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Review

Transformation: a tool for studying fungal pathogens of plants

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Abstract. Plant diseases caused by plant pathogenic fungi continuously threaten the sustainability of global crop production. An effective way to study the disease-causing mechanisms of these organisms is to disrupt their genes, in both a targeted and random manner, so as to isolate mutants exhibiting altered virulence. Although a number of techniques have been employed for such an analysis, those based on transformation are by far the most commonly used. In filamentous fungi, the introduction of DNA by transformation typically results in either the heterologous (illegitimate) integration or the homologous integration of the transforming DNA into the target genome. Homologous integration permits a targeted gene disruption by replacing the wild-type allele on the genome with a mutant allele on transforming DNA. This process has been widely used to determine the role of newly isolated fungal genes in pathogenicity. The heterologous integration of transforming DNA causes a random process of gene disruption (insertional mutagenesis) and has led to the isolation of many fungal mutants defective in pathogenicity. A big advantage of insertional mutagenesis over the more traditional chemical or radiation mutagenesis procedures is that the mutated gene is tagged by transforming DNA and can subsequently be cloned using the transforming DNA. The application of various transformation-based techniques for fungal gene manipulation and how they have increased our understanding and appreciation of some of the most serious plant pathogenic fungi are discussed.

Key words. Restriction-enzyme-mediated integration; *Agrobacterium tumefaciens*-mediated transformation; reporter gene; transposon-mediated mutagenesis.

Introduction

It is clear that food production and distribution must increase concurrently in order to meet the demands of the world's rapidly expanding population. This needs to be accomplished in light of global climatic changes and without expanding, too dramatically, existing cultivated acreage. As if not complex enough, this situation is compounded further by the understated impact plant-pathogenic fungi exert on global crop production. The International Society for Plant Pathology has stated that collectively, crop diseases reduce global production by more than 10% every year (http://www.isppweb.org). On an individual basis, fungal pathogens possess the ability to inflict enormous losses that can result in serious socioeconomic hardship. For example, the reappearance of wheat and barley scab (causal agent *Fusarium graminearum*) in North America resulted, for 1993 alone, in yield and quality losses estimated at $1 billion [1]. Rice blast disease caused by *Magnaporthe grisea* consistently reduces the yield of rice, the world's most staple crop [2], while potato late blight by *Phytophthora infestans*, causative agent of the Irish potato famine, has become prevalent once again with the reemergence of more aggressive, fungicide-resistant strains [3]. The advent of such chemically tolerant pathogens is cause for concern because only

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a limited number of measures, alternative to chemical control, are available for many crops. In addition, the literature contains numerous papers that describe either the appearance of strains that have evolved to infect a novel host or the migration of pathogens to areas, previously unaffected. Besides the losses in crop production, certain groups of fungal pathogens also pose a health hazard to humans and animals through the food chain by producing mycotoxins in infected crops [4]. For instance, the genus *Fusarium* is one of the most creative producers of toxic compounds known, and approximately 20 *Fusarium* species are known to be toxigenic. The Food and Agriculture Organization of the United Nations has estimated that 25% of the world's crops are tainted with mycotoxins with substantial impact to food contamination [5].

The development of new and innovative ways to diagnose, predict and restrict plant diseases has therefore become a constant challenge for the science of plant pathology (study of plant disease). This interdisciplinary field has progressed dramatically in the last decade in parallel with the transition of biological sciences as a whole, in large part due to the introduction of various molecular genetic and genomic tools. One such tool entails the modification of the fungal gene through the integration of non-homologous DNA, which ultimately reveals the role played by the targeted gene in initiating plant disease. The efficacy of fungal gene manipulation has improved substantially in recent years as a result of two technical innovations in particular, restriction-enzyme-mediated integration (REMI) [6, 7] and the *A. tumefaciens*-mediated transformation (ATMT) protocol [8].

Rather than compiling a detailed list of all plant pathogens subjected to various molecular genetic analyses via transformation, in writing this review, our primary objective is to describe those transformation techniques that have advanced our understanding of the mechanisms of pathogenicity in some of the most serious plant pathogens of today's agriculture.

Basics of fungal transformation

The potential of transforming fungal cells with foreign DNA was initially realized following the publication [9] of a procedure that described the generation of protoplasts of bakers yeast (*Saccharomyces cerevisiae*). Since the groundbreaking transformation of *leu*2 mutant *S. cerevisiae* protoplasts with the *LEU*2 gene [10], attempts to improve the efficiency of yeast transformation have been ongoing with considerable success, and a large array of vectors and protocols have now been developed to manipulate yeast genetically via transformation. The number of reports describing the successful transformation of filamentous fungi has also steadily increased since the first transformation of *Neurospora crassa* [11].

