The distal GATA sequences of the sid1 promoter of *Ustilago maydis* mediate iron repression of siderophore production and interact directly with Urbs1, a GATA family transcription factor

Zhiqiang An¹,², Baigen Mei¹,³, Walter M. Yuan¹ and Sally A. Leong¹,⁴,⁵

¹Department of Plant Pathology, University of Wisconsin and
²USDA-ARS Plant Disease Resistance Research Unit, 1630 Linden Drive, Madison, WI 53706, USA
³Present address: Department of Biological Resources Development, ChemGenics Pharmaceuticals Inc., Building 300, One Kendall Square, Cambridge, MA 02139, USA
⁴Present address: Promega Corp., 2800 Woods Hollow Road, Madison, WI 53711, USA
⁵Corresponding author

The sid1 and urbs1 genes encode L-ornithine N⁵-oxygenase and a GATA family transcription regulator, respectively, for siderophore biosynthesis in *Ustilago maydis*. The basic promoter and iron-regulatory sequences of the *U.maydis* sid1 gene were defined by fusing restriction and *Bal31* nuclease-generated deletion fragments of the promoter region with the *Escherichia coli* β-glucuronidase (GUS) reporter gene. Sequences required for basal expression of sid1 mapped within 1043 bp upstream of the translation start site and include the first untranslated exon and first intron. Sequences needed for iron-regulated expression of sid1 were localized to a 306 bp region mapping 2.3 and 2.6 kb upstream of the ATG. The 306 bp region contains two G/TGATAA sequences, consensus DNA binding sites of GATA family transcription factors. Deletion or site-directed mutation of either or both GATA sequences resulted in deregulated expression of sid1. *In vitro* DNA binding studies showed that Urbs1 binds to the 3' -GATA site in the 306 bp iron-responsive region. However, deletion of 1.1 kb between the distal GATA sites and the basal promoter region led to deregulated expression of GUS, indicating that these GATA sequences are by themselves insufficient to regulate sid1. *In vitro* DNA binding and *in vivo* reporter gene analysis revealed that siderophores are not co-repressors of Urbs1.

**Keywords:** DNA looping/electrophoretic mobility shift assay/epitope tagging/fungi/zinc finger

**Introduction**

Iron is one of the most abundant elements in nature, but its availability to living organisms is very limited due to the extremely low solubility of ferric ion ([Fe⁴⁺] = 10⁻¹⁸ M at pH 7) in aerobic environments (Griffiths, 1987; Williams, 1990). Ferrous iron under physiological conditions tends to oxidize, hydrolyze and polymerize, forming insoluble ferric hydroxide and oxyhydroxide polymers (Williams, 1990). On the other hand, too much cellular iron is harmful because iron possesses unfilled d atomic orbitals and is able to undergo changes in oxidation state involving one electron. This easy access to two oxidation states allows iron to react with oxygen and produce hydroxyl radicals (Halliwell and Gutteridge, 1984; Imlay and Linn, 1988). Hydroxyl radicals react at an extremely high rate with most organic molecules found in cells, in particular, attacking and destroying cell membranes and DNA (Halliwell and Gutteridge, 1984).

To acquire iron from the environment and, at the same time, avoid toxicity from excess cellular iron, organisms have developed various elaborate biological systems to coordinate iron transport and homeostasis (Griffiths, 1987). In plants, iron is transported in the sap in the form of complexes with low molecular weight chelators, such as citrate and malate. In mammals, iron is mobilized to different tissues via the iron binding protein transferrin. In egg white, iron is stored in the iron binding protein ovotransferrin. Most microorganisms produce and secrete a class of low molecular weight ferric iron chelating agents termed siderophores to gather environmental iron (Bagg and Neillands, 1987; Mei and Leong, 1994). In general, siderophore biosynthetic genes are negatively regulated by iron (Bagg and Neillands, 1987; Mei and Leong, 1994). One of the few microorganisms which do not produce siderophores is the yeast *Saccharomyces cerevisiae* (Klausner and Dancis, 1994). In *S.cerevisiae*, several factors, including two membrane-bound ferric reductases FRE1 (Dancis et al., 1992) and FRE2 (Georgatsou and Alexandraki, 1994), a ferric ion transporter, FTR1 (Stearman et al., 1996) and a membrane-associated multicopper oxidase/ferrroxidase FET3 (Askwith et al., 1994; DeSilva et al., 1995), work together to mediate iron uptake under iron starvation conditions. Like siderophore biosynthetic genes, FRE1, FRE2, FTR1 and FET3 are negatively regulated by iron (Dancis et al., 1992; Askwith et al., 1994; DeSilva et al., 1995). This regulation is mediated by AFT-1 (Yamaguchi-Iwai et al., 1996), a transcription activator that binds to the promoters of these genes in the absence of iron.

