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Tagging pathogenicity genes in *Ustilago maydis* by restriction enzyme-mediated integration (REMI)

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Abstract In the maize pathogenic fungus *Ustilago maydis* integration of transforming DNA at homologous or heterologous sites is often accompanied by duplications of the DNA. We show that it is possible to generate single-copy integration events with high efficiency by restriction enzyme-mediated integration (REMI). In about 50% of cases, a plasmid that contains a single *Bam*HI site is integrated at chromosomal *Bam*HI sites, if *Bam*HI is added to the transformation mixtures. In the other cases it appears that integration events have also occurred preferentially at *Bam*HI sites, but without restoration of the recognition sites. Using REMI we have generated approximately 1000 insertion mutants. Pathogenicity tests demonstrated that about 1–2% of these mutants were unable to induce symptoms when tested *in planta*. For two of the mutants we have shown that the phenotype is linked to the insertion event.

Key words *Ustilago maydis* · Pathogenicity genes · Insertional mutagenesis · Restriction enzyme-mediated integration (REMI)

Introduction

Ustilago maydis is the causative agent of corn smut disease. The fungus exists as a haploid form, which is nonpathogenic, and as a pathogenic dikaryon that results from fusion of cells of different mating types (see Christensen 1963; Banuett 1992). Associated with the establishment of the infectious form is a dramatic transition from yeast-like to filamentous growth. The successive steps of this developmental program are controlled by the *a* and *b* mating type loci. The *a* locus encodes a pheromone-based recognition system that governs cell recognition and cell fusion (Bölker et al. 1992); after fusion this system remains active and is necessary to trigger and maintain filamentous growth in conjunction with the multiallelic *b* locus (Banuett and Herskowitz 1989; Bölker et al. 1992; Spellig et al. 1994). The *b* locus encodes two homeodomain proteins, bE and bW, which function as central regulators for pathogenic development (Schulz et al. 1990; Kronstad and Leong 1990; Gillissen et al. 1992). Initiation of this process requires that different alleles of the *bW* and *bE* genes are combined (Gillissen et al. 1992). Only in this situation can heterodimers of bE and bW be formed (Kämper et al. 1995). These heterodimers are presumed to regulate a set of pathogenicity genes.

The cloning and molecular analysis of the *b* locus made possible the construction of haploid strains that express an active *bE-bW* gene complex and are therefore pathogenic (Kronstad and Leong 1989; Gillissen et al. 1992; Bölker et al. 1995). Such strains can be used to isolate pathogenicity mutants because mating is no longer required to generate the pathogenic phase. If such mutants were generated by chemical or UV treatment, identification of the mutated genes would pose serious problems, because it involves complementation with a cosmid library. This is an extremely time-consuming process, since several thousands of transformants have to be tested *in planta* for restoration of pathogenicity. It would therefore be desirable to establish a tagging mutagenesis protocol since this should facilitate the isolation of mutated genes.

In other systems, tagging mutagenesis has been achieved by inserting various DNA elements, such as transposons, T-DNA or plasmids, into the genome. An additional method has been developed for *Saccharomyces cerevisiae* and *Dictyostelium discoideum*. In these systems, the presence of a restriction enzyme during transformation efficiently targets the transforming

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DNA to corresponding restriction sites (Schiestl and Petes 1992; Kuspa and Loomis 1992).

For *U. maydis* it has been shown that transforming linear DNA molecules are integrated into the genome either randomly or at homologous sites (Wang et al. 1988; Kronstad et al. 1989; Fotheringham and Holloman 1989). Especially in heterologous insertion events the DNA was found to integrate predominantly in multiple tandem copies (Wang et al. 1988; Fotheringham and Holloman 1990). The use of circular plasmids in transformations reduces the transformation efficiency but has been reported to result in preferential single-copy integration at random sites (Fotheringham and Holloman 1990). We reasoned that, use of a circular plasmid without sequence homology to the *U. maydis* genome could improve the tagging of genes by random insertion. Applying this approach we found a high proportion of tandem copy integration events. This problem was overcome by using the method of restriction enzyme-mediated integration (REMI), which allowed efficient isolation of tagged genes in *U. maydis*

