with a nuclear localization signal (17) under the control of 35 S promoter was also prepared. The pNL5-GFP and pNL5-RFP plasmids were introduced into Arabidopsis protoplasts by polyethylene glycol treatment as described (17). Expression of the fusion constructs was monitored at various times after transformation by fluorescence microscopy using a Zeiss Axioskop fluorescence microscope, and the image was captured with a cooled charge-coupled device camera. The filter sets (Omega Optical Inc., Brattleboro, VT) were: X5116 (exciter, 474AF20; dichroic, 500DRL; and emitter, 510AFP3) for GFP and XF355 (exciter, 535DF35; dichroic, 570DRL; and emitter, 505DF0) for RFP. The data were processed using Adobe Photoshop software and presented in pseudocolor format.

**DNA and RNA Gel Blot Analyses**—For DNA gel blot analysis, the genomic DNA isolated from tobacco leaves was digested with EcoRI and HindIII, electrophoresed on a 0.8% agarose gel, and blotted onto Hybond-N nylon membrane (Amersham Biosciences). The probe was the 0.4-kb PCR fragment corresponding to the C-terminal region of NtFHA1. Prehybridization and hybridization were carried out in 5× SSC, 5× Denhardt’s solution, and 0.5% SDS at 60 °C overnight. The membranes were washed twice with 2× SSC and 1% SDS at room temperature and then washed with 0.1× SSC and 0.1% SDS at 60 °C for 30 min.

**RESULTS**

**The NtFHA1 Gene Encodes a Putative FHA Domain-containing Transcription Regulator in Tobacco**—A cDNA encoding a protein of 209 amino acids with structural features of a transcription factor was identified in Nicotiana tabacum (cv. xanthi). The cDNA is 819 bp in length, and its predicted molecular mass is 22,601 Da. Based on the predicted sequence, the protein contains 59 amino acids (residues 30–88) that show conserved structural features of the FHA domain in the N terminus and the acidic region (residues 153–185) as a putative transactivation domain in the C terminus, but lacks any known DNA-binding motifs (Fig. 1A). The cDNA clone was designated NtFHA1.

We searched the Arabidopsis data base for homologs of NtFHA1 and found two genomic sequences encoding highly homologous proteins (GenBank™/EBI accession numbers AF202905 and AF202906). The corresponding cDNAs were isolated from the Arabidopsis cDNA library by PCR and designated AtFHA1 and AtFHA2. AtFHA1 and AtFHA2 encode proteins of 253 and 320 amino acids, respectively. The expected molecular masses of AtFHA1 and AtFHA2 are 27,980 and 35,994 Da, respectively. Like NtFHA1, they contain the FHA domains in the N terminus and the acidic domains in the C terminus and lack any DNA-binding domain. The amino acid sequence of NtFHA1 and comparison of the sequence with
The FHA domain and the acidic region of the compared sequences are boxed in black, and the residues conserved among two of the compared sequences are boxed in light gray. The FHA domain and the acidic region are indicated by brackets and overlining, respectively. The conserved residues of the FHA domain are marked with arrowheads above the sequences. C, schematic representation of the yeast Fhl1 protein in comparison with NtFHA1. D, sequence alignment of the FHA domains of the two proteins are shown in Fig. 1 (C and D, respectively). Yeast Fhl1, consisting of 936 amino acids, contains two large acidic domains at the N and C termini and the FHA domain and a DNA-binding forkhead domain in the center. The amino acid sequence of the FHA domain of NtFHA1 shows 46% identity to that of Fhl1.

**Fig. 1. Structure and amino acid sequence comparison of NtFHA1 with related sequences in other species.** A, schematic representation of the NtFHA1 protein. The FHA domain, the acidic domain, and the residue numbers are indicated. B, deduced amino acid sequence of NtFHA1 and alignment with AtFHA1 and AtFHA2 of Arabidopsis. The numbers on the right indicate amino acid positions. Gaps, which were introduced to maximize alignment, are indicated by dashes. The residues conserved among all three of the compared sequences are boxed in light gray. The FHA domain and the acidic region are indicated by brackets and overlining, respectively. The conserved residues of the FHA domain are marked with arrowheads above the sequences. C, schematic representation of the yeast Fhl1 protein in comparison with NtFHA1. D, sequence alignment of the FHA domains of the two proteins are shown in Fig. 1 (C and D, respectively). Yeast Fhl1, consisting of 936 amino acids, contains two large acidic domains at the N and C termini and the FHA domain and a DNA-binding forkhead domain in the center. The amino acid sequence of the FHA domain of NtFHA1 shows 46% identity to that of Fhl1.

