Isolation of the Acetyl-CoA Synthase Gene from the Corn Smut Pathogen, *Ustilago maydis*

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The acetyl-CoA synthase (*acuA*) gene from *Ustilago maydis* has been isolated using a fragment of the *acu5* gene from *Neurospora crassa* as a heterologous probe. The *U. maydis acuA* gene transformed *Acu*[−] mutants of *U. maydis* to acetate utilization. Transformation was accomplished by integration of the vector sequences into the chromosomal DNA.

INTRODUCTION

Isolation of the genes expressed during the interaction of a fungal pathogen with its host plant and a knowledge of their regulation will be central to the elucidation of mechanisms unique to the parasitic habit. However, before molecular genetic analysis of such genes can be done, a selection of gene markers needs to be available for gene transfer systems and to aid manipulation of the expression of particular genes of interest.

The basidiomycete maize pathogen, *Ustilago maydis*, has many advantages for studying both genetic and molecular genetic aspects of pathogenicity. It is amenable to manipulation in the laboratory, growing on defined media as small uninucleate haploid colonies. In addition, the genetics and sexual cycle of this pathogen are relatively well understood, allowing strains to be crossed and recombinants to be recovered (Banuett & Herskowitz, 1988; Holliday, 1974).

Integrative and multicopy plasmid vectors have been described for the transformation of *U. maydis* (Tsukuda *et al.*, 1988; Wang *et al.*, 1988). In both cases, the *Escherichia coli* hygromycin B phosphotransferase (*hygB*^R) gene was employed as a selectable marker. However, unlike many other fungi used in molecular genetic studies, homologous wild-type or mutant genes have not yet been developed as selectable markers for gene transfer. The only homologous selectable gene described so far for *U. maydis* is the *pyr3* gene encoding dihydroorotase (Banks & Taylor, 1988).

Here we report the isolation of the acetyl-CoA synthase (*acuA*) gene of *U. maydis* and its use as a selectable marker in the transformation of *U. maydis* mutants unable to utilize acetate as the sole carbon source.

METHODS

Escherichia coli strain and plasmids. *E. coli* strain DH1 (*recA palA hsr hsm*⁺ *endo 1B relA1*) was transformed according to Hanahan (1983). pC₂B1.0 carrying a *Bam*HI fragment of the coding region of the *acu5* gene of *Neurospora crassa* was provided by I. Connerton, University of Reading, UK. The plasmid pUC9 was used as the cloning vector throughout.

U. maydis strain and culture conditions. All manipulations were done using an isolate obtained from the CAB International Mycological Institute, Kew, UK, no. 103761. Cultures were maintained on, and sporidia prepared from, a glucose (10 g l^{−1})/yeast extract (2 g l^{−1}) medium (GYE). Minimal medium was as described by Holliday (1974), except that the carbon source (5 g l^{−1}) and 50 mM-MOPS (final concentration) were added after autoclaving. Mutants unable to utilize acetate as a carbon source were isolated by selection for resistance to fluoroacetate on minimal medium containing 5 g fluoroacetate l^{−1} and 1 g glucose l^{−1}. All solutions were adjusted

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to pH 6.5 and solid media contained 2% (w/v) agar. Cultures were incubated at 25 °C and liquid cultures were shaken at 150 r.p.m.

Isolation and manipulation of DNA. Total *U. maydis* DNA was isolated from sporidia grown in GYE medium for 48 h. The sporidia were harvested from 100 ml cultures by centrifugation at 1000 *g* for 20 min, then washed with 0.6 M-KCl. Protoplasts were prepared by incubating sporidia with 10 ml Novozyme 234 (10 mg ml⁻¹) and Cellulase R10 (10 mg ml⁻¹) in 0.6 M-KCl, for 45–60 min at 30 °C. The protoplasts were centrifuged at 1000 *g* for 20 min and then washed once with 0.6 M-KCl. DNA was isolated from the protoplasts as described by Rothstein (1985), with the exception that phenol/chloroform extraction was performed twice. This procedure yielded approximately 20 µg DNA.

Standard methods were used for other DNA manipulations (Maniatis *et al.*, 1982).

Conditions for Southern transfer and colony hybridization. The transfer of *U. maydis* genomic DNA from agarose to nylon filters (Hybond-N, Amersham) was in accordance with the manufacturer's instructions. Conditions to obtain specific hybridization between the *N. crassa* fragment and *U. maydis* DNA were optimized as described by Howley *et al.* (1979). Colony hybridization was as described by Maniatis *et al.* (1982) and the conditions used were similar to those for Southern hybridization. Analysis of DNA from transformants was done using standard procedures (hybridization in 5 × SSC at 65 °C and washes to 0.1 × SSC, 0.1% SDS; 1 × SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate).

