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REVIEW

Approaches to functional genomics in filamentous fungi

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The study of gene function in filamentous fungi is a field of research that has made great advances in very recent years. A number of transformation and gene manipulation strategies have been developed and applied to a diverse and rapidly expanding list of economically important filamentous fungi and oomycetes. With the significant number of fungal genomes now sequenced or being sequenced, functional genomics promises to uncover a great deal of new information in coming years. This review discusses recent advances that have been made in examining gene function in filamentous fungi and describes the advantages and limitations of the different approaches.

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Introduction

In recent years there has been a surge of interest in functional genomics research in filamentous fungi and oomycetes. This has been facilitated by several important advances. Firstly, the development of various transformation systems has steadily increased the number of filamentous fungi, and the variety of fungal tissue types, that are amenable to genetic manipulation. Secondly, techniques for random mutagenesis and targeted gene mutation have been adapted and developed as efficient tools for investigating gene function in fungi. Thirdly, the genomes of a significant number of fungi have been, or are presently being, sequenced (http://www.genomesonline.org/). Despite the multiple tools now available, the initiation of a new functional genomics project is not always straightforward.

There are many possible combinations of transformation systems and functional genomics strategies available, not all of which are standard practice in filamentous fungi. Tailoring the right strategy for a particular project is a complicated task with many variables to consider. While *Agrobacterium*-mediated transformation (AMT) has been developed as a powerful tool for both random and targeted gene disruption and is increasingly being seen as the system of choice for many fungi (reviewed in [1]), there are other methods that offer advantages in specific situations. Also, some successful transformation systems may not be suitable for particular uses. Frequency of homologous recombination, for example, is highly dependent on the mode of transformation [2]. In this review we provide an overview of the different approaches to transformation and manipulation of gene expression in filamentous fungi and describe strategies that have been used successfully for fungal functional genomics.

There are a number of reviews on transformation and functional genomics of fungi in the literature. Some of these reviews are restricted to quite specific areas of this topic. AMT and its application to functional genomics in fungi has been comprehensively reviewed recently [1]. A review of RNA silencing in fungi, still *in press* at the time of writing, by Hitoshi Nakayashiki has just been completed [3]. While our review covers some of the material in those papers, we have sought to give a broader, comparative overview of the approaches to functional genomics in filamentous fungi and to indicate the directions in which this research is heading. Other general reviews are also available [4, 5] and this review updates that work with recent advances.

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Achieving stable, homokaryotic transformation

Most functional genomics methods depend upon the production of stable transformants. In recent years, efficient transformation systems have been developed for a wide range of filamentous fungi (reviewed in [1, 4, 5]). Successful transformation of fungi has been achieved by: $CaCl_2$/polyethylene glycol [6-8], electroporation [9-15], particle bombardment [2, 6, 10, 16, 17], and *Agrobacterium tumefaciens*-mediated transformation (AMT) [1].

Transformation of protoplasts using $CaCl_2$/polyethylene glycol (PEG) typically occurs in 15-30 min incubation at room temperature in the presence of 10-50 mM $CaCl_2$ and high concentrations of PEG. Electroporation is also usually used to transform protoplasts, though sometimes germinating conidia are used. During electroporation, protoplasts or conidia are exposed briefly to a high amplitude electric field, which permeabilizes the membrane permitting uptake of foreign DNA. Although used widely, a major limitation of these methods is that they usually require the production of protoplasts, a process that suffers from inconsistencies between the enzyme batches used to digest fungal cell walls and the ability of cell walls from different fungi to be digested [16, 18].

For particle bombardment (biolistics), gold or tungsten beads are coated with the transforming DNA and used to bombard the intact fungal tissue [2, 18-21]. The flexibility of target tissues used for biolistics has advantages in strains that produce little material suitable for protoplast production [18, 22]. Biolistics is also useful in systems where protoplasting has not been developed and there is little known about the components of the cell wall [23, 24]. Many researchers have reported similar or increased frequency of transformation, complexity of integration events and mitotic stability compared with protoplast mediated transformation [2, 18-21, 25], although direct comparisons are difficult due to different target tissues, fungal genera and optimisation protocols.

A recent trend has been towards functional genomics approaches based on AMT. AMT has been shown to produce a significantly higher frequency of transformation and more stable transformants with less complicated insertional mutants than biolistics [26, 27]. Under the right conditions, *A. tumefaciens* is able to transfer DNA (T-DNA) to a very wide range of fungi and fungal tissues. Fungi that have been recalcitrant to transformation by other systems have been successfully transformed by co-cultivation with *Agrobacterium* [27]. AMT is a relatively simple system to work with, does not require the production of protoplasts, and is suitable for both gene-replacement by homologous recombination [1, 28, 29] and insertional mutagenesis by random integration [30, 31].

Despite these advances, the development of a new transformation system is often not as straightforward as the literature suggests, which is biased towards success. Even for AMT there are so many variables that need to be optimised for individual systems that the selection of a particular combination of parameters that work may often be more fortuitous than systematic. Once a transformation system has been successfully developed, there may still be major barriers to overcome before it is possible to analyse gene function. A potential problem for genetic analysis is the multinucleate nature of filamentous fungi. The presence of multiple nuclei can confound methods, such as gene replacement and insertional mutagenesis, which rely on the isolation of homokaryotic transformants derived from a single transformation event to study loss of function mutants [32].