*Ustilago maydis* produces two cyclic hexapeptide siderophores, ferrichrome and ferrichrome A (Budde and Leong, 1989). The first step in ferrichrome and ferrichrome A biosynthesis is catalyzed by ornithine N⁵-oxygenase (Wang et al., 1989; Mei et al., 1993). The gene encoding ornithine N⁵-oxygenase (*sid1*) has been characterized (Mei et al., 1993). Siderophore biosynthesis in *U.maydis* is negatively regulated by the iron concentration in the growth medium. Urbs1, a transcription regulator of siderophore biosynthesis in *U.maydis*, contains two zinc finger motifs characteristic of the GATA family of transcription factors (Voisard et al., 1993). Disruption of *urbs1* leads to constitutive expression of *sid1* (Mei et al., 1993), suggesting that Urbs1 may directly interact with the *sid1* promoter to repress biosynthesis of siderophores in *U.maydis* when cellular iron is not limiting.
In this communication we describe the structural analysis of the sid1 promoter and document the specific interaction of Urb1 with sequences in this promoter. Two GATA motif I.6 kb upstream of the transcription initiation site in the sid1 promoter are required for iron-regulated expression of sid1. In vitro electrophoretic gel mobility shift assay indicated that Urb1 interacts directly with one of the GATA sequences. Finally, a model involving DNA looping for the regulatory action of Urb1 on sid1 expression is discussed.

Results

sid1 promoter analysis

To delineate the functional domains in the sid1 promoter region, seven convenient restriction fragments in the sid1 promoter region (pCG2.8, pCGC, pCG2.0, pCG1.6, pCGPN, pCGS1 and pCGPS) were fused with the GUS reporter gene (Gallagher, 1992), which encodes the E.coli β-glucuronidase in the U.maydis self-replicating vector pCM54 (Tsukuda et al., 1988; Figure 1A). Both wild-type UM001 (strain 518) and a urbs1 disruption mutant UMC015 were transformed with these constructs and β-glucuronidase activities of the transformants were determined on plates. The results of these experiments are summarized in Figure 1A. A 0.7 kb fragment between 2.3 (pCG2.0) and 3 (pCG2.8) kb upstream of the translation start site was required for iron-regulated expression of sid1. The basic promoter activity was mapped within 1.27 kb (pCGS1) upstream of the translation start site. Sequences containing the 654 bp intron which is located between the transcription and translation start sites of sid1 were also required for basal expression of the gene (pCGPN and pCGPS). Deletion of the −2285 to −1627 region (pCGC), which is located downstream of the 700 bp iron-responsive sequence and upstream of the basic promoter of sid1, abolished iron-regulated expression of sid1. Fusions between GUS and promoter fragments in the wrong orientation were constructed as controls. None of these constructs gave GUS activity (Figure 1A).

To further define the promoter region of sid1, a set of progressive deletions of the 3 kb sid1 promoter region was created by Bal31 nuclease treatment (p1007–p1044; Figure 1B). The sequence for basal expression of the gene was further mapped within a 228 bp fragment between −1271 (pCGS1) and −1043 bp (p1028) upstream of the translation start site (Figure 1A and B). Sequences required for iron-regulated expression of sid1 were localized to a 306 bp region mapping 2.3 (pCG2.0) and 2.6 (p1011) kb upstream of the ATG (Figure 1A and B). This 306 bp fragment contains two G/TGATA sequences separated by 23 bp (Figure 1C).

To eliminate copy number and position effects, three Bal31 nuclease-generated promoter–GUS constructs (p1010, p1013 and p1028; Figure 1B) were introduced into the sid1 locus of U.maydis by gene replacement. GUS activity of strain UMS115, which has the reporter gene under the control of the 2.7 kb sid1 promoter region, was repressed 16-fold in cells grown on high iron medium (Figure 1D). When a fragment containing the two upstream G/TGATA sequences was removed from the sid1 promoter in strain UMS115, GUS activity became constitutive and was no longer regulated by iron concentration in the medium (Figure 1D). In strain UMS117, the 228 bp fragment required for basal expression of sid1 was deleted. As expected, no GUS activity was detected in this strain (Figure 1D). Results from these experiments confirmed the observations made with fusion constructs in the self-replicating vector and GUS plate assay.

