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Mini review

REMI (Restriction Enzyme Mediated Integration) and its impact on the isolation of pathogenicity genes in fungi attacking plants

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Abstract

Development of molecular techniques for phytopathogenic fungi aims at the identification of fungal genes whose products are essential for successful infection of the host plant. Initial approaches have relied on isolating candidate genes and generating null-mutations by homologous recombination. Unfortunately, the results of this strategy have not been overly successful. This has led to a search for alternatives which allow an unbiased identification of pathogenicity genes. One method, which has proved successful in several systems, is a tagging mutagenesis procedure termed restriction enzyme mediated integration (REMI). In this mini-review we describe this procedure and review its features and results of its use when applied to the identification of fungal genes required for disease development *in planta*.

Abbreviations: REMI – restriction enzyme mediated integration; RFLP – restriction fragment length polymorphism; GFP – green fluorescent protein.

Introduction

Plant pathogenic fungi have made it into the molecular area only within the past 15 years when DNA transformation protocols and dominant selectable markers were developed for them. In principle, these methods allow the identification of genes in a pathogen which are necessary to cause disease. However, using chemical mutagenesis the experimentalist is faced with large numbers: first, a mutant needs to be identified from a large pool and this is in most cases done by testing individual clones. Even if successful, confirmation that the correct gene was isolated would require complementation with a wild-type cosmid or plasmid library. Whereas feasible for mutants affected in nutritional requirements or morphology, this procedure would be extremely laborious to identify pathogenicity genes because several hundred transformants would have to be generated and tested for restoration of pathogenicity in individual infections. Finally, gene isolation would require recovery of the cosmid or parts of it. Alternatively, one could use sib selection (Vollmer and Yanofsky, 1986), a method where smaller pools of plasmids or cosmids are transformed. Once the pool containing the complementing gene is identified, increasingly smaller subpools are generated and tested until a single clone is identified. Both procedures are time consuming and laborious. For this reason, less labour intensive alternative methods were sought. Unfortunately, reliable heterologous transposon tagging techniques are not yet available for filamentous fungi. However, this may change soon since recent experiments indicate that the *impala* transposable element from *Fusarium oxysporum* might be exploited for gene tagging (Daboussi, 1997; Hua-Van et al., 1998).

An alternative method, which proved very efficient in the yeast *Saccharomyces cerevisiae*, has relied on the generation of transposon-tagged cosmid pools in *E. coli*

which were then subsequently introduced into yeast by homologous recombination (Burns et al., 1994). This method has not yet been attempted in plant pathogenic fungi and, in any case, its success would depend on an efficient homologous recombination system. In several plant pathogenic fungi, homologous recombination events are rare and the majority of integrations occur at ectopic sites (Kües and Stahl, 1990). Very few studies have addressed the mechanism of such illegitimate recombination events but, if one assumes that integrations occur at random, this provides a way of generating insertion mutants where the transforming DNA physically marks the insertion site. Consequently, insertional mutagenesis protocols should greatly facilitate the cloning of the respective gene by recovering the plasmid together with flanking sequences. While successful in some systems (Diallinas and Scazzocchio, 1989; Tilburn et al., 1990; Kang and Metzenberg, 1993; Itoh and Scott, 1994; Giasson and Kronstad, 1995) this approach often has posed severe problems because mutagenesis plasmids were not integrated in single copies but rather in tandem repeats. In these cases rescue attempts where DNA of the desired mutant strains was cut with a restriction enzyme not cleaving within the plasmid sequences proved difficult because of the large plasmid size, concomitant low transformation efficiency and instability in *E. coli*. Although single plasmids can be released from concatemers by cleavage within the vector, a mixture of plasmids is generated and those containing flanking sequences represent a minority. Nevertheless, if the organism of choice displays a favourable frequency of single inserts compared to multiple inserts, insertional mutagenesis does provide for efficient mutagenesis and gene reisolation. An advancement of this technique which may, depending on the system, increase the number of chromosomal sites where integration can occur, has been the establishment of REMI (Restriction Enzyme Mediated Integration) in *S. cerevisiae* (Schiestl and Petes, 1991).