In transformed multinucleate yeast, where the cells are heterokaryotic for the transformation event, homokaryotic tissue is produced within a single round of colony growth, probably by random assortment of nuclei during budding [33]. By growing colonies on selective media that kills cells that are homokaryotic for untransformed nuclei, transformed, homokaryotic tissue is easily isolated. A similar effect may work in filamentous fungi as it has long been known that the proportion of genetically different nuclei within hyphae is responsive to selective pressure [34]. In filamentous fungi, selection can also produce homokaryotic tissue from transformed tissue that must originally have been heterokaryotic [35], though this may not always happen [18, 36]. Where gene disruption results in a loss of function that impairs growth, selection for transformants is likely to produce heterokaryotic tissue as tissue that is homokaryotic for either mutant or wild-type nuclei will suffer a selective disadvantage [29, 37]. This problem might be overcome if it is possible to transform uninucleate tissue or to cycle transformed tissue through a uninucleate stage [18, 29, 38].

Difficulties producing homokaryotic transformants from multinucleate tissue can be avoided by using techniques that are not compromised by the presence of the wild-type gene. If the target gene sequence is known, it is possible to use gene expression knockdown strategies, such as RNAi, that rely on inactivation of mRNA rather than gene mutation [39, 40]. However, for random mutagenesis strategies which seek to find unknown genes through phenotypic changes, the presence of untransformed nuclei or nuclei with different integration loci, may prove more challenging.

Marker genes for transformation

A wide range of genes have been found to be suitable as selectable markers for fungi. The *hph* gene (hygromycin

B resistance) is the most commonly used selection system because of its effectiveness in most, but not all, systems. Other selective agents such as phleomycin, sulfonylurea, nourseothricin, bialophos, carboxin, blasticidin S and benomyl have also been used [5, 41-44]. An alternative to drug resistance genes for transformation of fungi is to use auxotrophic markers such as *pyrG* (a homologue of the *Saccharomyces cerevisiae ura3* gene). Mutants that lack *pyrG* are auxotrophic for uracil so vectors containing *pyrG* allow selection on uracil-deficient media [45]. Additionally, *pyrG*-deficient mutants are resistant to 5-fluoro-orotic acid (5FOA) which is toxic in prototrophs. Positive/negative selection genes such as *pyrG* provide the possibility of conducting a series of sequential transformation by using Blaster cassettes [46].

Blaster cassettes, based on URA3, have allowed sequential transformation in yeast and have now been developed for use in filamentous fungi [47]. In the blaster cassette, the *ura3/pyrG* gene is flanked by two small direct repeat sequences. Carried on the transforming DNA, the blaster cassette allows selection for transformants on medium lacking uracil. Once transformants are isolated, the *ura3/pyrG* gene can be excised by recombination between the two direct repeat sequences, returning the transformants to uracil prototrophy. Transformants that have excised the *ura3/pyrG* gene are selected in the presence of 5FOA and can be re-transformed with another vector carrying the blaster cassette.

It is important to note that there is a problem using URA3 as a marker for gene disruption. The virulence phenotype of *Candida albicans* has been shown to vary dependent upon the position of the URA3 marker gene [48]. The activity of the enzyme encoded by URA3 is to some extent loci dependent and has an influence on virulence. This phenomenon is a problem for studies using URA3 in studies seeking to correlate gene knockout with virulence.

Random insertional mutagenesis (gene tagging)

By inserting DNA into the genome, either through transformation or by the *in vivo* movement of mobile genetic elements, a genome-wide series of random, tagged mutations can be created. Depending on the composition of the genetic element, it is possible to disrupt genes, to tag promoters or enhancers, or to up-regulate genes. Transformed isolates are selected and examined for phenotypic changes of interest. On the assumption that the phenotype change is due to genetic disruption wrought by the transferred DNA, the genomic region contiguous with the inserted T-DNA is retrieved either by PCR-based methods such as inverse PCR and TAIL-PCR or by plasmid rescue [31]. If there are many tandem integrations, semi-random PCR can be used to recover genomic DNA [49].

Ideally, a gene tagging system should have: a high transformation frequency; random integration as a single copy, at a single locus without rearrangement or deletion of either the transferred DNA or the genomic DNA; and, since all transformation methods induce unexpected genomic lesions, a straightforward method, such as sexual crosses, to test the correlation of the mutant phenotype with the presence of the DNA tag. If genetic segregation cannot be used, another strategy to rapidly check that the integration site correlates with the phenotype is to use plasmid rescue to create a gene disruption vector. The rescued plasmid can then be used to disrupt the same locus in a wild-type strain and do a complementation test [50].