A typical procedure for transforming filamentous fungi involves the preparation of fungal protoplasts, delivery of the transforming DNA therein, and selection of the generated transformants. The first step involves the digestion of the fungal cell walls using a mixture of hydrolytic enzymes. The resulting protoplasts are mixed with transforming DNA in the presence of various chemicals, typically including calcium chloride, polyethylene glycol (PEG) and a buffering agent, which cultivate the introduction of the DNA into the fungal protoplasts. Protoplast generation can be circumvented in two other transformation methods, biolistic transformation and ATMT, whereby intact fungal cells are either bombarded with gold particles coated with DNA or coincubated with *A. tumefaciens* carrying transforming DNA on a binary vector. The recent transformation of *Erysiphe graminis* (the causal agent of barley powdery mildew), an obligate fungus that can only grow on and in living hosts, demonstrates the utility of biolistic transformation as a tool for studying this and other obligate pathogens [12].

In contrast to the availability of autonomously replicating vectors for *S. cerevisiae*, most vectors for transforming filamentous fungi do not replicate in the host and instead integrate into the host genome via homologous or illegitimate recombination. The frequency of individual recombination events depends on multiple factors, including the methods and conditions for transformation, the transformation host, and the degree and length of sequence homology that exists between the transforming DNA and the host genome. To identify the transformants, it is imperative that a gene conferring a selection advantage to the transformed cells be included within the transforming vector. Initial studies focused upon the conversion of auxotrophic fungal strains (requiring certain additional nutrients to grow) to a state of prototrophy [10, 13–17]. An alternative selection process was presented when genes conferring antibiotic resistance and typically bacterial in origin were included in the transforming vector [18]. Once integrated, the vector presents the resulting transformants with the ability to grow in the presence of a specific antibiotic that is otherwise toxic to the untransformed, wild-type strain. Such dominant selectable markers are now commonplace in fungal transformation procedures, with genes conferring resistance to hygromycin B, geneticin, benomyl, phleomycin, carbendazim or bialaphos having been identified. For an excellent review of the history, methods and requirements of fungal transformation, we refer to Fincham [19].

Gene disruption via transformation

Random, insertional mutagenesis

The objectives of insertional mutagenesis are to achieve an efficient and random disruption of the genome of a tar-

get organism and to ultimately reisolate those genes tagged by the transforming DNA. Homologous recombination (assimilation of two DNA sequences possessing sequence homology) is considered to be the exception, rather than the rule in many filamentous fungi, with recombination events entailing little or no homology and classified as 'illegitimate', occurring most frequently. It is this latter property, whereby integration occurs at ectopic sites, that has permitted the random mutagenesis of pathogen genes using the transforming DNA as a mutagen.

The basic random mutagenesis protocol involves mixing of the foreign plasmid, containing a dominant selectable marker, with prepared fungal protoplasts in the presence of a buffered medium, typically containing polyethylene glycol (PEG). Termed PEG-mediated transformation, Dufresne et al. [20] employed such an approach when they generated mutants of the common bean pathogen *Colletotrichum lindemuthianum* and subsequently showed how significant *clk1*, a gene encoding serine/threonine protein kinase, was in the early steps of the disease.

REMI

The efficiency of insertional mutagenesis mediated by transformation was significantly improved when a new technique called REMI was introduced. This technique is based on the finding that the addition of a restriction enzyme into the transformation mix (fig. 1B) can significantly increase the efficiency of transformation [21]. Treating the host genome with the same restriction en-

