Identification of *cis*-Active Elements in *Ustilago maydis mig2* Promoters Conferring High-Level Activity During Pathogenic Growth in Maize

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The *Ustilago maydis mig2* cluster comprises five highly homologous genes that display a pronounced plant-specific expression profile. A 350-bp *mig2-5* promoter fragment contained all elements sufficient to confer differential promoter activity. Mutational analysis of this region, fused to the green fluorescent protein reporter gene, allowed dissecting core promoter elements required for high-level promoter activity from elements conferring inducible expression in planta. In particular, the presence of several 5'-CCA-3' motifs within a short stretch of the *mig2-5* promoter was decisive for inducible promoter activity. On this basis, we reconstituted an artificial promoter whose inducible activity specifically relied on multiple CCA motifs. In addition, we identified a novel *mig2* homologous gene, *mig2-6*, that is not part of the *mig2* cluster, but displayed the strongest differential expression profile among *mig2* genes. The deletion of all six *mig2* genes did not compromise the ability to induce tumor formation in infected maize plants. Comparative sequence analysis including the *mig2-6* promoter revealed an over-representation of the consensus motif 5'-MNMNWNCCAMM-3'. We discuss putative transcriptional activators involved in *mig2* regulation.

The basidiomycete fungus *Ustilago maydis* causes smut disease in its host plants, maize and teosinte. *U. maydis* infects young meristematic tissue aboveground and triggers the formation of tumors on all aerial parts of infected plants (Christensen 1963). In these tumors, massive fungal proliferation and the formation of diploid teliospores occurs. Pathogenicity of *U. maydis* is intimately linked to the sexual life cycle and initiated with the fusion of haploid sporidia to dikaryotic hyphae. Prerequisite for mating compatibility are different *a* and *b* mating type loci (Kahmann et al. 2000). The *a* locus contains the pheromone (*mfal*,2) and receptor (*pral*,2) genes that enable cell recognition and fusion (Bölker et al. 1992; Spellig et al. 1994). The *b* loci encode the bE and bW homeodomain proteins that, in nonallelic combinations, dimerize to an active transcription factor required for filamentous growth and pathogenicity (Brachmann et al. 2001; Gillissen et al. 1992; Kämper et al. 1995; Kronstad and Leong 1990; Romeis et al. 2000; Schulz et al. 1990). The individual stages of pathogenic development have been described in detail (Banuett

and Herskowitz 1996; Kahmann et al. 2000; Snetselaar and Mims 1994) and include the formation of infection structures on the leaf surface, intra- and intercellular growth of branched hyphae through plant tissue, karyogamy, and the formation of sporogenous hyphae resulting in the release of single diploid cells that mature to black-pigmented teliospores. During growth in planta, *U. maydis* maintains a biotrophic relationship with its host.

Various *U. maydis* genes have been identified that are specifically expressed during distinct stages of pathogenic development. These include genes of the *b* pathway that are expressed upon mating (Bohlmann 1996; Brachmann et al. 2001, 2003; Romeis et al. 2000; Schauwecker et al. 1995; Urban et al. 1996; Wösten et al. 1996), as well as genes whose regulation is controlled during the interaction with the plant host. The *U. maydis pig* genes, many of which are clustered in the *p*-locus, display a plant-regulated expression pattern. Pig protein sequences include similarities to a disulfide isomerase and permeases of the major facilitator family (Aichinger et al. 2003). The *U. maydis ssp1* gene coding for a protein with similarity to fatty acid dioxygenases was specifically expressed during teliospore formation (Huber et al. 2002). The *mig1* gene and, more recently, the *U. maydis mig2* cluster containing five highly homologous genes arranged as direct repeats within a 7.1-kb DNA region, were identified by virtue of their strong induction during the biotrophic growth phase (Basse et al. 2002). The *mig* genes encode small, secreted, cysteine-containing, hydrophilic proteins. In particular, Mig2 proteins display a highly conserved C-terminal region of approximately 100 amino acids that are characterized by eight invariant Cys residues. Mig protein sequences lack homology in the NCBI database and deletion mutants of either *mig1* or the entire *mig2* cluster are not affected in their ability to trigger tumor formation in infected plants. Based on their extracellular localization, presumed compact structure, and plant-specific expression profile, these proteins resemble fungal avirulence proteins (Basse et al. 2000, 2002; Lauge and De Wit 1998). Studies based on fluorescence microscopy using *U. maydis* strains expressing the green fluorescent protein reporter gene (*eGFP*) under control of the endogenous *mig1* or *mig2-1* promoters, respectively, revealed that *eGFP* expression is induced immediately upon fungal penetration and is maintained at a high level until the formation of teliospores, whereas infection structures on the leaf surface displayed no or only weak GFP fluorescence (Basse et al. 2000, 2002). Furthermore, neither starvation conditions, which have been reported to mimic fungal gene expression in planta (Coleman et al. 1997; Pieterse et al. 1994; Van den Ackerveken et al. 1994), nor growth in the presence of tumor extracts induced the *mig1* or *mig2-1* promoters under cul-

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ture conditions (axenic growth). The *mig2* genes slightly differed in their expression kinetics but markedly differed in their inducible expression levels. Transcripts of *mig2-1*, *mig2-4*, and *mig2-5* already were detected 2 days post inoculation (dpi) and peaked 2 days later, whereas transcripts of *mig2-2* and *mig2-3* were not detected before 4 dpi and subsequently declined. Highest transcript levels were detected for *mig2-5*, followed by *mig2-4*, *mig2-2*, *mig2-1*, and *mig2-3*. Investigation of *mig1* and *mig2-1* promoters provided evidence for positively as well as negatively acting *cis*-elements in close proximity to each other. For *mig1*, the region decisive for inducible promoter activity was confined to positions –519 to –148, and for *mig2-1* positively acting promoter elements mapped in the region from –428 to –203 (Basse et al. 2000, 2002).

Here, we present a detailed mutational analysis of the *mig2-5* promoter in *U. maydis*. This provided insight into the promoter structure and allowed the identification of repeated sequence motifs crucial for inducible promoter activity in planta, as well as the reconstitution of an artificial promoter conferring high-level activity in planta. In addition, we have identified a novel *U. maydis* gene related to *mig2* genes, which displayed the most pronounced plant-inducible expression pattern.

RESULTS

mig2-5 Promoter deletion analysis.