The REMI mutagenesis technique

In this method, linearized plasmid DNA is transformed into the organism of choice in the presence of a restriction enzyme which generates compatible (or incompatible) ends. As a result, plasmid integration occurs at the corresponding sites in the genome, often by regenerating the recognition sites for the enzyme used at both ends (Figure 1). Another, highly favourable side effect of this technique can be a significantly higher

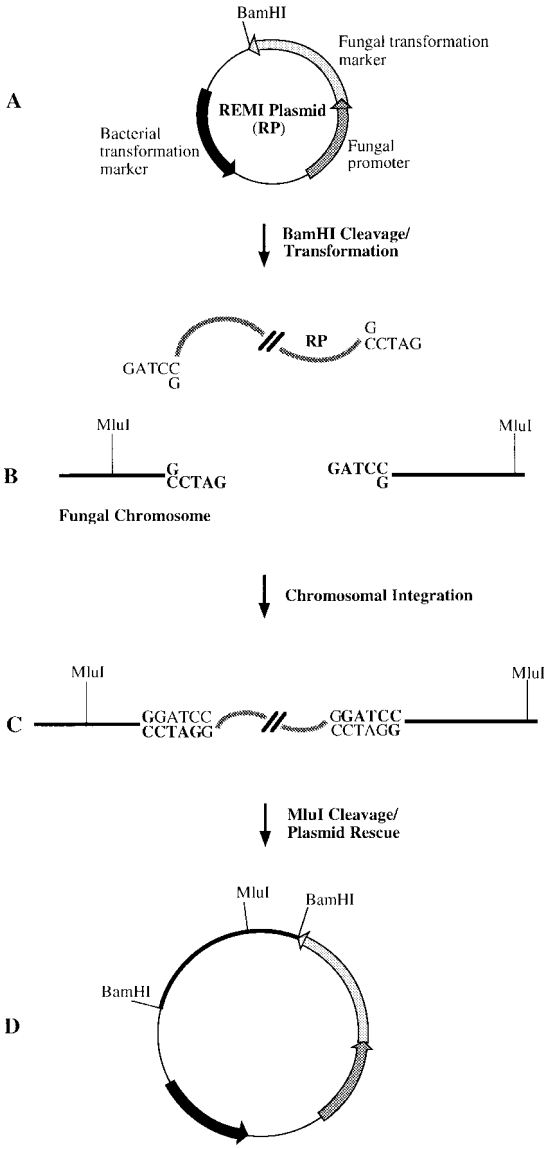

Figure 1. REMI transformation and plasmid rescue. *A.* The REMI plasmid (RP) contains a fungal promoter to drive transcription of a fungal transformation marker. The bacterial transformation marker, often the gene conferring ampicillin resistance, is needed for plasmid rescue. Ideally, the REMI plasmid lacks homology to the genome of the fungal recipient. *B.* The REMI plasmid (circular or linearized) is transformed in the presence of a restriction enzyme (e.g., *Bam*HI) resulting in cleavage of the transforming plasmid and at respective chromosomal sites. *C.* Free ends of cleaved plasmid and genome join together. *D.* Plasmid rescue is achieved by plasmid excision from genomic DNA together with flanking sequences (e.g., by using *Mlu*I) and circularization with DNA ligase prior to subsequent transformation in *E. coli.*

