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Activation of the Ustilagic Acid Biosynthesis Gene Cluster in *Ustilago maydis* by the C_2H_2 Zinc Finger Transcription Factor Rua1▽

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The phytopathogenic basidiomycetous fungus *Ustilago maydis* secretes, under conditions of nitrogen starvation, large amounts of the biosurfactant ustilagic acid (UA). This secreted cellobiose glycolipid is toxic for many microorganisms and confers biocontrol activity to *U. maydis*. Recently, a large gene cluster that is responsible for UA biosynthesis was identified. Here, we show that expression of all cluster genes depends on Rua1, a nuclear protein of the C_2H_2 zinc finger family, whose gene is located within the gene cluster. While deletion of *rua1* results in complete loss of UA production, overexpression of *rua1* promotes increased UA synthesis even in the presence of a good nitrogen source. Bioinformatic analysis allowed us to identify a conserved sequence element that is present in the promoters of all structural genes involved in UA biosynthesis. Deletion analysis of several promoters within the cluster revealed that this DNA element serves as an upstream activating sequence (UAS) and mediates Rua1-dependent expression. We used the yeast one-hybrid system to demonstrate specific recognition of this DNA element by Rua1. Introduction of nucleotide exchanges into the consensus sequence interfered with Rua1-dependent activation, suggesting that this sequence element acts as a direct binding site for Rua1.

Many fungi have the ability to synthesize complex secondary metabolites (15). These bioactive compounds are not essential for viability and are often produced only under certain environmental conditions or at selected times during the life cycle of the organism. Many secondary metabolites help the fungal cells to cope with environmental stress or to compete with other microorganisms.

Under conditions of nitrogen limitation, the basidiomycete *Ustilago maydis* produces large amounts of extracellular glycolipids (4). Glycolipids are amphipathic substances and thus act as biosurfactants. Some of the known extracellular glycolipids also display antibiotic activity. *U. maydis* is unique among fungal producers of glycolipids since it secretes two structurally unrelated classes of glycolipids. The mannosylerythritol lipids (MELs) are heavier than water and form an extracellular oil (9). MELs are highly potent surface-active substances but not toxic for other organisms (19). In addition, *U. maydis* produces the cellobiose lipid ustilagic acid (UA) (see Fig. 2A), which has broad antibacterial and antifungal spectra (11). The enzymes involved in biosynthesis of these glycolipids are in both cases encoded by genes found at adjacent locations on the chromosome. While a small gene cluster of five genes on chromosome 7 is responsible for MEL production, a large gene cluster on chromosome 23, encompassing 45 kb and containing 12 open reading frames (ORFs), is involved in UA production (13, 29). The UA gene cluster contains all genes necessary for the production of this glycolipid, including genes encoding two cytochrome P450 monooxygenases (*cyp1* and *cyp2*), an acyl- and an acetyl-transferase (*uat1* and *uat2*), a complete single-chain fatty acid synthase (*fas2*), a glycosyl transferase (*ugt1*), two hydroxylases (*uhd1* and *ahd1*), and a potential export protein of the ABC-transporter family (*atr1*) (see Fig. 2E). By mutational analysis and mass spectrometric analysis of secreted intermediates, we were able to deduce the complete biosynthesis pathway of the cellobiose lipid UA (29).

In fungi, genes involved in secondary metabolism are often arranged in clusters which are coregulated (15). In some cases, the transcription factor responsible for coregulation is itself encoded by one of the cluster genes, e.g., the *Aspergillus flavus* transcription factor AflR, which regulates aflatoxin and sterigmatocystin biosynthesis (35), and the regulator of gliotoxin production GliZ in *Aspergillus fumigatus* (3).

Here, we show that the zinc finger protein Rua1, whose gene is part of the UA biosynthesis gene cluster, acts as a transcription factor and regulates the activity of all structural genes involved in UA biosynthesis. We show that Rua1 binds directly to a short conserved DNA sequence motif, which is present in the promoters of all cluster genes and which confers nitrogen-dependent expression of UA biosynthesis.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. *Escherichia coli* strain DH5α and *Saccharomyces cerevisiae* strain DF5 his1-1 (8) were used for all DNA manipulations. Construction of plasmids was performed using standard procedures (24). *U. maydis* strains FB1 (*a1 b1*), FB2 (*a2 b2*) (2), and MB215 (*a2 b13*) (12) were grown as described before (21). The *U. maydis* Δ*rua1* deletion strain and the strains in which *rua1* is expressed under the control of either the constitutive P_{otef} promoter (MB215-P_{otef}::*rua1*) or the arabinose-inducible P_{crg} promoter (MB215-P_{crg}::*rua1*) were generated according to the published protocol for PCR-based generation of gene replacement mutants (5, 17). The sequences of the primers used for generation of mutants are available from the authors on request. All mutants were confirmed by Southern analysis, and deletion phenotypes were verified by complementation. Transformation of *U. maydis* was per-

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formed as described previously (26). For selection of transformants, PD plates containing 200 mg/ml hygromycin or 2 μg/ml carboxin were used.

To induce glycolipid production, strains were grown at 30°C in liquid yeast extract-peptone-sucrose (YEPS) medium to logarithmic phase and then shifted to nitrogen starvation medium containing 1.7 g/liter yeast nitrogen base (YNB; Difco) and 5% glucose as a carbon source. Glycolipids were isolated after cultivation of cells for 4 days at 30°C on a rotary shaker. The P_{otef}::*rua1* strain was grown in liquid YEPS medium to logarithmic phase prior to glycolipid extraction.

Localization of Rua1. For construction of *U. maydis* strains expressing green fluorescent protein (GFP)-Rua1, the *rua1* gene and shortened versions of *rua1* were amplified and cloned downstream of the GFP gene in plasmid p123, which carries an enhanced GFP (eGFP) reporter under the control of the strong constitutive P_{otef} promoter (28). Prior to transformation, plasmids were linearized with SspI. Integration into the *ip* locus of *U. maydis* was verified by Southern blot analysis.

