Research Note

The *Ustilago maydis* *ubc4* and *ubc5* Genes Encode Members of a MAP Kinase Cascade Required for Filamentous Growth

David L. Andrews, John D. Egan, María E. Mayorga, and Scott E. Gold

Department of Plant Pathology, University of Georgia, Athens 30602, U.S.A.

Accepted 6 April 2000.

Ustilago maydis, the causal agent of corn smut disease, displays dimorphic growth in which it alternates between a budding haploid saprophyte and a filamentous dikaryotic pathogen. We are interested in identifying the genetic determinants of filamentous growth and pathogenicity in *U. maydis*. To do this we have taken a forward genetic approach. Earlier, we showed that haploid adenylate cyclase (*uac1*) mutants display a constitutively filamentous phenotype. Mutagenesis of a *uac1* disruption strain allowed the isolation of a large number of budding suppressor mutants. These mutants are named *ubc*, for *Ustilago* *bypass* of *cyclase*, as they no longer require the production of cyclic AMP (cAMP) to grow in the budding morphology. Complementation of a subset of these suppressor mutants led to the identification of the *ubc4* and *ubc5* genes, which are required for filamentous growth and encode a MAP (mitogen-activated protein) kinase kinase kinase and a MAP kinase kinase, respectively. Evidence suggests that they are important in the pheromone response pathway and in pathogenicity. These results further support an important interplay of the cAMP and MAP kinase signal transduction pathways in the control of morphogenesis and pathogenicity in *U. maydis*.

Additional keywords: BYR2, *fuz7*, *nrc-1*, *STE11*.

The fungal pathogen of maize, *Ustilago maydis*, causes corn smut disease. Pathogenicity depends on the transition from the haploid, noninfectious, yeastlike form to the infectious, filamentous dikaryon. The successful formation of the filamentous form occurs when there is mating between haploid cells bearing different alleles at both the *a* and *b* mating type loci (Banuett 1995, and references therein). The correlation of the relationship between pathogenic infection and the morphogenetic switch to the filamentous form provides a useful

paradigm that we have exploited to more fully understand the relationship between filamentous growth and pathogenicity.

The initial step in understanding the relationship of filamentous growth and pathogenicity was made possible by the complementation of a constitutively filamentous mutant (Barrett et al. 1993), which led to the isolation and characterization of the *Ustilago* adenylate cyclase (*uac1*) gene (Gold et al. 1994). Haploid strains in which the *uac1* gene was disrupted with a gene for resistance to hygromycin possessed a stable filamentous phenotype and were nonpathogenic (Barrett et al. 1993). It was found that the exogenous addition of cyclic AMP (cAMP) affected the morphogenesis of both the *uac1* mutant and wild-type cells. Namely, budding growth was restored to the filamentous mutant and bud-site selection and cytokinesis was altered in the wild type.

From these observations it was possible, through mutagenesis of the filamentous *uac1* disruption mutant, to isolate second site suppressor mutants (designated *ubc*, for *Ustilago* *bypass* of *cyclase*) that no longer exhibit the filamentous phenotype. The complementation of several of these mutations has been accomplished with a visual screen in which complemented mutants regain the filamentous phenotype, a phenomenon easily seen on solid media (Gold et al. 1994; Mayorga and Gold 1998).

Genomic clones had previously been identified that defined four specific and non-overlapping *ubc* mutant complementation groups. Initially, the *ubc1* gene was isolated and identified as encoding the regulatory subunit of the cAMP-dependent protein kinase (Gold et al. 1994). This gene was shown to be critical for virulence (Gold et al. 1997). Later, three additional complementing clones (*ubc2*, *ubc3*, and *ubc4*) were isolated (Mayorga and Gold 1998). The *ubc2* sequence does not identify a clear homolog or function (M. Mayorga and S. Gold, *unpublished*) but, as discussed below, the structure of its product suggests that it is involved in protein-protein interactions. Characterization of the *ubc3* gene indicated that it encodes a homolog of the yeast pheromone-responsive MAP kinases Fus3p and Kss1p (Mayorga and Gold 1999). This gene was also identified by a homology-based approach and designated *kpp2* (Muller et al. 1999). Here we report the identification of the *ubc4* gene as a MAP kinase kinase kinase (MAPKK kinase). Additionally, we present data on the complementation of another subset of *ubc*