percentage of single-copy integration events as well as an increase in transformation rate. The REMI technique was originally developed for *S. cerevisiae* and subsequently applied in *Dictyostelium discoideum*. It is in these organisms only that fundamental aspects of the end-joining events leading to integration have been analyzed in some detail (Schiestl and Petes, 1991; Kuspa and Loomis, 1992). For *S. cerevisiae*, it was shown that transformation of cells with *Bam*HI-cut vector (lacking homology to the yeast genome) along with *Bam*HI enzyme elevates transformation frequency about 7-fold compared to the situation where the restriction enzyme is omitted. Integration events occurred at *Bam*HI sites in 80–90% of all cases and these events were termed restriction enzyme mediated events (Schiestl and Petes, 1991). For subsequent terminology, we designate integration events where the recognition sites for the enzyme used are restored at the plasmid insertion site as REMI events. REMI events occurred with similar efficiency in cells carrying mutations in *RAD51*, *RAD52* and *RAD57* genes encoding double-strand repair functions required for homologous recombination. Conversely, the *RAD50* gene product was required for REMI events, although it is dispensable for homologous integration (Schiestl et al., 1994). These studies demonstrate that REMI and homologous recombination events are under different genetic control. The specific role of the *RAD50* gene product in REMI events might reflect its ability to stabilize interactions involving very small regions of homology (Schiestl et al., 1994). In a recently published study, Manivasakam and Schiestl (1998) demonstrate that different restriction enzymes vary significantly in their ability to mediate fragment integration into the yeast genome. Increases in transformation efficiency were observed with only a few enzymes and this required the presence of matching 5′ or 3′ overhangs. The same authors also provide additional insight into end-processing events leading to the integration of fragments with incompatible ends.

In *Dictyostelium*, REMI greatly stimulated transformation provided that the linearized plasmid ends and the ends generated by the restriction enzyme match in sequence. REMI events occurred in about 70% of the cases and uptake of the DNA as well as of the restriction enzyme was achieved by electroporation of intact cells (Kuspa and Loomis, 1992). Most mutations were tagged and integration apparently occurred at random sites as about 1 in 400 mutants displayed developmental defects due to the inactivation of individual genes within a large set of different genes. Many of these mutants have been characterized in molecular terms and the corresponding genes were isolated (Dynes et al., 1994; Shaulsky et al., 1995; Chen and Devroetes, 1997; Nagasaki et al., 1998). The randomness of REMI insertions has been further addressed by Kuspa and Loomis (1994). In this study REMI has been used to map the *Dictyostelium* genome using the so-called REMI-restriction fragment length polymorphism (RFLP) technique which is based on the ability to introduce random changes in the genome that induce polymorphisms useful for mapping. REMI insertion sites were spread throughout the genome with little preference for certain areas (Kuspa and Loomis, 1994).

In several non-phytopathogenic fungi REMI has also been successfully established. These include the ascomycete *Candida albicans*, a human pathogen (Brown et al., 1996). Transformation efficiencies were markedly increased and most transformants harbored single-copy insertions at the corresponding restriction site, making REMI an ideal tool for mutagenesis. Since *C. albicans* is diploid, however, insertional events will rarely lead to phenotypic alterations. However, REMI might be used in promoter studies or to generate gain of function mutations if the REMI vector carries a promoter allowing readthrough into sequences flanking the insertion site. In *Aspergillus nidulans*, insertional mutagenesis in the presence of a restriction enzyme increased the transformation efficiency but did not lower the number of independent multiple insertion events. Mutants carrying single insertions were recovered by subsequent backcrosses with wild-type strains (Karos and Fischer, 1996). In *Coprinus cinereus*, a saprophytic basidiomycete, transformation rates were elevated up to 7-fold in the presence of optimal concentrations of restriction enzymes and single-copy plasmid integrations were increased (Granado et al., 1997). However, the number of REMI events in which the plasmid actually marked the inactivated gene of interest was quite variable and relatively low compared to those in the other systems mentioned (Brown et al., 1996; Schiestl and Petes, 1991; Kuspa and Loomis, 1992).