Northern analysis. Total RNA was isolated for Northern blot analysis (25). Wild-type (WT) strain MB215 and the *rua1* deletion strain were grown at 30°C to logarithmic phase in YNB medium containing 5% glucose and 0.2% ammonium sulfate. After centrifugation, cells were resuspended in the same volume of fresh medium containing 5% glucose but lacking a nitrogen source. RNA was prepared at the time points indicated. Northern blots were prepared using standard procedures (24). For hybridization, internal probes of the respective genes that have been amplified by PCR were used.

Isolation of glycolipids. Extracellular glycolipids were extracted from suspension cultures (0.5 ml) with 1 volume of ethyl acetate. The ethyl acetate phase was evaporated, and glycolipids were dissolved in methanol. Glycolipids were analyzed by thin-layer chromatography (TLC) on silica plates (Silica gel 60; Merck) with a solvent system consisting of chloroform-methanol-water (65:25:4, vol/vol). The plate was dried thoroughly, and sugar-containing compounds were visualized by spraying them with a mixture of glacial acetic acid–sulfuric acid–*p*-anisaldehyde (50:1:0.5, vol/vol) and heating them at 150°C for 3 min (12).

Generation of truncated promoter constructs. For the construction of the plasmids containing the different promoter fragments, DNA sequences upstream of the *atr1*, *cyp1*, and *cyp2* genes were amplified by PCR and inserted into the plasmid p123-mCherry, containing the mCherry gene as a reporter gene (27, 28), thereby replacing the constitutive P_{otef} promoter. All plasmids contained a carboxin resistance (*ip*) cassette as a selectable marker in *U. maydis*. For integrative transformation into the *ip* locus of *U. maydis* MB215-P_{crg}::*rua1* strains, plasmids were linearized with SspI.

Yeast one-hybrid system. *S. cerevisiae* strain YM4271 (32, 33) was used for yeast one-hybrid analysis. Cells were grown in liquid yeast extract-peptone-dextrose (YEPD) medium or minimal medium lacking the appropriate uracil or leucine amino acids at 30°C to logarithmic phase. To generate the reporter plasmid, DNA fragments from the *atr1* promoter were amplified by PCR and inserted into the XhoI or HindIII sites of pLacZi (Clontech, Mountain View, CA). Plasmids pAD2-*rua1*, pAD2-*rua1*$_{N\Delta1\text{-}307}$, and pAD2-*rua1*$_{N\Delta1\text{-}576}$ were created as follows. The *rua1* gene and the N-terminally shortened versions of *rua1* were amplified by PCR and cloned into pAD2 as described previously (16). Cells were cotransformed as follows. Harvested cells were resuspended in 100 μl LiT (10 mM Tris-Cl, pH 7.4, 100 mM lithium acetate) and transformed with either 1 μg of the corresponding plasmid DNA or 5 μl salmon sperm DNA (10 mg/ml). Cells were covered with 500 μl LiT-polyethylene glycol (PEG) (LiT with 1 g/ml PEG 3350) and slowly rotated for 1 h. The mixture was supplemented with 50 μl dimethyl sulfoxide (DMSO) and treated with heat shock at 42°C for 15 min. Cells were plated on YEPD medium lacking the appropriate amino acids.

For quantitative measurements, β-galactosidase activity was determined using early logarithmic-phase cultures (optical density at 600 nm [OD_{600}] of ~0.5) of yeast cells transformed with the indicated plasmids. Samples (3 ml) were withdrawn from the cultures, and cells were harvested, washed with 1 ml Z buffer (100 mM Na_2HPO_4-NaH_2PO_4, pH 7.0, 10 mM KCl, 2 mM $MgSO_4$), and resuspended in 250 μl of Z buffer. The cells (100 μl) were permeabilized by three cycles of freezing and thawing. To each sample, 700 μl Z buffer, 20 μl β-mercaptoethanol, and 160 μl ONPG (*o*-nitrophenyl-β-D-galactopyranoside; 4 mg/ml in Z buffer) were added. Following a 10-min incubation, 400 μl of 1 M Na_2CO_3 was added to stop the reactions. After clarification by centrifugation, the A_{420} was measured, and β-galactosidase activity was calculated as Miller units (23).

Microscopy. For microscopic analysis, logarithmically growing cells were placed on agarose cushions and directly analyzed using a Zeiss Axiovert 200 microscope (Göttingen, Germany). Images were taken using a cooled charge-coupled-device (CCD) camera (Hamamatsu Orca-ER, Bridgewater, NJ) with an exposure time of 100 to 1.000 ms.

RESULTS

The *rua1* gene contains a cysteine-rich region distantly related to the classic C_2H_2 zinc finger motif. The UA biosynthesis gene cluster contains a gene that encodes a protein carrying a characteristic cysteine-rich region at its C-terminal end. A BLAST search revealed that this domain displays some similarity to zinc finger transcription factors of the C_2H_2 family (Fig. 1). This suggested that this protein might be involved in regulation of UA biosynthesis. Therefore, this gene has been termed *rua1* (regulator of ustilagic acid production) (29).

The deduced polypeptide sequence of *rua1* encodes a protein of 757 amino acid residues, which contains two potential C-terminal zinc finger domains (Fig. 1A). Database comparison revealed that Rua1 belongs to a small family of fungal proteins characterized by two putative zinc finger domains (Fig. 1B). Members of this subfamily were found both in ascomycetes and in basidiomycetes, with *U. maydis* containing a total of four related proteins. Two of them, Um06470 and Um05351 (for the MUMDB entry number, see http://mips.gsf.de/genre/proj/ustilago), are of unknown function but are very closely related to Rua1. A third *U. maydis* protein (Um03541) is similar to *Schizosaccharomyces pombe* Meu26, which was isolated in a screen for meiotically upregulated genes (31) (Fig. 1B).

We termed the two putative zinc finger domains Znf I and Znf II. In Rua1, they are located in the C-terminal part of the protein, comprising the positions between amino acids 663 to 690 and 718 to 749, respectively. Interestingly, the amino acid sequence of the zinc finger domains resembles that of C_2H_2 zinc fingers but at the same time deviates significantly from the classic C_2H_2 motif (34). Therefore, the Rua1 zinc finger was not predicted if database searches such as those using Interpro, SMART, or Pfam were performed. It might be surprising that for some members of the Rua1 family of zinc finger proteins, one of the histidines is exchanged for an aspartic acid. However, recently, it has been shown for type 2 B-box domain proteins that aspartic acid might also be involved in zinc atom coordination (22). Thus, Rua1 and the other related fungal proteins constitute a novel subfamily of C_2H_2-like zinc finger transcription factors (Fig. 1).