Epitope tagging of urbs1 and detection of Urbs1 protein in U.maydis

To confirm that urbs1 encodes an expressed protein in U.maydis, the influenza hemagglutinin antigen (YPYDVPDYA)3 was translationally fused as an epitope at the C-terminus of Urb1. The epitope-tagged Urb1 was constructed in the U.maydis self-replicating vector pCM54 to give pAN15. Biological activity of the epitope-tagged Urb1 was tested by its ability to complement the urbs1 gene disruption mutant UMC015. On high iron medium, UMC015 gives an orange colony phenotype due to the constitutive production of siderophore by this strain. In comparison, UMC015/pAN15 transformants, which carry the epitope-tagged Urb1, displayed a complemented wild-type white colony phenotype on high iron medium (data not shown). This indicates that the epitope-tagged Urb1 is biologically active in U.maydis. pAN15 replicates as a multicopy plasmid; therefore, epitope-tagged Urb1 is expressed in multiple copies in UMC015/pAN15 transformants. To avoid copy number effects, UMUrbs1126, in which the wild-type urbs1 allele was replaced by the epitope-tagged allele, was constructed. Siderophore production by UMUrbs1126 in both low and high iron medium was first determined qualitatively using the color plate assay. Wild-type strain 518, siderophore constitutive production mutant UMC015 and an urbs1 NTG mutants UMC002 were included as controls. The results showed that siderophore production in 518 and UMUrbs1126 is negatively regulated by iron, as indicated by their white colony phenotype on high iron medium (data not shown). In contrast, the two urbs1 mutants, UMC015 and UMC002, displayed an orange colony phenotype on high iron medium (data not shown). Siderophore production by these strains was also determined quantitatively in both low and high iron media (Table 1). These studies further indicate that epitope-tagged Urb1 was biologically functional in U.maydis. A protein of ~110 kDa, the predicted molecular weight of epitope-tagged Urb1, was detected in U.maydis expressing epitope-tagged Urb1 (UMC015/pAN15) by immunoprecipitation/Western blotting using a monoclonal antibody (anti-Ha) to influenza hemagglutinin antigen and protein A–Sepharose (Figure 2). No signal was detected in cells expressing wild-type Urb1 (UMC015/pSC3; Figure 2). Urb1126 was detected at similar levels in both low-iron and high-iron growth conditions (Q.Zhao and S.A.Leong, unpublished findings).

Evidence for direct interaction of Urb1 with the sid1 promoter by electrophoretic gel mobility shift analysis

Since the 306 bp DNA fragment containing the iron-responsive sequence(s) in the sid1 promoter region has two GATA sites and urbs1 encodes a GATA family transcription factor with two putative zinc finger domains, Urb1 may act as a transcription repressor of sid1 by binding to GATA motifs in the sid1 promoter. In vitro
Sid1 promoter analysis. (A) Restriction fragments of the 2.9 kb SspI–PvuII sid1 promoter were fused to the GUS reporter gene carried on the U. maydis replicating vector pCM54 and transformed into U. maydis wild-type (528) and an urbs1 disruption mutant (UMC015). GUS activity was determined colorimetrically as described in Materials and methods, on E medium containing no added iron or 10 μM FeSO₄. + indicates that strong activity (colonies turned dark blue) and – indicates that no activity (colonies remained white) was seen within 6 h after application of X-gluc to colonies. This assay is only a qualitative measure of promoter activity. (B) A set of progressive deletions of the 3 kb sid1 promoter region created by Bal31 nuclease treatment were fused to the GUS reporter gene. Transformation and GUS activity assay were the same as in (A). (C) Sequence of the iron-responsive region. (D) GUS activities of gene replacement strains carrying the wild-type and deleted sid1 promoters fused to GUS. β-Glucuronidase activities were determined fluorometrically as described in Materials and methods, in whole cell extracts obtained from UMS113, UMS115 and UMS117 cells cultured on minimal liquid medium containing no added iron or 10 μM FeSO₄. Assays were done in triplicate on five independent gene replacement strains for each construct. Activities were corrected by subtracting the fluorescence observed using an extract of the untransformed strain 518, which gave similar levels of background fluorescence at 0 and 30 min to that observed at time 0 for extracts of the gene replacement strains. The filled boxes for p1011 (A) and UMS113 (D) indicate the location of GATA sequences required for iron-regulated expression of GUS, and the GATA boxes are underlined in the sequence showed in (C).
Table I. Siderophore production\(^a\) by wild-type and urbs1 mutants

<table>
<thead>
<tr>
<th></th>
<th>Low iron</th>
<th>High iron</th>
<th>Low iron/high iron ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>518</td>
<td>0.46 ± 0.02</td>
<td>0.03 ± 0.00</td>
<td>15</td>
</tr>
<tr>
<td>UMC015</td>
<td>0.88 ± 0.04</td>
<td>0.26 ± 0.02</td>
<td>3</td>
</tr>
<tr>
<td>UMC002</td>
<td>0.71 ± 0.09</td>
<td>0.26 ± 0.01</td>
<td>3</td>
</tr>
<tr>
<td>UMurbs1(^*)</td>
<td>0.25 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>7</td>
</tr>
</tbody>
</table>

\(^a\)Cells were grown for 48 h at 28°C in low iron medium with or without 10 \(\mu\)M FeSO\(_4\). Culture supernatants were treated with an equal volume of ferric perchlorate solution and the absorbance was read at 495 nm (Budde and Leong, 1989). Analyses were done in triplicate. Data were normalized to equal cell growth using an absorbance at OD\(_{600}\) of 1.0.

DNA binding studies were conducted with the 306 bp GATA-containing DNA fragment and whole cell extracts of UMC015, a urbs1 disruption mutant, and UMC015, carrying a replicating plasmid expressing the wild-type Urbs1 (pSC3) or epitope-tagged Urbs1 (pAN15). To induce Urbs1 to be in an active conformation for siderophore gene repression, whole cell extracts were isolated from cultures grown in high iron medium (10 \(\mu\)M FeSO\(_4\)). As shown in Figure 3, the 306 bp fragment was shifted to give one major band by extracts obtained from strains expressing urbs1 and epitope-tagged urbs1, but not by extracts from the urbs1 null mutant. To determine whether the shifted bands were due to Urbs1, a monoclonal antibody (anti-Ha) to the influenza hemagglutinin epitope was included in the gel shift reactions. As shown in Figure 4, the addition of increasing amounts of antibody reduced the level of the shifted bands in the assays containing the tagged Urbs1 but not in assays containing wild-type Urbs1, indicating that Urbs1 is associated with the gel shift complex. No supershifted band was observed under the conditions of electrophoresis employed.