General requirements for REMI

The REMI vector molecule should contain a plasmid backbone for replication and selection in *E. coli*. To detect transformants in the fungus of choice, the vector should contain either a dominant selectable marker or a marker complementing an auxotrophic mutation (Figure 1). The presence of a selectable dominant

marker gene which lacks homology to the genome in which it is inserted may be advantageous in systems where homologous recombination frequency is high (Lu et al., 1994; Shi et al., 1995; Bölker et al., 1995a), although no rigorous investigations have addressed this point. The vector should also contain unique sites for the enzyme used in the REMI procedure. For DNA and restriction enzyme uptake the method established for transformation (either electroporation or the use of protoplasts) of the respective organisms should be maintained and modified only by addition of restriction enzyme. Titration of the restriction enzyme is required to determine the optimal concentration for obtaining large numbers of transformants and to minimize cytotoxic effects. Not all enzyme preparations act the same and it appears advisable to obtain highly concentrated stocks to minimize adverse effects caused by storage buffers of enzymes which may lower transformation efficiency (Kahmann, unpubl.).

Mutants are selected first according to the desired phenotype. From such strains DNA is isolated and subjected to Southern analysis using the REMI vector as probe. This step provides information about the number and complexity of insertion events, i.e., single or tandem integrations, and should indicate the presence of REMI events. The integrated plasmid is excised from genomic DNA together with flanking sequences, circularized with DNA ligase, and transformed into *E. coli* using electrocompetent cells to increase recovery efficiency (Figure 1). The targeted gene then can be sequenced directly using primers complementary to the vector backbone. Alternatively, plasmids or cosmids containing the wild-type gene can be isolated using the sequences flanking the REMI vector in the reisolated plasmid as probe. These wild-type sequences can be used to complement the defect of the original REMI mutant. The latter approach may be advisable when the insertion event has occurred in an intergenic region where it is not readily clear which gene is affected in function.

Due to mutational events arising either through nucleases that contaminate the lytic enzymes used for generating protoplasts or from DNA cleavage by the added restriction enzyme followed by repair of the cleaved ends without integration of the REMI vector, it is mandatory to prove that the phenotype observed is caused by integration of the vector at that particular site. This can be done by different procedures, which may not all be applicable to the system under study.

(a) If the system is amenable to genetic analysis, cosegregation of the phenotype and the selectable marker on the REMI vector in progeny from a cross with wild-type strains may be the method of choice.
(b) If the system is efficient for gene replacement, the plasmid and flanking sequences reisolated from the original REMI mutant can be linearized with the enzyme used for plasmid recovery and reintroduced into a wild-type strain by homologous recombination. Verification involves Southern analysis using the original REMI mutant as control. Once the mutant has been recreated, its phenotype is compared to the original REMI mutant.
(c) Finally, a link between insertion and mutant phenotype can be established by complementation of the original REMI mutant with a cosmid or plasmid carrying the respective region from a wild-type strain.

REMI in phytopathogenic fungi

Due to the establishment of molecular techniques in a number of phytopathogenic fungi, mutagenesis tagging by REMI has become an attractive method of choice to provide for an unbiased identification of pathogenicity genes.

Cochliobolus heterostrophus. REMI has been performed to obtain tagged mutations at the *Tox1* locus of the ascomycete *Cochliobolus heterostrophus*, a maize pathogen (Lu et al., 1994). Transformation of a linearized plasmid in the presence of a compatible restriction enzyme strongly stimulated the transformation efficiency in this fungus. *C. heterostrophus* race T produces T-toxin, a linear polyketide which is of great economic interest because it provides for high virulence toward Texas male sterile (T) cytoplasm corn (Yoder et al., 1993). *C. heterostrophus* transformants obtained by REMI were screened for loss of T-toxin production using a microbial bioassay (Lu et al., 1994). In 2 of 1306 transformants, the production of T-toxin was abolished due to REMI events at two different positions that both mapped at the *Tox1* locus (Lu et al., 1994). This allowed the identification of a gene which encodes a polyketide synthase engaged in T-toxin production (Yang et al., 1996). In addition to this REMI mutant, several other mutants defective in mating, conidiation, altered color

and colony morphology were present among the transformants. The phenotypic alteration was linked to the insertion event in 30–50% of all transformants, illustrating a reasonably high tagging efficiency.