Rua1 is necessary for regulation of the UA biosynthesis gene cluster. To address the physiological function of Rua1 and its involvement in cluster regulation, we constructed a deletion mutant for *rua1* by replacing the open reading frame with a hygromycin resistance cassette. Wild-type and *rua1* deletion strains were cultivated for 4 days under nitrogen limitation and tested for glycolipid production by thin-layer chromatography (TLC). Whereas wild-type cells synthesized large amounts of both MEL and UA glycolipids, production of UA was completely abolished in *rua1* deletion strains. Secretion of MEL glycolipids, however, was unaffected in *rua1* mutants (Fig. 2B).

To determine whether deletion of *rua1* influences the transcriptional level of the cluster genes, Northern blot analyses were performed. In wild-type strains, all UA biosynthesis genes are strongly upregulated under conditions of nitrogen starvation (29). Six representative genes of the UA biosynthesis gene cluster were tested for expression in *rua1* deletion strains. Upon nitrogen starvation, no expression of these genes could

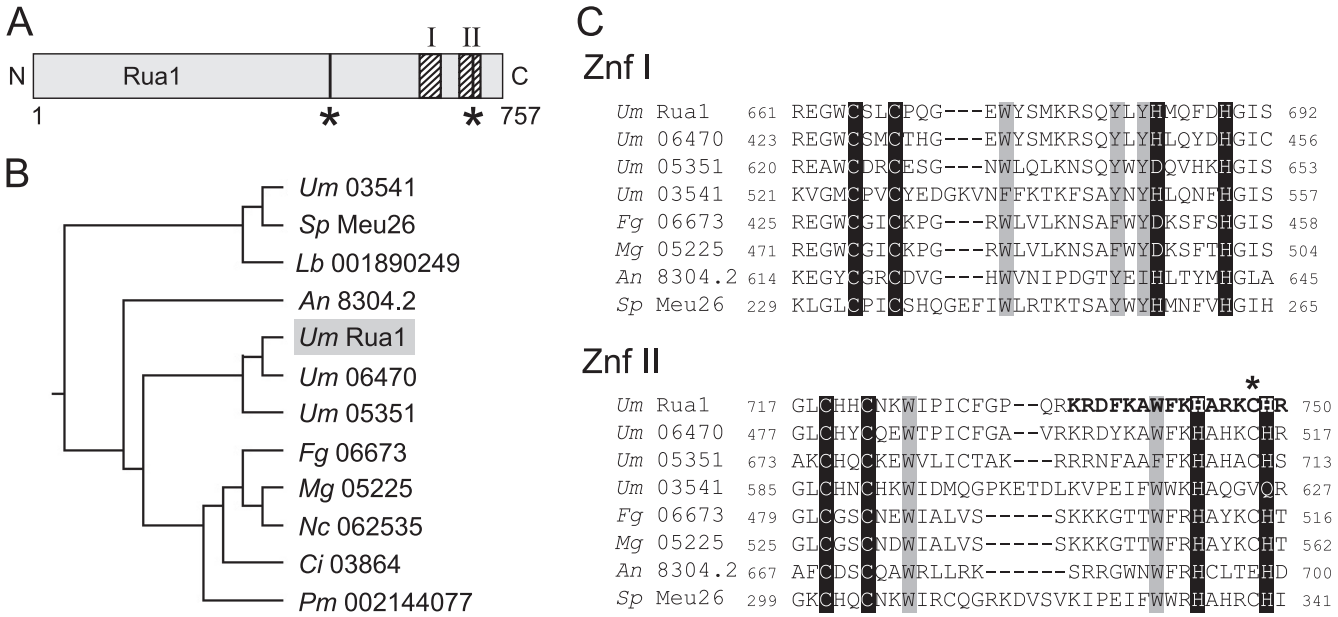

FIG. 1. Domain organization of the transcription factor Rua1. (A) Scheme of the domain organization of Rua1. The two C-terminal zinc finger domains are indicated by hatched areas. Predicted nuclear localization motifs are marked by vertical bars and asterisks. (B) Database comparison of the C-terminal region reveals that Rua1 is related to a yet uncharacterized subfamily of fungal proteins. The tree was constructed with CLUSTALW. The GenBank accession numbers are as follows: for *U. maydis* (*Um*) 03541, XP_759688; for *Schizosaccharomyces pombe* (*Sp*) Meu26, BAB60877; for *Lacaria bicolor* (*Lb*) 001890249, XP_001890249; for *A. nidulans* (*An*) 8304.4, CBF80270; for *U. maydis* (*Um*) Rua1, XP_762605; for *U. maydis* (*Um*) 06470, XP_762617; for *U. maydis* (*Um*) 05351, XP_761498; for *Fusarium gramineum* (*Fg*) 06673, XP_386849; for *Magnaporthe grisea* (*Mg*) 05225, XP_359552; for *Neurospora crassa* (*Nc*) 062535, XP_963086; for *Coccidioides immitis* (*Ci*) 03864, XP_001244423; and for *Penicillium marneffei* (*Pm*) 002144077, XP_002144077. (C) Sequence alignment of both Rua1 zinc finger domains (ZnF I and ZnF II). The Cys and His residues predicted to be involved in coordination of zinc are shaded in black. Highly conserved amino acids are shaded in gray. The C-terminal nuclear localization motif overlaps with the Znf II sequence (bold letters); the amino acid exchanged in the *rua1*^{C747R} mutant strain is marked with an asterisk.

be observed (Fig. 2C). This supports the notion that Rua1 plays a major role in regulation of the UA biosynthesis cluster genes.