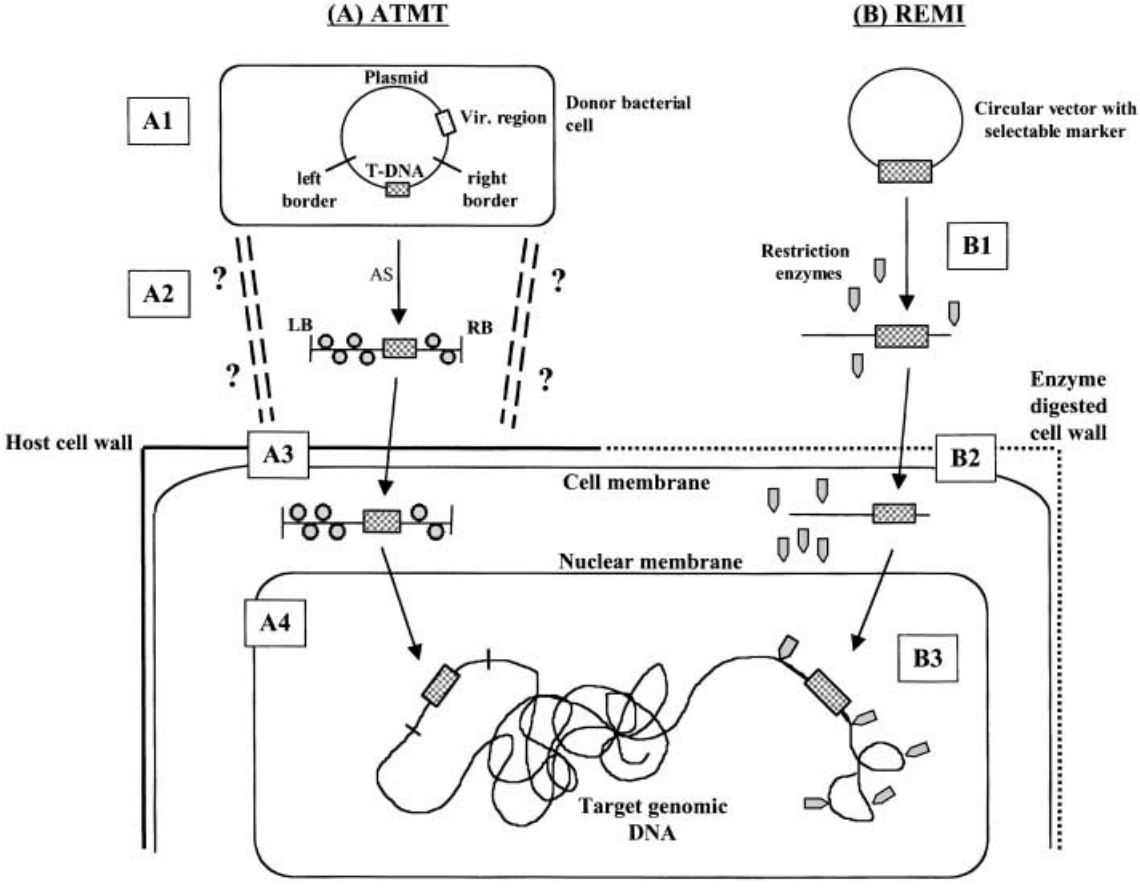

Figure 1. Diagrammatic representation of two forms of transformation: (*A*) *Agrobacterium tumefaciens*-mediated transformation (ATMT) and (*B*) restriction enzyme mediated integration (REMI). (*A1*) In ATMT, the process is initiated by inserting a plasmid, equipped with a selectable marker gene (▨) situated between the left (LB) and right (RB) border of the transforming T-DNA sequence, into the donor bacterial cell. (*A2*) In the presence of specific compounds such as acetosyringone (AS), it is believed (?) that the bacterial cell adheres to the host cell wall. A single-stranded T-DNA complex (T-DNA + Vir proteins [○]) is then produced. The presence of the left and right borders is essential for this process. (*A3*) A channel permits the transport of the complex through the host cell wall and membrane. (*A4*) The T-DNA crosses the nuclear membrane into the nucleus where it then randomly integrates itself into the host genomic DNA. In REMI, the selectable marker is inserted into a circular vector that is then linearized (*B1*) by the action of a specific restriction enzyme. (*B2*) The linear vector is transported across the cell membrane of the host protoplast (host cell wall has to be removed with hydrolytic enzymes) and through the nuclear membrane in the presence of the same restriction enzyme. (*B3*) The host DNA is digested by the restriction enzyme, and the transforming DNA, compatible with the cut ends of the host genome, inserts itself into these sites.

zyme used to linearize the introduced plasmid generates a high number of exposed ends ('potential integration sites') that are compatible with the already cleaved ends of the transforming vector throughout the host genome, thereby resulting in a significantly higher rate of transformation. This has been reported in *Aspergillus nidulans* [27] and *Colletotrichum graminicola* [23] where, in comparison to a transformation procedure utilizing linearized plasmid only, the presence of restriction enzymes induced a 20- to 60-fold and 27-fold increase, respectively, in the frequency of transformation.