The *mig2* cluster of *U. maydis* comprises five highly homologous genes that are arranged as direct repeats and separated by intergenic regions in the range of 751 to 882 bp (Basse et al. 2002). Among the *mig2* genes, *mig2-5* is most strongly upregulated during biotrophic growth; therefore, we selected the 870-bp intergenic region between *mig2-4* and *mig2-5* for a detailed promoter analysis. For this purpose, this region was fused to *eGFP* at the predicted translational start ATG and ectopically integrated in single copy into the *ip* locus of the *U. maydis* strain SG200 (*a1mfa2bE1bW2*) to provide for a defined promoter context. This strain is solopathogenic and causes infections when injected alone into a corn plant without the need for a mating partner (Bölker et al. 1995). The 870-bp 5' untranslated sequence (UTS) of *mig2-5* conferred high GFP fluorescence in planta, whereas no fluorescence was microscopically detected during axenic growth. In contrast, construct pJD1, which is deleted in the region between –670 and –152, was unable to confer GFP fluorescence in planta, demonstrating the loss of *cis*-active promoter elements (Fig. 1A).

To delimit the intergenic region between *mig2-4* and *mig2-5* into parts required for conferring inducible *eGFP* expression in planta promoter truncations were introduced from the 5' end. This showed that the region from positions –351 to –1 conferred the same promoter activity as the entire intergenic region (Fig. 1A). Inducible promoter activity conferred by a fragment shortened to 240 bp was reduced, and was abolished in promoter fragments ≤160 bp. Conversely, an internal deletion encompassing positions –370 to –234 resulted in only slightly reduced promoter activity, suggesting the presence of upstream elements, which can substitute for this region. Three overlapping deletions between –234 to –152 severely compromised inducible *mig2-5* promoter activity, while the region from –161 to –122 was less critical for induction (Fig. 1A). Successive promoter truncations from the 5' end conferred enhanced promoter activity during axenic growth as assessed by fluorimeter analysis; however, GFP activity levels were not high enough for detection by fluorescence microscopy in planta, as exemplified by strain JT1 (Fig. 1A). Promoter activities of the various reporter strains additionally were assessed by Northern analysis using *eGFP* as probe. Due to integration in the *ip* locus, all transformants harbored the endogenous

mig2-5 gene. This allowed normalizing *eGFP* transcript levels by comparison with native *mig2-5* expression levels (Fig. 1B) and confirmed the strongly reduced promoter activities in strains JD1, JT1, JT2, JD15, JD16, and JD18 in planta, as well as in strain JF1 during axenic growth. Together, these results pointed to a decisive role of the region from positions –234 to –161 for induced promoter activity, but also showed that adjacent regions contributed to *eGFP* expression during fungal growth in planta.

Mapping the transcriptional start positions of *mig2* genes and requirement of the core region for *mig2-5* promoter activity.

To gain insight into the core promoter structure of *mig2* genes, transcriptional start sites were determined by RNA ligase-mediated rapid amplification of 5' cDNA ends (5' RACE) analysis. This revealed multiple transcription start sites for *mig2* genes with a preference between positions –62 and –55 for *mig2-2*, *mig2-4*, and *mig2-5* (Fig. 2A). With the exception of *mig2-3*, representing a pseudogene (Basse et al. 2002), the sequence context of all determined transcriptional start sites was CCACA^T/_AC (Fig. 2B), and thus matched the conserved mammalian initiator (Inr) sequence C_T/TAN^A/_TC_T/C_T (Javahery et al. 1994; Lo and Smale 1996). Approximately 40 bp further upstream, a putative TATA-like element (TLE) is found in the promoters of all *mig2* genes (Fig. 2B). Upstream of this element, the sequence CGCC is present in *mig2-4* and *mig2-5* promoters resembling the eukaryotic TFIIB recognition sequence (Lagrange et al. 1998). Together, this suggests that the *mig2-5* core promoter comprises at least the initial 106 bp of the 5' UTS (Fig. 2B).

To address whether parts of the basal *mig2-5* promoter contributed to inducible activity, we initially replaced the region from –86 to –1 by the weak *mfa1* basal promoter (*mfa1*_{bas}) (Urban et al. 1996). The control construct pJM1 in which *mfa1*_{bas} was fused to *eGFP* conferred elevated axenic GFP fluorescence compared with construct pJF1 (Fig. 1); however, this was not detectable in planta (Fig. 3). In contrast, construct pJM26 carrying the *mig2-5* promoter region from positions –351 to –86 fused to *mfa1*_{bas} conferred full inducibility in planta, indicating that *mfa1*_{bas} can replace the initial 86 bp of the *mig2-5* promoter. To assess the requirement of the putative TLE for *mig2-5* promoter activity, the sequence 5'-ATAAAA-3' was substituted in construct pJF1. The resulting construct pJSTATA conferred drastically reduced GFP fluorescence in planta, and this also was reflected at the transcriptional level (Figs. 3 and 4B). Consistently, *mig2-5* promoter fragments lacking the region between –119 and –1 conferred markedly reduced GFP fluorescence in combination with *mfa1*_{bas}, in which a TLE is absent (strains JM25 and JM17).

Cis-regulatory regions in the *mig2-5* promoter required for induced expression.

The contribution of the region from –161 to –119 for inducible reporter gene activity in strains carrying *mig2-5* promoter (*P*_{*mig2-5*})-*mfa1*_{bas} fusions was corroborated by successive 3' end truncations to position –162 (Fig. 3). Based on the alignment of *mig2* promoter sequences (Fig. 2B), three regions in the *mig2-5* promoter, from positions –130 to –120 (box I), –155 to –139 (box II), and –189 to –173 (box III), show a high degree of conservation. To address their influence on *mig2-5* regulation, these boxes were substituted by random sequences. Although substitution of both boxes I and II only moderately affected GFP fluorescence in planta, the substitution of box III interfered more severely with *mig2-5* promoter activity. When the substitution in box III was combined with those in boxes I and II (strain JS7), GFP fluorescence in planta was almost abol-

ished (Fig. 4A). Furthermore, delimiting the substitution in box III to positions -189 to -181 in combination with substituted boxes I and II (strain JS8) had the same severe effect on promoter activity. The substitution from -189 to -181 affected two 5'-CCA-3' motifs that were present in nine copies between -230 and -169. Inspection of the sequence context of CCA motifs led to the consensus sequence 5'-CCAMC-3', which was present in five copies (Fig. 4C, numbers 1, 3, 6, 8, and 9) in the *mig2-5* promoter from positions -233 to -166. Although substitution of three of the CCAMC motifs (numbers 1, 3, and 6) only partially diminished *mig2-5* promoter activity in strain JS10, GFP fluorescence was no longer detected in planta in

strain JS12, in which six of the CCA motifs (numbers 1, 2, 3, 4, 6, and 7) within positions -215 to -169 were substituted by the sequence 5'-GTC-3' (Fig. 4A). This underlined the importance of CCA-containing motifs for inducible *mig2-5* promoter activity. The region from -234 to -209 (box IV), which contained two inverted repeats including the CCAMC motif, markedly contributed to GFP fluorescence in planta (Fig. 4A and C). The consequences of these substitutions on *eGFP* transcript levels in planta also were assessed by Northern analysis (Fig. 4B). This confirmed the severely reduced *mig2-5* promoter activities in strains JS7, JS8, and JS12; however, whereas substitutions in strains JS3 and JS11 had only a limited effect on