Magnaporthe grisea. The filamentous ascomycete *Magnaporthe grisea* infects a large number of grasses including rice, wheat, barley and millet. Strains of either mating type are infectious in the field where they typically reproduce asexually (Talbot, 1995). In two initial screens, 800 and 600 transformants, respectively, derived from transformation of circular plasmids were generated in the presence or absence of restriction enzymes (Shi and Leung, 1995; Shi et al., 1995). From these collections, ten mutants with defects in sporulation, pathogenicity and auxotrophy were recovered. By demonstrating cosegregation with the selectable marker, all mutant phenotypes were linked to the insertional event (Shi and Leung, 1995; Shi et al., 1995). Although the presence of a restriction enzyme apparently was not necessary to obtain single-copy insertions, its presence stimulated transformation efficiencies up to 10-fold (Shi et al., 1995). In a significant proportion of transformants, plasmids integrated at the corresponding restriction site in the host genome but, due to repair processes, the cleavage sites for the restriction enzyme were lost. In transformations where the ends of the REMI vector and the enzyme added were incompatible, integration still occurred, although transformation frequencies were not increased. REMI events occurred with a frequency up to 72% depending on the restriction enzyme used. Very recently, a comprehensive screen of 5538 *M. grisea* transformants mainly generated by REMI using a spectrum of restriction enzymes has produced 27 stable pathogenicity mutants (Sweigard et al., 1998). Among these, 18 were tagged and all except one were obtained by REMI. Disease mutants were classified into 5 groups ranging from no disease symptoms, various types of lesion deficiencies and conidiation defects. Some of the pathogenicity genes were studied in more detail. *pth1* mutants displayed a rare lesion phenotype. The *PTH1* gene product displays homology to *GRR1*, a regulatory gene involved in glucose repression in *S. cerevisiae* (Bailey and Woodword, 1984). The *PTH2* gene was affected in two independent mutations, suggesting REMI insertions may not occur randomly. The corresponding mutants produced no pathogenicity symptoms. *PTH2* reveals strongest homology to carnitine acetyl transferase of *S. cerevisiae* and, thus, might be involved in translocating fatty acyl-CoA into the mitochondrial matrix for subsequent oxidation. The *pth3* disruption mutant displayed histidine auxotrophy and was severely attenuated in pathogenicity. The sequence of the gene revealed strong similarity to imidazole glycerol phosphate dehydratase. *PTH4* encodes a cAMP dependent kinase which had been isolated before by other means (Mitchell and Dean, 1995). This gene is essential for appressorial penetration (Mitchell and Dean, 1995; Xu et al., 1997). These four pathogenicity genes were originally identified in a *M. grisea* strain pathogenic on barley but not on rice. Subsequently, all four genes were shown to be also required for pathogenicity of a *M. grisea* strain that infects rice (Sweigard et al., 1998).

Ustilago maydis. The hemibasidiomycete *Ustilago maydis* is a maize pathogen and causes tumors in its host. *U. maydis* is a dimorphic facultative pathogen. Haploid sporidia grow yeast-like and only after fusion of two compatible sporidia a filamentous dikaryon is generated which represents the infectious form. Cell fusion, pathogenicity, and the morphogenic switch are controlled by two unlinked mating type loci, termed *a* and *b* (Kahmann et al., 1995). The *a* locus with the alleles *a1* and *a2* encodes a pheromone-based cell-recognition system which governs cell fusion (Bölker et al., 1992). The multiallelic *b* locus encompasses two divergently transcribed genes *bE* and *bW*, encoding a pair of homeodomain proteins. *bE* and *bW* dimerize when they are derived from different alleles and it is this protein complex which triggers subsequent pathogenic development (Gillissen et al., 1992; Kämper et al., 1995). To screen systematically for genes that are essential for pathogenicity, the need to form the dikaryon had to be circumvented. This was accomplished by constructing a haploid *U. maydis* strain which expresses an active bE1/bW2 heterodimer and proved to be pathogenic without need for a mating partner (Bölker et al., 1995a). The REMI plasmid pSMUT which lacks homology to the *U. maydis* genome was generated by screening short *S. cerevisiae* DNA sequences that are able to function as promoters in *U. maydis* and drive transcription of the bacterial gene for hygromycin resistance (Bölker et al., 1995a). Transformation in the presence of restriction enzymes did not affect transformation efficiences but strongly favored single-copy insertions (Bölker et al., 1995b). An initial investigation of 1000 insertion mutants has demonstrated REMI events in about