In current years, numerous papers, including two comprehensive reviews [6, 7], have been published that describe the effective application of REMI to study gene functionality in fungal phytopathogens. Examples of the genes cloned by REMI include the AAL-toxin-synthesizing gene of *Alternaria alternata* ('stem canker' of tomato) [24] that is required for successful infection, and the polyketide PM-toxin of the maize pathogen *Mycospharella zeae-maydis* [25]. In the rice pathogen *Magnaporthe grisea*, from the 5538 REMI-generated transformants collected in one comprehensive study, 27 mutants exhibited a defect in their ability to cause disease, from which several genes, all possible fungicide targets, have been cloned [26]. A similar study of the same pathogen identified an additional five mutants from a REMI-constructed library [27]. Other fungal species that have been targeted and from which mutants have been created include *Colletotrichum magna* (cucurbit pathogen) [28], *Ustilago maydis* (maize pathogen) [29], *Gibberella fujikuroi* (maize and cereal pathogen) [30], *C. graminicola* (maize pathogen) [23, 31], *Fusarium oxysporum* (melon pathogen) [32] and *Cochliobolus heterostrophus* (maize pathogen) [33].

In the absence of a restriction enzyme, the transforming vector is typically integrated in tandem arrays [29]. REMI, in contrast, favors single-vector insertion into each genomic locus [7] and moreover, a high percentage of the created transformants possess a single copy of the integrated DNA, thereby facilitating its rescue along with parts of the tagged gene [6]. Yet the ability of REMI to instigate random integrations throughout the host genome is uncertain. Sweigard et al. [26] reported that they tagged the same locus twice out of a population of 5538 transformants, and Lu et al. recorded the same with the maize pathogen *Cochliobolus heterostrophus* [33]. It has been hypothesized that the physical packing of the genome at the time of integration could be responsible for this nonrandomness. For example, the highly transcribed areas of the genome are likely to be more receptive to restriction digest than those corresponding to transcriptionally inactive areas because their chromatin structure is more relaxed to permit gene expression [7].

Typically, the double-stranded break generated in the chromosome by the restriction enzyme will be repaired either through incorporation of the vector or religation mediated by the cellular DNA repair system. However, a disadvantage of REMI lies in the fact that the processes of DNA repair are not always efficient enough to accurately complete this task. This can lead to the occurrence of unsolicited deletions or rearrangements [26, 30] during transformation. In the above-mentioned study [26], one third of the *M. grisea* mutants generated by REMI were found to be untagged with the selectable marker gene, and a similar result was described with *Cochliobolus heterostrophus* [33]. In *U. maydis* (maize pathogen) the figure may be as high as 50% of generated transformants [29], which concurs with the conclusions reached by Kahmann [6]. In each study, inaccurate DNA repairs after the restriction of the host genome have been presented as possible explanations. This problem can be alleviated somewhat, though, if the concentration and type of restriction enzyme used to cleave the host genome are fully optimized.

The generation of such untagged mutations can be a substantial nuisance when it comes to cloning the gene causing the mutant phenotype. For fungi with a sexual stage, genetic crosses can be employed to identify those untagged mutants in which the mutant phenotype fails to cosegregate with the selectable marker on the inserted plasmid. However, for fungi lacking a sexual stage, cloning of tagged gene(s) and subsequent functional complementation by transformation are necessary to verify whether a given mutant has been tagged. When REMI generates a high percentage of mutants with multiple copies of inserted vector, such a verification process can be quite time consuming. Nevertheless, transformation through REMI has proven itself in dissecting the mechanisms of pathogenesis of several phytopathogens, one of the most prominent being *M. grisea* [26, 34, 35].

ATMT

Transformation mediated through the plant pathogenic bacteria *A. tumefaciens* (ATMT) has long been used to transfer genes to a wide variety of plants and has also been used extensively as a tool for the insertional mutagenesis of *Arabidopsis thaliana* [36–38]. Recently, several fungi have been transformed using the ATMT approach [8, 39–43].

In natural *A. tumefaciens* infection of plant cells, the bacterial cells synthesize cellulose filaments that adhere the pathogen to the wall of a wounded host cell. The bacterium then initiates a complex process that concludes with the transfer and incorporation of a piece of single-stranded bacterial DNA into the host genome. The net effect of this is that the host cell is manipulated to synthesize compounds that *A. tumefaciens* can employ as a nutrient source. The transferred fragment of bacterial DNA is a segment of DNA located on the bacterial plasmid.