Materials and methods

Strains and plasmids

Cloning in *Escherichia coli* was done in DH5α (BRL). The *Ustilago maydis* strain CL13 is *a1bW2bE1* and was generated by replacing the *bW1* allele of strain FB1 (*a1b1*) with the *bW2* allele (Bölker et al. 1995). This haploid strain induces tumors when injected into susceptible plants. pSMUT is a derivative of pSP72 (Promega; Krieg and Melton 1987) carrying the coding region of the bacterial hygromycin resistance gene from plasmid pCM54 (Tsukuda et al. 1988) fused to a 477 bp *Rsa*I fragment of *Saccharomyces cerevisiae* DNA that functions as promoter in *U. maydis* (Bölker et al. 1995). A cosmid library of strain FBD11 (*a1a2b1b2*) (Banuett and Herskowitz 1989) was constructed in pUMcos by cloning partially cleaved *Mbo*I fragments into the *Bam*HI site of this vector. pUMcos was derived from pScos1 (Stratagene; Wahl et al. 1987) by replacing a *Hin*dIII-*Sma*I fragment containing the neomycin resistance gene with an *Eco*RV-*Sma*I fragment from pCBX122 (Keon et al. 1991) that confers carboxin resistance in *U. maydis*.

Molecular techniques

Standard molecular techniques were used according to Sambrook et al. (1989). *U. maydis* DNA was prepared according to Hoffman and Winston (1987). For Southern blots, 3 μg of DNA were cleaved, separated on 0.8% agarose gels, transferred to Nylon membranes (Pall B) and hybridized to pSMUT labelled with ^{32}P using the Megaprime labelling kit (Amersham).

U. maydis growth and transformation procedures

Liquid cultures were grown in YEPS (Tsukuda et al. 1988) at 28° C. Transformation of *U. maydis* was performed as described by Schulz et al. (1990). To mixtures containing 50 μl protoplasts in STC, 4–5 μg circular plasmid DNA in 5 μl H_2O, 50 U *Bam*HI (Boehringer, high concentration) and 1 μl heparin (15 mg/ml) were added. After 10 min incubation on ice 500 μl polyethylene glycol (40% w/v) in STC were added, the mixture was incubated on ice for an additional 15 min and then spread directly on regeneration plates. Plates contained 10 ml of 1.5% agar in YEPS, 1 M sorbitol, 400 μg/ml hygromycin overlaid with 10 ml of 1.5% agar in YEPS, 1 M sorbitol. Individual transformants appeared after 3–4 days at 28° C, they were purified on selective medium before further use. For plasmid rescue from *U. maydis*, 1 μg of chromosomal DNA was cleaved with *Mlu*I and religated in a volume of 200 μl, transformation was done using a Biorad electroporation unit, following the protocol supplied by the manufacturer.

Pathogenicity test

To assay pathogenicity, 6–8-day-old corn seedlings (Early Golden Bantam), grown in a greenhouse under controlled conditions (14 h light, 28° C; 10 h dark, 20° C), were infected with pure cultures as described by Holliday (1974). For pathogenic strains, at least one of 4 plants infected showed anthocyanin biosynthesis and developed tumors after 5–8 days (Tum^+ phenotype); for nonpathogenic lines none of 20 plants infected induced tumors or showed anthocyanin streaks (Tum^- phenotype).