**Transactivation Activity of NtFHA1**—We tested whether NtFHA1 has the ability for transcription activation using the yeast system. Various effector constructs were transformed into the yeast strain that contains two reporter genes (LEU2 and LexA) under the control of multiple LexA operators (Fig. 2A). As effectors, fusion proteins between the LexA DNA-binding domain and full-length NtFHA1 or its deletion mutants were expressed under the control of ADH1 promoters. Transcription activation was monitored by β-galactosidase activity and yeast growth in medium lacking leucine. The LexA DNA-binding domain alone, used as a negative control, did not exhibit any transactivation activity, whereas the positive control LexA-B42, a fusion protein between the LexA DNA-binding domain and the B42 activation domain, showed strong β-galactosidase activity and growth in Leu−. Full-length NtFHA1 was able to transactivate expression of the reporter genes, whereas NtFHA1 deletion mutants such as LexA-D1, LexA-D2, and LexA-D3 did not show any detectable transactivation activity. These results demonstrate that NtFHA1 is functional as a transcription activator.

**Nuclear Localization of NtFHA1**—Cellular localization of NtFHA1 was examined by constructing the NtFHA1-GFP fusion protein, the expression of which is controlled by the cauliflower mosaic virus 35 S promoter. As a positive control for nuclear targeting, RFP fused with a nuclear localization signal (NLS) (17) was expressed under the control of the same promoter. DNA constructs encoding NtFHA1-GFP and NLS-RFP were simultaneously introduced into protoplasts isolated from Arabidopsis seedlings. After incubation at 25 °C, expression of the introduced genes was examined under a fluorescent microscope with two different filters to capture the image of GFP and RFP. After a 24-h incubation, the green fluorescent signal completely overlapped with the red fluorescent signal in the nucleus, exhibiting colocalization of NtFHA1-GFP and NLS-RFP in the nucleus. This result demonstrates that NtFHA1 is targeted to the nucleus (Fig. 2B).

**Genomic Organization and Expression of the NtFHA1 Gene**—DNA gel blot analysis was performed with tobacco genomic DNA digested with restriction enzymes (Fig. 3A). The probe was the 0.4-kb PCR fragment corresponding to the C-terminal region of the NtFHA1 cDNA. EcoRI digestion resulted in three hybridizing bands, whereas HindIII digestion showed four hybridizing bands. Considering that N. tabacum is amphidiploid between Nicotiana tomentosiformis and Nicotiana sylvestris, these results indicate that the tobacco genome contains two copies of the NtFHA1-related sequences (Fig. 3A). We examined expression of the NtFHA1 mRNA in plant tissues...
at different developmental stages using RNA gel blot analysis and semiquantitative reverse transcription-PCR. In tobacco, the 1.0-kb NtFHA1 transcript was detected in all of the tissues examined, with the highest level found in open flowers (Fig. 3B). During seed germination, the NtFHA1 mRNA was detected at 2 days of incubation in MS (Murashige and Skoog) medium after 3 days of cold treatment, and the level decreased at 3–5 days of incubation and then increased at 7–9 days of incubation (Fig. 3C). During pollination, the NtFHA1 mRNA was detected in unpollinated ovaries just before anthesis at the level that was maintained until 3 days after pollination, and the mRNA level decreased from 5 days after pollination to a barely detectable level at 7 days after pollination (Fig. 3D). The NtFHA1 mRNA level fluctuated during the cell cycle in tobacco BY2 cells (Fig. 3E). Suspension-cultured tobacco BY2 cells were synchronized by a 24-h treatment with aphidicolin, an inhibitor of DNA polymerases. After release from the aphidicolin block, mitotic index and the NtFHA1 mRNA levels were monitored during the cell cycle. As controls, the histone H4 gene and the cyclin B1 gene were used as markers of S phase and M phase, respectively. The level of NtFHA1 mRNA was maintained at a similar level through S phase and G2 phase until mid-M phase, and then the transcript level decreased to a very low level during G phase.