Corresponding author: Scott E. Gold; Telephone: 1-(706) 542-1259; Fax: 1-(706) 542-1262; E-mail: sgold@arches.uga.edu

Current address of María E. Mayorga: Microbia Inc., 840 Memorial Drive, Cambridge, MA 02139, U.S.A.

Nucleotide and/or amino acid sequence data have been deposited at the GenBank data base as accession number AF197562.

mutants leading to the cloning of a fifth *ubc* gene (*ubc5*). Sequence analysis indicated that *ubc5* is the *fuz7* gene, encoding a *U. maydis* MAP kinase kinase, that had previously been identified by a homology-based approach and shown to be required for pheromone-responsive morphogenesis (Banuett and Herskowitz 1994). Additionally, it was found that the *mfa1* gene is still induced in *fuz7* mutants, suggesting that the *fuz7* gene is dispensable for this aspect of pheromone response (Regenfelder et al. 1997).

U. maydis haploid strains used for this study were 3/5 and 3/6 (*ubc4*, *uac1* double mutant strains), and 3/4 and 3/33 (*ubc5*, *uac1* double mutant strains). The production, growth conditions, and genetic manipulations of these strains were as described (Gold et al. 1994; Mayorga and Gold 1998). Plasmids, the cosmid library, as well as DNA manipulations (other than the Tn5 disruption procedure described below), *U. maydis* transformations, and morphological analyses used for this study were as described in Mayorga and Gold (1998). The cosmid clone (cosubc4) that complemented strains 3/5 and 3/6 was previously isolated (Mayorga and Gold 1998). Complementation of the *ubc5*, *uac1* double mutant strains 3/4 and 3/33 was accomplished by methods previously reported (Gold et al. 1994; Mayorga and Gold 1998) and led to the isolation of the cosmid clone cosubc5.

To locate the functional *ubc4* and *ubc5* genes on their respective cosmids, we used transposon-mediated gene disruption similar to that used previously (Gold et al. 1994) but employing the broad-host-range Tn5 derivative Tn5-B20 (Simon et al. 1989) to infect *Escherichia coli* SK6501 (Aldea et al. 1989), which had previously been transformed with either cosubc4 or cosubc5. Mutagenized cosmids were isolated and introduced via electroporation into *E. coli* DH10B (Gibco BRL, Gaithersburg, MD).

To localize the *ubc4* gene, approximately 100 kanamycin-ampicillin resistant *E. coli* colonies were selected for cosmid isolation and subsequent restriction analysis with *Bgl*III. This enzyme cut the cosubc4 cosmid into eight restriction fragments of different sizes. Also, this enzyme cuts Tn5-B20 twice, liberating a 2.25-kb fragment, while leaving the remaining 1.5-kb and 4.25-kb segments attached to the flanking cosmid DNA. Comparison of *Bgl*III-digested, Tn5-inserted cosubc4 with the wild-type cosubc4 allowed the point of insertion to be estimated by the shift in relative sizes of the resulting bands (data not shown). This analysis enabled 26 cosmids to be selected that appeared to carry Tn5-B20 insertions at locations randomly distributed within cosubc4. Protoplasts of *U. maydis* *ubc4*, *uac1* mutant strains 3/5 and 3/6 were transformed with purified preparations of these mutagenized cosmids and plated. The transformants were visually scored for dimorphic phenotype. If the *ubc4* gene was not interrupted by the transposon, the transformants were complemented and

exhibited filamentous colonies. However, when the gene was interrupted, no complementation occurred and yeastlike colonies were observed. This procedure yielded two Tn5 insertion cosmid clones that gave only yeastlike colonies. These were designated cosubc4-Tn5/17 and cosubc4-Tn5/38 and were the subject of further study. Figure 1 shows the morphology of strain 3/5 (*ubc4 uac1*) transformed with cosubc4 with and without selected Tn5 insertion mutations.