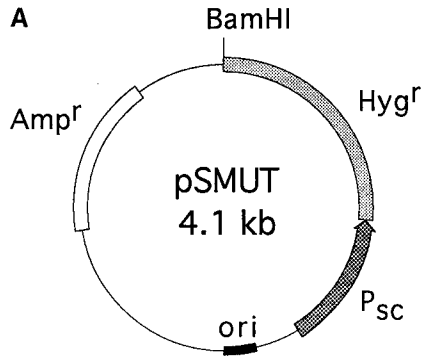
Results

Characterization of the insertion vector pSMUT

Since all plasmids used previously in *U. maydis* transformations contain *U. maydis* sequences for the expression of the selectable marker genes, they have the potential to integrate at homologous sequences. To avoid such integration events we recently constructed the plasmid pSMUT (Bölker et al. 1995), which does not contain any *U. maydis* sequences (Fig. 1A). In this plasmid, expression of the bacterial hygromycin gene is driven by a *S. cerevisiae* sequence selected from a random pool of *Rsa*I fragments for promoter function in *U. maydis*. pSMUT does not cross-hybridize with genomic DNA of *U. maydis* (see Fig. 2, lane CL13). The nucleotide sequence of the 477 bp promoter fragment was determined (Fig. 1B). The entire promoter sequence has a low G content and is repetitive in nature. The repeated element consists of a 9 bp consensus ($ACTTCTAC^{A}/_{C}$). Significant similarities to sequences in the database could not be detected, but the repetitive sequence contains an element (CTTC) that resembles the motifs CATC and CTTT, at which transcription has been shown to start in other *U. maydis* promoters (Spanos et al. 1992).

Insertional mutagenesis

pSMUT was used to transform CL13 by conventional transformation with supercoiled DNA, and by a modified transformation procedure in which 50 units *Bam*HI were added to the transformation mixtures (see Materials and methods). Using supercoiled pSMUT DNA



B

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  1 ACTTCGACT ACTCCTACT ACCACTATC ACTTCTACT
 37 ACTTCTACC ACATCGACT ACTCCTACA ACCTCTACC
 73 ACTTCTACA ACTTCTACA ACTTCTACA ACTTCTACA
109 ACTTCTACA ACTTCTACA ACTTCTACA ACTTCTACA
145 ACTTCTACA ACTTCTACA ACCTCTACC ACTCCTACA
181 ACCTCTACC ACATCGACT ATTCCTACA ACCTCTACT
217 ACTTCTACA ACTTCTACC ACTCCTACA ACTTCTACA
253 ACTTCGAGC ACTTCTACC ACGCCTACA ACCTTTACT
289 ACTTCTACC ACTTCGACT ACTCCTACC ACTTCAAGC
325 ACCTTGACC ACTTCAACT ACTTCAACT ACTTCTACT
361 ACGCCTACA ACCTCTACC CCTTCAAGT AGTTCTAGC
397 ACTTCTACT ACGCCTACA ACTTCTATC ACTTCTACC
433 ATTTCTACC GCTCCCACG ACCTCTACA ACTTCAAGC
469 ACTTTCAGT

Consensus: ACTTCTAC A/C
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Fig. 1A, B Plasmid used for insertion mutagenesis. **A** Schematic map of pSMUT. The plasmid backbone is derived from pSP72, the coding region for hygromycin resistance originates from pCM54 (Tsukuda et al. 1988), and the *filled arrow* indicates a *Rsa*I fragment from *Saccharomyces cerevisiae* that shows promoter activity in *Ustilago maydis*. In the polylinker region pSMUT contains unique sites for *Xho*I, *Hin*dIII, *Sal*I, *Acc*I, *Xba*I, *Bam*HI, *Cla*I and *Bgl*II. **B** Nucleotide sequence of the 477 bp *Rsa*I fragment that functions as a promoter in *U. maydis*

without restriction enzyme, 5–10 transformants per μg DNA were obtained, which is consistent with published values (Wang et al. 1988; Fotheringham and Holloman 1990). In transformations in which *Bam*HI was added to the supercoiled DNA, 5–25 transformants were obtained per μg DNA. Thus, the addition of the restriction enzyme did not affect the transformation efficiency.

To analyse the integration events, DNA was prepared from six randomly chosen transformants obtained with supercoiled DNA, and from 10 randomly chosen transformants from the transformation to which *Bam*HI had been added. The DNAs were subjected to Southern analysis after cleavage of separate samples with *Bam*HI and *Mlu*I. pSMUT has a single *Bam*HI site but lacks *Mlu*I sites (see Fig. 1A). The analysis of the transformants generated with circular DNA without *Bam*HI revealed multiple tandem insertion events in all cases. This is indicated by the presence of a strongly hybridizing *Bam*HI fragment which has the size of pSMUT, and by the appearance of at least two additional fragments that could represent the junctions with *U. maydis* DNA (Fig. 2A). Since the amounts of DNA loaded were about equal in all lanes, the intensity of the pSMUT-sized band is a direct measure of copy number and we estimate that from two (Fig. 2A, lane KHB1398) to 20 copies (Fig. 2A, lane KHB1396) are present in these transformants. Strains KHB1396 and KHB1400 show a number of weakly hybridizing fragments in addition to the putative junctions, which most probably indicates plasmid instability or reintegration events (Fig. 2A). The analysis with *Mlu*I reveals that the integration events have occurred at 1 or 2 locations per strain (Fig. 2C), although we presently cannot exclude the possibility that some of these copies exist extrachromosomally, as has been described by Fotheringham and Holloman (1990). Attempts to reisolate plasmids together with flanking sequences from such strains always yielded only pSMUT alone. This can be explained by assuming recombination within the large *Mlu*I fragments containing multiple plasmid copies (Fig. 2C).