To determine whether the GATA boxes in the 306 bp fragment are the \textit{in vitro} DNA binding sites for Urbs1, a 63 bp oligonucleotide (oligonucleotide 1 in Figure 5A) encompassing the two distal GATA sites in the iron-responsive sequence of the \textit{sid1} promoter region with whole cell protein extracts of UMC015, UMC015/pSC3 and UMC015/pAN15. UMC015 is an \textit{urbs1} disruption mutant. pAN15 carries the \textit{urbs1} tagged gene on the \textit{U.maydis} replicating vector pCM54 and pSC3 carries wild-type \textit{urbs1} on the same vector. Electrophoretic gel mobility shift assays were done as described in Materials and methods.
was examined by including unlabeled oligonucleotides in the DNA–protein binding reactions. The binding signal between the labeled probe and Urb1 was reduced by the addition of excess unlabeled probe in the reaction (Figure 5B).

The role of the two GATA motifs in the 63 bp oligonucleotide probe was assessed by mutation of one or both sites in the probe (oligonucleotides 2, 3 and 4 in Figure 5A). Mutation of the 5′-GATA site had no apparent effect on the in vitro binding of Urb1 to the probe, while mutation of the 3′-GATA site or mutation of both GATA sites eliminated binding (Figure 5C). To test the in vivo roles of the two GATA sites, three sid1 promoter–GUS constructs which carry mutations at the 5′-GATA (pAY2), 3′-GATA (pAY3) or both (pAY4) sites were introduced into the sid1 locus of U.maydis by gene replacement to give UMS202 (5′-GATA to CTGA), UMS203 (3′-GATA to CTGA) and UMS204 (double mutation). GUS activities expressed by these three strains were determined in both low and high iron growth conditions. UMS113, which has the reporter gene under the control of the wild-type sid1 promoter region, was included as a control. The results are shown in Table II. When the 5′-, 3′- or both GATA sites were mutated to CTGA in the sid1 promoter region in strains UMS202, UMS203 and UMS204, GUS activity was no longer tightly regulated by iron concentration in the medium, as indicated by the low low/iron:high iron ratio (Table II). Mutation of a single GATA site led to partial deregulation of GUS activity, while mutation of both sites led to complete deregulation of GUS activity. In contrast, the GUS activity in strain UMS113, which has the reporter under the control of the wild-type sid1 promoter, was negatively regulated by iron concentration in the medium (Table II).

**Discussion**

This study represents the first comprehensive analysis of a U.maydis gene promoter. The complexity of the sid1 promoter provides a unique opportunity to understand better how U.maydis promoters and transcription factors function. By using GUS as a reporter, three regions in the 3 kb promoter have been defined to be important for expression of sid1. The first region is a 306 bp fragment encompassing two GATA sequences and mapping 1.6 kb from the start of transcription. Deletion of the GATA sequences resulted in deregulated expression of sid1 by iron. This result indicated that these GATA sequences might be iron-responsive elements and the DNA binding sites for Urb1. Since these two GATA motifs are distantly located from the transcription start site, we refer to them as the ‘distal GATA sites’. A second region of 228 bp located 211 bp upstream of the start of transcription is required for basal level expression of the gene. A CAAT box and pyrimidine-rich sequence are both located in this region (Mei et al., 1993). A third region encompassing the first exon and intron of sid1 is also required for basal level expression of the gene. This finding is not surprising, since introns contribute to basic promoter activity as well as transcription regulation in other genes (Crestani et al., 1993; Brown and Taylor, 1994; Corrochano et al., 1995). However, a definitive role for the intron in expression of sid1 will require further mutational studies.

**In vitro** electrophoretic gel mobility shift analysis using synthetic oligonucleotides containing the wild-type and mutated distal GATA sites indicate that Urb1 binds specifically to the distal 3′-GATA sequence located 1.6 kb

---

**Table II. β-Glucuronidase activity produced from wild-type and mutant sid1 promoters fused to the GUS gene**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Low iron</th>
<th>High iron</th>
<th>Low iron/high iron ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>UMS113</td>
<td>15.7 ± 1.8</td>
<td>1.0 ± 0.04</td>
<td>16</td>
</tr>
<tr>
<td>UMS202</td>
<td>13.7 ± 3.3</td>
<td>4.9 ± 0.9</td>
<td>3</td>
</tr>
<tr>
<td>UMS203</td>
<td>17.9 ± 1.7</td>
<td>10.4 ± 1.6</td>
<td>2</td>
</tr>
<tr>
<td>UMS204</td>
<td>19.8 ± 3.0</td>
<td>18.8 ± 1.7</td>
<td>1</td>
</tr>
</tbody>
</table>