Fig. 1. *mig2-5* Promoter deletion analysis. **A**, Schematic representation of the various promoter deletion constructs and their activities in the respective transformants 4 days post inoculation (dpi) (in planta) and during growth in complete medium (CM) (axenic). The numbers indicated on top of the individual promoter constructs refer to deleted regions. Positions refer to the translational start ATG (+1). Relative green fluorescent protein (GFP) activities in culture were determined by fluorimeter analysis. Mean value ($\bar{X}$) and standard error (SE) were calculated from four independent measurements of at least two independent transformants for each construct. Fluorescence levels during biotrophic growth were evaluated by microscopy and are indicated by + and - symbols, where - = no detectable GFP fluorescence and + to +++++ = increasing fluorescence levels from faintly detectable to brightly fluorescent. Only levels $\geq$ +++ allowed a detection of fluorescent hyphae in cells underneath the epidermal layer. At least two independent transformants were investigated during growth in planta. **B**, *eGFP* transcript levels in planta were determined by Northern analysis using RNA from infected plant tissue 3 dpi with the strains indicated (for each, one representative strain was selected). JF1ax. refers to RNA from strain JF1 grown in liquid CM. Northern blots were probed with a 32 P-labeled *eGFP* fragment. Transcripts of *eGFP* appeared as doublet, which might reflect different end processing. Radioactive signals were quantified and normalized by comparison with signals obtained after hybridization with the *mig2-5* gene (bottom). The calculated ratio of strain JD1 (negative control) was defined as background. Ratios of the remaining strains were adapted accordingly, with the ratio of strain JF1 (positive control) set to 1. Staining with methylene blue reflects the amounts of total RNA loaded.

GFP fluorescence in planta, *eGFP* transcript levels were barely detected (Fig. 4B). This discrepancy might be explained by the observation that only a minor fraction of hyphae of these strains showed elevated GFP fluorescence in planta, suggesting that boxes III and IV were not decisive under all conditions during biotrophic growth. Together, these results suggest a strong requirement of CCA-containing motifs in *mig2-5* regulation, as well as an interplay between distinct regions on the *mig2-5* promoter. Furthermore, the finding that none of the introduced deletions or substitutions conferred a marked increase in promoter activity during axenic growth may indicate

that the strong increase in *mig2-5* transcript levels in planta is mediated by positively acting *cis*-elements.

Reconstitution of a heterologous promoter conferring plant-inducible expression.

Prompted by the finding that regions essential for *mig2-5* promoter activity contained multiple CCA motifs, we asked whether their copy number determined the extent of plant-inducible expression. A single copy of the 122-bp fragment from -240 to -119 (Fig. 4C) fused to *mfal*_{bas} conferred moderate promoter activity, which was further reduced by a 5' truncation of

Fig. 2. Mapping of transcriptional start positions of *mig2* genes and comparison of *mig2* promoter sequences. **A**, Survey of transcriptional start site positions in *mig2* 5' untranslated regions (UTRs). Numbers (bold) assigned to transcriptional start sites determined by RNA ligase-mediated rapid amplification of 5' cDNA ends (5' RACE) analysis refer to the start ATG (+1). Numbers of sequenced cDNAs is indicated in parentheses. For RACE analysis, RNA was isolated from infected plant tissue 2 days post inoculation with a mixture of strains FB1 and FB2. **B**, DNA alignment of *mig2* 5' UTRs. Numbers refer to the translational start ATG (+1). Gaps have been inserted to increase the number of identities (boxed and light gray). Putative TATA-like elements are marked in dark gray. The two most frequent transcriptional start sites of *mig2* genes are marked in dark gray and denoted by asterisks. Conserved sequence stretches (boxes I to IV) are framed.

this fragment to position -221 resulting in loss of two CCA motifs (Fig. 5A, strains JM20 and 21). In contrast, fusion of four copies of the 122-bp fragment to *mfaI*_{bas} strongly enhanced inducible promoter activity (Fig. 5A, strain JTTC2). Based on the arrangement of CCA motifs in close proximity in box IV (Fig. 4C), we examined whether the multimerization of this box was sufficient to confer strongly increased promoter activity in planta. To this end, constructs pJTTC5 and pJTTC4 were generated. These harbored four and six direct copies of box IV (26 bp) extended to maintain a spacing of half a helical turn. This arrangement was fused to the initial 160 bp of the *mig2-5* promoter to provide for the native promoter context. The control construct pJTTC6 had the same arrangement as in pJTTC5, but with the three 5'-CCA-3' triplets of box IV substituted by the sequence 5'-GTC-3'. Remarkably, GFP fluorescence intensity reached in strain JTTC5 was comparable with strain JF1 in planta, whereas the control strain JTTC6 displayed only weak fluorescence in planta (Fig. 5A and C). Furthermore, reporter gene activity was even more pronounced in strain JTTC4, harboring six copies of box IV (Fig. 5A). These differences also were reflected at the transcriptional level (Fig. 5B). Northern analysis additionally revealed that transcript levels of strain JTTC6 were not elevated compared with strain JT2 (Fig. 1A), emphasizing that inducible promoter activity strictly relied on the presence of CCA motifs. As expected, promoters reconstituted either from *mfaI*_{bas} or the native *P*_{*mig2-5*} from -160 to -1 conferred increased basal activities (Fig. 5A and B); however, these levels were not high enough to account for strong GFP fluorescence in planta (Fig. 5A; C, strains JT2 and JTTC6). During axenic growth, two additional larger transcripts were detected with the *eGFP* probe in strain JTTC5. These are presumed to arise from the reconstituted promoter (Fig. 5B).

Identification of an additional gene related to genes in the *mig2* cluster and comparative expression analysis.

The complete genome sequence of *U. maydis* from the Broad Institute revealed the presence of an additional *mig2* homologue,

which we designated *mig2-6*. The C-terminal 81 amino acid sequence of Mig2-6, with a predicted open reading frame (ORF) sequence encoding 404 amino acids, was 58% identical to its closest homologue, Mig2-4, and shared the eight invariant cysteine residues (data not shown). Searches based on BLAST and PSI-BLAST revealed no homologous entries in the NCBI data base. The *mig2-6* gene resides on chromosome XXI and, thus, is unlinked from the *mig2* cluster, which resides on chromosome XXII of *U. maydis* strain 521. This raised the question of whether *mig2-6* followed the same regulation as the known *mig2* genes. Therefore, we compared expression profiles of *mig2-6* with those of *mig2-2* and *mig2-5*, which previously have been shown to vary in their expression kinetics during biotrophic growth (see above). As judged from Northern analysis, *mig2-6* was not expressed in haploid strains FB1 and FB2 during axenic growth. In contrast to *mig2-2* and *mig2-5*, expression of a weak signal already was detected during filamentous growth of dikaryotic hyphae resulting from a cross of strains FB1 and FB2. Furthermore, whereas *mig2-5* transcript levels culminated 2 dpi, those of *mig2-6* further increased between 2 and 5 dpi. Thus, the expression kinetics of *mig2-6* is related to *mig2-2* (Fig. 6). Strikingly, expression of *mig2-6* exceeded *mig2-5* expression more than sevenfold in tumor tissue (Fig. 6, lane 5). In particular, a comparison between *mig2-6* transcript levels in 5-day-old tumor tissue and during filamentous growth on solid complete medium (CM) charcoal revealed an approximately 2,000-fold increase (Fig. 6, lanes 3 and 5). Thus, among all *mig2* genes, *mig2-6* displays the strongest expression levels in planta. Transcription start sites of *mig2-6* mapped to positions -33 and -35 which, in an alignment of basal promoter sequences, coincided with the two preferred start positions of *mig2-1* to *mig2-5* genes (Fig. 2B and data not shown).