In transformants that were generated in the presence of *Bam*HI, a completely different pattern of integration events is observed, except for transformant KHB3788, which exhibits integration of tandem copies (Fig. 2B). In all other transformants a single *Bam*HI fragment hybridizes to the pSMUT probe. Based on the intensity of the hybridizing fragments we estimate that a single copy of pSMUT has been inserted in all cases. Note that five transformants yield a fragment of pSMUT size, indicating insertions into genomic *Bam*HI sites, which have been maintained during insertion. The four remaining transformants each display a hybridizing *Bam*HI fragment that is larger than pSMUT and most probably arose by insertion into a *Bam*HI site without regeneration of that site. Two other transformants (KHB2997 and KHB2563), which were later found to be pathogenicity mutants, showed a pattern comparable to that found in the randomly chosen samples (Fig. 2B). The pattern observed in the *Mlu*I digest shows that different insertion sites have been used in individual transformants (Fig. 2D). Since single integration events were observed in 90% of the restriction enzyme-mediated integrations, REMI is the method of choice for gene tagging in *U. maydis*.

Tagging pathogenicity genes by REMI

So far, we have generated 928 insertion mutants of CL13 by transformation with pSMUT in the presence of *Bam*HI as described (see Materials and methods). Each transformant was grown in liquid culture and used to infect 4 plants initially. Strains that showed a Tum$^-$ phenotype in the first screen were rescored by infecting another 20 plants each. Only such strains were considered nonpathogenic that induced neither anthocyanin biosynthesis in the infected tissue nor the formation of plant tumors. By these criteria, 13 of the mutant strains tested were nonpathogenic.