Gene tagging by direct DNA transfer

Direct DNA transfer has been, and is still being, used successfully as a method for random mutagenesis in fungi [50-53]. As DNA that is not homologous to the recipient genome appears to integrate approximately at random [54], thousands of individual transformants collectively provide a library of tagged mutations. This method has worked successfully for the discovery of new genes correlated to a particular mutant phenotype. However, the generation of thousands of mutants by generally low efficiency transformation methods is not always feasible. Also, plasmid integration may result in a high frequency of tandem-repeat integrations. Tandem integrations probably occur through an initial integration at a chromosomal break or nick, followed by homologous recombination of the second plasmid at the first integration site [55]. Such tandem-repeat copies make it more difficult to clone the tagged gene [55, 56].

Restriction enzyme mediated integration (REMI) has been used to increase the frequency of insertion mutagenesis in fungi and to increase the frequency of single-copy integrations (reviewed in [57]). REMI involves transforming protoplasts or spores with a linearised plasmid and the restriction endonuclease (RE) used to linearise the vector. The RE gains access to the nucleus and induces double stranded breaks in the genome. The ends of these breakages recombine with the linearised plasmid, integrating the vector into the genome at the recognition site of the RE [58].

By increasing the target sites for recombination, REMI usually improves the overall transformation rate and frequency of single copy integration events [49, 55, 59-64] although that is not always the case [50, 65]. Conditions of transformation contribute significantly to the variable success rate of REMI. Although the enzyme type and concentration have an important impact on the number of transformants and the frequency of single-copy integrations, there are no clear rules for selecting either [56, 64, 66], and the

concentration of RE that produces the maximum number of transformants is enzyme and host dependent [66-68]. REMI can give rise to a significant number of different integration events including single insertion with deletion of flanking RE sites, ectopic integration in the absence of an appropriate RE site, tandem insertion and large genome deletions or inversions [50, 56, 64, 65, 68, 69]. The high frequency with which the mutant phenotype is not linked to the integrated DNA greatly complicates the use of this system for gene discovery.

T-DNA tagging

T-DNA tagging is a form of insertional mutagenesis that relies on AMT to mutate the recipient genome at random sites by integration of T-DNA carrying a selectable marker (reviewed in [1]). T-DNA tagging has been used successfully to find new genes [30, 70] and T-DNA tagging projects on fungi have recently been initiated in many laboratories around the world [31, 68]. AMT is very suitable for insertional mutagenesis as it can cause a relatively high frequency of transformation [71-73] and often creates single-copy integrations [27, 31]. Also, T-DNA appears to integrate approximately at random [31, 73], although integration may be targeted towards transcribed regions [70, 74] and promoters in particular [70]. Retrieval of genomic regions flanking the T-DNA is facilitated by the general lack of major truncation or rearrangement of the T-DNA, although small deletions can occur at both borders of the T-DNA. Vector sequence from outside the T-DNA may also be present [31, 75, 76].

The frequency of single-copy integrations can be quite variable [27, 31, 73, 76, 77]. Altering the frequency of single-copy integrations can be achieved by inducing or not inducing the *Agrobacterium* with acetosyringone prior to co-cultivation and by increasing the duration of co-cultivation [75]. The effect of induction varies between fungi, with induction causing either higher [31] or lower [73, 75] frequencies of single-copy integrations. A longer period of co-cultivation may also lead to a lower frequency of single-copy transformants [75].

In plants and fungi, AMT has been shown to cause mutations that are unlinked to the site of T-DNA integration [70, 78, 79]. The majority of AMT transformants may contain small (~100 bp) genomic deletions [79]. Even with single-copy T-DNA integrations, small or large genomic rearrangements are frequently observed. These include major chromosomal translocations in a significant proportion of tagged mutants [78, 79]. For this reason it is important that putatively T-DNA tagged mutants be tested to see if the T-DNA insertion site is linked to the mutant phenotype.

Promoter and enhancer trapping

Creating loss-of-function mutants through random insertion has several limitations. Mutations in essential or redundant genes may not be recovered and loss-of-function provides relatively unsubtle information about the mutated gene. Vectors for random insertional mutagenesis may be improved by the addition of elements that allow the detection of promoter activity or increase the transcription of contiguous genes. Such strategies are commonly used in plants [80-82] and have been used successfully on filamentous fungi with AMT and REMI [57, 83, 84]. Such a strategy will still pick up loss-of-function mutants but it will also pick up additional mutants that may be of interest.

For enhancer traps, a reporter gene, such as *gfp* (green fluorescent protein), downstream from a minimal promoter that is insufficient for its expression, is positioned near the end of the transferred DNA. Insertion of the DNA near an enhancer element will allow expression of the reporter gene from the minimal promoter. Similarly, a promoter-less reporter gene can be used to tag promoters and monitor their expression. In a gene trap the reporter gene has a 5' splice acceptor sequence, and is only expressed when integrated into introns. Additionally, gain-of-function mutants can be generated and tagged by placing a strong promoter near the end of the transferred DNA in the orientation that will cause over-expression of flanking genes. It is possible to include combinations of these functions on one random mutagenesis construct [85].

Transposon tagging

Tagged mutations can also be produced by movement of mobile genetic elements (such as transposons) within the genome of the fungus. For a comprehensive review of transposable elements in filamentous fungi see Daboussi and Capy 2003 [86]. Transposon tagging can rely on endogenous transposons or highly active, engineered transposons can be transferred into heterologous hosts. The main advantage of a transposon mutagenesis strategy is that a high efficiency transformation system is not required. Some fungi are recalcitrant to transformation and the processes involved in transformation can be time consuming and are known to create genetic mutations unlinked to the transferred DNA. As transposition is relatively free from genomic lesions and rearrangements, it might be a useful method for high-throughput mutagenesis in some systems [87].

