Agrobacterium-mediated transformation as a tool for functional genomics in fungi

Michielse, C.B.; Hooykaas, P.J.J.; Hondel, C.A.M.J.J. van den; Ram, A.F.J.

Curr Genet (2005) 48: 1–17
DOI 10.1007/s00294-005-0578-0

REVIEW ARTICLE

Caroline B. Michielse · Paul J. J. Hooykaas
Cees A. M. J. J. van den Hondel · Arthur F. J. Ram

Agrobacterium-mediated transformation as a tool for functional genomics in fungi

Received: 18 January 2005 / Revised: 10 March 2005 / Accepted: 26 March 2005 / Published online: 12 May 2005

Abstract In the era of functional genomics, the need for tools to perform large-scale targeted and random mutagenesis is increasing. A potential tool is *Agrobacterium*-mediated fungal transformation. *A. tumefaciens* is able to transfer a part of its DNA (transferred DNA; T-DNA) to a wide variety of fungi and the number of fungi that can be transformed by *Agrobacterium*-mediated transformation (AMT) is still increasing. AMT has especially opened the field of molecular genetics for fungi that were difficult to transform with traditional methods or for which the traditional protocols failed to yield stable DNA integration. Because of the simplicity and efficiency of transformation via *A. tumefaciens*, it is relatively easy to generate a large number of stable transformants. In combination with the finding that the T-DNA integrates randomly and predominantly as a single copy, AMT is well suited to perform insertional mutagenesis in fungi. In addition, in various gene-targeting experiments, high homologous recombination frequencies were obtained, indicating that the T-DNA is also a useful substrate for targeted mutagenesis. In this review, we discuss the potential of the *Agrobacterium* DNA transfer system to be used as a tool for targeted and random mutagenesis in fungi.

Communicated by J. Heitman

C. B. Michielse · P. J. J. Hooykaas
C. A. M. J. J. van den Hondel · A. F. J. Ram
Institute of Biology, Clusius Laboratory,
Fungal Genetics Research Group, Leiden University,
Wassenaarseweg 64, 2333 AL Leiden, The Netherlands

C. A. M. J. J. van den Hondel · A. F. J. Ram (✉)
TNO Quality of Life, Microbiology,
Utrechtseweg 48, 3500 AJ Zeist, The Netherlands
E-mail: ram@rulbim.leidenuniv.nl
Tel.: +31-71-5274938
Fax: +31-71-5274999

Present address: C. B. Michielse
Department Human Genetics,
University Medical Centre Nijmegen,
P.O. Box 9101, 6500 HB Nijmegen,
The Netherlands

Keywords Fungal transformation · Insertional mutagenesis · Targeted mutagenesis · *Agrobacterium tumefaciens*

Introduction

Fungal biology will enter the era of functional genomics as more sequence data for entire fungal genomes become available. Currently, ten fungal genomes have been completely sequenced and at least 100 fungal genome-sequencing projects are underway (http://www.genomesonline.org). Obviously, the need for tools to perform large-scale functional genomics is increasing. Numerous tools have been developed for the yeast *Saccharomyces cerevisiae* (reviewed by Bader et al. 2003), which was the first fungal genome to be fully sequenced. Most of these tools, which include computational annotation of gene function, transcriptional profiling, and protein profiling using 2-D gel analysis and/or mass spectrometry, have already been adapted or can potentially be adapted for filamentous fungi (Mannhaupt et al. 2003; Sims et al. 2004). However, systematic mutational analysis of every predicted gene, either by targeted mutagenesis or random mutagenesis, is more difficult in filamentous fungi. In contrast to *S. cerevisiae*, targeted mutagenesis in filamentous fungi usually requires gene disruption cassettes with large homologous DNA-flanking regions (≥1,000 bp). Such constructs give a homologous recombination frequency of 0.5–30.0% (Asch and Kinsey 1990; Hynes 1996), which makes construction of a complete set of deletion mutants of all predicted genes a laborious task. Several methods have been described to obtain large homologous DNA flanks in gene-disruption cassettes for filamentous fungi. Recently, an overview of different methods to generate various types of gene-disruption cassettes was published (Wendland 2003) and we refer readers to this comprehensive review for more information. For random mutagenesis, methods such as transposon mutagenesis (reviewed by Daboussi and