Overexpression of the NtFHA1 FHA Domain Causes a Growth Defect in Yeast—To obtain clues to the function of the FHA domain of NtFHA1, we overexpressed the domain using a galactose-inducible system in yeast. The DNA construct containing the NtFHA1 FHA domain fused to the LexA DNA-binding domain for nuclear targeting or the construct containing the LexA DNA-binding domain alone as a vector control was transformed into yeast (Fig. 4A). Additionally, the FHA domain constructs with alanine substitutions of the highly conserved residues of the FHA domain (Mut34 and Mut55) were also overexpressed (Fig. 4A). The transformed yeast strains carrying the FHA domain or the vector showed normal growth in medium containing 2% glucose that repressed expression of the constructs. However, in medium containing 4%
galactose, the strain overexpressing the FHA domain exhibited considerably slower growth kinetics than the vector control (Fig. 4B). Overexpression of the FHA domain did not affect cell viability because colony formation per A unit was very similar for FHA domain and vector control cultures when plated on glucose medium (data not shown). As shown in Fig. 4B, this growth defect phenotype was dependent on the integrity of the FHA domain, as it was abrogated by alanine substitution of the highly conserved residues Arg-34 and Ser-55 (Fig. 4B). The abrogation of the growth defect indicates that these residues may be directly involved in the binding of a target protein that is crucial for the growth defect phenotype in yeast. The corresponding proteins for the FHA domain, Mut34, and Mut55 constructs were expressed at similar levels, as shown by immunoblotting using the anti-β-tubulin antibody (Fig. 4).

Slower G₁-S Transition in NtFHA1 FHA Domain-overexpressing Yeast Cells during the Cell Cycle—To examine whether the NtFHA1 FHA domain reduces cell growth in a specific phase of the cell cycle, aliquots of liquid cultures expressing the vector control or the NtFHA1 FHA domain before and 6 and 12 h after addition of galactose. Before and 6 h after addition of galactose, when the growth defect was not apparent, both cultures showed essentially identical cell cycle profiles; the majority of cells had a 2n DNA content (corresponding to G₂/M). However, induction of

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**Fig. 4.** Yeast growth phenotypes caused by overexpression of the FHA domain of NtFHA1. A, schematic diagrams of constructs for overexpression. GAL1p and BD represent the GAL1 promoter and the LexA DNA-binding domain, respectively. B, yeast cell growth. Yeast strains containing the various constructs were grown for 24 h in 2% glucose or 4% galactose and 2% sucrose. At 2-h intervals, aliquots were removed, and cell growth was monitored by absorbance measurements. C, Western blot analysis of FHA domain expression. 30 µg of total proteins isolated from yeast cells grown to 0.8 A units in glucose or galactose were subjected to immunoblotting with the monoclonal antibody (LexA-BD) against LexA DNA binding domain and the anti-β-tubulin antibody.

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**Fig. 5.** Cell cycle analysis of growth defects in FHA domain-overexpressing yeast cells. A, 1-ml aliquots were removed from growing vector control- and FHA domain-expressing cultures (W303-1A background) in 4% galactose medium at the time points indicated and analyzed by flow cytometry. B, vector control and FHA constructs induced for 10 h in 4% galactose were diluted to 0.2 A units, synchronized in G₁ by α-factor, and released into galactose medium. Aliquots removed at the indicated times were analyzed by flow cytometry. Asynch., asynchronized.
FIG. 6. Interchangeability between FHA domains of NtFHA1 and Fhl1. A, schematic diagrams of constructs for overexpression and yeast cell growth. Yeast strains containing various constructs separately or in combination were grown for 24 h in 2% sucrose and 1 or 4% galactose. GAL1p, GAL1 promoter; BD, LexA DNA-binding domain. B, Western blot analysis of expression of Fhl1 and the FHA domain. 30 μg of total proteins isolated from yeast cells grown to 0.8 A units in galactose were subjected to immunoblotting with the anti-LexA DNA-binding domain antibody (LexA-BD) and the anti-β-tubulin antibody. The arrowhead and filled square indicate the Fhl1 protein and the FHA domain, respectively. C, RNA gel blot analysis of FHL1 mRNA expression. D, schematic diagrams of the chimeric Fhl1 construct (cFHL1) for overexpression and resulting yeast cell growth. Yeast strains containing various constructs separately or in combination were grown for 24 h in 2% sucrose and 1 or 4% galactose.
However, expression of cFhl1 by the mutant when their expression was induced by 2% galactose. This is probably due to the galactose-induced overexpression of the Fhl1 protein, which has been shown to inhibit yeast growth (Fig. 6A). However, expression of cFhl1 by the FHL1 promoter fully restored the growth of the mutant to a degree similar to expression of wild-type Fhl1 on a multicopy plasmid (Fig. 7).