half of the transformants and a frequency of 1.5% non-pathogenic mutants (Bölker et al., 1995b). In extending this study, a comprehensive investigation comprising 6000 *U. maydis* REMI transformants was performed (Kahmann et al., unpubl.); 2.8% of these mutants were unable to induce disease symptoms. Of 122 non-pathogenic mutants analysed, 95 harbored single-copy insertions, and 57 represented REMI events, thus corroborating the initial results. Plasmids were recovered and retransformed to regenerate the mutation and from this a tagging efficiency of approximately 50% was determined. Two of the non-pathogenic mutants were auxotrophic for the amino acids arginine and methionine, respectively (Braun et al., unpubl.). This is consistent with previous studies (Holliday, 1961) which show that auxotrophic *U. maydis* strains carrying mutations in *ad-1*, *me-1*, and *leu-1* fail to initiate disease symptoms when both mating partners carry the mutated allele. Another mutant requiring *p*-amino benzoic acid was severely attenuated in pathogenicity (Braun et al., unpubl.). One insertion had occurred concomitantly with a large deletion in the promoter region of the *prf* gene and this mutant was strongly attenuated in pathogenicity (Basse and Kahmann, unpubl.). Prf is a HMG box-like transcription factor necessary for transcriptional regulation of the *b*-genes (Hartmann et al., 1996). The putative gene for long-chain fatty acid CoA ligase in *U. maydis* was independently tagged twice, suggesting some preferential insertion sites. However, in both cases loss of pathogenicity was unlinked to the insertional event (Böhnert et al., unpubl.). A large number of other pathogenicity mutants is currently under molecular investigation.

Pyrenophora teres. The ascomycete *Pyrenophora teres* is a leaf pathogen causing net blotch of barley. Transformation efficiencies of linearized plasmids were at least 5-fold increased when a compatible restriction enzyme was added. As in *U. maydis*, transformation in the presence of restriction enzymes resulted in single-copy insertions in 95% of the transformants whereas transformation with circular plasmids preferentially favored tandem insertion events. The tagging efficiency in these mutants remains to be determined. Vectors used for transformation carried bidirectionally oriented promoter-less reporter genes at the site for plasmid linearization, thus allowing determination of promoter strength at the insertion site (Maier and Schäfer, unpubl.).

Cladosporium fulvum. In the fungal pathogen *Cladosporium fulvum*, which causes tomato leaf mold, REMI did not increase transformation efficiency but reduced the number and complexity of integration events. However, REMI events were not detected and complementation of pathogenicity mutants by the corresponding wild-type loci was unsuccessful, possibly due to the presence of additional mutations. This indicates a low degree of linkage, making the method unsuitable for this fungus (Spanu, personal commun.).

Colletotrichum lindemuthianum. Although the presence of a restriction enzyme compromised transformation efficiencies in *Colletotrichum lindemuthianum*, a pathogen of cucumber, Southern analysis of transformants generated by the REMI method indicated an increased number of integration events compared to the situation where the restriction enzyme was absent during transformation (Redman and Rodriguez, 1994). However, it remains to be shown whether true REMI events have occurred and whether tagged genes can be retrieved.