Capy 2003; Kempken and Kuck 1996) and restriction enzyme-mediated integration (REMI; reviewed by Brown and Holden 1998; Kahmann and Basse 1999; Maier and Schafer 1999) have been developed for fungi. However, both methods have their disadvantages that limit their application for mutagenesis purposes. Transposon mutagenesis developed for various fungi indicates that transposition is not a completely random process and that a preference for non-coding regions exists (Firon et al. 2003; Ladendorf et al. 2003; Li Destri Nicosia et al. 2001). In addition, the occurrence of chromosomal rearrangements after transposon mutagenesis has been reported (Hua-Van et al. 2002). A significant limitation of REMI is that a substantial portion (30–50%) of the mutations generated appears to be untagged by the transforming DNA (Bolker et al. 1995; Linnemannstons et al. 1999; Lu et al. 1994; Sweigard et al. 1998). Furthermore, it seems that integration of the introduced DNA is not completely at random and that a preference for highly transcribed genomic regions exists (Lu et al. 1994; Sweigard et al. 1998).

Bundock et al. (1995) published a novel method for the transformation of the yeast *S. cerevisiae*. This method is commonly used for plant-cell transformations and is based on the ability of *Agrobacterium tumefaciens* to transfer a part of its DNA (transferred DNA; T-DNA) to eukaryotic cells (reviewed by Zhu et al. 2000; Zupan et al. 2000). Subsequently, de Groot et al. (1998) demonstrated that the *Agrobacterium*-mediated transformation (AMT) system can be used for the transformation of several filamentous fungi. After these initial publications, many other researchers successfully used *A. tumefaciens* to transform various fungi, including members of the Ascomycetes, Basidiomycetes, and Zygomycetes (see Table 1). Furthermore, it is also posssible to transform Oomycetes with this transformation system. Importantly, AMT has been developed as a transformation system for fungi such as *Agaricus bisporus*, *Calonectria morganii*, *Fusarium circinatum*, and *Helminthosporium turcicum* (see Table 1 for references), which were recalcitrant to other more conventional fungal transformation methods. In addition, AMT appeared to be a more efficient transformation method, in comparison with the protoplast-based transformation method (Amey et al. 2002; de Groot et al. 1998; Fitzgerald et al. 2003; Meyer et al. 2003). The ability of *A. tumefaciens* to transfer its DNA to fungi belonging to various classes is indicative of the potential of this transformation system for fungal biotechnology.

AMT has been shown to have several advantages over conventional transformation methods. Intact cells, such as conidia, vegetative, and fruiting body mycelia (Table 1; Fig. 1), can be used as starting material, thereby eliminating the need to generate protoplasts. The isolation of protoplasts is laborious; and both yield and viability are dependent on the quality of the enzyme preparation and batch used for generating protoplasts. AMT generates a high percentage of transformants with a single-copy integrated DNA, which facilitates the isolation of tagged genes. Integration also appears to occur at random (Abuodeh et al. 2000; Bundock et al. 2002; Combier et al. 2003; de Groot et al. 1998; Degefu and Hanif 2003; Leclerque et al. 2003; Michielse et al. 2004a; Mullins et al. 2001; Rho et al. 2001). Additionally, it has been shown that the T-DNA is an efficient substrate for homologous recombination (Bundock et al. 1999; Michielse et al. 2005). These properties of AMT make this transformation method a valuable tool for systematic mutational analysis in fungi, either by targeted integration or by insertional mutagenesis. AMT has been used as a tool for insertional mutagenesis in plants, such as *Arabidopsis thaliana*, *Nicotiana* species, and *Oryza sativa* (Jeon et al. 2000; Koncz et al. 1989, 1992; Krysan et al. 1999).