Previous investigation of *mig1* regulation had indicated that *mig1* was subject to repression by *U. maydis* histone deacetylase 1 (Hda1) (Torreblanca et al. 2003). This prompted us to investigate expression of *mig2-2*, *mig2-5*, and *mig2-6* in *hda1* mutant strains. Indeed, transcripts of the *mig2-5* and *mig2-6*

Fig. 3. 3' End analysis of *mig2-5* promoter sequences. Schematic representation of various promoter reconstitution constructs and their activities in the respective transformants 4 days post inoculation (in planta) and during growth in complete medium (axenic). The numbers (referred to the start ATG [+1]) mark truncated or substituted regions (black box in construct pJSTATA). The *mfaI*_{bas} promoter is hatched. Relative green fluorescent protein (GFP) activities in culture were determined by fluorimeter analysis. At least two independent transformants were investigated during biotrophic growth. The rating of GFP activity in planta was referred to strain JF1, which was included as standard.

genes were detected in $\Delta hda1$ strains under culture conditions (Fig. 6). In contrast to the strong *mig2* expression levels achieved in planta, the extent of *mig2-5* and *mig2-6* expression in $\Delta hda1$ strains was drastically reduced. In particular, the relative signal intensities obtained with the *mig2-6* probe in $\Delta hda1$ strains were increased only slightly over the signal during filamentous growth on CM charcoal (Fig. 6; lanes 3, 7, and 8).

Generation of *U. maydis* mutant strains deleted in all *mig2* genes.

U. maydis $\Delta mig2$ strains deleted in the entire *mig2* cluster were not affected in pathogenic development and the ability to

trigger tumor formation (Basse et al. 2002); therefore, we asked whether the existence of *mig2-6* had a redundant function in these mutants. For this purpose, an *mig2-6* replacement construct was introduced into the compatible *U. maydis* strains BUB7 $\Delta mig2$ and FB2 $\Delta mig2$. The resulting $\Delta mig2 \Delta mig2-6$ mutants were not affected in their ability to mate and develop dikaryotic hyphae on solid charcoal-containing medium (data not shown). Maize infection experiments revealed that crossings of $\Delta mig2 \Delta mig2-6$ strains still were able to trigger tumor formation with similar frequencies as induced by combinations of compatible $\Delta mig2$ or the respective wild-type strains (Table 1). To test the effect of the absence of *mig2-6* alone, we addi-

Fig. 4. Identification of *cis*-regulatory elements in the *mig2-5* promoter. **A**, Schematic representation of the various promoter substitution constructs and their activities in the respective transformants 4 days post inoculation (dpi) (in planta) and during growth in complete medium (axenic). Relative green fluorescent protein (GFP) activities in culture were determined by fluorimeter analysis. At least two independent transformants were investigated during biotrophic growth. The rating of GFP activity in planta was referred to strain JF1, which was included as standard. The numbers (referred to the start ATG [+1]) indicated on top of the individual promoter constructs refer to substituted regions (black boxes). **B**, *eGFP* transcript levels in planta were determined by Northern analysis using RNA from infected plant tissue 3 dpi with the strains indicated. Northern blots were probed with a 32 P-labeled *eGFP* fragment. Radioactive signals were quantified and normalized (JF1 = 1) by comparison with signals obtained by hybridization with the *mig2-5* gene. Staining with methylene blue reflects the amounts of total RNA loaded. **C**, Distribution of repetitive motifs in the *mig2-5* promoter from -240 to -119 referred to the start ATG (+1). Elements matching the consensus 5'-CCAMC-3' are denoted by arrows. CCA base triplets are boxed in light gray (+ strand) and dark gray (- strand) and consecutively numbered from 1 to 9. Boxes I to IV are marked by brackets. The two inverted repeats of box IV are framed.

Fig. 5. Reconstitution of a plant-inducible promoter. **A**, Schematic representation of the various promoter constructs and their activities in the respective transformants 4 days post inoculation (dpi) (*in planta*) and during growth in complete medium (CM) (axenic). Relative green fluorescent protein (GFP) activities in culture were determined by fluorimeter analysis. At least two independent transformants were investigated during biotrophic growth. The rating of GFP activity in planta was referred to strain JF1, which was included as standard. *mig2-5* Promoter regions were fused to either *mfaI*_{bas} or a proximal *mig2-5* promoter element from -160 to -1 referred to the start ATG (+1). The number of multiple elements is indicated on the left. **B**, *eGFP* transcript levels in planta were determined by Northern analysis using RNA from infected plant tissue 4 dpi with the strains indicated. JTTC5ax. refers to RNA from strain JTTC5 grown in liquid CM. Northern blots were probed with a ³²P-labeled *eGFP* fragment. Radioactive signals were quantified and normalized (JF1 = 1) by comparison with signals obtained by hybridization with the *mig2-5* gene. Staining with methylene blue reflects the amounts of total RNA loaded. **C**, Comparison of GFP fluorescence in strains JF1, JT2, JTTC5, and JTTC6 during biotrophic growth. For each, one representative picture is shown. Maize tissue samples were assayed by differential interference contrast light microscopy (DIC) and epifluorescence (GFP). Hyphae are marked by arrows. Exposure time for fluorescence photography is indicated in the upper left corner of the panels. All pictures were prepared using Adobe Photoshop and applying identical processing functions, with only those that could be applied equally to all pixels of the image being used. The bar (10 μ m) refers to all panels.

tionally inoculated maize plants with the combination BUB7-Δ*mig2*Δ*mig2*-6 × FB2Δ*mig2*-6. Again, tumor formation frequency was not affected compared with control combinations in the presence of *mig2*-6. In addition, for all combinations, the formation of teliospores was detected in infected tumor tissue (data not shown). In summary, this indicated that *mig2* genes are not essential for pathogenic development of *U. maydis*.