Taken together, these studies illustrate the efficiency of the REMI method in the creation of tagged mutants and its use in the identification of pathogenicity genes. Although this method worked appropriately in the phytopathogenic fungi *C. heterostrophus*, *M. grisea*, and *U. maydis* and looks promising in *P. teres*, attempts with other fungi were less successful. The most severe problems arise when additional mutations unlinked to REMI events are generated and when deletions or inversions accompany the insertion of the REMI plasmid (Sweigard, 1996; Böhnert and Basse, unpubl.). As long as the chromosomal rearrangements occur close to the site of plasmid integration the plasmid marker and the mutant phenotype will be linked and the gene responsible for the pathogenicity defect can be isolated from cosmids overlapping the region affected by the rearrangement. Mutations which are not tagged by the REMI plasmid, and which likely arise from mutagenic effects of the restriction enzyme alone are usually discarded because the effort to clone the gene by complementation is too time consuming.

Perspectives and conclusions

For most fungal plant pathogens, infection of a plant host and subsequent development *in planta* requires a multitude of discrete steps including attachment to the

plant surface, generation of infection structures, penetration, growth within the plant tissue and formation of either sexual or asexual spores. Most of these processes are associated with alterations in fungal morphology. In broad terms all genes involved in these processes could be designated pathogenicity genes. A more restrictive designation would probably eliminate from this group of genes all those which cause growth defects or defects in development during axenic culture of the respective fungus. However, this distinction is possible only in those cases where development of the respective fungus does not depend on an association with the host plant. In part, this may be the reason why so many different genes affect pathogenicity. Summarizing their functions to date would most certainly be premature because in none of the systems under investigation has saturation mutagenesis been achieved. Nevertheless, the genes which affect pathogenic development clearly point to certain general directions in terms of classes of genes which are to be expected and which are likely to be identified in all systems: housekeeping genes including genes of primary metabolism, regulatory genes, genes encoding extracellular enzymes and products which may be needed to degrade certain plant metabolites to allow fungal growth, or proteins which may protect the fungus from recognition by the plant's defence system (Yoder and Turgeon, 1996).

As it stands, mutagenesis by REMI offers distinct advantages over conventional mutagenesis in plant pathogenic fungi because it provides easy identification of genes through the tag introduced. Nevertheless, in most systems only about 50% of the genes appear to be tagged and to determine linkage may require a substantial amount of additional work. Also, to generate enough transformants for saturation mutagenesis may be a formidable task in particular in such systems where transformation frequency is not increased by adding the restriction enzyme to the transformation mix. An additional caveat may be the apparent nonrandomness of insertion events evident in some fungi which may or may not be overcome through the use of a series of different restriction enzymes. We expect that additional tools like efficient transposon mutagenesis procedures will have to be developed to cope with these problems. As with all insertional mutagenesis procedures, genes with redundant function cannot be isolated and for essential genes only insertions in regulatory regions are to be expected which may not cause a strong phenotype. This illustrates that REMI cannot provide a substitute for additional techniques of gene identification involving, for example, differential methods.

Besides the use of REMI for insertional mutagenesis, a variety of specific additional applications requiring single insertions are conceivable: In the slime mold *Polysphondylium*, REMI has been applied to introduce the gene encoding green fluorescent protein (*gfp*) into the genome by transforming a mixture of plasmids which allow the generation of N-terminal fusions with GFP in all three reading frames (Fey and Cox, 1997). By monitoring *gfp* expression, the temporal and developmental expression pattern of the tagged gene can be determined. Additionally, in those cases where GFP is fused to a functional protein domain, the spatial *gfp* expression pattern can allow the localization of this protein in living cells (Fey and Cox, 1997). Such an approach is particularly attractive to identify stage-specific genes in phytopathogenic fungi, i.e., fungal genes whose expression is confined to the biotrophic phase. These genes are of special interest because they might play crucial roles during the infection process. In an attempt to identify *in planta* induced genes in *U. maydis*, REMI has been successfully combined with an enhancer trap method (Hill and Wurst, 1993) using *gfp* as a reporter (Figure 2; Aichinger and Kahmann, unpubl.).