FIG. 7. Complementation of the yeast fhl1 null mutation by chimeric Fhl1 containing the NtFHA1 FHA domain. The FHL1 cDNA, chimeric Fhl1 containing the NtFHA1 FHA domain (cFHL1), and cFhl1 with a deletion of 33 amino acids (Fig. 1D) in its NtFHA1 FHA domain (cFHL1 Δ33) were fused under the control of either the GAL1 or FHL1 native promoter on a multicopy plasmid and introduced into the fhl1 deletion mutant (W303 background). Complementation assay was also carried out with Fhl1 and cFhl1 under the control of the FHL1 promoter on a low copy plasmid (pAS310). Cultures were grown to 0.8 × 10^6 units, and 10 μl were plated in a series of 10-fold dilutions on 2% glucose (left panel) and on 2% galactose and 2% sucrose (right panel). wt, wild-type.

LexA antibody, and the results show that the Fhl1 protein was expressed at similar levels in strains expressing either FHL1 and the vector or FHL1 and FHA, standardized by the levels of β-tubulin (Fig. 6B). The FHL1 mRNA levels were also similar in these strains, being expressed at a higher level compared with the strain that did not contain FHL (Fig. 6C). To test whether the FHA domains of NtFHA1 can replace the corresponding domain of Fhl1, the construct for chimeric Fhl1 in which its FHA domain was replaced with the NtFHA1 FHA domain (cFhl1) was introduced into yeast (Fig. 6D). Overexpression of cFhl1 resulted in growth retardation, similar to Fhl1 expression. Simultaneous expression of chimeric Fhl1 and the NtFHA1 FHA domain additively inhibited yeast growth (Fig. 6D).

Complementation of the fhl1 Deletion Mutation by Chimeric Fhl1 Containing the NtFHA1 FHA Domain.—We further tested the functional similarity between the FHA domains of NtFHA1 and Fhl1 using a complementation assay (Fig. 7). FHL1 is a nonessential gene in yeast, but its inactivation greatly impairs the growth of the mutant strains (3). The fhl1 deletion mutant was constructed in the haploid W303 by replacing the open reading frame of the FHL1 gene with a HIS3 cassette. The cDNA fragments encoding wild-type Fhl1 and chimeric Fhl1 in which its FHA domain was replaced with the FHA domain of NtFHA1 (cFhl1) were fused with either the GAL1 promoter or the native FHL1 promoter on a multicopy plasmid. These recombinant plasmids were introduced into the yeast fhl1 Δ mutant, and were examined whether expression of the chimeric Fhl1 protein could rescue the slow growth phenotype of the fhl1 Δ mutant and, in particular, whether expression of the chimeric Fhl1 mutant complemented with vector (fhl1 Δ/vector), the wild-type yeast cultures expressing the NtFHA1 FHA domain (wt/pGAL1::FHL1) or the Fhl1 FHA domain (wt/pGAL1::FHL1), the fhl1 Δ mutant complemented with vector (fhl1 Δ/vector), and the fhl1 Δ mutant complemented with chimeric Fhl1 with deletion of 33 residues in the NtFHA1 FHA domain (fhl1 Δ/pFHL1::cFHL1Δ33) all accumulated less amounts of 25S and 18S rRNAs (Fig. 9A), as quantified by the rRNA/rRNA and rRNA/actin mRNA ratios for each rRNA (data not shown). The fhl1 Δ mutant complemented with cFhl1 (fhl1 Δ/pFHL1::cFHL1) restored the amounts of 25S and 18S rRNAs in the wild-type yeast cultures expressing the FHL1 promoter, demonstrating that the NtFHA1 FHA domain is critical in cFhl1 function for complementing the fhl1 Δ mutant phenotype. Interestingly, both Fhl1 and cFhl1 could only partially complement the slow growth phenotypes of the fhl1 Δ mutant to the same degree on a low copy plasmid, indicating that FHL1 gene expression is regulated by its position in the genome. Taken together, these results further demonstrate that the FHA domains of NtFHA1 and Fhl1 are interchangeable for normal function of Fhl1 in yeast.