*Wild-type sid1 promoter and sid1 promoters containing distal GATA mutations fused with GUS were introduced into the sid1 locus by gene replacement. The genotype of each strain is described in Table III. Assays were done in triplicate on three independent transformants and the experiment was repeated three times. Assay conditions for GUS activity are described in Figure 1 and Materials and methods.*
family transcription factor Nit2 shows a clear preference for binding sites containing two closely apposed GATA elements (Xiao and Marzluf, 1996). Moreover, GATA-1 interactions with palindromic GATA sites are considerably stronger than with those having single GATA elements (Trainor et al., 1996); yet strong binding of Urbs1 to the 5’ distal GATA site was not observed. As a result, we believe that this GATA site may not play an essential role in regulation of sid1. Mutation of this site and analysis of its ability to interact specifically with Urbs1 are underway to confirm this interpretation.

Based on the results available, we propose a model predicting that Urbs1 mediates the formation of a DNA loop in the 1.6 kb promoter region of sid1 which involves the distal GATA and the proximal GATA sites or the two GATA sites in the intron (Figure 6A and D). DNA looping has been reported in the regulation of a large number of prokaryotic and eukaryotic genes (Bellomy and Record, 1990; Schleif, 1992). One of the best studied examples is the AraC-regulated BAD operon in E.coli (Dunn et al., 1984; Martin et al., 1986; Huo et al., 1988). In eukaryotic systems, DNA looping has been shown to play a role in silencing of the mating type genes in the yeast Saccharomyces cerevisiae (Hofoiinn et al., 1989) and enhancer-mediated control of the prolactin gene in the rat (Cullen et al., 1993). Sequences required for basal expression of sid1 are located between the upstream and downstream GATA sites (Figure 6). Formation of a loop between the upstream and downstream DNA sites could impede the activity of basic transcription factors either through steric hindrance or direct interaction of transcription factors with Urbs1 (Herschbach et al., 1994) or by forming a topologically isolated sequence that can no longer bind transcription factors. Mutational studies of the proximal and intronic GATA sites are in progress to assess their role in regulation of sid1. However, loop formation might not involve the interaction of Urbs1 bound to both downstream and distal GATA sites, but rather the interaction of Urbs1 bound to distal GATA sites with another factor bound to the downstream promoter region. In this regard, it is interesting to note that GATA-1 can interact physically with the transcription factors Sp1 and EKLF and that Sp1 is able in vivo to recruit GATA-1 to a promoter in the absence of GATA binding sites (Merika and Orkin, 1995). A comprehensive analysis of the DNA looping model for the regulatory function of Urbs1 is underway to investigate these possibilities.

Since the function of Urbs1 as a transcription repressor of sid1 is mediated by cellular iron concentration, iron may play a direct or indirect role in the conformation, function and/or cellular localization of Urbs1. Efforts are underway to investigate the functional association of iron with domains of Urbs1 purified from U.maydis and to study the subcellular location of Urbs1 and the in vivo binding of Urbs1 to DNA in iron-starved and iron-replete cells. Results from two experiments suggest that siderophores do not act as co-repressors of Urbs1. In vitro DNA binding analysis showed that neither deferrated nor ferrated ferrichrome (present at 1 μg/ml) affected in vitro binding of Urbs1 and the distal GATA site of the sid1 promoter (data not shown). Moreover, expression of the GUS gene in UMS113 was negatively regulated by the iron concentration of the medium (Figure 1D), indicating
that siderophores are not involved in iron-mediated regulation of the sid1 promoter by Urbs1. Since the sid1 structural gene is replaced by the GUS reporter gene, no siderophores are produced in strain UMS113.

Materials and methods

**Ustilago maydis and E. coli strains**

The strains used in this paper are listed in Table III. UMC015, a gene disruption mutant of urbs1, was constructed as follows. The internal 2 kb Stul fragment in pSC3 (Voisard et al., 1993) was replaced by blunt end ligation with the 2 kb XbaI-HindIII fragment of pUble20 containing the Tn3-phleo U.maydis expression cassette (Gold et al., 1994) to give pUWAN41. The XbaI-BamHI fragment of urbs1 in the genome of strain 518 (UM001) was replaced by double cross-over with the XbaI–BamHI fragment of pUWAN41 containing the Tn3-phleo cassette. Gene replacements were scored by screening for the orange phenotype of urbs1 mutants on high iron medium (Voisard et al., 1993) and confirmed by Southern hybridization analysis.