DISCUSSION

Our investigation allowed the identification of a *cis*-acting element in *U. maydis mig2* promoters specifically activated during the biotrophic phase. In particular, we have shown that

Fig. 6. Expression analysis of *mig2* genes in dependency on the developmental stage and in regulatory *Ustilago maydis* mutants. RNA was isolated from *U. maydis* strains indicated above. Strains or strain combinations denoted with “ax.” were cultivated either in liquid complete medium (CM) (lanes 1, 2, and 7, 8) or on solid CM charcoal medium (lane 3) before RNA isolation. Lanes 4 to 6, RNA was isolated from either infected maize tissue 2 days post inoculation (dpi) (lane 4) or tumor tissue 5 dpi (lane 5) with strains FB1×FB2, and from 10-day-old noninfected maize leaf tissue (lane 6). Each gel was loaded with identical amounts of the same RNA preparations. Northern blots were hybridized with identically concentrated ³²P-labeled gene-specific fragments of *mig2*-2, *mig2*-5, and *mig2*-6. Radioactive signals were quantified and normalized by comparison with signals obtained after hybridization with the constitutively expressed *U. maydis ppi* gene. Pictures (*mig2*-2, *mig2*-5, *mig2*-6, and *ppi*) were prepared using ImageQuant applying identical processing functions and only those that could be applied equally to all pixels of the image were used. Staining with methylene blue reflects the amounts of total RNA loaded.

Table 1. Pathogenicity of *Amig2* strains

Inoculum	Inoculated plants ^a	Plants with tumors/% ^b
BUB7Δ <i>mig2</i> Δ <i>mig2</i> -6#1 × FB2Δ <i>mig2</i> Δ <i>mig2</i> -6#1	20	14/70
BUB7Δ <i>mig2</i> Δ <i>mig2</i> -6#1 × FB2Δ <i>mig2</i> Δ <i>mig2</i> -6#2	20	10/50
BUB7Δ <i>mig2</i> Δ <i>mig2</i> -6#2 × FB2Δ <i>mig2</i> Δ <i>mig2</i> -6#1	20	9/45
BUB7Δ <i>mig2</i> Δ <i>mig2</i> -6#1 × FB2Δ <i>mig2</i> -6	20	10/50
BUB7Δ <i>mig2</i> × FB2Δ <i>mig2</i>	18	8/44
BUB7 × FB2	17	8/47

^a Plants were inspected 7 days post inoculation.
^b Number of plants possessing at least one tumor and respective percentage.

iterated elements containing a 5'-CCA-3' motif account for inducible *mig2*-5 promoter activity. Furthermore, we identified another *mig2* homologue, *mig2*-6, that is unlinked to the *mig2* cluster genes but displays the most pronounced plant-specific expression profile among *mig2* genes. Regulation of *mig2* genes is additionally controlled by *U. maydis* Hda1; however, gene induction in *hda1* mutant strains was only weak compared with the strong increase mediated by positively acting *cis*-elements. Despite their extensive upregulation during pathogenic growth, *mig2* gene products are not essential for pathogenic development of *U. maydis* in the host plant maize.

Identification of cis-acting regions in the mig2-5 promoter.

The system applied to assess inducible *mig2*-5 promoter activity relied on ectopic integrations of *P_{mig2-5}-eGFP* fusions within the *ip* locus. This system maintained promoter specificity as judged from a comparison of promoter activities in strain JF1 during biotrophic and axenic growth, and in strain JD1, which served as negative control (Fig. 1A). With the help of this system, we showed that elements specifying strongly inducible promoter activity resided within a 350-bp fragment, which could be further delimited to 240 bp without losing the ability to confer inducible expression. By deletion analysis, elements required for inducible expression were located between –234 and –161, while adjacent regions on either side contributed to inducible promoter activity.

Promoter reconstitution experiments based on the *mfaI*_{bas} promoter revealed that sequences from –86 to –1 bp of the *mig2*-5 promoter were dispensable for inducible gene expression, whereas the region between –119 and –86 significantly contributed to inducible promoter activity. This region contained a TLE that was present in all *mig2* core promoters and whose spacing to the predicted Inr element was consistent with eukaryotic core promoter structures (Smale and Kadonaga 2003). The TLE appeared to be specifically required for strongly elevated *mig2*-5 promoter activity in planta (Fig. 3A), suggesting that inducible *mig2*-5 promoter activity relies on cooperation with a putative TATA box-binding protein.

Cooperation of distinct cis-regions in the mig2-5 promoter.

To get insight into common *mig2* promoter sequence requirements and prompted by the highest *mig2*-6 transcript levels in planta, we compared the presence and sequence context of CCA motifs in the *mig2*-6 and *mig2*-5 promoter sequences between –500 and predicted TLEs (Fig. 2). This showed the presence of 17 CCA motifs within the *mig2*-6 region and 29 copies in the *mig2*-5 region. Remarkably, the 5' sequence context of the CCA motif was more conserved in the *mig2*-6 promoter region, leading to the consensus sequence MNMNW NCCAMM (Fig. 7). If one mismatch was allowed, this consensus was fulfilled for 12 of the *mig2*-6, 9 of the *mig2*-5, but only 4 of the 11 CCA-containing motifs in the critical *mig2*-1 promoter region from –428 to –203 (Basse et al. 2002). This underscores that the number of this consensus motif correlates with the *mig2* promoter activity in planta. Furthermore, we no-

ticed the iteration of five of these motifs separated from each other either by one or half a helical turn in the *mig2-6* promoter sequence (Fig. 7; positions –289 to –234). This suggests additional criteria to achieve high-level promoter activity in planta and leads to a number of testable predictions aimed to further pinpoint *mig2* promoter sequence requirements. In addition to *mig2* promoter elements specifying the expression strength in planta, *mig2* promoters also must account for differences in expression kinetics observed among *mig2-2*, *mig2-5*, and *mig2-6* genes. Because the *mig1* promoter conferred a similar plant-specific expression profile (Basse et al. 2000), we asked for the presence of the *mig2-6* promoter consensus motif. Indeed, the *mig1* promoter contained 15 CCA-containing motifs between –519 and –100, and 4 of these matched the MNMNWNCCAMM sequence with not more than one mismatch (data not shown). This suggests that common transcription factors are involved in *mig1* and *mig2* regulation.