Defective rRNA Processing in FHA Domain-overexpressing Yeast Cells.—The Fhl1 protein is important for optimal growth and rRNA processing in yeast, leading to a reduced steady-state level of mature 25S, 18S, and 5.8S rRNAs in the fhl1 deletion mutant strain (3). We investigated whether the growth inhibition by overexpressing FHA domains of NtFHA1 and Fhl1 is related to impaired pre-rRNA processing in yeast by examining the steady-state levels of mature rRNA and pre-rRNA intermediates by Northern blot analysis (Figs. 8 and 9). To assess the levels of mature rRNA, 15 or 1.5 μg of total RNA were loaded per lane, and the membrane was hybridized with probes corresponding to 25S and 18S rRNAs of yeast and, as controls, with rRNA(Puc1) and actin probes (Fig. 9A). Compared with the vector control (fhl1 Δ/vector), cultures overexpressing the NtFHA1 FHA domain (wt/pGAL1::FHL1) or the Fhl1 FHA domain (wt/pGAL1::FHL1), the fhl1 Δ mutant complemented with vector (fhl1 Δ/vector), and the fhl1 Δ mutant complemented with chimeric Fhl1 with deletion of 33 residues in the NtFHA1 FHA domain (fhl1 Δ/pFHL1::cFHL1Δ33) all accumulated less amounts of 25S and 18S rRNAs (Fig. 9A), as quantified by the rRNA/rRNA and rRNA/actin mRNA ratios for each rRNA (data not shown). The fhl1 Δ mutant complemented with cFhl1 (fhl1 Δ/pFHL1::cFHL1) restored the amounts of 25S and 18S rRNAs.
and 18 S rRNAs to the wild-type levels.

Three oligonucleotide probes to detect the accumulation of yeast pre-rRNA intermediates were synthesized: ITS1-1 and ITS1-2 (designed to bind to the internal transcribed spacer 1 of yeast pre-rRNA) and ITS2 (designed to bind to the internal transcribed spacer 2) (Fig. 9B) (18, 19). Total RNA was prepared from wild-type cells expressing the vector control, the NtFHA1 FHA domain (wt/vector), the Fhl1 FHA domain (wt/vector) or from the fhl1Δ mutant complemented with vector (fhl1Δ/vector), the fhl1Δ mutant complemented with cFhl1 (fhl1Δ/pFHHL1::cFHL1), or the fhl1Δ mutant complemented with chimeric Fhl1 with a deletion of 33 residues in the NtFHA1 FHA domain (fhl1Δ/pFHHL1::cFHL1Δ33), all of which were grown in 2% galactose and 2% sucrose medium to 0.8 A units. The RNA gel blots containing 15 and 1.5 μg of total RNA/lane were hybridized with probes of 25 S rRNA, 18 S rRNA, actin (ACT1) mRNA, and tRNA^Phe. B, the positions of the ITS1-1 (5'--GAAACGGTTTTAATTGTCCTATAAC--3'), ITS1-2 (5'--TGTTACCTCTGGGCCC--3'), and ITS2 (5'--GGCCAGCAGATTCTCAAGTTA--3') oligonucleotide probes in the 35 S rRNA primary transcript are indicated. Northern blot hybridization was carried out using end-labeled ITS1-1, ITS1-2, and ITS2 probes corresponding to part of the ITS1 and ITS2 sequences of 35 S primary pre-rRNA. 15 μg of total RNA were used for each lane. The unidentified pre-rRNA intermediate detected by the ITS1-2 probe is indicated by the asterisk.

Fig. 9. RNA gel blot analysis of pre-rRNA processing. A, total RNA was extracted from wild-type yeast cells overexpressing the vector (wt/vector), the NtFHA1 FHA domain (wt/GAL1::FHA), or the Fhl1 FHA domain (wt/GAL1::FHA) or from the fhl1Δ mutant complemented with vector (fhl1Δ/vector), the fhl1Δ mutant complemented with cFhl1 (fhl1Δ/pFHHL1::cFHL1), or the fhl1Δ mutant complemented with chimeric Fhl1 with a deletion of 33 residues in the NtFHA1 FHA domain (fhl1Δ/pFHHL1::cFHL1Δ33), all of which were grown in 2% galactose and 2% sucrose medium to 0.8 A units in 2% galactose and 2% sucrose. The RNA gel blots containing 15 and 1.5 μg of total RNA/lane were hybridized with probes of 25 S rRNA, 18 S rRNA, actin (ACT1) mRNA, and tRNA^Phe. B, the positions of the ITS1-1 (5'--GAAACGGTTTTAATTGTCCTATAAC--3'), ITS1-2 (5'--TGTTACCTCTGGGCCC--3'), and ITS2 (5'--GGCCAGCAGATTCTCAAGTTA--3') oligonucleotide probes in the 35 S rRNA primary transcript are indicated. Northern blot hybridization was carried out using end-labeled ITS1-1, ITS1-2, and ITS2 probes corresponding to part of the ITS1 and ITS2 sequences of 35 S primary pre-rRNA. 15 μg of total RNA were used for each lane. The unidentified pre-rRNA intermediate detected by the ITS1-2 probe is indicated by the asterisk.

and 18 S rRNAs to the wild-type levels.