Three BglI nuclease-generated sid1 promoter–GUS fusion constructs from p1010, p1013 and p1028 were introduced into the sid1 locus by double cross-over to give strains UMS113, UMS115 and UMS117, respectively. These constructs were done as follows. The XbaI–SpeI fragment which contains upstream DNA flanking the sid1 locus from pUWAN2 was cloned at the SacII site of pBSK(+) by blunt end ligation to give pUWAN110. The PvuII–EcoRI fragment which contains the downstream DNA flanking the sid1 locus from pUC(7.5) was cloned into the XhoI site of pUWAN110 by blunt end ligation to give pUWAN111. The XhoI fragments which contain various sid1 promoter sequences fused to GUS from p1010, p1013 and p1028 were cloned into the NotI site of pUWAN111 by blunt end ligation to give pUWAN113, pUWAN115 and pUWAN117, respectively. The KpnI–XbaI fragments of pUWAN113, pUWAN115 and pUWAN117 were co-transformed with the HindIII fragment of pUble20, which contains the Tn3-phleo U.maydis expression cassette, into UMS1100, which carries a hygβ marker at the sid1 locus. Gene replacement events were scored by screening for phleoR, hygβ, white colony phenotype on high iron medium (Voisard et al., 1993) and confirmed by Southern hybridization analysis.

Three BalI nuclease-generated sid1 promoter–GUS fusion constructs in the sid1 locus were constructed by the same strategy as used for the construction of UMS113, UMS115 and UMS117. Briefly, The SpeI–PvuII fragments containing site-directed mutations in the GUS reporter gene were fused to the sid1 promoter of pUWAN111 by blunt end ligation to give pUWAN112. The SpeI–PvuII fragments of pUWAN112, pUWAN115 and pUWAN117 were used for transformation into E. coli DH5α. The strains used in this paper are listed in Table III. UMC015, a gene disruption mutant of urbs1, was constructed as follows. The internal 2 kb Stul fragment in pSC3 (Voisard et al., 1993) was replaced by blunt end ligation with the 2 kb XbaI-HindIII fragment of pUble20 containing the Tn3-phleo U.maydis expression cassette (Gold et al., 1994) to give pUWAN41. The XbaI-BamHI fragment of urbs1 in the genome of strain 518 (UM001) was replaced by double cross-over with the XbaI–BamHI fragment of pUWAN41 containing the Tn3-phleo cassette. Gene replacements were scored by screening for the orange phenotype of urbs1 mutants on high iron medium (Voisard et al., 1993) and confirmed by Southern hybridization analysis.

**Subclones of the sid1 locus**

The 2.2 and 7.5 kb HindIII fragments of sid1 (Wang et al., 1989; Mei et al., 1993) were cloned into the HindIII site of pUC18 to give pUCHI12 and pUCHI7 respectively. The 6.4 kb BglII fragment was cloned into the BamHI site of pCM54 (Tsukuda et al., 1998) to give pCM51. The 8.7 kb EcoRI fragment of sid1 was cloned into the EcoRI site of pUWAN1 to yield pUWAN1. pUWAN1 was created by removing the pUC18 116 bp Spel–AscI fragment, Klenow filling and religation.

**Construction of GUS reporter gene fusions with various restriction fragments of the sid1 promoter region**

The 1.8 kb XbaI–BamHI fragment of pNOM123 (a gift of R.P.Oliver) containing the promoterless GUS structural sequence was cloned into the XhoI and BamHI sites of pCM54 backbone (Tsukuda et al., 1998) to give pG. DNA restriction fragments isolated from pUCHI12 were blunt ended and cloned into the unique BamHI site of pUC18 by blunt end ligation to give pCGI16 (1.6 kb NcoI–PvuII), pCGP5 (0.9 kb NcoI–PvuII), pCGPS (0.5 kb SacI–PvuII) and pCG20 (2.1 kb HindIII–PvuII). pCG28 (2.8 kb Spel–PvuII) or pCGI (1.2 kb SacI–PvuII). pCGC was constructed by deleting the 1 kb internal HindIII–SacI fragment of pCG28. pCG28 (–), pCGC (–), pCG20 (–) and pCG16 (–) were controls having the respective fragments present in the opposite orientation.

Plasmids p1007–p1044 were a set of progressive deletions of the 2.9 kb Spel–PvuII sid1 promoter region fused to GUS created by BalI nuclease treatment. p104, the plasmid used for the BalI digestion, was constructed as follows. The Spel–PvuII fragment from pUWAN2 was cloned into the EcoRV site of pBSK(+) (Stratagene, La Jolla, CA) to give pCGI16 (1.6 kb NcoI–PvuII), pCGP5 (0.9 kb NcoI–PvuII), pCGPS (0.5 kb SacI–PvuII) and pCG20 (2.1 kb HindIII–PvuII). pCG28 (2.8 kb Spel–PvuII) or pCGI (1.2 kb SacI–PvuII). pCGC was constructed by deleting the 1 kb internal HindIII–SacI fragment of pCG28. pCG28 (–), pCGC (–), pCG20 (–) and pCG16 (–) were controls having the respective fragments present in the opposite orientation.