Termed the T-DNA ('transfer' DNA), this DNA segment is bound by left and right border sequences (fig. 1A), each of which is composed of a 25-bp imperfect direct repeat. The existence of these borders on the T-DNA is imperative for successful infection. With regard to transformation studies, what is most convenient is that any DNA, irrespective of its source, engineered to fit between the T-DNA left and right borders can be efficiently integrated into the host of choice. Also situated on the plasmid is the *virulence* (*vir*) region that encodes the production of several Vir proteins, which are essential for infection. These proteins detect the presence of a wounded plant cell (by perceiving such plant phenolics as acetosyringone) and also mediate T-DNA transfer into the host cell by forming a T-DNA-protein complex (fig. 1A) which facilitates transfer of the T-DNA complex through the host cell wall [44]. Subsequently, the T-DNA passes into the nucleus and integrates itself into the host DNA. Although it is speculated that a similar mechanism operates in the *A. tumefaciens* infection of fungal cells, given the requirement for acetosyringone (an 'infection-inducing' compound for plant transformation) for fungal transformation, the process of infection requires further investigation to determine whether this is the case.

For insertional mutagenesis, this technique offers huge potential as an alternative tool to REMI. One of the principal advantages of ATMT over conventional transformation techniques is the versatility it provides in choosing which starting material to transform. In our ATMT system [43] we transformed conidia of *F. oxysporum* and *Verticillium dahliae* and avoided the problems (low yield and viability) typically associated with the time-consuming process of protoplast isolation. Indeed, *A. tumefaciens* can transform protoplasts in addition to hyphae, spores and even blocks of mushroom mycelial tissue [8, 45]. With approximately 300–500 hygromycin B-resistant colonies collected in the *F. oxysporum* system per 1×10^6 fungal conidia treated [43], ATMT can be used to rapidly generate a large library of fungal transformants, though the efficiency appears to be highly dependent on the fungal species transformed. More significantly, 50–80% of transformants possess a single T-DNA insert per genome [43], something that is important when it comes to rescuing the tagged gene from fungi lacking a sexual stage, such as *F. oxysporum* and *V. dahliae*.

Targeted mutagenesis

The typical research objective of a fungal plant pathologist is to determine what processes are essential for the target fungus to cause plant disease. The random mutagenesis procedure described above is one form of transformation that can be used to accomplish such a task. Alternatively, researchers can take a more direct approach by specifically disrupting a predetermined gene that is conceived to be essential for pathogenicity, thereby resolutely disproving/proving its role in pathogenicity. Termed gene replacement, the method comprises the direct substitution of the wild-type gene with its mutant allele, which has been disrupted by the insertion of a selectable marker (e.g. antibiotic resistance) within its coding region, via homologous recombination mediated by transformation. By generating a precise mutation in the studied genome, it can provide a powerful means for assessing gene function.

A number of approaches have been used to isolate fungal genes for targeted mutagenesis: (i) polymerase chain reaction (PCR) amplification of the genes homologous to known pathogenicity genes in other fungi [46–49], (ii) PCR cloning of genes encoding purified pathogenicity factors [50] and (iii) isolation of genes differentially expressed during infection [51, 52]. In the conventional gene replacement approach the chosen gene, once cloned into a suitable vector and disrupted with a selected marker gene, is introduced into the host genome via direct PEG-mediated DNA transfer. While the process can accurately knock out a target gene, the frequency of such an event can be quite low if the frequency of ectopic (heterologous) integration is high. In such cases, large numbers of transformants need to be screened to determine which one possesses the desired mutation. This inconvenience can be mitigated if *A. tumefaciens* is used to reintegrate the mutant allele back into the chromosomal locus. For example, in the commercially important yeast, *Kluyveromyces lactis*, the precise gene replacement event occurred in only 1% of transformants that were produced by a traditional method of direct DNA transfer (via electroporation of protoplasts) [53]. Employing *A. tumefaciens* to replace the gene increased this frequency to 71% [53].

A number of genes in several fungal pathogens have been identified to be important for pathogenicity via targeted mutagenesis, including genes for mitogen-activated protein (MAP) kinase [47, 49–54], ATP-binding cassette (ABC) transporter [55], heterotrimeric GTP-binding protein [48], adenylate cyclase [46] and regulating catabolite derepression [56]. The plant cuticle and cell wall are major physical barriers against fungal infection. To breach these barriers, fungal pathogens produce an array of enzymes. Although genes encoding many of these enzymes have been cloned from several fungi [57], their role in pathogenicity has been difficult to establish experimentally due to the genetic redundancy of genes for individual enzymes [58–60]. The production of cell-wall-degrading enzymes is subjected to catabolite (glucose) repression and requires a kinase for derepression, termed *SNF1* in yeast [61]. Mutations in the *SNF1* homolog of *Cochliobolus carbonum* (maize pathogen), *ccSNF1,* by gene replacement cause pleiotropic effects, including a