Three oligonucleotide probes to detect the accumulation of yeast pre-rRNA intermediates were synthesized: ITS1-1 and ITS1-2 (designed to bind to the internal transcribed spacer 1 of yeast pre-rRNA) and ITS2 (designed to bind to the internal transcribed spacer 2) (Fig. 9B) (18, 19). Total RNA was prepared from wild-type cells expressing the vector control, the NtFHA1 FHA domain, or the Fhl1 FHA domain or from the fhl1Δ mutant complemented with vector (fhl1Δ/vector), the fhl1Δ mutant complemented with cFhl1 (fhl1Δ/pFHHL1::cFHL1), or the fhl1Δ mutant complemented with chimeric Fhl1 with a deletion of 33 residues in the NtFHA1 FHA domain (fhl1Δ/pFHHL1::cFHL1Δ33), all of which were collected from yeast cells grown to 0.8 A units in 2% galactose and 2% sucrose. The RNA gel blots containing 15 and 1.5 μg of total RNA/lane were hybridized with probes of 25 S rRNA, 18 S rRNA, actin (ACT1) mRNA, and tRNA^Phe. B, the positions of the ITS1-1 (5'--GAAACGGTTTTAATTGTCCTATAAC--3'), ITS1-2 (5'--TGTTACCTCTGGGCCC--3'), and ITS2 (5'--GGCCAGCAGATTCTCAAGTTA--3') oligonucleotide probes in the 35 S rRNA primary transcript are indicated. Northern blot hybridization was carried out using end-labeled ITS1-1, ITS1-2, and ITS2 probes corresponding to part of the ITS1 and ITS2 sequences of 35 S primary pre-rRNA. 15 μg of total RNA were used for each lane. The unidentified pre-rRNA intermediate detected by the ITS1-2 probe is indicated by the asterisk.

With 32P-labeled ITS1-2, two major bands were detected corresponding to 27 S 3' pre-rRNA and an unidentified pre-rRNA, the size of which is slightly larger than the 20 S RNA (Fig. 9B) (18, 19). Overexpression of the FHA domains of NtFHA1 and Fhl1 resulted in high accumulation of the 35 S and unidentified pre-rRNAs, with no visible accumulation of 20 S pre-rRNA, indicating that the rRNA-processing steps from 35 S to 20 S pre-rRNA are significantly delayed in these cells. Additionally, they accumulated the 23 S pre-rRNA, which is considered an aberrant product formed by cleavage of 35 S pre-rRNA at site A3, bypassing processing sites A0–A2, often observed when pre-rRNA processing is perturbed (18).
the 27 SA₂ RNA, indicating that rRNA processing is significantly delayed (Fig. 9B). This pattern of pre-rRNA accumulation was very similar to that of the fhl1Δ mutant. Expression of cFhl1 under the control of the native FHL1 promoter in the fhl1Δ mutant restored the pre-rRNA accumulation pattern to the pattern of wild-type, whereas expression of cFhl1Δ33 under the control of the FHL1 promoter exhibited the same pattern of pre-rRNA processing as the fhl1Δ mutant.

When the 32P-labeled ITS2 probe was used, the wild-type control showed bands corresponding to 27 SA27 SB and 7 S pre-rRNAs, with no visible detection of 35 S RNA (Fig. 9A and B) (18, 19). However, yeast cells overexpressing the FHA domains of NtFHA1 and Fhl1 exhibited increased levels of 35 S and 27 SA27 SB pre-rRNAs and significantly decreased levels of 7 S pre-rRNA, similar to the pattern of the fhl1Δ mutation. Expression of cFhl1 in the fhl1Δ mutant restored the rRNA-processing pattern to that of wild-type, whereas the fhl1Δ mutant complemented with cFhl1Δ33 still showed the same pattern of pre-rRNA accumulation as the fhl1Δ mutant. Taken together, these results demonstrate that overexpression of the FHA domains of NtFHA1 and Fhl1 results in the same rRNA-processing defects as deletion of the FHL1 gene. In these cells, RNA processing is defective in multiple steps, indicating that FHL1 is involved in the general regulation of rRNA processing rather than being involved in a specific step of processing. Complementation of the fhl1Δ mutant by cFhl1 (but not by cFhl1Δ33) demonstrated that the intact FHA domain is critical for cFhl1 in controlling rRNA processing in yeast and that the FHA domain of NtFHA1 functionally substitutes for the corresponding domain of Fhl1 in yeast. These results also show that the cellular levels of Fhl1 are critical for normal function. The slow growth phenotypes of the FHA-domain- or Fhl1-overexpressing cells and the fhl1Δ deletion mutant are likely caused by defective rRNA processing and reduced accumulation of mature rRNAs.