Sequence data were obtained from both cosubc4-Tn5/17 and cosubc4-Tn5/38 with primers (Tn5ISR, 5'-GCCGCACGATGAAGAGCAG-3' and Tn5LacZ, 5'-GTAAAACGACGGGATCCAT-3') designed to extend from the termini of the Tn5-B20 inserted sequence and into the disrupted cosmid sequence. These two insertions were in opposite orientations and sequence data from the Tn5-B20 primers in both isolates had high homology to a region conserved in genes encoding MAPKK kinases from other organisms.

The *ubc4* genomic clone was sequenced. A single, continuous open reading frame encoding 1,166 amino acids was detected with regions of high homology to previously isolated MAPKK kinases. Although there were potential 5' and 3' consensus splice sequences, there was no clear, conserved, internal site for lariat formation, suggesting that introns are lacking in *ubc4*. In addition, 1,000 bp 5' of the ATG start codon and 500 bp 3' of the TAG stop codon were sequenced (GenBank accession number AF197562). One TATA-like sequence was present starting at base 826 and several CAAT-like sequences (upstream of the TATA sequence) were present within the region 5' of the presumed translational start site. The functional relevance of these sequences was not tested. No clear polyadenylation consensus sequence was present in the 500 bp 3' of the translational stop. Within this 5,000-base sequence the insertion sites of the Tn5 transposon for cosubc4-Tn5/17 and for cosubc4-Tn5/38 were between bases 3739 and 3740 (between amino acids 913 and 914) and between bases 2965 and 2966 (between amino acids 655 and 656), respectively. Both mutations would be expected to disrupt the MAPKK kinase catalytic domain function.

BLAST (Altschul et al. 1990, 1997) searches clearly show that Ubc4 is most closely related to MAPKK kinases Ste11p (Rhodes et al. 1990) and BYR2 (Styrkarsdottir et al. 1992; Wang et al. 1991), which are involved in mating responses in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively, and to the *nrc-1* MAPKK kinase from *Neurospora crassa* (Kothe and Free 1998), which is involved in conidiation. The overall structure of Ubc4 indicates two regions of significant amino acid sequence conservation. The strongest conservation lies in the C-terminal catalytic domain (Fig. 2A), indicating that Ubc4 is a typical member of the Ste11p family of MAPKK kinases (Jouannic et al. 1999). Second, a region of weaker homology is found in the N-

Fig. 1. Characterization of morphological complementation and localization by disruption of the complementing gene in cosubc4 and cosubc5 by Tn5 mutagenesis. **A–H**, Liquid medium assay of transformant strains grown 18 h in PDB (potato dextrose broth) containing 50 µg of hygromycin B per ml for plasmid maintenance. Bar in **A** = 50 µm. **A–D**, Strain 3/5 (*ubc4 uac1*) transformants containing (**A**) phyg101, (**B**) cosubc4, (**C**) cosubc4-Tn5/17, (**D**) cosubc4-Tn5/15. **E–H**, Strain 3/4 (*ubc5 uac1*) transformants containing (**E**) phyg101, (**F**) cosubc5, (**G**) cosubc5-Tn5/3A, (**H**) cosubc5-Tn5/2. **I**, Plate assay of transformant strains grown 24 h on charcoal medium. Strain 3/5 or 3/4 was transformed with plasmids indicated. Plasmids cosubc are cosubc4 and cosubc5 for strains 3/5 and 3/4, respectively. Plasmids cosubc::Tn5- are noncomplementing plasmids, cosubc4-Tn5/17 and cosubc5-Tn5/3A for strains 3/5 and 3/4, respectively. Plasmids cosubc::Tn5+ are complementing plasmids, cosubc4-Tn5/15 and cosubc5-Tn5/2 for strains 3/5 and 3/4, respectively.