reduction in cell-wall-degrading enzyme activities and their transcripts, reduced growth on certain complex and simple carbon sources, and reduced virulence. This result confirms the hypothesis that *ccSNF1* is necessary for the expression of both cell-wall-degrading enzymes and for virulence [56]. Targeted mutagenesis has also been effectively used to study the process of transformation [62] and pathogenesis [55] of *Botrytis cinerea*, the ability of *Cercospora kikuchii* to infect soybean [63], the importance of the trichothecene toxin-producing gene to the virulence of the wheat and rye pathogen, *Gibberella zeae* [64], the role of *pgx4*, a gene encoding polygalacturonase, in pathogenicity of the tomato pathogen *F. oxysporum* [58], the effect of the *magB* gene (a GTP-binding protein expressing gene) on the growth and development of the rice pathogen *M. grisea* [34] and the significance of the mitogen-activated-protein kinase gene, *PTK1*, in causing *Pyrenophora teres* disease in barley [65].

Complementing our program of random mutagenesis of the vascular wilt-causing pathogens *V. dahliae* and *F. oxysporum*, we have also applied a targeted mutagenesis system based on *A. tumefaciens* to help us further understand their mechanisms of pathogenesis. In this system (fig. 2), we mutagenized a target gene cloned into a binary vector (e.g. pDHt) in vitro, using purified transposase (Epicentre Technologies, Madison, WI, USA) and a modified *Escherichia coli* Tn5 transposon carrying a dominant marker gene for selecting fungal transformants. As the marker gene has already been inserted within the Tn5 transposon, the action of the added transposase will result