The transposon Impala has been highly studied for use as a gene tagging element in fungi [88]. Impala transposes by a cut and paste method. It encodes a single transposase enzyme that recognizes inverted terminal repeat sequences on the transposon and mediates the excision and reintegra-

tion of the transposon without the involvement of host proteins. Consequently, transposons like Impala can typically be used in heterologous hosts [89]. The frequency of transposition of Impala can vary quite widely between strains, possibly dependant on the genomic position of the element [90]. While Impala does not have a reintegration hotspot within the genome (it reintegrates at a TA dinucleotide) and can excise from one chromosome and reintegrate into a different chromosome, it is not known to what extent reintegration is random [87, 90]. Transposition does not always result in reintegration and the frequency of lost elements can be quite high (~10-30% for Impala [90, 91]). Rarely, genomic rearrangements can result from aberrant transposition [92].

Transposons have been used successfully to mutate and tag unknown fungal genes. A homologue of the *Neurospora crassa* nitrate metabolism regulator *nit4* was isolated from *Tolypocladium inflatum* by the endogenous transposon Restless [93]. However, usually, a transposition tagging system will consist of an ectopically integrated construct containing a marker gene inactivated by a transposon carrying a second selectable marker gene. Excision of the transposon can be selected through restoration of the first marker gene and stable reintegration can be selected through the presence of the second marker gene. Heterologous transposition of Impala has been used to identify novel essential genes in *Aspergillus fumigatus* [87]. In that study, as transposition frequency depends on position effect, strains were evaluated for transposition frequency. A strain with a very high transposition rate of Impala was selected and that strain was used to generate a library of over two thousands individual transposition events. Of that library, 1.2% had a copy of Impala integrated in an essential gene. Impala has also been used successfully to tag genes in *A. nidulans* and *Magnaporthe grisea* [94, 95].

Targeted gene disruption/replacement

If a gene of interest has been identified, one strategy to investigate its function is to disrupt its expression and determine whether the phenotype is altered. In fungi, targeted gene disruption or replacement (knockout) can be achieved by homologous recombination. For gene disruption, the fungus is transformed with a disruption cassette consisting of sequences from the target gene flanking a selectable marker gene. The cassette is then inserted into the recipient genome at the target gene by homologous recombination. Homologous recombination can also be used to replace part of the endogenous gene, with exogenous sequences, for example promoter exchange. Recovered transformants will contain either, or both, DNA integrated by homologous recombination or by ectopic, illegitimate integration.

The efficiency of gene targeting in filamentous fungi is dependent on the length of the homologous sequence, the extent of homology, the transformation method and the genomic position of the target gene [2, 96]. Unlike homologous recombination in *S. cerevisiae*, where about 100 bp of homologous sequence is often sufficient, filamentous fungi generally require at least 1 kb of homologous sequence, and in some cases much more for gene targeting at a practical frequency [97-99]. For efficient gene targeting, the level of homology between the target sequence and the homologous region of the transferred DNA needs to be very high, almost 100%, and sequence variation may even preclude recombination of homologous sequences from different strains [2].

Gene targeting by homologous recombination in filamentous fungi has been achieved by all the transformation methods described. Although there are few comparisons available, it appears that AMT and particle bombardment may be particularly suited to homologous recombination [2]. AMT appears to be relatively efficient, often leading to a high frequency of gene targeting mediated by fungal recombination/repair proteins [100, 101]. The generally higher frequency of recombination achieved by AMT may be due to the fact that the T-DNA is linear and single-stranded, a preferred substrate for homologous recombination [72, 102], and is accompanied by *Agrobacterium* proteins that specifically interact with DNA-associated proteins in the recipient cell [74]. Electroporation, has also been used successfully for gene targeting although the frequency of homologous recombination is lower than AMT [2, 103].

Where the frequency of homologous recombination is low, it may be necessary to screen large numbers of transformants to find those with targeted gene integration. A positive and negative selection system, whereby ectopic integration results in non-viable transformants, can be used to enrich for the targeted events sought. A gene, such as *amdS* which confers sensitivity to fluoro-acetamide, is included in the construct, outside the sequences homologous to the target gene [99]. Homologous recombination, but not illegitimate integration, removes the negative selection gene allowing selection of targeted gene integration. The thymidine kinase gene from the herpes simplex virus and the *oliC31* mutation, which confers sensitivity to triethylin, have also been used for negative selection with significant increases in efficiency [28, 45, 104]. However, false positives were still produced in these studies through ectopic integration of only a part of the construct.