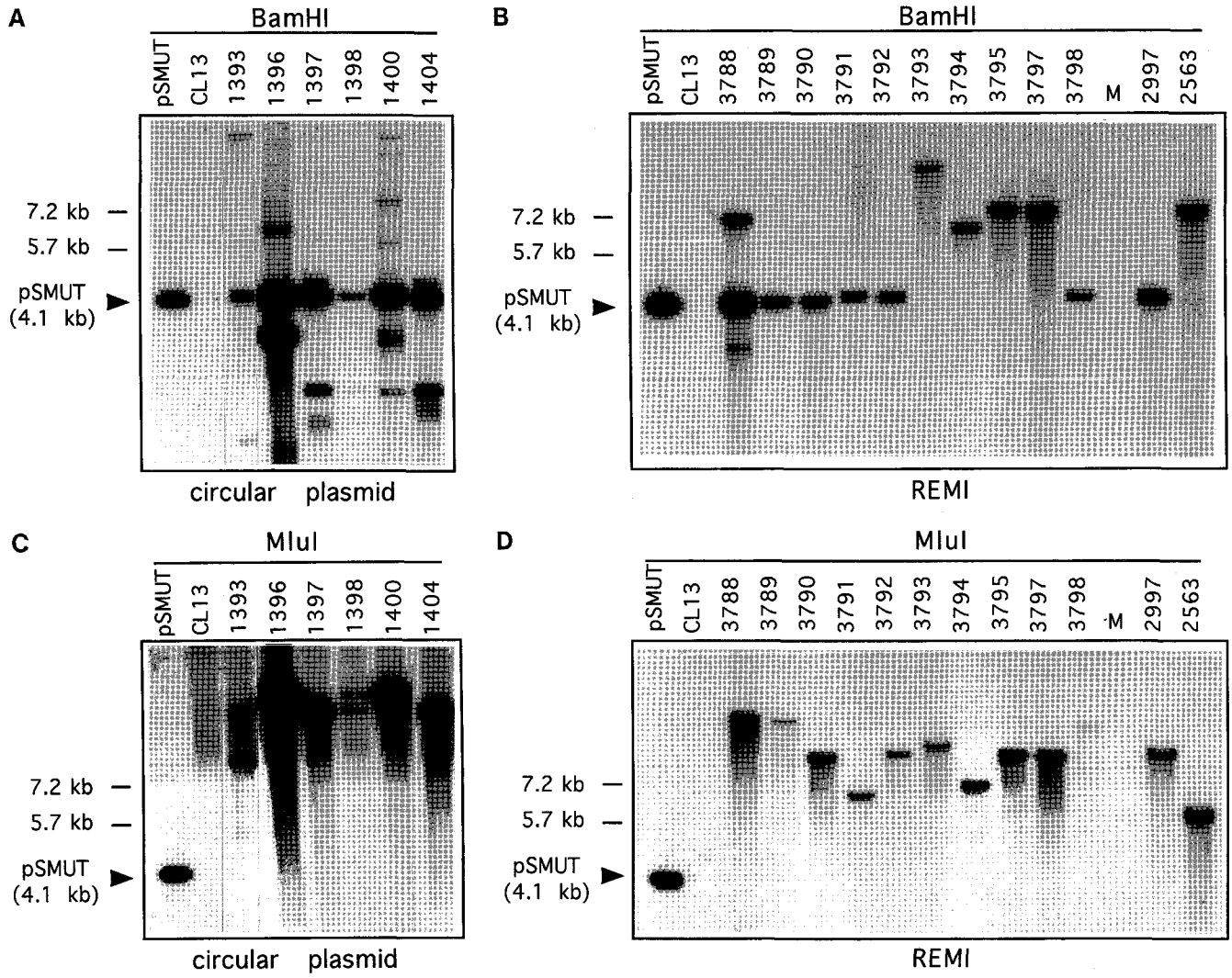

Fig. 2A–D Southern analysis of plasmid integration events. DNA from individual transformants was isolated and cleaved with enzymes indicated at the top of each panel. The numbers above each lane are KHB strain numbers, CL13 DNA serves as nontransformed control, pSMUT (1 ng DNA cleaved with *Bam*HI) serves as a hybridizing size marker. In lane M, λDNA cleaved with *Bst*EII was used as size marker. Below each panel the transformation protocol used is indicated. In all cases pSMUT was used as hybridization probe. **A, C** Integration events obtained by transformation with a supercoiled plasmid. **B, D** Integration events obtained by transformation with a supercoiled plasmid in the presence of the restriction enzyme *Bam*HI

This shows that pathogenicity defects arise with a frequency of 1.4%.

To investigate whether the loss of pathogenicity is linked to the insertion event, two nonpathogenic mutants were subjected to a more detailed analysis. To this end DNA was prepared from mutants KHB2563 and KHB2997 (see Fig. 2B) and the integrated plasmids plus flanking sequences were recovered after cleavage with *Mlu*I (see Fig. 2D), religation and transformation in DH5α as described in Materials and methods. Plasmids recovered were designated p2563 and p2997, respectively; their restriction maps are shown in Fig. 3. While in p2997 the insertion event had regenerated *Bam*HI sites at both junctions, in p2563 the *Bam*HI site in one of the junctions had been lost. Sequencing across this junction revealed that the integration of pSMUT had occurred at a *Bam*HI site, in the fusion sequence, however, one G/C base pair had been deleted (Fig. 3). Next, p2563 and p2997 were linearized with *Mlu*I and retransformed in CL13 to re-establish the mutation by homologous recombination. For each plasmid, 12 transformants were isolated, their DNA was prepared and analysed by Southern blot (not shown). For p2997, homologous recombination had occurred in 4 of the 12 transformants, regenerating mutant strains indistinguishable from KHB2997. When these strains were used to infect plants, no tumor induction occurred. This confirmed that the Tum$^-$ phenotype of KHB2997 was linked to the insertion.