The program PSORT (http://psort.ims.u-tokyo.ac.jp/form.html) predicts two putative nuclear localization motifs, which are indicated in Fig. 1A. The amino acid sequences of these motifs are R_{468}KHKVDAVRQSAVRSRR$_{484}$ and R_{735}KRDFKAWFKHARKCHR$_{750}$. To verify the predicted nuclear localization of Rua1, different GFP-Rua1 fusion proteins were expressed under the control of the constitutively active P_{otef} promoter. All these constructs were introduced into a *rua1* deletion strain and analyzed for functionality. While a C-terminal full-length Rua1-GFP fusion was not functional, the respective N-terminal GFP fusion was able to trigger normal amounts of UA production in the Δ*rua1* strain (data not shown). If the first 307 N-terminal amino acids of Rua1 were removed, no induction of UA production was observed (data not shown). If either the full-length N-terminal GFP-Rua1 fusion protein or the truncated version (GFP-Rua1$_{308-757}$) was expressed in *U. maydis* cells, nuclear localization was observed, indicating that Rua1 is targeted to the nucleus (Fig. 2D), most presumably due to its C-terminal nuclear localization domains. Together, this suggests that the N-terminal region of Rua1 is important for the function of Rua1 but not involved in nuclear localization.

Expression of *rua1* is sufficient to support UA biosynthesis under noninducing conditions. To test whether expression of Rua1 is also sufficient to trigger gene regulation of the UA biosynthesis genes, we expressed the ORF of *rua1* under the control of the constitutive P_{otef} promoter (P_{otef}::*rua1*) in a strain deleted for the endogenous copy of *rua1*. Glycolipid production in these strains was analyzed by TLC. Cells were grown in either nitrogen-free minimal medium (YNB −N) or complete medium (YEPS). Secreted glycolipids were extracted from the culture with ethyl acetate and analyzed by TLC. Remarkably, expression of *rua1* under the control of the constitutive P_{otef} promoter resulted in UA production even in noninducing rich medium (Fig. 3A). One has to point out that MEL production was not observed in this strain under these conditions. Nitrogen depletion induced production of comparable amounts of UA both in the WT and in the strain constitutively expressing Rua1 (Fig. 3B). Taken together, these data indicate that Rua1 is both necessary and sufficient to trigger UA biosynthesis. Since production of MEL glycolipids was not affected in Rua1-expressing strains, Rua1 appears to act as a cluster-specific regulator of UA biosynthesis.

Regulation of Rua1 expression. To elucidate the regulation of Rua1, we also analyzed the transcription of *rua1* by Northern blot analysis. While no expression of *rua1* could be observed if a rich nitrogen source was offered, nitrogen limitation resulted in strong *rua1* expression (Fig. 2C). These Northern data thus confirm our previous results observed with microarray analysis (29).

During construction of a deletion mutant for the gene *cyp2*,

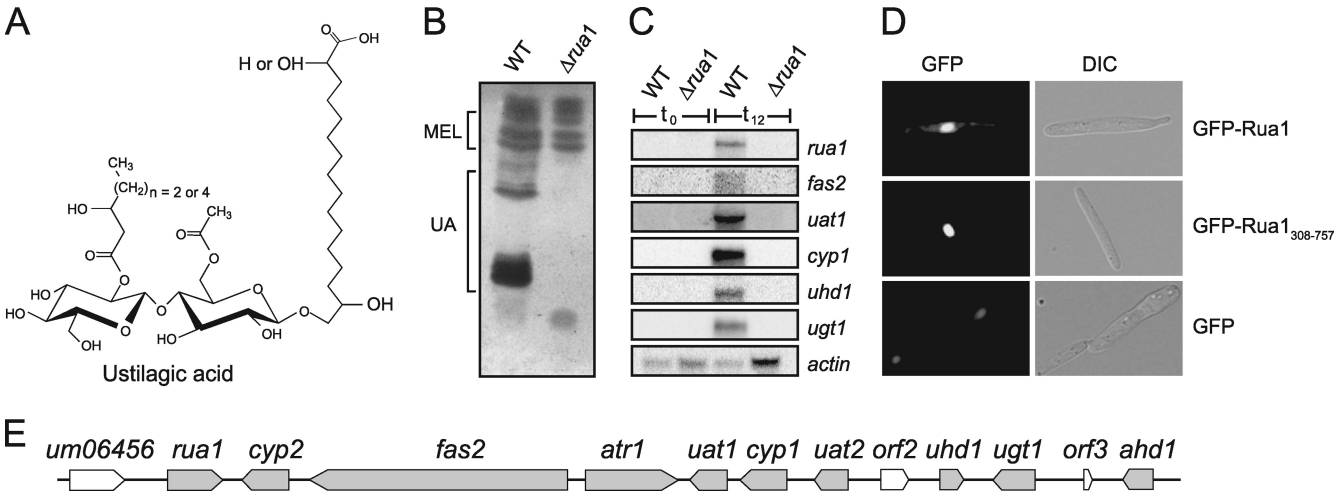

FIG. 2. Rua1 is essential for UA biosynthesis. (A) Molecular structure of ustilagic acid (UA). UA consists of cellobiose O-glycosidically linked to the ω-hydroxyl group of 15,16-dihydroxypalmitic acid or 2,15,16-trihydroxypalmitic acid. The cellobiose moiety is esterified with an acetyl group and a short-chain β-hydroxy fatty acid. (B) TLC analysis of extracellular glycolipids produced by wild-type and Δ*rua1* mutant strains. Deletion of *rua1* results in complete loss of UA secretion, while production of MELs is not affected. (C) Total RNA of wild-type and *rua1* deletion strains was prepared before (0 h) and 12 h after shifting of cells to nitrogen-limited medium. Under these conditions, transcription of cluster genes is strongly induced in wild-type strains but is completely abolished in *rua1* mutant strains. The *actin* gene is constitutively expressed and serves as a loading control. (D) Rua1 localizes to the nucleus. *U. maydis* FB2 strains expressing full-length eGFP-Rua1 or the N-terminally truncated version eGFP-Rua1$_{308-757}$ were analyzed by differential interference contrast (DIC) light microscopy (right column) and epifluorescence (left column) to detect eGFP. Both fusion proteins show strong localization to the nucleus. Expression of the unfused eGFP reporter gene serves as a control (GFP). (E) Genetic organization of the UA biosynthesis gene cluster. For gene designations, see reference 29.

which is adjacent to *rua1* and codes for a P450 monooxygenase involved in subterminal hydroxylation of hexadecanoic acid (29), we inadvertently introduced a point mutation into the coding region of *rua1*. This mutation affects a conserved cysteine at position 747 between the two core histidines of the second C_2H_2-like zinc finger (Fig. 1C, marked with an asterisk). Interestingly, this mutant was unable to produce UAs, indicating that the function of *rua1* was affected by this point mutation. Northern blot analysis revealed that the nitrogen-dependent transcription of the cluster genes was abolished in the mutant strain, which expresses *rua1*^{C747R} (Fig. 4A and data not shown). Interestingly, expression of *rua1*^{C747R} itself was also abolished in the mutant strain, suggesting that transcription of *rua1* may be subject to positive feedback regulation (Fig. 4A).