Another possible strategy for increasing the efficiency of gene targeting in fungi with a very low frequency of homologous recombination is to increase the expression of genes involved in homologous recombination [105] or decrease the expression of genes involved in nonhomologous

end-joining (NHEJ) [106]. Homologous recombination in *S. cerevisiae* is dependent on the RAD51 protein [107]. In *A. nidulans*, over expression of the *rad51* homologue *uvsC* resulted in an improvement in targeting efficiency through homologous recombination [105]. However, elevated expression of *uvsC* also suppressed colony growth rate. It has also been suggested that high levels of UVSC protein could potentially increase the frequency of homeologous recombination, leading to increased genomic instability [105]. Knockout of *mus-51 and mus-52* genes required for NHEJ in *N. crassa*, allowed a frequency of homologous recombination of 100%. *N. crassa mus-51* and *mus-52* mutants did not show defects in growth or morphology, suggesting that this might be a viable strategy.

High throughput gene targeting strategies

There is a growing need for more efficient ways to achieve targeted gene disruption, amenable to high throughput systems, in order to make use of the plethora of information yielded by fungal genome sequencing projects. In *S. cerevisiae*, where homologous recombination can be achieved with very short homologous sequences, PCR has been used as a quick method to generate gene targeting cassettes (reviewed in [108]). Coupled with complete genome sequence data and direct DNA transfer, this development allows a high throughput approach to investigating gene function without the need for cloning. With some modifications, this approach is now being applied to filamentous fungi.

Overlap or fusion PCR is a rapid method for creating gene targeting cassettes with large (>1 kb) homologous regions suitable for recombination in filamentous fungi (Figure 1). PCR is first used to amplify the flanking regions of the targeted gene and to amplify a selectable marker cassette. The primer for one segment has a tail complementary to the primer used to amplify the adjacent segment of the final product. The first round products are generally gel purified and mixed in equimolar ratios, then reamplified using primers complementary to each end of the final product [109]. This method has been used to create constructs for gene replacement in *A. awamori* [99] and for targeted *in vivo* promoter exchange [110]. A variation of the technique, in which overlapping segments were present on both primers at a join (termed double-jointed PCR), was used to create linear constructs for biolistic transformation of *Cryptococcus neoformans* [111] and for protoplast transformations of *A. nidulans, A. fumigatus and Fusarium graminarium* [112].

Another rapid method of creating linear gene disruption constructs is ligation-mediated PCR. The flanking regions of the target gene are amplified using PCR primers which include restriction sites compatible with those in the selection cassette. Purified products are digested with restriction enzymes and directionally ligated to the digested selection cassette, then the desired product is gel purified and amplified [113]. This method has an advantage over overlap PCR in that the selection cassette is not reamplified so has less opportunity to accumulate mutations that may disable it. Additionally, the number and size of primers required

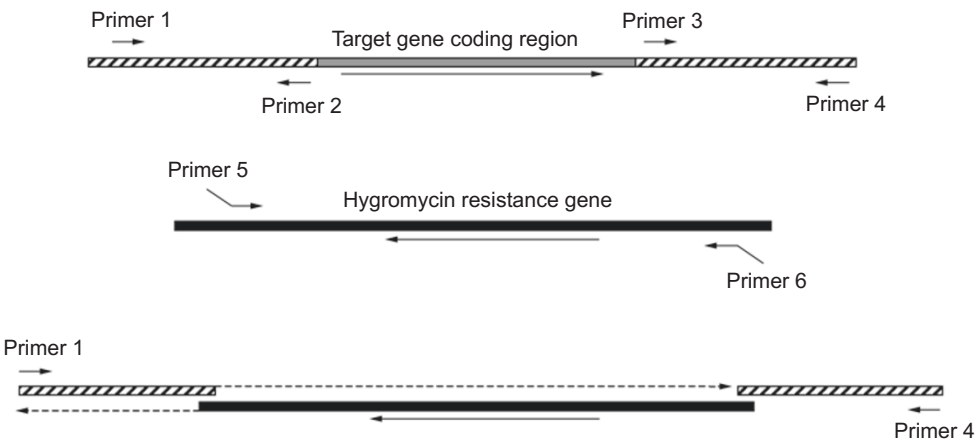

Figure 1 Creation of a gene replacement construct by overlap PCR. The flanking regions (= 1 kb) of the target gene are amplified using primers 1-4. The selectable marker cassette, including constitutive promoter and terminator sequences, is amplified using primers 5 and 6, which have tails complementary to primers 2 and 3 respectively. PCR products are gel purified and mixed at equal molarity. During the final PCR the overlapping sections allow the fragments to act as primers for each other, and the whole construct is amplified using primers 1 and 4. If the product is to be cloned, restriction sites can be included in tails on primers 1 and 4. A long template polymerase such as Expand Long Template (Roche) is required.

is reduced. The use of these PCR methods to create linear constructs is particularly suited to direct DNA transfer methods such as particle bombardment or protoplast transformation. Gene targeting cassettes created by PCR can also be inserted into binary vectors for use in AMT [99].

In vitro transposon tagging

Gene discovery and the creation of gene disruption constructs can be performed simultaneously by transposon arrayed gene knockout (TAGKO) [114]. In TAGKO, a cosmid library is created from the whole fungal genome (Figure 2). Transposon Tn7 carrying a selectable marker gene integrates, by an enzyme-catalysed (transposase) *in vitro* reaction, into a pool of cosmids or a single cosmid, randomly disrupting genes. Sequencing primed by transposon sequences identifies disrupted genes, allowing selection of cosmids carrying disruptions in genes of interest, for gene disruption by homologous recombination. Restriction digestion of selected cosmids releases a linear insert suitable for transformation using protoplast or electroporation methods, and phenotypic characterisation of disruption mutants can then follow. The process can be used to create a range of mutations in a single gene or to disrupt genes from throughout the genome.