terminal region that is conserved only in the three fungal MAPKK kinases mentioned above (Fig. 2B). Interestingly, this region has been shown to be critical for genetic interaction of *STE11* with *STE50* in *S. cerevisiae* (Wu et al. 1999). The *STE11-1* allele has a mutation that converts the proline at amino acid 300 (Fig. 2B) to serine, partially eliminating the need for Ste50p. This suggests that this region may have a common regulatory role in these fungal proteins.

As mentioned above, the *ubc5* gene was isolated by its ability to complement strains 3/4 and 3/33. Because we had

identified the *ubc2* (Mayorga and Gold 1998; M. Mayorga and S. Gold, *unpublished data*), *ubc3* (Mayorga and Gold 1998, 1999), and *ubc4* (Mayorga and Gold 1998; this report) genes as likely members of a pheromone-responsive MAP kinase cascade, we anticipated that *ubc5* might also encode a member of, or a protein interacting with, this cascade. This seemed particularly likely because the morphology of mutants possessing the *uac1* disruption in combination with any one of the *ubc2*, *ubc3*, *ubc4*, or *ubc5* gene mutations was clearly distinct from wild type but the double mutants were morphologically

A

Ubc4	700	IKWHK G ALIGAGSFGNVFLG M NAKTGLLMAVKQ V ELP-----
STE11	436	--WLKGACIGSGSFGSVV L G M NAITGELMAVKQ V EIKNNIGVPTDNNKQANSDENNEQE
BYR2	392	IKWIRGALIGSGSFGQV L G M NASSELMAVKQ V ILD-----
<i>nrc-1</i>	388	SMWMK G SLIGQSGSFGSV L ALHAITGELMAVKQ V ETP-----
Ubc4	737	-----S--GDSHL D ORKK G MLEALEREIKLLKSLEHENIVQY L DSFADD S HLNIF
STE11	494	EQQEKIEDVGAVSHPKTNQNI I IKMDAL O HEM N LLKELHHENIVTYGASQEGGN L NIF
BYR2	429	-----SV---SESKDR A K L DALAGEIALLOEL S HEHIVQY L GSN L NSDHLNIF
<i>nrc-1</i>	425	-----PGADSK N DARKK S MEAL K REITLL D LOHPNIVQY L GCS S SAEY L NIF
Ubc4	785	LEYVPGGSIVALLRNYGA F EEPLVRNFVRQILNGLSFLHNRGIMHRDIKGANILVDNK G G
STE11	554	LEYVPGGSVSSMLN N YGP F EE S LITNFTROILIGVAYLHK N IHRDIKGANIL D IK G C
BYR2	476	LEYVPGGSVAGLLTMYGS F EE T LVNFIKQTLKGLV L HSRGIVHRDIKGANILVDNK G K
<i>nrc-1</i>	475	LEYVPGGSVQTM L D O YGAL P ESLVR S FVRQILQGL S YHNRDIHRDIKGANILVDNK G T
Ubc4	845	IKISDFGISK K VESDLV I ATNKSGAGGGGAGGAHRPS L QGSVFWM A PEVVKQTSY T IK A
STE11	614	VKI I DFGISK K I S PLNKKQ N K-----RAS L QGSVFWM S PEVVKQT A TT A K A
BYR2	536	IKISDFGISK K ELN S TSTKT-----GGA-----RPS F QGS S FWM A PEVVKQTMHT E K T
<i>nrc-1</i>	535	IKISDFGISK K LEAT N IL-----NGAN N NKH R PS L QGSVFWM A PEVVKQTSY T R K A
Ubc4	905	DIWSLGCLV V EMISGTH P W A ELNQM Q ALFQIGMGR-KPS L PDEISNECRDFLEK T FEL D Y
STE11	660	DIW S TGCV I EMFTGKH P EP F DSOM Q AIFKIG-TNTT P EP S W A ISEG N FLRK A FEL D Y
BYR2	585	DIWSLGCLV I EM L TSKH P YP N CDOM Q AIFRIG-ENIL P EP S NISS A IDFLEK T F A DC
<i>nrc-1</i>	586	DIWSLGCLV E MMTGTH P EP F CT O L Q AIFKIGG S K A SPT I PD N ASE F A K QFL A Q T F E ID H
Ubc4	964	NNRPSADELL N H A FM
STE11	719	QYRPSA E LL O HP W L
BYR2	644	NLRPTA S ELL S HP F V
<i>nrc-1</i>	646	NKRPSADE L ML S P F L