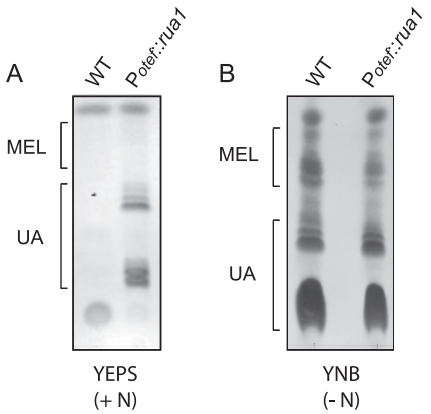

FIG. 3. Constitutive expression of *rua1* results in UA production. (A) *U. maydis* MB215 (WT) and MB215 Δ*rua1* P$_{otef}$::*rua1* (P$_{otef}$::*rua1*) cells were grown in rich medium (YEPS) containing nitrogen (+ N). While wild-type cells grown with sufficient nitrogen were unable to secrete glycolipids under these conditions, constitutive expression of *rua1* resulted in significant UA secretion. (B) The same strains were grown in minimal medium (YNB) lacking nitrogen (− N).

Promoters of Rua1-regulated genes share a conserved DNA sequence element. To identify potential regulatory sequences within the promoter regions of the coregulated cluster genes, an *in silico* search for conserved sequence motifs was performed using the MEME algorithm (http://meme.sdsc.edu/meme4_1/cgi-bin/meme.cgi). This analysis revealed a short consensus sequence ([T/G][G/T]CGCAT[A/T][C/T][C/T][G/A]) (Fig. 4B) which can be found in either orientation in the promoter regions of all cluster genes in at least one copy. Interestingly, the *rua1* promoter itself does not contain this element (Fig. 4C).

To determine whether the predicted sequence elements contribute to the regulation of the cluster genes, we performed a deletion analysis. The putative promoter regions used were either full-length or truncated at their 5′ ends to drive expression of the mCherry reporter gene. All constructs were integrated as single copies into the *ip* locus of the *U. maydis* strain MB215-P$_{crg}$::*rua1* by homologous recombination. In this strain, *rua1* is expressed under the control of the arabinose-inducible P$_{crg}$ promoter (Fig. 4D). Strains were first grown in glucose medium and then transferred to liquid medium containing arabinose as the sole carbon source to induce *rua1* expression. Expression of the mCherry gene was followed by fluorescence microscopy to determine which of the truncated promoters could still be induced by Rua1 (Fig. 4D).

A truncated version of the *atr1* promoter including the region comprising bp −667 to bp −1 still showed full promoter