TAGKO has been used successfully for targeted gene disruption [103, 115]. Electroporation of 11 TAGKO constructs into *Mycosphaerella graminicola* protoplasts produced disruption mutants at a targeting frequency of 15-28%, which is very high for that method of transformation [103]. The high frequency of gene targeting probably reflects the length of homologous regions in TAGKO constructs (~40 kb) [103]. However, much higher frequencies of homologous recombination (44%) can be achieved in *M. graminicola* by AMT [101]. *In vivo* recombination has been used to rapidly convert BACs to binary vectors [116], conversion of TAGKO cosmids to binary vectors might create a very efficient gene targeting system based on TAGKO-AMT [103].

In a system similar to TAGKO, the GPS-1 genome-priming system (New England Biolabs) has been used to create gene targeting constructs. In this system, a gene of interest is ligated into the A/T cloning vector pGEM (Promega) [117]. *In vitro* transposition of the pGPS1 transprimer 1 transposon, modified to carry the selectable marker gene *pyr4*, is used to randomly mutate the gene of interest. The constructs are amplified in *E. coli*, the transposon disruption is mapped, and the vector is linearized ready for direct DNA transfer to the target fungus.

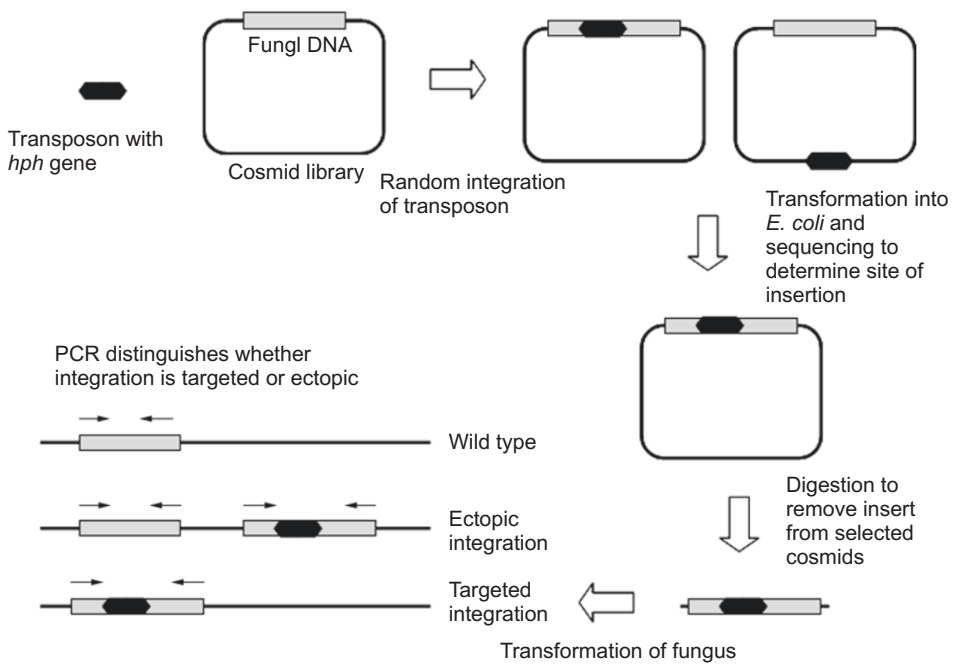

Figure 2 Transposon arrayed gene knockout (TAGKO). The transposon integrates randomly into cosmids *in vitro*. Mutated cosmids are transformed into *E. coli*, and the transposon insertion sites are determined by sequencing adjacent to the transposon. Selected cosmids are digested with endonucleases and the linear fragment is used for fungal transformation. Ectopic and targeted integration can be distinguished by PCR.

Laboratory information management

With whole genome sequencing and advances in high throughput gene disruption, mutagenesis and phenotypic characterization of an entire organism have become possible. In many systems, logistical rather than technical difficulties limit this approach. The creation and analysis of many thousands of strains require careful coordination and standardisation both of experimental steps and in data collection. PACLIMS (Phenotype Assay Component Laboratory Information Management System) is an online data management system that was designed to support the multi-institutional production of fifty thousand random insertion mutants of *M. grisea* [118]. PACLIMS uses barcoding to track the biological material and control the analysis through a carefully planned protocol. PACLIMS builds up an online, searchable database and is open source software that is currently freely available from: http://paclims.sourceforge.net.

Post-transcriptional gene silencing

Targeted gene disruption or replacement (knockout), as described above, has been the technique of choice for creating null mutants of specific genes in many fungi. Gene knockout often requires significant length of homologous sequence for integration and even then, the frequency of targeted integration can be very low in some systems. Further, the consequent phenotype may be lethal or it may not be observed in heterokaryotic tissue. An alternative method, that overcomes some of the problems associated with knockouts, is to disrupt gene expression at a post-transcription level (gene knockdown) by targeting the mRNA rather than the gene.