**DISCUSSION**

*NtFHA1* encodes a novel transcription activator in tobacco that contains the FHA domain, a highly diverse protein-protein interaction domain. There are two homologs of *NtFHA1* in *Arabidopsis*, the FHA domains of which show 91–94% sequence identity to the FHA domain of NtFHA1. In this study, we have shown that the FHA domain of NtFHA1 shares the conserved structure and function with the FHA domain of yeast Fhl1, regulating rRNA processing and cell growth in yeast, implying that its function in plant cells is similar to the function of the FHA domain of Fhl1 in yeast. The FHA domain was first identified in a group of forkhead transcription factors and has since been identified in many signaling proteins, including proteins kinases, protein phosphatases, proteases, kinesins, and zinc finger proteins in yeast and animals. This existence of FHA domains in a wide variety of proteins suggests that they are involved in many different cellular processes, although a specific cellular target of these FHA-domain-containing proteins is largely unknown. In the plant kingdom, *Arabidopsis* contains 15 FHA domain-containing open reading frames, of which the biological function of only KAPP (7, 11) and zeaxanthin epoxidase (20, 21) has been characterized. Thus, the role of the FHA domain in those open reading frames is entirely unknown, except that the FHA domain of KAPP is involved in phosphorylation-dependent binding to multiple plant receptor-like kinases (12).

Our results demonstrate that overexpression of the FHA domain of NtFHA1 causes a severe growth defect in yeast by slowing down the G₁–S transition in the cell cycle, similar to the result of overexpression of Fhl1, its FHA domain, or chimeric Fhl1 containing the NtFHA1 FHA domain instead of its own. The growth defect phenotype is associated with defective rRNA processing and reduced rRNA accumulation, as shown by the RNA gel blot analysis using oligonucleotide probes corresponding to internal transcribed spacers 1 and 2 (Fig. 9). The results demonstrate that multiple steps of rRNA processing are defective in the FHA domain-overexpressing yeast cells, including processing from 35 S to 20 S pre-rRNA, from 35 S to 27 SA₂ pre-rRNA, and from 27 SA₂/27 SB₁/27 SB₂₁ to 7 S pre-rRNA and 25 S mature rRNA, and that these defects exactly match those of the fhl1Δ mutant (Fig. 9). Our results are also consistent with the results of Hermann-Le Denmat et al. (3), who observed accumulation of 35 S and 27 S pre-rRNAs in the fhl1Δ mutant by pulse-chase labeling. Based on our results, Fhl1 seems to be involved in an overall regulation of rRNA processing, controlling multiple steps. In addition, the defects we have seen here do not lead to complete blockage of rRNA processing, unlike many mutants in which accumulation of 35 S pre-rRNA and the aberrant 23 S species is often associated with complete depletion of 18 S and 25 S/5.8 S mature rRNAs (22–24). This delayed as well as defective processing of pre-rRNAs apparently contributes to the reduced accumulation of both 18 S and 25 S mature rRNAs and the slower growth rate of the FHA domain-overexpressing cells and the fhl1Δ mutant.

Chimeric Fhl1 driven by the native FHL1 promoter could rescue the slow growth phenotype and defective rRNA processing of the yeast fhl1Δ mutant, whereas deletion of 33 amino acids containing 4 conserved residues of the FHA domain from cFhl1 completely abolished the complementing activity. Thus, the intact NtFHA1 FHA domain in cFhl1 is absolutely required for rescuing the fhl1Δ mutant phenotypes. These results also demonstrate that the NtFHA1 FHA domain mimics the function of the FHA domain of Fhl1 in yeast, probably interacting with cellular components involved in regulation of rRNA processing. The FHL1 gene was originally isolated as a multicopy suppressor of RNA polymerase III mutations and later recloned as a multicopy suppressor of a mutation of RNA polymerase III transcription factor IIIc (3). Thus, the Fhl1 protein plays a role in the control of RNA polymerase III transcription and rRNA maturation, presumably by acting as a transcription regulator of genes specifically involved in these processes.