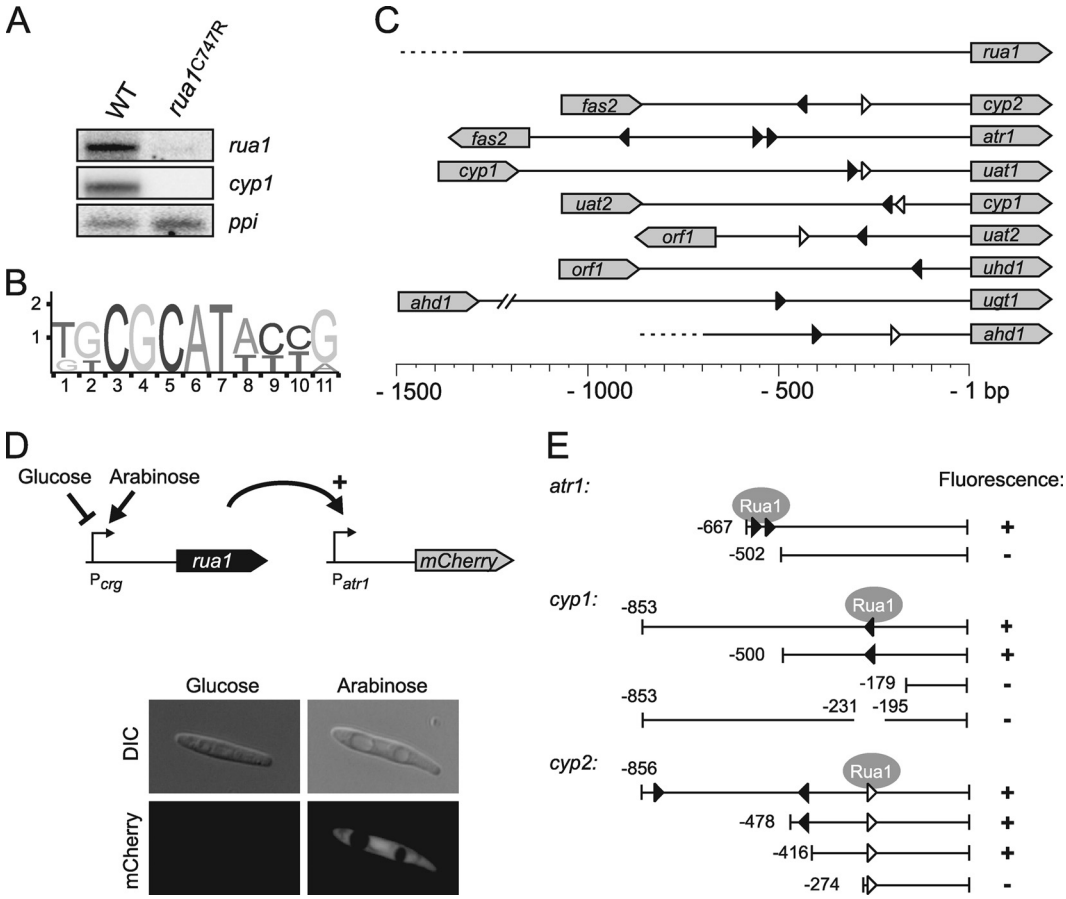

FIG. 4. Autoregulation of Rua1 and localization of the Rua1 DNA binding motif. (A) Total RNA of wild-type MB215 and *rua1* mutation strain MB215 *rua1*^{C747R} was prepared 12 h after shifting of cells to nitrogen-limited medium. While wild-type cells show strong expression of the UA biosynthesis cluster genes, indicated by *cyp1* expression, *rua1*^{C747R} cells were not able to express *cyp1*. Also, the *rua1* gene was not expressed in this mutant strain, indicating autoregulation of Rua1. The *ppi* gene is constitutively expressed and serves as a loading control. (B) A common motif within the promoter regions of the UA cluster genes was identified by MEME analysis. (C) Localization of the predicted motif within the 5′ untranslated regions of the UA biosynthesis genes. The triangles indicate the direction of the motif; black triangles indicate that the sequence matches with the predicted motif; white triangles allow for a single mismatch within the variable region. (D) Schematic diagram of the constructs used to narrow down the core region for Rua1-dependent promoters. Different promoter regions were fused to the mCherry reporter gene and transformed into a *U. maydis* strain expressing Rua1 under the control of the inducible P_{crg} promoter. *U. maydis* strains expressing the mCherry gene were analyzed by DIC light microscopy (upper column) and epifluorescence (lower column). (E) Schematic diagram of the various promoter deletion constructs and their activities. The numbers indicate the lengths of the selected 5′ untranslated regions of the respective genes or refer to deleted regions. mCherry gene activity was determined by microscopy.

activity while a fragment shortened to bp −502 showed no activity. This indicates that for *atr1*, the region between bp −667 and −502 is critical for regulation by Rua1. Interestingly, this region contains two copies of the predicted motif (Fig. 4E). Next, we selected the 853-bp intergenic region between *cyp1* and *uat2* for a detailed promoter analysis. The whole 853-bp 5′ untranslated sequence as well as the 500-bp shortened region conferred high mCherry fluorescence upon Rua1 induction, whereas no fluorescence was detected when the promoter region was shortened to 179 bp. If the region between positions −231 and −195, which contains the predicted motif, was deleted, promoter activity was completely abolished, supporting our view that this motif acts as an upstream activating sequence (UAS) which is critical for induction by Rua1 (Fig. 4E).

In the *cyp2* promoter, the sequence from bp −478 to bp −1 was sufficient to confer Rua1 dependent expression to the same extent as the entire intergenic region. Surprisingly, Rua1 regulation was still observed if the promoter was shortened to 416 bp and thus did not contain a consensus motif predicted within this region. However, a more degenerate sequence motif containing a nonconserved nucleotide within the flanking area is located further downstream. Therefore, we tested if the promoter region comprising bp −274 to −1, which contains this motif at the very left border, still supports Rua1-dependent mCherry gene expression. Surprisingly, no activity was induced, suggesting that the function of the consensus motif may in this case depend on its immediate sequence context, which was altered in this reporter construct (Fig. 4E).

Rua1 specifically recognizes the conserved upstream activating sequence present in the promoters of all UA biosynthetic cluster genes. To verify that Rua1 acts as a transcriptional factor and is capable of binding DNA, we tried to perform *in vitro* DNA binding assays. Unfortunately, expression of Rua1 in *E. coli* resulted in the formation of inclusion