RNA interference (RNAi) is a sequence-specific post-transcriptional gene-silencing phenomenon [119]. RNAi can be induced by the presence of foreign nucleic acid sequences such as RNA viruses and transgenes. An essential, rate-limiting step is the formation of dsRNA which is cleaved by dicer to produce small interfering RNA (siRNA) [120, 121]. mRNA with sequence identity to the siRNA is specifically degraded by an RNA-induced silencing complex (RISC) [122]. In some organisms, an RNA-dependent RNA polymerase (RdRP) uses antisense siRNA to prime the conversion of endogenous mRNA into dsRNA amplifying the silencing signal [120]. RNAi has recently been used as a functional genomics tool in fungi. However, RNAi is still a relatively undeveloped technique that does not always reduce expression to a point where a phenotype change is seen.

RNAi has been used successfully for gene knockdown in filamentous fungi [3]. In its most effective form, an ectopically integrated RNAi construct codes for double stranded RNA, identical to part of the endogenous sequence being targeted [120, 123-125]. The linear RNAi knockdown transcript contains an inverse repeat of the target sequence that folds up into a "hairpin" in which the inverse repeat is double stranded. The repeat sequences are typically about 500 bp of coding sequence separated by a spacer sequence of about 250 bp [126]. A substantial decrease in efficiency was found when using repeat sequences of 200 bp rather than 600 bp or 900 bp [125]. The inclusion of an intron in the spacer region may greatly increase the silencing efficiency, possibly through enhanced export of the hairpin from the nucleus during splicing [125].

RNAi silencing vectors for rapid cloning of target sequences are available [127]. High throughput cloning of target sequences for RNAi in filamentous fungi can now also be achieved using Gateway® (Invitrogen) *in vitro* recombination technology. In this system, site-specific recombination directs the incorporation of PCR amplified target sequences into an intron-containing, RNAi expression cassette (developed by Ellen Fox, Don Gardiner and Barbara Howlett, unpublished).

Gene knockdown has some advantages when compared to gene knockout. Knockdown requires relatively short stretches of sequence information. This is a major advantage for organisms for which there is little sequence information. As RNAi works at the mRNA level, its efficacy is not compromised by the presence of non-transformed nuclei or multi-copy genes due to aneuploidy. Where there is some sequence conservation between genes, one or a few RNAi constructs can be used to knockdown an entire family of genes. This ability can be especially useful where gene redundancy would otherwise mask the phenotype of a gene knockout. To avoid the problem of lethal knockouts, RNAi can be used to achieve down regulation or conditional promoters can be used to control knockdown [125].

There are also some limitations to RNAi. Firstly, RNAi usually does not abolish expression of the target gene and the extent of knockdown of transcript is highly variable making it necessary to screen transformants for high levels of knockdown [124, 126]. Also, the threshold of expression knockdown required before a change in phenotype is observed varies between target genes. This problem can be mitigated to some extent by simultaneously targeting a second gene, as a control, by incorporating two target sequences in a chimeric RNAi hairpin construct [41, 126]. For this strategy, the second gene could be a transgenic reporter gene such as *gfp*. The simultaneous silencing of the reporter and endogenous gene would also be amenable to high throughput genetic analyses [41].

Another potential disadvantage is that, as only a short sequence is required for RNAi, genes other than those

targeted might be silenced. Testing for the possibility of off-target effects is simpler for organisms for which there is a complete genome sequence. Transcripts for those sequences that are most similar can be assessed for down regulation, to determine off target effects. In addition, integration of the silencing construct might directly influence the phenotype by randomly knocking out genes. Several knockdown transformants displaying the same phenotype adequately demonstrate that this has not occurred. Finally, knockdown transformants can not be complemented as the endogenous gene itself has not been altered. It is possible to overcome this by re-transformation with a full gene sequence that has the codons altered to avoid targeting by the RNAi construct. This concept is now more achievable with the ability to obtain synthetic gene constructs. Also, an RNAi complementation method for mammalian cells has been achieved by knockdown using small hairpin RNA targeting untranslated regions (UTR) of the endogenous gene with complementation by a simultaneously expressed gene lacking the UTRs [128]. Alternatively, an inducible promoter driving expression of the RNAi construct can be used [126].

Other targeted gene strategies

Although the targeted creation of loss-of-function mutants is the most common method for examining gene function, other methods have also been applied to filamentous fungi. Most of these methods depend upon modifying the gene *in vitro* and then transforming it, either into the same or a different genetic background. The advantage of these techniques is that they provide specific additional information that is not obtainable through loss-of-function mutation. Introducing the gene into a different genetic background such as into yeast or *E. coli*, allows the gene and its product to be examined in isolation from endogenous proteins. Genes can also be examined within a specific, defined context. For example, the function and activity of fungal pathogenicity genes have been examined by transferring the genes to a host plant and expressing them *in planta* [129] for example, functional cloning of *avr* genes from *Cladosporium fulvum* has been achieved by expressing a cDNA library in tomato [130]. Similarly, the function of fungal fungicide resistance genes can be examined by expressing them in a sensitive strain of the same fungus [131].