Plasmids p1007–p1044 were a set of progressive deletions of the 2.9 kb Spel–PvuII sid1 promoter region fused to GUS created by BalI nuclease treatment. p104, the plasmid used for the BalI digestion, was constructed as follows: The Spel–PvuII fragment from pUWAN2 was cloned into the EcoRV site of pBSK(+) (Stratagene, La Jolla, CA) to give pCGI16 (1.6 kb NcoI–PvuII), pCGP5 (0.9 kb NcoI–PvuII), pCGPS (0.5 kb SacI–PvuII) and pCG20 (2.1 kb HindIII–PvuII). pCG28 (2.8 kb Spel–PvuII) or pCGI (1.2 kb SacI–PvuII). pCGC was constructed by deleting the 1 kb internal HindIII–SacI fragment of pCG28. pCG28 (–), pCGC (–), pCG20 (–) and pCG16 (–) were controls having the respective fragments present in the opposite orientation.

**Construction of epitope-tagged urbs1**

To construct the epitope-tagged urbs1 allele, a NotI site was introduced at the C-terminus of urbs1 by PCR using two synthetic oligonucleotides: P42 (5′-CCAGATCTCAGCCGGCCCTGACGACAGAAGCCCATCGGCC-3′), corresponding to the C-terminal sequence of urbs1 and also containing a NotI site, a stop codon and a BalI site; P43 (5′-GTGCGTCCATGCGACACAG-3′), corresponding to a region upstream of the C-terminus of urbs1 and spanning a NotI site. PCR products from oligonucleotides P42 and P43 using pSC6 (Voisard et al., 1993) as template were digested with NotI and BalI and ligated to the large

---

**Table III. Ustilago maydis and E. coli strains used in this study**

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Source</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ustilago maydis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>518</td>
<td>Holliday, 1974</td>
<td>Wild-type a2 b2</td>
</tr>
<tr>
<td>UMC002</td>
<td>Voisard et al., 1993</td>
<td>urbs1 a2 b2</td>
</tr>
<tr>
<td>UMC015</td>
<td>this study</td>
<td>a2 b2 urbs1:phleo3</td>
</tr>
<tr>
<td>UMFSH008</td>
<td>Mei et al., 1993</td>
<td>a2 b2 sid1::hyg4</td>
</tr>
<tr>
<td>UMS113</td>
<td>this study</td>
<td>a2 b2 sid1::GUS phleo5</td>
</tr>
<tr>
<td>UMS115</td>
<td>this study</td>
<td>a2 b2 sid1::GUS phleo5</td>
</tr>
<tr>
<td>UMS117</td>
<td>this study</td>
<td>a2 b2 sid1::GUS phleo5</td>
</tr>
<tr>
<td>UMS202</td>
<td>this study6</td>
<td>a2 b2 sid1-202::GUS phleo6</td>
</tr>
<tr>
<td>UMS203</td>
<td>this study6</td>
<td>a2 b2 sid1-203::GUS phleo6</td>
</tr>
<tr>
<td>UMS204</td>
<td>this study6</td>
<td>a2 b2 sid1-204::GUS phleo6</td>
</tr>
<tr>
<td>UMMurb1tag2</td>
<td>this study</td>
<td>a2 b2 urbs1::lag hyg6</td>
</tr>
</tbody>
</table>

**Escherichia coli**

| DH5α | BRL Inc., Gaithersburg, MD | f80lacZ DM15 endA1 recA1 hsdR17 (rK– mK+) supE44 thi-1 gyrA96 r V· F· ΔlacZΔM15::Tn10 dcm69 |

---

4Gene replacement strains carrying site-directed mutations of the distal GATA sequences in the sid1 promoter. Mutations 202, 203 and 204 are shown in Figure 5.

1748
Ncol–BglII fragment of pSC6 to give pSC6Not. The 114 bp Not fragment from pGTEPI containing a triple influenza hemagglutinin epitope was then inserted in-frame at the Not site of pSC6Not to give pUWAN13. The epitope-tagged urbs1 was subsequently cloned into the U.maydis self-replicating vector pCM54 by replacing the XbaI–BglII fragment of pSC3 (Voisard et al., 1993) with the XbaI–BglII fragment of pUWAN13 to give pAN15. Orientations of inserts in all the constructs were determined by restriction enzyme digestion and/or DNA sequencing.

Site-directed mutagenesis

The Muta-Gen phagemid in vitro mutagenesis kit from BioRad (Hercules, CA) was employed. The 4.4 kb BamHI fragment of pUWAN2, which contains the sid1 promoter region, was cloned into the BamHI site of pTZ18U to give pUWAN81. The mutagenic primers used to introduce mutations in the GATA sites were oligonucleotides 2, 3 and 4 in Figure 5A for mutation in 5′-GATA, 3′-GATA and both GATA sites, respectively. Mutations were confirmed by DNA sequencing.

Reagents and transformation techniques

A microfuge tube-based procedure (Wang et al., 1988; Voisard et al., 1993) was employed for U.maydis transformation. Standard E.coli procedures were used for all bacterial work (Ausubel et al., 1994). Restriction endonucleases and modifying enzymes were from NEB (Beverly, MA). All reagents from other sources are identified in the text.

Complementation of urbs1 mutants by the C-terminus-tagged urbs1

Wild-type strain S18, urbs1 disruption mutant (UMC015) and urbs1 NTG mutant (528) were transformed with C-terminus-tagged urbs1 (pGTEP1) and the non-tagged urbs1 (urbs1B) by the lithium acetate transformation method. Growth on plates and in liquid media was used to select transformants. Orientation of inserts in all the constructs was determined by restriction enzyme digestion and/or DNA sequencing.