Another attractive extension of REMI is signature tagging mutagenesis, a method developed to identify virulence factors in pathogenic bacteria (Hensel et al., 1995). In this method, tagging mutagenesis is carried out with a large pool of transposons harboring individual sequence flags. Consequently, each transformant is characterized by a unique DNA sequence motif. Subsequently, a pool of transformants is injected into its host and the bacteria successful in colonizing the tissue are recovered. Sequence flags of mutants which fail to colonize the host will consequently be lost. This can be visualized by probing the sequence flags recovered against the original pool of transformants. Provided that disease development is not clonal and that a fair percentage of the inoculum is successful in plant colonization, it is conceivable to combine signature tagging mutagenesis and REMI to isolate pathogenicity genes from a pool of fungal mutants, thus circumventing cumbersome individual pathogenicity tests.

It is evident that with such tools as REMI available the pace of fungal pathogenicity gene isolation will increase steadily. The next step will be to ascribe functions to genes expressed during pathogenic development, especially in those cases where the gene

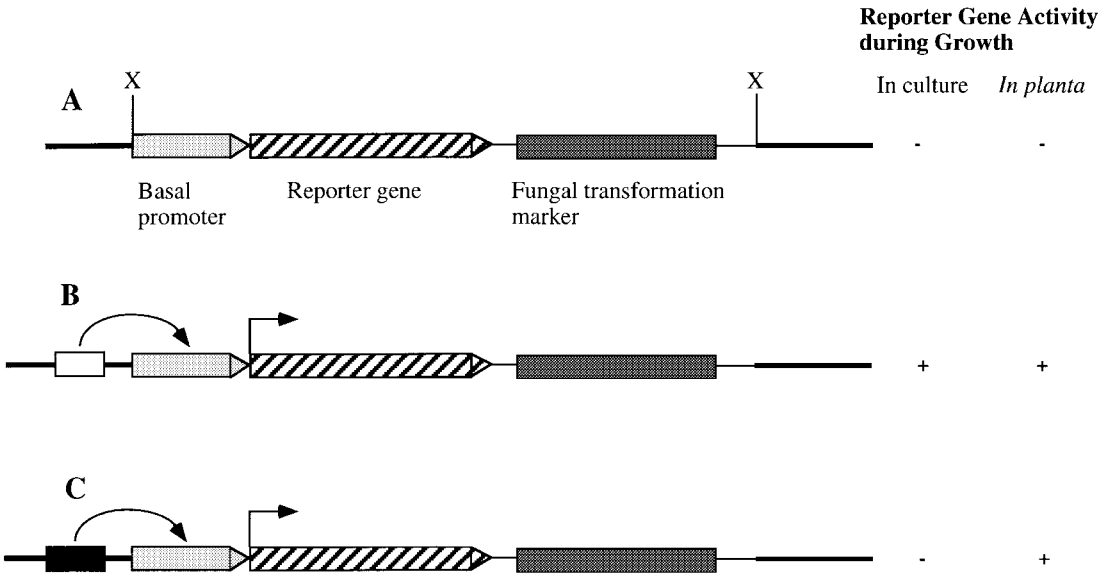

Figure 2. Combination of REMI with enhancer trapping. The modified REMI vector contains a basal promoter, which is fused to a reporter gene and a fungal transformation marker. The basal promoter is inactive alone but confers high levels of expression when fused to an enhancer sequence. The REMI vector is linearized prior to transformation in the presence of restriction enzyme (X). The following scenarios are conceivable: *A.* The vector is inserted in a non-transcribed region resulting in no reporter gene activity. *B.* The vector is inserted in an expressed region leading to constitutive reporter gene activity. *C.* The vector is inserted near an enhancer element mediating specific expression, e.g., during growth *in planta* (Aichinger and Kahmann, unpubl.). *E. coli* plasmid backbone and genomic sequences are indicated by thin and thick lines, respectively.

identified lacks homologs in databases. This will require elaborate cytology to define the stage during pathogenesis which is affected, protein localization, the identification of interaction partners and biochemical analyses. It will be the combination of these techniques which will further our understanding of plant–fungal interactions and which will eventually allow us to differentiate between common and specific infection strategies shared and adopted by fungal pathogens of plants.

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