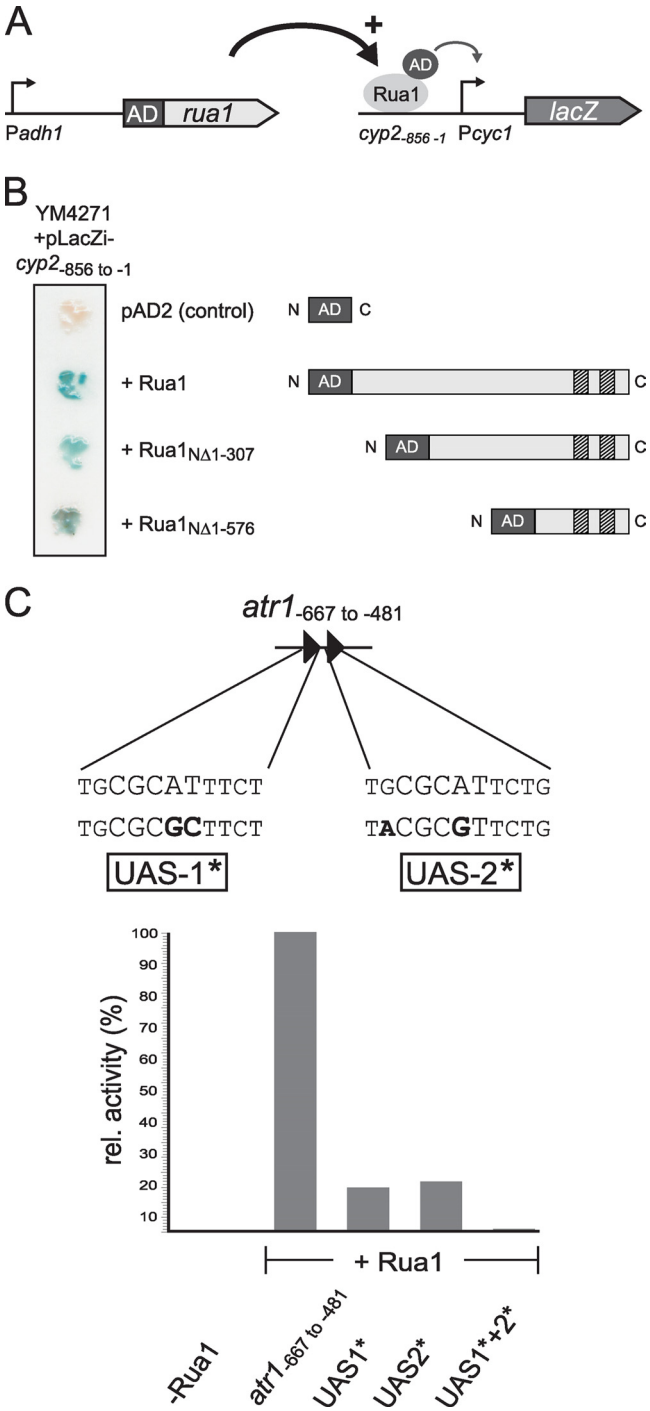

FIG. 5. Rua1 binds to a conserved DNA motif within the promoter region of the UA cluster genes. (A) Schematic diagram of constructs used in the yeast one-hybrid system. A chimeric protein where Rua1 or N-terminally shortened versions $Rua1_{N\Delta1\text{-}307}$ and $Rua1_{N\Delta1\text{-}576}$ were N-terminally fused to the activation domain of Gal4 was constructed and expressed under the constitutive yeast alcohol dehydrogenase promoter (P_{ahd1}). As a reporter construct, the 5′ untranslated region of the *cyp2* gene (P_{cyp2}) containing the Rua1 binding site was introduced upstream of the minimal promoter of the iso-1-cytochrome *c* (P_{cyc1}) gene, which was fused to the reporter gene *lacZ*. (B) Yeast one-hybrid transcription activation analysis of Rua1 or N-terminally shortened versions of Rua1. *S. cerevisiae* strain YM4271 was cotransformed with the Gal4 activation domain alone or Gal4-AD-Rua1 fusion proteins and the reporter plasmid. Transcription activation was visualized by adding X-Gal which was cleaved by β-galactosidase, yielding an insoluble blue product. (C) Schematic diagram of the construct used in the yeast one-hybrid system. An ~200-bp fragment of the *atr1* promoter ($atr1_{-667\text{ to }-481}$), containing two times the motif identified by MEME analysis, was used for Rua1 binding studies. The motifs found in this area, proposed to act as upstream activating sequences (UAS-1 and UAS-2) for Rua1, are indicated. Black letters indicate nucleotides that have been mutated in this study to give UAS-1* and UAS-2*. For quantification, β-galactosidase activity was determined as described in Materials and Methods and is indicated in Miller units. Shown are relative activities compared to the activity of the 200-bp *atr1* UAS, which was set to 100%.

bodies that could not be solubilized. Therefore, we used the yeast one-hybrid system to test whether the conserved sequence motif is recognized by Rua1 *in vivo*. To this end, the activation domain of the yeast Gal4 protein was fused either to full-length Rua1 or to N-terminally truncated versions ($Rua1_{N\Delta1\text{-}307}$ and $Rua1_{N\Delta1\text{-}576}$). A plasmid expressing only the Gal4 activation domain served as a control. Plasmids were transformed into *S. cerevisiae* strain YM4271, which harbors a reporter construct in which the regulatory region of the *cyp2* promoter was placed upstream of the minimal promoter of the iso-1-cytochrome *c* (*CYC1*) gene, which itself drives the reporter gene *lacZ* (Fig. 5A). The activity of the chimeric promoters was quantified by growing *S. cerevisiae* on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) containing solid medium. Blue coloring of colonies indicated β-galactosidase activity. The results shown in Fig. 5A demonstrate that full-length Rua1 was clearly able to activate β-galactosidase activity. This indicates that Rua1 expression is sufficient to stimulate expression of the chimeric promoter in yeast. Furthermore, the N-terminally shortened versions of Rua1 were also able to activate reporter gene activity, indicating that the C-terminal zinc finger domain of Rua1 is sufficient to recognize the putative upstream activating sequence. In contrast, control strains which express only the Gal4 activation domain showed no β-galactosidase activity.

To further prove that Rua1 directly binds to the predicted sequence motif, we tested whether mutation of conserved residues within the consensus sequence interferes with Rua1 activation. To this end, a fragment of the upstream region of *atr1* was analyzed by yeast one-hybrid experiments. This region of about 200 bp ($atr1_{-667\text{ to }-481}$) carries two copies of the predicted motif, named UAS-1 and UAS-2. *S. cerevisiae* cells that express the Rua1-AD fusion protein were transformed with reporter plasmids containing either the wild-type version of the 200-bp fragment derived from the *atr1* promoter or mutated versions carrying mutations within the core element of the predicted motif, named UAS-1* and UAS-2* (Fig. 5B). The mutated UAS elements were tested either singly or jointly. To quantify the amount of transcription of the *lacZ* gene, we measured β-galactosidase activity in cultures of the transformed cells. The results are shown in Fig. 5C and revealed that strong β-galactosidase activity could be observed only when both wild-type motifs were present. Nucleotide exchanges within one of the predicted motifs resulted in strongly reduced β-galactosidase activity while no β-galactosidase ac-

tivity was detected in cells transformed with the DNA fragment carrying mutations in both motifs. Taken together, these results confirm that the predicted sequence motif acts as a binding site for the C_2H_2 transcription factor Rua1, which acts as activator for transcription of the respective cluster genes.

DISCUSSION

We have identified the C_2H_2 zinc finger transcription factor Rua1, which is both necessary and sufficient to trigger expression of the biosynthetic gene cluster for the cellobiose lipid UA. The *rua1* gene itself is part of the cluster and is located at its leftmost end. Since nitrogen limitation induces expression of the UA biosynthesis gene cluster, we assume that this regulation is mediated by Rua1, most probably by nitrogen-dependent expression of Rua1. We showed that Rua1 localizes to the nucleus and that it recognizes a conserved sequence element found in the promoter sequences of all cluster genes, with the only exception being the *rua1* gene itself. While deletion of *rua1* abolished transcription of all cluster genes tested, constitutive expression of *rua1* resulted in constitutive UA production even under noninducing conditions, i.e., in the presence of a rich nitrogen source (Fig. 3A).