The importance of CCA-containing motifs for inducible promoter activity was explicitly confirmed for *mig2-5*. The region upstream of position –160 comprising boxes III and IV appeared to contain the elements crucial for inducible promoter activity. This was particularly evident from the deletion spanning positions –211 to –172 (Fig. 1, strain JD16) as well as from the substitutions of boxes III (–189 to –173) and IV (–225 to –233). Boxes III and IV contain multiple CCA motifs that are present in nine copies in the region from –230 to –169. The context of five

of these motifs was consistent with the CCAMM motif derived from the *mig2-6* promoter (Fig. 4C). Only a substitution of the majority of CCA motifs within the region from –215 to –169 abolished inducible *mig2-5* promoter activity (Fig. 4A, strain JS12), suggesting recognition of additional CCA motifs that deviate from the CCAMM sequence. The palindromic motif identified within the *mig2-5* promoter region (box IV) was only poorly conserved in the other *mig2* promoters (Fig. 2B). For this reason, we consider it unlikely that such an arrangement is prerequisite for inducible *mig2-5* promoter activity. The importance of CCA motifs was further substantiated by their requirement for strongly inducible promoter activity conferred by multimerization of box IV. In contrast, CCA-containing motifs were absent in boxes I and II (region from –155 to –120), which only contributed to inducible promoter activity. In addition, the clustering of CCA motifs in the *mig2-5* promoter upstream of position –370 may provide an explanation for the weak effect on inducible promoter activity obtained by the deletion spanning positions –370 to –234 (Figs. 1 and 7).

Presently, only a small number of transcriptional regulators specifically operating in plant-pathogenic fungi during host interactions are known. The *Cladosporium fulvum* Avr9 gene is regulated by the protein NRF1, which is a member of the GATA family transcription factors, characterized by a zinc finger DNA-binding domain (Perez-Garcia et al. 2001). Recent analysis of the Avr9 promoter revealed two regions, each con-

<i>mig2-1</i>		<i>mig2-5</i>		<i>mig2-6</i>	
–443 (–)	CCAGGTCCAAGTTGA	–494 (–)	GACCGCCAGCACTC	–413 (–)	CGCAACCCAAGAAAC
–437 (–)	ACGATACCAGGTCCA	–473 (+)	TGATAGCCAGCCAGC	–385 (–)	GCATTCCCACAGTTG
–428 (–)	CACTTGCCAACGATA	–469 (+)	AGCCAGCCAGCCAGC	–371 (–)	ATCGTCCCACATTCG
–417 (–)	GACAGTCCATGCACT	–465 (+)	AGCCAGCCAGCCGCT	–337 (–)	AAGGTACCACTGCGC
–386 (–)	GGAGCGCCAAATGCA	–455 (–)	GAGATGCCAGCGGCT	–289 (–)	TCCAACCCACCCTGT
–381 (+)	GGCGCTCCACTCAAC	–426 (–)	CTGAGGCCACGATCG	–284 (–)	CTGCTTCCAACCCAC
–358 (+)	TAATTGCCAACAGTC	–415 (+)	AGCTGTCCAAGATA	–254 (–)	ATCACGCCATCTACA
–290 (–)	TATCAACCACAAGGC	–390 (–)	ATTC AACCAAAGAG	–244 (–)	ATCTTCCCACATCAC
–276 (+)	CTGTCACCACGTACT	–382 (–)	GGCGAGCCATTCAAC	–234 (–)	ACCCACCCAAATCTT
–260 (+)	TCATTTCCATCGACC	–376 (+)	GCTCGCCCACTCCGT	–230 (–)	TCACCCACCCCAA
–237 (+)	ACACATCCACTTCAC	–361 (–)	GGAGCACCACAGCAC	–219 (+)	GATGTCCCACAGAG
–226 (+)	TCACAACCACAACCT	–317 (–)	AACGCTCCACTTCCT	–171 (–)	ACACAGCCACTCGAA
–212 (+)	TCCTTGCCAAGGCTG	–310 (+)	AGCGTTCCAACCCAC	–142 (–)	CAACTGCCAACGGCG
–186 (+)	ATGAGGCCATCAAAC	–305 (+)	TCCAACCCACACCTT	–135 (–)	AAACTGCCAACTGCC
–136 (+)	GCGATTCCACTGGGC	–285 (+)	CGAGATCCAGTAGCC	–127 (+)	AGTTTGCCAAATGAG
–132 (–)	ACGAGCCCAGTGGA	–278 (+)	CAGTAGCCACATATT	–98 (–)	GCACATCCACGGATC
		–269 (+)	CATATTCCACGCATT	–86 (+)	CTTCGACCAACAAGG
		–256 (–)	ACGAATCCAGAAATG		
		–249 (+)	TCGTTTCCAGTATGT		
		–228 (–)	GGCCACCAACTTGA		
		–225 (–)	CAAGGCCCACTTGA		
		–215 (+)	TTGCAACCATCTTTT		
		–204 (+)	TTTTCCCCACCCATT		
		–200 (+)	CCCCACCCATTGGAC		
		–194 (–)	TGGCGTCCAATGGGT		
		–190 (+)	TGGACGCCAACCGTG		
		–181 (–)	AGGTCACCACGGTTG		
		–171 (+)	CCTGTCCCACCTAGC		
		–115 (+)	GATGCGCCATTCTAT		

$C_4 N_6 A_6 N C_{17} C_{17} A_{17} C_7$
 $A_3 A_6 T_9 A_7 A_6$
consensus

Fig. 7. Distribution of consensus motifs in *mig2* promoter sequences. Sequence contexts of CCA containing motifs in regions from –500 to positions of predicted TATA-like elements are aligned in 5' to 3' orientation. Position numbers refer to the first C nucleotide in the CCA sequence. Orientations are indicated by sense (+) and antisense (–). Nucleotides matching the *mig2-6* promoter consensus sequence are highlighted in gray. Numbers in bold refer to elements matching the *mig2-6* promoter consensus in not more than one mismatch. Numbers assigned to nucleotides in the *mig2-6* promoter consensus sequence refer to the number of matches.

taining two TAGATA motifs in inverted orientation, to be crucial for inducibility of *Avr9* promoter activity by nitrogen starvation in *Aspergillus nidulans* (Snoeijs et al. 2003). In *Fusarium solani*, a binuclear zinc cluster transcription factor (Cys_6Zn_2) recently has been described to bind within a 40-bp regulatory element of the *PDA1* promoter that was sufficient to mediate expression of the pisatin demethylase gene in response to pisatin stimulation (Khan et al. 2003). Furthermore, the expression of cutinase genes in *F. solani* f. sp. *pisi* is mediated by two binuclear zinc cluster transcription factors (Li et al. 2002). However, this type of transcription factor binds to CCG palindromic sequences and, thus, cannot account for binding repeated CCA-containing motifs.

