Isolation, characterization and sequence of a gene conferring resistance to the systemic fungicide carboxin from the maize smut pathogen, *Ustilago maydis*

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**Summary.** A gene which confers resistance to the systemic fungicide carboxin (Cbx) has been isolated from the maize pathogen, *Ustilago maydis*, by transferring a plasmid gene library from a Cbx-resistant mutant strain into a sensitive strain and selecting for expression of the resistance gene. Five plasmids, rescued from transformants which exhibited enhanced resistance to Cbx, were shown to have DNA inserts with common restriction enzyme fragments. All the plasmids transformed a sensitive *U. maydis* strain to Cbx resistance. The gene (Cbx-) sub-cloned on a 3.2 kb EcoR1-HindIII fragment, transformed *U. maydis* to Cbx resistance at frequencies similar to those obtained with the bacterial Hygromycin B resistance (HygB-) gene. The sequence of the Cbx- gene showed a high degree of homology to succinate dehydrogenase (EC 1.3.99.1) iron-sulphur subunit genes from other organisms.

**Key words:** Basidiomycete – Fungicide resistance – Succinate dehydrogenase – Iron-sulphur protein – Complex II

**Introduction**

Carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carbox-anilide) is a systemic fungicide which is highly active against basidiomycete fungi and is particularly effective in controlling smut diseases (Edgington et al. 1966). It inhibits respiration by preventing the oxidation of succinate by the tricarboxylic acid cycle enzyme, succinate dehydrogenase (Sdh). This enzyme is composed of two subunits, an iron-sulphur protein (Ip) and a flavoprotein (Fp) which, together with two other integral membrane proteins, make up Complex II (succinate-ubiquinone reductase) of the respiratory electron transport chain. Although its precise mechanism of action is unclear, Cbx appears to prevent the transfer of electrons from succinate to ubiquinone by inhibiting the reoxidation of the non-heme iron redox centres of the Ip subunit (Ackrell et al. 1977). A role, in the inhibitory mechanism, for membrane proteins associated with Sdh has been inferred because the soluble form of Sdh is unaffected by Cbx (Ulrich and Mathre 1972).

Mutations at two nuclear loci (oxr-1 and oxr-2) in *Ustilago maydis* lead to increased resistance to Cbx (Georgopoulos et al. 1972, 1975). The oxr-1B mutation, which confers a relatively high degree of resistance, has also been shown to reduce the sensitivity of complex II to Cbx (Georgopoulos et al. 1972). This suggests that resistance in this mutant is due to conformational changes at the site of action of Cbx.

Recent advances in the molecular genetics of *U. maydis* make it possible to rapidly move DNA in and out of cells and to gain the expression of genes transferred into the host cell (Banks and Taylor 1988; Hargreaves and Turner 1989; Tsukuda et al. 1988; Wang et al. 1988). The key to this success has been the development of efficient gene transfer systems for *U. maydis*, and a number of plasmid and cosmid vectors are available for this purpose (Kronstad and Leong 1989; Tsukuda et al. 1988; Wang et al. 1988). The inclusion of an *U. maydis* autonomously replicating sequence in transformation vectors has led to a significant increase in the frequency of transformation (Tsukuda et al. 1988). Furthermore, the ability to conduct gene replacements and gene disruptions in *U. maydis* greatly enhances the versatility of this organism for molecular genetic studies (Fotheringham and Holloman 1989; Kronstad et al. 1989). These developments make *U. maydis* a suitable organism for studying the molecular genetic basis of both plant-fungal pathogen interactions and the mode of action of fungicides.

Here we report the isolation and characterisation of a gene which confers resistance to Cbx and its use as a selectable marker for gene transformation.
Materials and methods

Strains and chemicals. E. coli strain DH5α (F−, endA1, hsdR17 (rK−, mK−), supE44 thi−, recA1, gyrA96 relA1, φ80lacZ M15) was obtained from Gibco-BRL, Life Technologies Ltd Paisley, Scotland and used for all DNA manipulations. Reagents for cloning in bacteriophage M13 were purchased from Boehringer Mannheim Lewes, UK, and clones were constructed according to the manufacturer’s instructions. The U. maydis Cbx-resistant strain 92, which carries the otx-1B mutation, has been described previously (Georgopoulos and Ziogas 1977). Strain 103761 was obtained from the CAB International Mycological Institute. Cbx was a gift from Dr. G. A. Carter and Hygromycin B (HygB) was purchased from Calbiochem Novabiochem, UK, Ltd and Nottingham, UK.