B

Ubc4	271	IRNFFGQRPPSELINTNLADFF S TD S KALQRTARRSIYGRASAST R SK R
STE11	292	IR O IF N QRPPSEFISTNL A G V FP H TD M KRL O K T M R ES--FRHSARLS I A
BYR2	207	-----QRPPSELIN S RIS D FF D HQ P K L LE K TIS N S R R N LS I R T S---
<i>nrc-1</i>	195	IR O FG L RPPSEL I ASDL T S V FP D HS R E A DR T AR L SM R RSAR L SR V N R

Fig. 2. Ubc4 is a mitogen-activated protein kinase kinase (MAPKK) kinase related to pheromone-responsive and conidiation-specific MAPKK kinases of fungi. Comparison of (A) Ubc4 carboxy terminal catalytic region and (B) N-terminal conserved region with other MAPKK kinases. Accession numbers: Ubc4 (GenBank accession no. AF197562), *STE11* (GenBank accession no. X53431; Rhodes et al. 1990), BYR2 (GenPept accession no. P28829; Stykarsdottir et al. 1992; Wang et al. 1991), and *nrc-1* (GenBank accession no. AF034090; Kothe and Free 1998).

indistinguishable one from another. Therefore, it seemed a reasonable hypothesis that *ubc5* was functionally related to *ubc2* through *ubc4*. One candidate gene that *ubc5* might encode was the previously reported MAP kinase kinase *fuz7* (Banuett and Herskowitz 1994). Strengthening this line of thought, earlier we had shown that a *fuz7* mutation suppressed the filamentous phenotype of the *uac1* mutant (Mayorga and Gold 1999). In fact, the *uac1 fuz7* double mutant was morphologically indistinguishable from the other *uac1* double mutants involving *ubc2*, *ubc3*, or *ubc4*.

With primers (OSGfuz7F, nucleotides -325 to -306 5'-CAGCATCGACAAGACTACG-3'; and OSGfuz7R, nucleotides 1498 to 1517 5'-CAGATCGATGCCTAGAGTGG-3') based on the published *fuz7* gene sequence (Banuett and Herskowitz 1994) a 1.8-kb polymerase chain reaction (PCR) fragment was amplified from several complementing cosubc5 clones that corresponded in size to the expected product derived from the *fuz7* gene. One of these (cosubc5-5.1 from 3/33) was analyzed further. Southern analysis of cosubc5 digested with *HindIII* was performed with the 1.8-kb *fuz7* PCR fragment and identified the expected 0.8-kb internal *fuz7* fragment (data not shown). Through Tn5-B20 mutagenesis, insertions were identified in this hybridizing band. One of these insertion mutants was designated cosubc5-Tn5/3A and sequencing from the *lacZ* primer of Tn5-B20 confirmed that the insertion was within the *fuz7* gene between nucleotides 683 and 684 (within the codon for amino acid D108), five amino acids upstream from the SEL active site, thus presumably disrupting the MAPK kinase gene function (data not shown). Evidence that this was the functional gene responsible for complementation was obtained by the inability of cosubc5-Tn5/3A to complement strain 3/4 or 3/33 (*ubc5*, *uac1*; Fig. 1G). Additionally, Tn5 insertions within the cosmid but outside this hybridizing band were identified. One insertion cosmid analyzed further was designated cosubc5-Tn5/2. Sequencing out from the Tn5-B20 primers of the cosubc5-Tn5/2 showed that the insertion was not within the reported *fuz7* sequence. This plasmid was tested by transformation and found to remain fully capable of complementing strains 3/4 (Fig. 1H) and 3/33 (data not shown).