Motifs based on the CCA consensus have been described in a variety of other systems. These include binding proteins of the NF-Y/HAP2,3,4,5 family with members known from *Saccharomyces cerevisiae* (Olesen et al. 1987), *A. nidulans* (Steidl et al. 1999), and higher eukaryotes (Mantovani 1999). They strictly bind to the short consensus element CCAAT and, therefore, do not provide for the factors recognizing the CCAMM consensus sequence of *mig2* promoters. In contrast, members of the Myb transcription factor family, which also recognize CCA-containing motifs like the transcriptional activator P from *Zea mays* binding to the $CC^A/T_{-}ACC$ motif (Grotewold et al. 1994; Martin and Paz-Ares 1997), differ considerably within their DNA-binding specificities (Martin and Paz-Ares 1997). Fungal representatives of this family are the FlbD and Rca-1 proteins from *A. nidulans* and *Neurospora crassa*, respectively (Shen et al. 1998; Wieser and Adams 1995) involved in conidiophore development and morphogenesis. Finally, consensus motifs fitting the *mig2* CCAMM motif are bound by members of the Sp1/KLF-family, a class of C_2H_2 zinc finger domain transcriptional activators involved in development, cell differentiation, and tissue-specific gene expression in vertebrates and *Drosophila* (Philipsen and Suske 1999; Suske 1999).

This restriction of transcription factor classes, together with the availability of the *U. maydis* genome sequence, opens new perspectives to approach the identification of the *mig2* regulator. Explicitly, according to current PFAM domain analysis by the Broad Institute, the *U. maydis* sequence contains at least 9 potential candidates for proteins with a Myb-DNA-binding domain and 23 potential candidates for proteins with a C_2H_2 zinc finger domain related to Sp1 class transcription factors.

The gained insight into the *mig2* promoter structure has furthered our understanding of a regulatory system enabling extensive increase in gene expression during the transition from saprophytic to biotrophic growth, and has provided a basis for a candidate approach to identify the respective regulators.

MATERIALS AND METHODS

Strains and growth conditions.

Escherichia coli K12 strain TOP10 (Invitrogen, Karlsruhe, Germany) was used as host for plasmid amplification. *U. maydis* strains were grown at 28°C in YEPS (Tsukuda et al. 1988), potato dextrose medium (Difco, Sparks, MD, U.S.A.), CM, or minimal medium (Holliday 1974). Mating of compatible strains was performed on solid charcoal containing CM at room temperature for 48 h. Plant infections were done as described (Gillissen et al. 1992) with cv. Early Golden Bantam (Olds Seed, Madison, WI, U.S.A.). Plants were inoculated 6 days after seed potting.

U. maydis strains FB1 (*alb1*), FB2 (*a2b2*), BUB7 (*alb3*), SG200 (*almlfa2bE1bW2*), FB1Δhda1, FB2Δhda1, BUB7Δ*mig2*, and FB2Δ*mig2* have been described (Banuett and Herskowitz 1989; Basse et al. 2002; Bölker et al. 1995; Reichmann et al. 2002). The antibiotics carboxin, hygromycin B, and nourseo-

thricin used for *U. maydis* selection were purchased from Riedel de Haen (Hanover, Germany), Roche (Mannheim, Germany), and Werner BioAgents (Jena-Cospeda, Germany), respectively. All other chemicals were of analytical grade and were obtained from Sigma (Taufkirchen, Germany) or Roth (Karlsruhe, Germany).

DNA and RNA procedures.

Transformation of *U. maydis* followed the protocol of (Schulz et al. 1990). All plasmids were integrated homologously into the *ip* locus (Loubradou et al. 2001). *U. maydis* chromosomal DNA was prepared according to Hoffman and Winston (1987). RNA was isolated using the TRIzol reagent (Invitrogen). Radioactive labeling of DNA was performed with the NEBlot kit (New England Biolabs, Frankfurt a.M., Germany). Visualization and quantification of radioactive signals was done using a PhosphorImager (Storm 840; Molecular Dynamics, Sunnyvale, CA, U.S.A.) and the ImageQuant software (Molecular Dynamics). All other DNA manipulations followed standard procedures as described by Sambrook and Russel (2001). The correctness of all plasmid constructs was verified by sequencing (ADIS, MPI Köln, Germany). Nucleotide sequences were compared using the NCBI database with BLAST and PSI-BLAST (Altschul et al. 1997). Promoter sequences were analyzed using Clustal X (Thompson et al. 1997), the SeqVu program, and the TRANSFAC database. Genomic sequences of *U. maydis* and PFAM domain analysis were retrieved from the first release from the Broad Institute. Assignments of DNA sequences to *U. maydis* chromosomes were performed on the basis of the chromosome map provided in collaboration with the Bayer AG (Leverkusen, Germany). Designation of nucleotide combinations followed the IUPAC code: M = A/C , W = A/T .

DNA fragments.

Gene-specific fragments of *mig2-2* and *mig2-5* have been described (Basse et al. 2002) and were isolated as *EcoRI* fragments from pCR2.1-TOPO. A specific *mig2-6* ORF fragment was amplified from genomic DNA of strain FB1 using the gene-specific primer combination 5'-ACAGGTACTGGTGGCTATGGCG-3' and 5'-GTGAACGACAGGCATCTCTCTC-3' and cloned into pCR4-TOPO (Invitrogen) for subsequent reisolation as *EcoRI* fragment. The *ppi* (peptidyl prolyl isomerase) fragment was used as described (Bohlmann 1996). The *eGFP* fragment has been described (Basse et al. 2000). A 1,939-bp *ip* fragment was isolated from pSL-Cbx(-) (Brachmann 2001).

Plasmids and plasmid constructions.

For subcloning and sequencing, plasmids pBS(-)KSII (Stratagene, La Jolla, CA, U.S.A.), pSL1180 (Amersham Biotech, Freiburg, Germany), and pCR4-TOPO were used. Genomic DNA fragments of the *ip* locus were detected by Southern hybridization using the *ip* fragment. Plasmid pP with the genomic *PstI* fragment encompassing *mig2-4* and *mig2-5* (Basse et al. 2002) was used for amplification of all *mig2-5* promoter fragments.

The *mig2-5* promoter-*eGFP* reporter constructs pJF, pJD, pJS, and pJT were derived from pH3 (Basse et al. 2002). All plasmids contained a carboxin resistance (*ip*) cassette (Keon et al. 1991) as selectable marker in *U. maydis*. The *otef* promoter of pH3 was replaced by the *mig2-5* promoter, which was amplified from plasmid pP using *Pfu* Ultra DNA polymerase (Stratagene) and primers JF1 (5'-GCTCTAGATACGCTGCTC GATAAAG-3') and JF2 (5'-CATGCCATGGTTTCTCGCAGC GTGGCCTGAAG-3') containing *XbaI* and *NcoI* restriction sites, respectively. Plasmid pJF1 was transformed into strains