DNA manipulations. Genomic DNA for construction of the plasmid library was prepared from cultures of strain 92 grown in liquid YEPD medium (1% yeast extract, 2% bactopeptone, 2% dextrose). The cultures (400 ml) were inoculated with approximately 107 sporidia and grown overnight at 32°C with vigorous shaking (200 rev/min). Sporidia were collected by centrifugation at 1000 g for 10 min, washed once with KSC buffer (1 M KCl, 20 mM Na citrate, pH 5.8) and then resuspended in 20 ml of the same buffer. A further 20 ml of KSC buffer containing 25 mg Novozyme 234/ml was added and the mixture gently agitated at room temperature for 10 min. Protoplasts were collected by centrifugation, washed twice with KSC buffer, then once with KCE buffer (1 M KCl, 50 mM EDTA, pH 8.0). The protoplasts were resuspended in 20 ml of the same buffer and gradually pipetted into 40 ml of lysis buffer (3% Na N-lauryl sarcosine, 500 mM Tris-HCl, 200 mM EDTA, pH 9.0) maintained at 65°C and incubated for 30 min. At intervals the flask containing the lysate was gently swirled to prevent the formation of clumps. On cooling, Protease K was added (final concentration 100 µg/ml) and incubation continued for a further 30 min. The lysate was extracted with an equal volume of phenol-CHCl3 and the upper aqueous phase added to 0.6 vol isopropanol and left at 4°C overnight. The precipitated nucleic acids were collected by centrifugation, resuspended in 2 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) containing 100 µg RNase/ml and incubated at 37°C until the pellet dissolved. One ml of this preparation was layered onto a 12 ml NaCl gradient (5–25%, containing 3 mM EDTA and 1.3% ethanol (final concentration)) and centrifuged at 20000 rpm in a Kontron sw 5826 rotor overnight. Fractions containing high molecular weight DNA were pooled, diluted with an equal volume of water, and the DNA precipitated with isopropanol. The DNA was then resuspended in TE buffer. Mini-prep DNA extraction from the transformants, for plasmid rescue, employed protoplasts generated from 50 ml YEPD liquid cultures as described above. Protoplasts in 2.5 ml KCE buffer were added to 5 ml of lysis buffer, then 2.5 ml 7.5 M NH4Ac was added and the mixture stored at 4°C overnight. After centrifugation the supernatant was extracted once with phenol-CHCl3, and the nucleic acids precipitated with 0.6 vol isopropanol. The resulting pellet was resuspended in 100 µl TE buffer containing 100 µg RNase/ml. Five microlitres of this preparation was used to transform competent E. coli cells prepared according to the method of Luthe (1983) and centrifuged at 20000 rpm in a Kontron sw 5826 rotor overnight. Fractions containing high molecular weight DNA were pooled, diluted with an equal volume of water, and the DNA precipitated with isopropanol. The DNA was then resuspended in TE buffer. Mini-prep DNA extraction from the transformants, for plasmid rescue, employed protoplasts generated from 50 ml YEPD liquid cultures as described above. Protoplasts in 2.5 ml KCE buffer were added to 5 ml of lysis buffer, then 2.5 ml 7.5 M NH4Ac was added and the mixture stored at 4°C overnight. After centrifugation the supernatant was extracted once with phenol-CHCl3, and the nucleic acids precipitated with 0.6 vol isopropanol. The resulting pellet was resuspended in 100 µl TE buffer containing 100 µg RNase/ml. Five microlitres of this preparation was used to transform competent E. coli cells prepared according to the method of Hanahan (1983). Plasmids used for transformation of U. maydis were prepared by the cleared lysate method, followed by cesium chloride density centrifugation (Maniatis et al. 1982), and plasmid mini-preps were done according to Morelle (1989). DNA sequence analysis was performed by the dideoxy – chain termination method of Sanger et al. (1977) using a DuPont Genesis 2000 automatic sequencer. Computer-assisted analysis of sequence results was performed using DNASIS PC software (Pharmacia, Milton Keynes, UK) and the Wisconsin package. Database comparisons were done using the FastA programme (Pearson and Lipman 1988).

Plasmid library construction. A genomic library was prepared by partially digesting DNA from strain 92 with the endonuclease Sau3A. The digested DNA was fractionated on an NaCl gradient as described by Kaiser and Murray (1985) and fragments between 5 and 9 kb were collected. These were ligated into a unique BamH1 site in the high-frequency U. maydis transformation vector pCM54 (Fotheringham and Holloman 1989; Fig. 1), which had been previously treated with alkaline phosphatase. Ligation conditions were as recommended by Revie et al. (1988) for dephosphorylated vectors and insert fragment sizes > 2 kb. Eighty separate sub-libraries were constructed, each containing between 15000 and 20000 colonies. The efficiency of fragment insertion into pCM54 ranged from 30–75%. Using an estimated haploid U. maydis genome size of approximately 106 kb (Fotheringham and Holloman 1989) and a probability of representing the complete genome of 0.99, the entire library was equivalent to approximately 3–4 times the size of the U. maydis genome. pCM54 regularly transformed U. maydis to HygB resistance at a frequency of approximately 5–10 x 105 transformants/µg DNA.

Transformation and selection procedures. Transformation of the Cbx-sensitive strain 103761 was as described by Hargreaves and Turner (1991) and involved treating protoplasts, in the presence of the transforming DNA, with polyethylene glycol and CaCl2. For selection, the protoplasts were plated in a 0.35% agarose overlay. An initial selection for HygB-resistant transformants was followed by a secondary screen for resistance to Cbx. To do this, HygB-resistent transformants that appeared after 3 days were removed from the selection plates by scraping off the agarose overlay and the colonies disrupted by forcing the agarose through a 19 g needle into YEPD liquid medium containing 200 µg HygB/ml. After incubating the cultures overnight, aliquots were plated onto YEPD medium containing 4.2 µM Cbx.

Growth and Sdh assays. Inhibition of sporidial growth was measured as described by White et al. (1978) using a yeast extract-supplemented liquid medium with succinate as the carbon source. Transformants were grown in media containing 5 µM Cbx prior to being transferred to a series of Cbx concentrations. Sporidia, for Sdh assays, were obtained from YEPD Liquid shake cultures (280 rev/min, 28°C) and processed as reported by White and Thorn (1975). The transformant cultures were supplemented with 4.2 µM Cbx to ensure maintenance of the plasmids during growth. Mitochondria were isolated from U. maydis sporidia according to White and Thorn (1980). Sdh activity was assayed spectrophotometrically employing DCPIP (2,6-dichlorophenolindophenol) as an electron acceptor (White and Thorn 1975). All inhibitors were added in ethanol (final concentration 1.3%), Reaction rates and I50 values (µM) were determined as reported by White et al. (1978).