We used the yeast one-hybrid system to demonstrate that Rua1 is able to activate gene expression in the heterologous yeast system. This strongly suggests that regulation of transcription by Rua1 occurs by direct binding to the conserved sequence elements and does not require other transcription factors or additional *U. maydis* proteins. Rua1 is both necessary and sufficient to trigger UA biosynthesis. Thus, environmental and nutrient regulation of UA biosynthesis is presumably directly mediated by the level of Rua1 present in the cell. This would predict that all environmental signals that affect UA biosynthesis are integrated at the *rua1* promoter. The *rua1* gene is characterized by an unusually long promoter region spanning more than 4.7 kb. This region could accommodate binding sites for a number of different transcription factors involved in environmental and nutritional regulation of *rua1* expression. However, one cannot exclude that *rua1* expression might also be subject to posttranscriptional control, as was described for the global nitrogen regulator AreA in *A. nidulans.* The AreA transcript is specifically degraded in response to intracellular glutamine (6). One could imagine that this is also true for Rua1. Under inducing conditions, the *rua1* transcript is stabilized, which results in UA biosynthesis. In contrast, the presence of a favored nitrogen source would result in transcript degradation. To distinguish between these potential regulatory schemes, the level of Rua1 expression should be followed by fusion of the promoter region to a reporter gene.

Truncation analysis of Rua1 indicated that the two zinc finger domains located at the C-terminal region are responsible for DNA binding. Interestingly, the amino acid sequences of both zinc finger domains deviate significantly from the C_2H_2 consensus sequence (34) and are not detected by database searches. This indicates that the zinc finger domain in Rua1, together with its relatives in other fungal species (Fig. 1), constitutes a novel fungus-specific subfamily of zinc finger transcription factors. Since some of these members have been identified in previous genetic screens but were annotated as proteins with unknown function, this finding sheds new light on their potential regulatory function. A prominent example is the meiotically upregulated gene *meu26* in *S. pombe* (31), which could now be regarded as a potential regulator of meiosis-specific genes.

Our data strongly suggest that DNA binding to the conserved motif within the promoter regions of the cluster genes is mediated by the zinc finger domains. This is supported by the point mutation located within the zinc finger region in the $rua1^{C747R}$ mutant strain, which is unable to secrete UA anymore. This mutation affects a conserved cysteine residue located directly adjacent to one of the histidines putatively involved in the coordination of zinc. For the regulatory gene *areA*, mediating nitrogen metabolite repression in *A. nidulans*, it was shown that a single amino acid exchange within the zinc finger loop at position 102 (Leu to Val) also leads to derepression of genes involved in nitrogen metabolism (20). Interestingly, in the $rua1^{C747R}$ point mutant, expression of *rua1* itself was affected, indicating that there might be some kind of autoregulation.

How expression of Rua1 is regulated remains to be elucidated. We suppose that regulatory proteins that sense global and specific nitrogen availability are involved. Candidate proteins for this regulation are the *U. maydis* AreA/NIT2 homolog Um10417 and the homolog of the pathway-specific activator NIT4, Um02808 (14). Alternatively, there might exist additional transcription factors that are specifically involved in the nutrient control of secondary metabolism genes. Initial experimental data indicate that nitrogen-dependent transcription of Rua1 is also regulated by another zinc finger transcription factor (Um02717), which we have termed Nit1 (B. Teichmann, L. Liu, and M. Bölker, unpublished data). It remains to be elucidated whether Nit1 is directly involved in nitrogen sensing. Interestingly, its deletion affects not only UA biosynthesis but also the production of MEL glycolipids.

The *cis*-acting element which acts as an upstream activating sequence and confers Rua1-specific transcription was identified by bioinformatic and mutational analysis. In the promoters of all structural genes involved in UA biosynthesis, at least one copy of the consensus sequence (T/G)(G/T)CGCAT(A/T)(C/T)(C/T)(G/A) was found. Interestingly, introduction of single point mutations in the tandem element upstream of the *atr1* gene resulted in loss of activation, indicating that both UASs act synergistically in gene activation *in vivo*. A similar observation has already been described for binding sites of AflR, the transcriptional factor of the *A. nidulans* sterigmatocystin biosynthesis gene cluster. Deletion of only one of the three AflR binding sites within the *stcU* promoter results in decreased gene expression (7).

The secreted cellobiose lipid UA has broad antibacterial and antifungal spectra (11). Coinoculation of wild-type *U. maydis* cells effectively prevents infection of tomato leaves by *Botrytis cinerea*, the causal agent of gray mold disease. Therefore, it has been proposed that *U. maydis* could be used as a biocontrol agent. UA production plays an essential role for this antagonistic activity since mutants that are unable to produce UA do not prevent *B. cinerea* infections (29). Interestingly, a similar glycolipid is produced by the basidiomycetous fungus *Pseudozyma flocculosa*, which is a natural inhabitant of the phyllosphere. *P. flocculosa* has already been described as a potent biocontrol agent against fungal pathogens of plants (1,

10). The elucidation of the regulation of the UA biosynthesis gene cluster in *U. maydis* may therefore also open new avenues for studying and improving biocontrol activity in the highly related fungus *P. flocculosa*.

Secondary metabolite genes are often found to be clustered and are in general not constitutively expressed (18, 30). To understand the role of transcription factors in cluster regulation, those involved in secondary metabolism in *Aspergillus* strains were classified into different groups (15). AflR was the first member of a class of pathway-specific transcription factors. The criteria by which AflR was sorted into this class also apply to Rua1: Rua1 recognizes and binds to a specific sequence found in all promoters of the UA biosynthesis genes. There is evidence of self-regulation of Rua1 because a point mutation in the *rua1* gene leads to a downregulation of itself, suggesting that Rua1 activates its transcription of its own gene. Deletion of *rua1* abolishes expression of UA cluster genes, whereas constitutive expression of *rua1* results in production of the secondary metabolite UA independently of nitrogen limitation. Thus, Rua1 acts as a central, highly pathway-specific transcription factor for UA biosynthesis. Secondary metabolites are often produced only under specific environmental conditions. The presence of a central regulator confers the advantage of allowing quick adaptation of cluster expression to different conditions by just changing the expression pattern of the pathway-specific transcription factor.

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