SG200, BUB7, and FB2 to yield strains JF1, JF2, and JF3, respectively. To generate deletion (pJD) or substitution (pJS) constructs, a polymerase chain reaction (PCR)-mediated mutagenesis approach was performed: overlapping primers in opposite direction, which comprised the deleted or substituted promoter regions, were used in combination with outer primers JF1 and JF2, respectively. Gel-purified fragments were annealed prior to a second PCR using primers JF1 and JF2. PCR products were cleaved with *Xba*I and *Nco*I and ligated into the respective sites of pHD3. In construct pJSTATA, the *P_{mig2-5}* sequence from –101 to –96 (referred to the start ATG) was substituted by 5'-GCTCGC-3'. For substitutions of boxes I and II (pJS4,7,8) the *P_{mig2-5}* sequence from –155 to –120 was replaced by 5'-GTTTATCGGAACTTTGCCTGCATTGGACGGACAATC-3' (substituted sequences are underlined). For the substitution affecting box III (pJS3,7), the *P_{mig2-5}* sequence from –189 to –173 was replaced by 5'-GCGATGTTGCGGATTC-3'. For the substitution in pJS8, the *P_{mig2-5}* sequence from –189 to –181 was replaced by 5'-GCGCGATGTTC-3'. For the substitution in pJS10, the *P_{mig2-5}* sequence from –205 to –167 was replaced by 5'-TGCGTCCATTGGACGCGATGCGTGGTGACCTGTACTATG-3' (substituted sequences are underlined). For the substitution affecting box IV (pJS11), the *P_{mig2-5}* sequence from –233 to –225 was replaced by 5'-CATCGATGC-3'. In construct pJS12 5'-CCA-3' motifs (Fig. 4A, numbers 1, 2, 3, 4, 6, and 7) were substituted by the sequence 5'-GTC-3'. For integrative transformation into the *ip* locus of *U. maydis* strain SG200, plasmids were linearized with *Ssp*I. Single integration events were confirmed by Southern analysis with the *ip* fragment as probe (Brachmann 2001).

Reconstituted *mig2-5* promoter constructs.

For the construction of pJM plasmids, *mig2-5* promoter fragments were cloned into the pUMa13 vector, which is derived from pSP72 and contains the *mfa1* basal promoter (positions –110 to –1 referred to the start ATG) as a *Bam*HI/*Nco*I fragment fused to *eGFP*. All plasmids contained a carboxin resistance (*ip*) cassette as selectable marker in *U. maydis*. The *mig2-5* promoter regions were amplified from plasmid pP (*Pfu Ultra*; Stratagene) using a forward primer containing an *Xba*I restriction site and a reverse primer containing a *Bam*HI restriction site. PCR products were cleaved with *Xba*I and *Bam*HI and ligated into the respective sites of pUMa13. For integrative transformation into the *ip* locus of *U. maydis* strain SG200, plasmids were linearized with *Ssp*I. Single integration events were confirmed by Southern analysis (Brachmann et al. 2001).

Multimerization of *mig2-5* promoter regions.

Plasmid pJTC2 was derived from pUMa13 and contained four tandem copies of the *mig2-5* promoter sequence from positions –240 to –119. The repeated fragment was amplified (*Pfu Ultra*) from pP using a forward primer containing an *Xba*I restriction site and a reverse primer containing an *Spe*I restriction site and integrated into the respective sites of pBS(–)KSII. The resulting plasmid was cleaved with either *Alw*NI/*Xba*I or *Alw*NI/*Spe*I. Ligation of isolated fragments of 1,009 and 2,202 bp, respectively, resulted in a plasmid containing two tandem copies of the *mig2-5* promoter fragment. The number of fragments was doubled following an additional round of this procedure. Finally, the multimer was excised as an *Xba*I/*Bam*HI fragment and integrated into pUMa13 to yield pJTC2.

For the construction of pJTTC4-6, the *mig2-5* promoter from –160 to –1, referred to the start ATG, was amplified with primers containing *Bam*HI or *Nco*I restriction sites at the 5' ends and ligated into the *Xba*I and *Nco*I sites of pJF1 to yield pJT2B. Complementary oligonucleotides, spanning the box IV

(26 nucleotides) sequence, extended by one nucleotide from the natural sequence context and a *Bgl*II restriction site at the 5' end and two nucleotides from the natural sequence context and a *Bam*HI restriction site at the 3' site, were annealed and ligated in the presence of *Bgl*II and *Bam*HI enzymes (0.5 units each). Multimers were integrated into the *Bgl*II and *Bam*HI sites of plasmid pSL1180. Resulting plasmids containing two or three tandem copies were cleaved with either *Dra*III/*Bgl*II or *Dra*III/*Bam*HI following the procedure applied for pJTC2 (discussed above) to double the number of copies. Multimers were excised with *Bgl*II/*Bam*HI from the resulting plasmids and integrated into the *Bam*HI site of pJT2B to yield plasmids pJTTC4-6. These contained four (pJTTC5 and pJTTC6) or six (pJTTC4) tandem copies of the box IV element with 5'-CCA-3' substituted by 5'-GTC-3' in pJTTC6.

Determination of *mig2* transcription start sites.

For determination of the transcription start sites of all *mig2* genes, the method of 5' RACE was applied using the GeneRacer Kit according to the manufacturer's protocol (Invitrogen). This method relied on the amplification of capped mRNAs. cDNA fragments from infected plant material 2 dpi were amplified using gene-specific primers against the individual genes. A second PCR run was performed with nested primers. Resulting distinct bands were purified by gel extraction and cloned into pCR4-TOPO for sequencing.

Deletion of *mig2-6*.

The generation of a deletion spanning the *mig2-6* ORF from positions 42 to 1,178 was performed by a PCR-based approach (Kämper 2004). DNA fragments of approximately 1 kb flanking the deleted region were amplified from chromosomal DNA of *U. maydis* strain 521 and ligated to a nourseothricin resistance cassette isolated as *Sfi*I fragment from pMF1-n (A. Brachmann, J. König, C. Julius, and M. Feldbrügge, *unpublished*). The resulting deletion construct was transformed into *U. maydis* strains FB2, FB2Δ*mig2*, and BUB7Δ*mig2*. Homologous integration events were confirmed by PCR as described (Kämper 2004). Strain BUB7Δ*mig2*Δ*mig2-6*#2 was obtained from the sexual progeny of a cross from strains BUB7Δ*mig2* and FB2Δ*mig2*Δ*mig2-6*.

Microscopy.

Microscopy and processing of images was performed as described (Basse et al. 2000). Inducible promoter activities were assessed according to relative GFP fluorescence intensities of hyphae within epidermal cells of infected leaves using strain JF1 as standard in all microscopic investigations. At least three leaf sections derived from individual plants were analyzed to assess promoter activity conferred by each construct. Relative fluorescence intensities were rated from – to +++++ (Fig. 1).

Fluorimeter measurements.

GFP fluorescence was determined using a Safire fluorimeter (TECAN) according to Kaffarnik and associates (2003). The fluorescence of the wild-type SG200 strain served as blank and was subtracted from each value for subsequent calculation of mean values and standard errors.

GenBank accession number.

The identified *mig2-6* gene has been deposited in the GenBank under accession number XM_403741.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

TRANSFAC database: www.gene-regulation.com
 Broad Institute: www.broad.mit.edu/annotation/fungi/ustilago_maydis