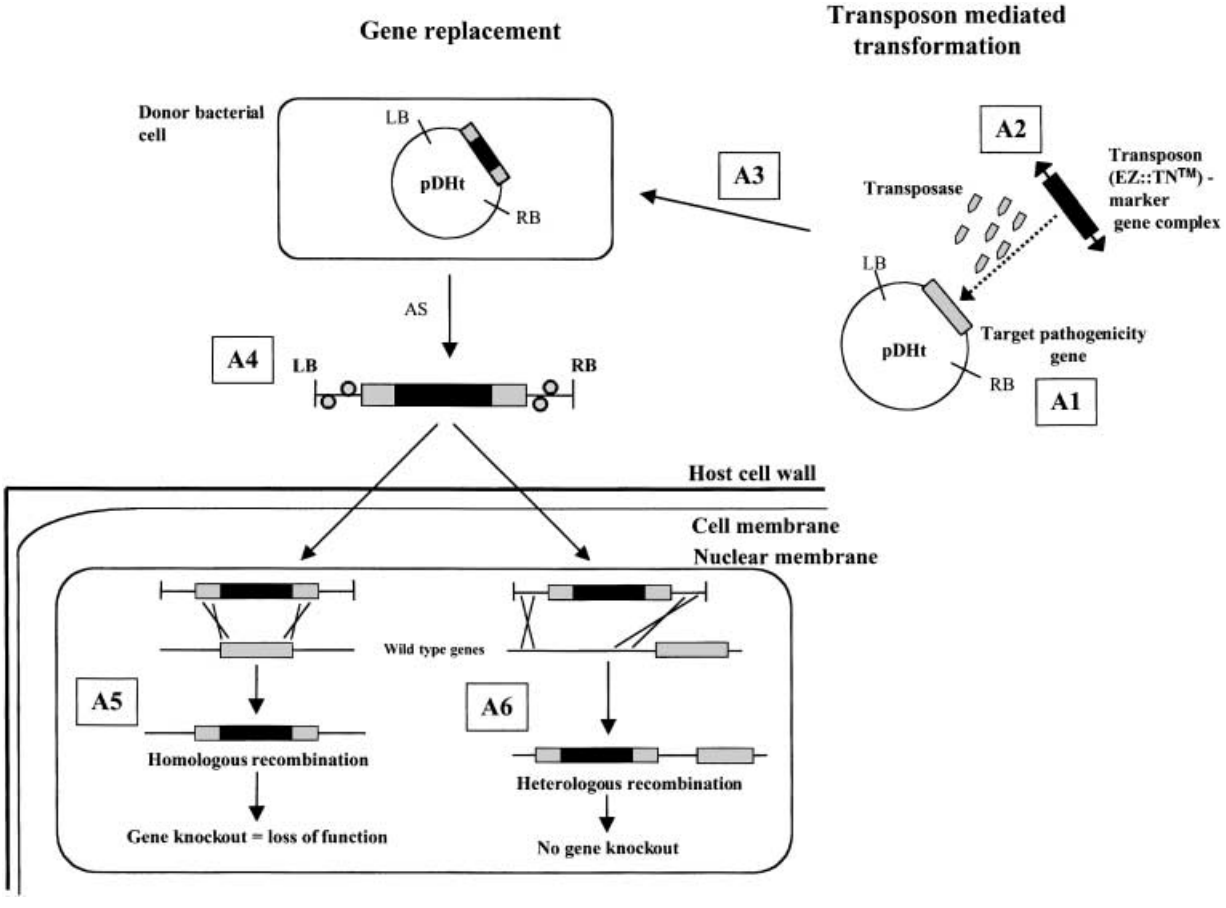

Figure 2. Representation of transposon-mediated transformation of a targeted gene and the ensuing process of gene replacement. The selected target gene (*A1*), situated between the left (LB) and right (RB) border of the T-DNA, is mutagenized by the insertion of the transposon-marker gene (e.g. antibiotic resistance) complex. This process is mediated by the associated transposase (*A2*). The resulting disruption vector is introduced into the donor *A. tumefaciens* cell (*A3*), which is then incubated in the presence of the host fungal cell and the infection inducing chemical acetosyringone (AS). The T-DNA-Vir protein ( ) complex (equipped with the transformed gene) crosses the host cell wall and passes through the nuclear membrane into the nucleus (*A4*). If homologous recombination occurs between the host gene and its transformed allele (*A5*), the function of the gene will be terminated, and the resulting phenotype of the transformant can be studied. If there is not sufficient compatibility between the transformed allele and the corresponding host gene, heterologous recombination (*A6*) will commence, ending in the ectopic integration of the disrupted gene into the host genome. Subsequently, by not interfering with the function of the originally targeted gene, any generated transformants will not display the desired phenotype. Note: Because the donor *A. tumefaciens* cell possesses a sufficient number of *vir.* genes, to permit T-DNA transfer, the Vir. region has been removed from pDHt, allowing larger sections of DNA to be cloned into the vector.

in the random insertion of the transposon-marker gene complex into the target gene, therefore generating multiple insertional mutant alleles. The mutant allele is then transferred into a donor *A. tumefaciens* cell, which can be used for gene replacement. As described in figure 2, the single-stranded T-DNA complex (here equipped with the mutant allele) is passed through the host cell wall and into the nucleus, where it inserts into the selected locus through double homologous recombination, resulting in a specific gene replacement event (gene knockout) and the loss of gene function. Utilizing this technique, we have mutagenized the *SNF1* gene homolog in *F. oxysporum*, and of the generated transformants, over 70% contained an accurate gene knockout. This suggests that the high efficiency of gene replacement observed in *K. lactis* is not unique to this fungus and that this technique has a broad application in the mutation of specific genes in various fungi.

Additional transformation-based tools for studying fungal pathogens

Transposons are mobile segments of DNA that possess the ability to integrate, exit and reintegrate themselves throughout the genome. By interrupting the functionality of the gene through insertion, their role is well suited to an insertional mutagenesis protocol. The *impala* transposon of *F. oxysporum* has been well characterized [66] and is one such element that could excise and reinsert in the genome of an unrelated fungal species. When it was introduced into *M. grisea* by transformation, it actively transposed and tagged genes important for pathogenicity [67], suggesting that the widespread use of this element for the random mutagenesis of plant fungal pathogens is a real possibility.

The use of specific reporter genes that permit a simplified, visual screening of created transformants by conferring a color change under appropriate conditions has been expanded such that they can serve as diagnostic tools in determining the presence/absence of fungal matter in planta [68–70]. The *E. coli* β-D-glucuronidase (GUS) reporter gene [71] has been used in the transformation of several phytopathogenic fungi [72–75] where in the presence of the enzyme substrate (X-glucuronide), hyphae of the transformed fungal strain take on a blue color, becoming clearly visible in plant tissue. Though widely adopted to examine plant-pathogen interactions, Thomma et al. [76] have reported that the GUS enzyme is not always an adequate marker for studying plant-pathogen interactions and that in particular, caution should be exercised when using this enzyme to quantify fungal biomass. For more qualitative studies, however, such as evaluating fungicide efficacy against important wheat pathogens (*F. culmorum*, 'foot rot' of wheat), the GUS system has proven its effectiveness [74].