Fhl1 was found to genetically interact with Ifh1, a 122-kDa protein with no homology to any proteins in the data base (25). Partial deletion of Ifh1 led to a slow growth phenotype with impaired pre-rRNA processing, similar to the fhl1Δ mutation. A full deletion of Ifh1 was lethal, but growth was restored in a strain deleted for both Ifh1 and Fhl1. Thus, Ifh1 is essential for growth, but only in the presence of a functional Fhl1 protein. Based on these data, a model was proposed in which Fhl1 is converted from a transcription repressor to an activator upon binding of Ifh1 (25). The phenotypes of yeast cells overexpressing Fhl1 observed in this study are consistent with the model; the growth defect phenotype may be caused by excessive production of Fhl1 repressors when Ifh1 is limited.

We have shown that NtFHA1 is targeted to the nucleus and has a transactivation activity; yet NtFHA1, AtFHA1, and AtFHA2 lack an obvious DNA-binding domain in their sequences, which separates them from other forkhead transcription factors in yeast and mammals. An intriguing possibility is that NtFHA1 interacts with other protein molecules with a DNA-binding domain to make a functional transcription complex. Obvious candidates would be protein factors containing the forkhead DNA-binding domain; however, no protein in the plant data base has been identified to contain the forkhead domain. Fkh2, a yeast forkhead transcription factor, forms a transcription complex with the MADS box protein Mcm1 to control cell cycle periodicity of SWI5 and CLB2 gene expression.
(26, 27). Identifying interacting partners of NtFHA1 would give an insight into understanding the function of this group of plant transcription regulators.

The slow growth phenotype caused by overexpression of the FHA domain requires an intact FHA domain because mutation of the conserved residues of the FHA domain (Arg-34 and Ser-55) abolishes the phenotype (Fig. 5). Furthermore, deletion of 33 residues containing 4 conserved residues of the FHA domain from cFhl1 diminishes its ability to complement the growth and rRNA-processing defects of the fhl1Δ mutant, indicating that the FHA domain plays a critical role in the regulation of rRNA processing. Recently, overexpression of the N-terminal FHA domain (FHA1) of Rad53 resulted in growth retardation of yeast cells due to G1 arrest of the cell cycle, and the phenotype was abrogated by mutation of the conserved residues (28). These residues were critical for phosphopeptide binding of the Rad53 FHA1 domain, and replacement of the RAD53 gene with alleles containing mutations in the FHA1 domain resulted in an increased DNA damage sensitivity in vivo (28). Thus, the FHA1 domain contributes to the checkpoint function of Rad53, possibly by associating with a phosphorylated target protein in response to DNA damage in G1. In the same way, the conserved residues of the NtFHA1 FHA domain, including Arg-34 and Ser-55, may constitute binding sites for phosphorylated target proteins. The target proteins for the FHA domains of Fhl1 and NtFHA1 are currently unidentified, but components of the transcription machinery involved in RNA polymerase III transcription and rRNA processing are likely candidates, given the function of Fhl1 in those processes.

Reduced accumulation of mature rRNAs in FHA domain- and Fhl1-overexpressing yeast cells is a likely reason for delayed progression from G1 to S phase during the cell cycle. It will be interesting to probe the molecular mechanism by which the defects in rRNA processing in these cells lead to delayed G1-S transition. It is known that cells can delay or block cell cycle transitions in response to perturbations in a variety of intracellular processes, including ribosome biogenesis. However, the nature of the connection between the cell cycle and ribosome biogenesis has remained unknown. Recently, overexpression of a dominant-negative form of Bop1 (Bop1Δ), a novel nuclear protein involved in rRNA processing and 60 S ribosome biogenesis, resulted in cell cycle arrest (29). Subsequently, it was found that the p53-dependent signaling pathway connects Bop1Δ-driven aberrant rRNA processing/ribosome biogenesis to cell cycle arrest (30). These results provide clear evidence that the signal transmitted to the cell cycle machinery is generated by a mechanism that monitors ribosome production in the nucleolus. Plant cells likely possess a similar checkpoint mechanism to respond to nucleolar stress such as perturbations in ribosome biosynthesis. Although the functions of NtFHA1 in plant cells remain to be revealed, the conserved nature of the FHA domains of NtFHA1 and Fhl1 indicates a possibility that NtFHA1 is involved in regulation of RNA polymerase III transcription and rRNA processing in plant cells, possibly through assembly of a functional transcription complex by interacting with other transcription factors. Molecular analysis of transcription and rRNA processing in transgenic or mutant plant cells in which NtFHA1 expression is blocked would give a clear idea of NtFHA1 function in plants. In addition, identifying the NtFHA1-interacting proteins may yield a new insight into the action mechanism of NtFHA1.

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Forkhead-associated Domains of the Tobacco NtFHA1 Transcription Activator and the Yeast Fhl1 Forkhead Transcription Factor Are Functionally Conserved
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