Preparation and transformation of *U. maydis* protoplasts. Sporidia were grown overnight in GYE medium (100 ml) and protoplasts were isolated and transformed as described by Wang *et al.* (1988), with some minor modifications. Sporidia were incubated with Novozyme 234 (5 mg ml⁻¹) for 20–30 min and protoplasts were recovered by centrifugation at 1000 *g* for 10 min. Following transformation, the protoplasts were plated out directly on to minimal medium containing sodium acetate and 1 M-sorbitol. On this medium it was not possible to clearly differentiate the transformed colonies from background growth, since sorbitol supports limited growth of *U. maydis*. After 5 d the colonies that grew were replica plated on to minimal medium containing sodium acetate alone and acetate-utilizing colonies appeared 4–5 d later.

RESULTS AND DISCUSSION

A random primed labelled (Feinberg & Vogelstein, 1983) 1.0 kb *Bam*HI fragment of the *N. crassa acu5* gene (Thomas *et al.*, 1988) was used as a hybridization probe against Southern blots of *U. maydis* genomic DNA (5 µg), digested with a number of different restriction enzymes. Using a stringency allowing hybridization of sequences with approximately 75% homology (30% formamide in the hybridization buffer at 35 °C and washes at 56 °C in 2 × SSC, 0.5% SDS), the *N. crassa* fragment hybridized to a single *Hind*III fragment (7.5 kb). At this stringency, a number of weaker background signals were also observed. *U. maydis* DNA (25 µg) was digested with *Hind*III and fragments 6.0–8.0 kb in size were isolated. A partial library (Nicholls, 1986) containing this *Hind*III fragment was constructed in the plasmid pUC9, and positive clones were identified by hybridization to the *N. crassa acu5* gene fragment. One clone (pUCH2) was recovered which contained a *U. maydis Hind*III fragment that gave a strong hybridization signal with the *N. crassa* probe. A partial map of this *U. maydis Hind*III fragment is shown in Fig. 1.

Acetate non-utilizing (*Acu*⁻) mutants were isolated by positive selection for resistance to fluoroacetate as described by Apirion (1965). Resistant colonies (*fac* mutants) appeared after incubation for 6–8 d. Only *fac* mutants unable to grow on media containing acetate as the sole carbon source were selected for transformation. Four separate *Acu*⁻ mutants (A7615, A7617, A76120 and A76121) were transformed with 1 µg of either pUC9 or pUCH2. Colonies capable of growing on acetate medium were obtained with all the mutants transformed with pUCH2, at frequencies ranging from 5 to 20 colonies per µg DNA. No growth occurred with any of the mutants when pUC9 was used as the transforming DNA. The transformants were mitotically stable when grown in the absence of acetate.

Six transformants from three different mutants (A7615, A7617 and A76121) were selected for Southern blot analysis. In the lanes containing undigested DNA from the transformants (Fig. 2a), radiolabelled pUC9 hybridized, with one exception (lane 5), to bands corresponding to undigested DNA. This indicates that transformation had occurred by integration of the vector sequences into the chromosomal DNA. No homology was detected between pUC9 sequences and *U. maydis* DNA (Fig. 2a, lane C). Digests of *U. maydis* DNA with *Sst*I were also probed with either pUC9 or an *Sst*I/*Hind*III fragment (6.8 kb) of the cloned *U. maydis Hind*III fragment

Fig. 1. A restriction endonuclease map of the *U. maydis* HindIII fragment carrying the *acuA* gene.

Fig. 2. Southern blot hybridization analysis of *U. maydis* *Acu*⁻ mutant A7615 (C) and six selected acetate utilizing transformants (1, AT76151; 2, AT76152; 3, AT76171; 4, AT76172; 5, AT761211; 6, AT761212). DNA from the *Acu*⁻ mutant and the transformants was probed undigested with radiolabelled pUC9 (a) or after *Sst*I digestion, with either pUC9 (b) or the 6.8 kb *Sst*I/*Hind*III fragment from the *U. maydis* *Hind*III insert (c).

(Fig. 2b, c). The hybridization signals obtained were consistent with the interpretation that integration of one copy or, in one case, two copies of the vector had occurred by either homologous or heterologous recombination. In the transformant where no hybridization to pUC9 was detected a double cross-over event seems to have led to the mutant gene being replaced by the wild-type gene, with the rest of the vector sequences being lost (Fig. 2b, lane 5). Although it is possible that this transformant arose as a result of a reversion of the *acuA* mutation in the recipient strain, this would seem unlikely since no reversions were observed when pUC9 was used alone as the transforming vector. The type of integration events observed here are in general consistent with those reported for other homologous gene transfer systems in fungi (Ballance & Turner, 1986; Banks & Taylor, 1988; de Graaff *et al.*, 1988; van Hartingsveldt *et al.*, 1987).

These results indicate that pUCH2 contains all the necessary control and regulatory sequences to gain expression of the acetyl-CoA synthase gene in *Acu*⁻ mutants of *U. maydis*. The availability of this and other homologous selectable genes for the transformation will aid the manipulation of gene expression in *U. maydis* using gene replacement or gene disruption techniques.

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