An alternative to the GUS reporter gene is the GFP (green fluorescent protein)-expressing gene, whose suitability as a reporter gene was first described by Chalfie et al. [77]. Originally extracted from the jellyfish *Aequorea victoria*, in vivo GFP activity has shown how essential it is for *M. grisea* conidia to disperse on the surface of the host, so that successful infection can be accomplished [78]. In *Colletotrichum gloeosporioides*, the localization of the gene product of *CgDN3*, an essential pathogenicity gene, has been determined [79] whereas in *Colletotrichum lindemuthianum* (bean pathogen) GFP-linked expression of the endopolygalacturonase gene, *clpg*2, found its product to be closely associated with conidia germination and appresoria formation on the leaf surface [80]. Both these stages are essential for successful infection and are potential targets for crop protectants. The potential of GFP-labeled fungal transformants as a tool to study the host-pathogen interaction of *U. maydis*-maize [81] and the population dynamics of filamentous fungi on the leaf surface have also been presented [82].

Applying a promoter trapping technology based on transformation, termed in vivo expressed technology (IVET), several gene promoters of *Histoplasma capsulatum* (causative agent of one of the most common human fungal infections) that are expressed exclusively during the infection process have been identified and are now being tested as possible targets for therapeutic agents [83]. The same procedure has also been applied to *Candida albicans* with similar results [84]. Though several posters at the 7th International Congress of Plant Pathology reported its initial use on *U. maydis* and *Pyrenophora teres* [46], the potential benefit of this technique in the study of fungal plant pathogens has yet to be realized.

Conclusion

The success of a random mutagenesis project mediated by transformation, irrespective of the method employed, is dependent on how effectively the process is designed and optimized. From the onset, a transformation system has four fundamental requirements. The primary one is to create transformants exhibiting a phenotypic alteration, which has been arrived at with a minimum amount of genomic disruption. The second is to design a screening procedure that will easily identify the desired phenotype. If for example, a large transformant library is to be created, a screening system must be developed that is rapid, sensitive and reproducible. Once the transformant has been selected, the third is related to how the tagged gene is to be rescued from the target genome, whereas the fourth refers to the verification process, that is proving that the phenotype of the transformant is indeed a direct result of the induced mutation. All four points are essential and must be considered carefully before the research is initiated. If REMI is to be

used, protoplasting temperature and the conditions at which the protoplasts are regenerated can all play a huge part in the success of the transformation protocol [85]. Similarly, with ATMT, transformation efficiency can be strongly influenced by experimental conditions [43]. In screening transformants for the desired phenotype, it is possible that the mutation may induce a lethal phenotype and the transformant will be lost in the screening process. This needs to be taken into consideration, as does the associated linkage between the mutation and the phenotype and how it is to be assessed. If the fungus possesses a sexual cycle, the latter point can simply be completed by segregation analysis. If the fungus is asexual however, complementing the transformant with an unmutated, wild-type allele of the mutated gene can only complete the process. This is why a single insertion of the transforming vector/sequence into the host genome is preferred over multiple insertions and why, in the case of the asexual pathogen *F. oxysporum*, ATMT has an advantage over other protocols by producing a high frequency of single insertion events in the generated transformants [43]. This is encouraging, because if two or more integrations occur, the complementation process can be quite time consuming. Nevertheless, in spite of the difficulties, in terms of studying the mechanisms of fungal pathogenesis, the benefits of transformation are well published. Since the earlier transformation studies were completed on the model fungus *S. cerevisiae*, significant insight has been gained into how the most severe fungal plant pathogens inflict the damage they do. This has resulted in improved crop protection systems, through more resistant cultivars and more efficient fungicides which have ultimately led to increased yield.

At present, the sequencing of the genomes of more than 100 microorganisms has been completed or is underway (http://www.tigr.org/tdb/mdb/mdbcomplete.html). Although plant pathogens are underrepresented in this group, with only two plant pathogenic bacterial genomes having been completely sequenced [86], this situation is likely to significantly improve in the near future, considering the community-wide efforts to promote the genome sequencing and/or large scale EST (expressed sequence tag) analysis of major plant pathogenic fungi. Information and technology resources derived from these sequencing efforts will undoubtedly transform the way we study these pathogens. DNA microarray-based assays will make it possible to monitor genome-wide changes in gene expression in both pathogens and their hosts during the various stages of infection. Since cellular activities are controlled not only at the transcriptional but also at the translational and post-translational levels, it will also be necessary to monitor the pattern of proteins synthesized under a given condition. Such a pattern could be determined using a combination of two-dimensional electrophoresis and mass spectrometry. The judicious application of these techniques will assist investigators in methodically identifying a large set of genes from pathogenic fungi which might play an important role in pathogenesis. To systematically characterize the function of these genes, the development of efficient gene knockout and modification tools is critical. ATMT exhibits huge potential as such a tool. The combination of genomic data with efficient gene manipulation tools should provide an excellent resource in the field of plant pathology with which to study the in planta functioning of the pathogen and further advance our understanding and appreciation of plant pathogenic fungi.

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