As stated previously, the *ubc2* through *ubc5* genes were found to encode components of a MAP kinase cascade. The core of a generic MAP kinase module consists of three kinases and in the case of the *S. cerevisiae* pheromone response pathway a scaffold protein (Ste5p) that holds the kinases in proximity to each other. In yeast these cassettes have been shown to be important in the transduction of extracellular signals and to be involved in regulation of pheromone response, polarized growth, and osmotic and nutritional sensing (Banuett 1998; Widmann et al. 1999).

We previously reported the identification of the *ubc3* gene as encoding a pheromone-responsive MAP kinase (Mayorga and Gold 1999). The phenotype of the *ubc3 uac1* double mutant is morphologically indistinguishable from those of the *ubc4 uac1* or the *ubc5 uac1* double mutant strains. The finding that the *ubc4* and *ubc5* genes encode a putative MAPKK kinase and a MAPK kinase, respectively, taken together with the previous analysis that showed the role of *fuz7* (*ubc5*) in pheromone response (Banuett and Herskowitz 1994), strongly suggests that the *ubc4* gene is the most upstream member of the pheromone-responsive MAP kinase cassette in *U. maydis*.

The putative Ubc4 protein also shares a high degree of structural similarity to the pheromone-responsive MAPKK kinases of the yeasts *S. cerevisiae* and *S. pombe*. The *ubc2* gene encodes a product with multiple recognizable protein-protein interaction motifs (M. Mayorga and S. Gold, *unpublished*). The *ubc2* gene is critical for pathogenicity (M. Mayorga and S. Gold, *unpublished*) while the *ubc3* and *fuz7* (*ubc5*) genes are virulence factors not absolutely required for disease development (Mayorga and Gold 1999; Banuett and Herskowitz 1994). We have thus isolated members analogous to the *S. cerevisiae* cascade at each level of the *U. maydis* pheromone-responsive MAP kinase cassette. This functional cassette is required for the filamentous phenotype of the *uac1* mutant and the individual members show important but variable roles in virulence and pathogenicity. This suggests that for specific members of the *U. maydis* cascade there may be genetic redundancy, as is found in *S. cerevisiae* for *KSS1* and *FUS3* in *FUS3* mutants (Elion et al. 1991; Cook et al. 1997; Madhani et al. 1997; Madhani and Fink 1998; Widmann et al. 1999). We have not tested the significance of the *ubc4* gene in pathogenicity but speculate that it will play a role relative to the presence or absence of such genetic redundancy. If there is a second MAPKK kinase that can substitute for *ubc4*, as can *SSK2* and *SSK22* in the yeast hyper-osmolarity sensing MAP kinase pathway (Widmann et al. 1999), it will likely be a relatively weak virulence factor like *ubc3* (Mayorga and Gold 1999). However, if no such redundancy exists, the *ubc4* null mutant may be defective in pathogenicity, like the *ubc2* mutant.

ACKNOWLEDGMENTS

This work was supported by U.S. Department of Agriculture National Research Initiative Grant nos. 95-37303-1706 and 97-35303-4871 to S. E. G. We would like to thank Lori Bennett for assistance early in this project with Tn5 mutagenesis of *ubc4*. We thank Flora Banuett for providing p18F-f7. We thank Alfredo Martinez-Espinoza and Gretchen Kuldau for critical review of this manuscript.

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