Preparation of U.maydis cellular extracts and SDS–PAGE of protein

U.maydis cells grown at 28°C in 250 ml low iron medium with 10 μM FeSO4 for 5 days were harvested and washed with HEPES buffer (20 mM HEPES, pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol and 10 mM phenylmethylsulfonyl fluoride). Washed cells were resuspended in 1.5 ml HEPES buffer and disrupted by passage through a French pressure cell at 12 000 p.s.i. and then centrifuged at 15 000 g for 20 min at 4°C. Total protein in the supernatant was determined by the Bradford method (Bradford, 1976). A total of 20 μg cellular protein was loaded for each sample and run on 8% SDS–polyacrylamide gels at 10 mA for 2 h. Gels were stained with Coomassie brilliant blue.

Immunoprecipitation of epitope-tagged Urbs1 and Western blotting

NaCl was added to 1 mg total cellular protein in 500 μl HEPES buffer to a final concentration of 500 mM. Five micrograms of anti-Ha (12CA5) monoclonal antibody (Boehringer Mannheim, Indianapolis), specific to the epitope, was added to TBS buffer (20 mM Tris, pH 7.5, and 500 mM NaCl), was then added to the mixture. The mixture was then rotated overnight at 4°C followed by the addition of TBS-equilibrated protein A-Sepharose (Pharmacia, Uppsala, Sweden) for 2 h at 4°C. The beads were washed five times with TBS buffer. After washing with TBS buffer, the beads were resuspended in 20 μl 2× SDS gel loading buffer and boiled for 5 min. The supernatant was subjected to SDS–PAGE.

Proteins in the gel were transferred onto nitrocellulose filters by electrophoresis at 4°C in Tris–glycine buffer at 50 V for 6 h. Protein standards transferred onto nitrocellulose filters were stained with Poncan S and marked. Remaining protein binding sites on the nitrocellulose filters were blocked with 5% non-fat dried milk in 1× TBS buffer at room temperature for 4 h, followed by immunoblotting with 5 μg anti-Ha (12CA5) monoclonal antibody in 1 ml 5% non-fat dried milk at room temperature for 8 h. The blots were washed with 1× TBS and incubated with 5 μl anti-mouse IgG alkaline phosphatase conjugate (Sigma, St Louis, MO) in 5 ml 5% non-fat dried milk at room temperature for 2 h. After the final wash with 1× TBS buffer, the filters were transferred to BCIP/NBT solution (Sigma) for enzymatic detection of conjugated antibody.

DNA binding assays

The 0.3 kb XbaI–HindIII DNA fragments from pT1011 used in the DNA binding assay were end-labeled with [γ-32P]dCTP and Klenow fragment. The sequences of the oligomers used in the DNA binding assay are listed in Figure 5A.

Oligomers were first end-labeled with [γ-32P]dATP and T4 DNA kinase and then annealed with cold complementary oligomers to generate double-stranded DNA probes. For each DNA binding reaction, labeled DNA (1–2 ng, 2–3×106 c.p.m.) was incubated in 20 μl DNA binding buffer containing 20 mM HEPES–KOH, pH 7.9, 50 mM KCl, 2 mM MgCl2, 0.5 μg/ml bovine serum albumin, 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 1 μg poly(dI·dC) (Peleg and Metzenberg, 1994) and 5 μg U.maydis protein extract. Electrophoresis was carried out at 10 V/cm for 2 h using a 1:l dilution of TBE (100 mM Tris base, 89 mM glycine, 0.1 mM EDTA, pH 8.0) as running buffer. Gels were pre-electrophoresed for 2 h before samples were loaded. Following electrophoresis, gels were transferred to Whatman 3 MM paper, dried under vacuum at 80°C and subjected to autoradiography.

β-Glucuronidase assay

Plate assays for β-glucuronidase activity in U.maydis (Gallagher, 1992; Naleway, 1992) were done colorimetrically on E medium (Wang et al., 1989) with or without the addition of 10 μM FeSO4. Cells were grown overnight in 1 ml liquid E medium and then 10 μl spotted on solid E medium. When the colony size reached ~3 mm in diameter, 2 μl X-glu (50 μg/ml in dimethylformamide) was added to the colony and the colonies were incubated at 28°C for color development. Quantitative assay of β-glucuronidase activity in whole cell extracts prepared from liquid cultures was done fluorometrically using 4-methylumbelliferyl β-glucuronide (MUG) as substrate (Gallagher, 1992). Cells from 5 ml culture (low iron with or without 10 μM FeSO4) were centrifuged at 3360 g for 5 min, washed with 5 ml 5% non-fat dried milk at room temperature for 8 h. The blots were washed with 1× TBS and 0.1% SDS–polyacrylamide gels at 10 mA for 2 h. Gels were stained with Coomassie brilliant blue.


Received on August 30, 1996; revised on December 5, 1996