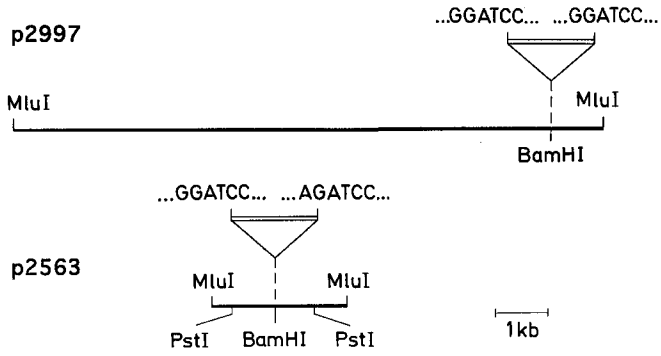

Fig. 3 Restriction maps of plasmids recovered from nonpathogenic insertion mutants. *U. maydis* DNA is shown as a *thick line*, the inserted pSMUT is represented by an *open bar*. Junction sequences are indicated

With p2563 this approach was unsuccessful. We therefore isolated cosmid clones using *Mlu*I-*Pst*I fragments from each of the two flanks of p2563 as probes (see Fig. 3). Several hybridizing cosmid clones were identified and one of them, p14F1, was used to transform the mutant strain KHB2563. Eleven transformants were tested *in planta*, and ten were able to induce tumors. This shows that in these cases complementation of the pathogenicity defect had occurred, establishing a link between the insertion event in KHB2563 and the phenotype observed for this strain.

Discussion

We have shown that the presence of restriction enzyme during the transformation of *U. maydis* results in preferential single-copy integration of plasmid DNA. Using *Bam*HI, we observed that plasmids carrying one *Bam*HI site were targeted to chromosomal *Bam*HI sites. Without the addition of *Bam*HI, circular plasmid DNA is predominantly integrated as tandem duplications. From the pattern of the restriction enzyme-mediated insertion events we can conclude that in about 50% of the cases both junctions regenerate the recognition site while in the other cases one or both restriction sites may be lost during the integration event. This may be due to degradation of ends prior to religation. The most valuable feature of the REMI method is the integration of just one copy of the plasmid. Why is it that the presence of restriction enzyme forces DNA to integrate in single copy? One explanation is that the incoming plasmid DNA remains in a state accessible to the nuclease, as long as it is not integrated and packaged tightly into chromatin. The nuclease could therefore act to generate linear plasmid molecules. Integration events may then occur into chromosomal sites which are accessible to the nuclease. The tight packaging of the chromatin would maintain chromosome integrity during this process. During integration events in the absence of nuclease, incoming DNA could multimerize prior to integration or be maintained extrachromosomally as described by Fotheringham and Holloman (1990). Concatamer formation has been observed with nearly all plasmids introduced into *U. maydis* (Wang et al. 1988; Schulz et al. 1990; Fotheringham and Holloman 1990).

Clearly, the use of REMI limits the number of sites accessible for integration, but restriction enzymes with different specificities could be used to extend the number of genes that can be tagged. In pSMUT, unique sites exist for the following enzymes, which could in principle be used for REMI: *Xho*I, *Hin*dIII, *Sal*I, *Acc*I, *Xba*I, *Cla*I and *Bgl*II.

The high frequency at which pathogenicity mutants were obtained deserves further comment. About 1.4% of all transformants were unable to induce disease symptoms. In *Dictyostelium discoideum* REMI mutants affected in development were found with a frequency of 0.27%, indicating that large numbers of genes participate in both processes. In *U. maydis* such genes could function directly in establishing infection. In addition, we may find genes whose products play an essential role in fungal metabolism during growth *in planta*. Such genes may not necessarily be required exclusively for fungal disease induction. It has been shown that *U. maydis* strains carrying mutations in *ad-1*, *me-1* and *leu-1* fail to initiate disease when both mating partners carry the mutated alleles (Holliday 1961). In this respect it is interesting that none of the 928 mutants analyzed so far are auxotrophs or show gross defects in growth or morphology on plates. This does not contradict the results of Holliday (1961), who showed that stable auxotrophs arise at a frequency of about 0.1%.

The finding that the two pathogenicity mutants that were analyzed in more detail are indeed tagged by the REMI method illustrates that molecular genetics can now be applied to the study of such complex processes as pathogenic development.

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