The yeast one-hybrid system has been used to isolate transcriptional activators of promoters from *Trichoderma reesei* [132, 133]. In two studies, a *Trichoderma* promoter has been cloned into a yeast replication vector upstream from a minimal promoter/*His3* selective marker gene cassette then transformed into yeast. In a second round of transformation, the yeast were transformed with a *Trichoderma* cDNA expression library and transformants expressing the *His3* marker gene were isolated on the assumption that *His3* expression was triggered by a cDNA transcription factor binding to the *Trichoderma* promoter. Transcription factors that control specific *Trichoderma* gene expression have been successfully retrieved from yeast transformants by this method [132, 133].

The interaction between two specific fungal gene products has been examined in the yeast two-hybrid system [134, 135]. In this system, the two genes are each cloned as fusions to either the activation domain or the binding domain of the GAL4 transcription factor on a yeast replicative vector and transferred into *S. cerevisiae*. Transcription activation of GAL4-activated reporter genes requires that the two GAL4 domains be brought together by specific interaction between the two proteins.

Where a cloned gene of interest is transformed for ectopic integration into the same species and strain it was originally retrieved from, its function is usually examined either through over-expression [37, 136, 137], or though transcription or translation fusions to marker genes such as *gfp* [138]. For over-expression studies, the coding region of the gene of interest can be cloned downstream from a strong heterologous promoter, or under the control of an inducible promoter such as *alcA* so there is some control over the level of over-expression [136, 139].

Enhancer and promoter elements can also be engineered and reintroduced ectopically to investigate their function. Transcriptional fusions to reporter genes have been used to define cis-acting promoter elements through the construction of a series of systematic modifications made to the promoter prior to its integration and expression in the host genome [140]. The effect of each mutation can be measured by changes in expression of the reporter gene. One problem with this approach is that the promoter may not be integrated into the same genetic context it was originally in and this could affect its expression [140].

From genomics to proteomics

There is an increasing interest in looking beyond the genome and investigating the functions and interactions of the proteins themselves. By using recombinant DNA approaches to fluorescently label endogenous proteins (e.g. GFP fusion proteins) or for epitope-tagging (translational fusion of an antigenic determinant to the protein of interest) the resources developed for studying the genome are now being used to study the proteome. Combining annotated sequence information with the method of targeted DNA integration achieved through homologous recombination, a library of yeast with an epitope-tag integrated at the

C-terminus of nearly every open reading frame has been created [141]. Although large-scale epitope-tagged libraries have not been made for any filamentous fungi, targeted epitope-tagging using high-throughput PCR methods have been achieved in at least one filamentous fungus [142].

High-throughput immunoprecipitation experiments with epitope-tagged libraries make it possible to look beyond transcription profiles and directly monitor protein location, post-transcriptional modifications and interactions with other proteins. Further, by creating fusions with fluorescent proteins such as GFP and applying advanced techniques such as fluorescence resonance energy transfer it is now possible to detect specific conformational changes within proteins [143]. In future we may see tagged proteins become widely used to study biochemical reactions *in vivo*, in real time.

Summary

Although a robotic, mass data collection approach may not be the inevitable endpoint of functional genomics in all filamentous fungi, it is clear that a major trend is towards high throughput strategies for whole genome analysis. That outcome can now be achieved in model systems by using entire genome sequences and linking rapid construct-building techniques, efficient transformation systems and phenotype assays supported by high throughput data management systems.

For those who are developing functional genomics in more recalcitrant fungi, there are many different approaches that can be taken. While REMI is still being used successfully for random mutagenesis, there is a clear trend away from REMI towards using T-DNA tagging for this purpose. T-DNA tagging generally appears to have a higher transformation frequency and is thought to create fewer genomic lesions that are unlinked to the DNA insertion site, although these do still occur. As a strategy for insertional mutagenesis, *in vivo* transposon tagging appears to be theoretically the most ideal approach. It does not require repeated transformation and to some extent it avoids the problems associated with mutations that are not linked to the tagged site. However, transposon tagging has not been taken up widely for fungal research, probably due to a perception that it lacks efficiency and also because it is more complicated to initially set up than a transformation-based mutation system. Similarly, the extra steps required to set up *in vitro* transposon tagging may be a barrier to the uptake of strategies such as TAGKO for random mutagenesis.

A major problem for both random mutagenesis and targeted gene disruption is the multinucleate nature of filamentous fungi. RNAi techniques may offer a solution to this problem, at least for gene targeting. However, RNAi is a relatively new technique that as yet has not been thoroughly developed for use in filamentous fungi, and does not work efficiently in every system. As RNAi has not been developed as a random mutagenesis strategy, something that seems technically feasible, the multinucleate state remains a problem for generating random, loss-of-function, mutants. It is surprising then, that more use has not been made of enhancer-traps and similar devices that extend the range of mutations that can be detected by insertional mutagenesis.

All the techniques described here have advantages and limitations and each may be more suitable in one system and less suitable in another. The particular approach taken will be dictated to some extent by laboratory-specific practices, by the practicalities of the individual organisms and the outcome that is desired. However, systematic planning is needed from an early stage to take advantage of the best developments for efficient experimentation that are now available.

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