In this review, we present a comprehensive overview of the *Agrobacterium* DNA transfer system and of the parameters which have been identified to play a role in *Agrobacterium*-mediated fungal transformation. In the final part, we discuss the potential of the *Agrobacterium* DNA transfer system as a tool for targeted and random mutagenesis.

Basics of AMT

The gram-negative bacterium *A. tumefaciens* is a plant pathogen, which causes crown gall tumors on plants (Stafford 2000). *A. tumefaciens* induces this tumorous growth by transferring a part of its DNA (T-DNA) which is located on its 200-kbp tumor-inducing (Ti) plasmid to the host. After integration into the host genome, genes that are naturally located on this T-DNA and encode enzymes for the production of plant growth regulators are expressed; and their expression results in uncontrolled growth of the plant cells. The Ti plasmid also contains another segment called the virulence region, which is composed of a large number of *vir* genes that are necessary for tumorigenicity (reviewed by Zhu et al. 2000; Zupan et al. 2000). Proteins encoded by the virulence region are involved in the formation, transport and possibly also integration of the T-DNA (reviewed by Hooykaas and Beijersbergen 1994; Zhu et al. 2000). The T-region of the Ti plasmid is surrounded by a 24-bp border repeat, which is the *cis*-acting signal for the DNA delivery system to plant cells (see below). But otherwise, all the sequences of the natural T-DNA can de deleted and replaced by other DNA sequences without a negative effect on trans-kingdom DNA transfer. For transformation of plants and fungi nowadays, the so-called binary vector system is used. In this binary system, the T-DNA and the virulence region are placed on two separate plasmids, which allows genetic manipulation of the small T-DNA containing the binary vector (Hoekema et al. 1983).

Table 1 Fungal species successfully transformed with *A. tumefaciens*

Fungal species	Selection marker	Starting material	Remarks	References
Ascomycetes				
Aspergilus awamori	*hph*, *BLE*, *A*a *pyrG*	Protoplasts, conidia	Targeted gene disruption of orotidine-5′-monophosphate decarboxylase (*pyrG*) and glucosamine/fructose-6-phosphate aminotransferase (*gfaA*)	de Groot et al. (1998); Gouka et al. (1999); Michielse et al. (2004b)
A. giganteus	*hph*	Conidia, germinated conidia		Meyer et al. (2003)
A. niger	*hph*, *Ao pyrG*	Conidia		de Groot et al. (1998); C.B. Michielse, unpublished data
Beauveria bassiana	*hph*	Conidia		Leclerque et al. (2003); Fang et al. (2004); dos-Reis et al. (2004)
B. dermatiditis	*hph*, *Hc ura5*	Yeast-like cells, germinated conidia		Brandhorst et al. (2002); Sullivan et al. (2002)
Botrytis cinerea	*hph*	Conidia		Rolland et al. (2003)
Calonectria morganii	*hph*	Conidia		Malonek and Meinhardt (2001)
Candida albicans	*hph*	Yeast cells		M. Furlaneto, personal communication
C. glabrata	*hph*	Yeast cells		M. Furlaneto, personal communication
C. tropicalis	*hph*	Yeast cells		M. Furlaneto, personal communication
Ceratocystis resinifera	*hph*	Germinated conidia	Targeted gene disruption of a polyketide synthase (*PKS1*)	Loppnau et al. (2004)
Claviceps pururea	*BLe*	Germinated conidia		Scheffer and Tudzynski at 22nd FGC, 2003
Coccidiodes immitis	*hph*	Protoplasts, germinated conidia		Abuodeh et al. (2000)
C. posadasii	*hph*	Germinated conidia	Targeted gene disruption of a 1,3-beta-glucan synthase (*FKS1*)	Kellner et al. (2005)
Colletotrichum gloeosporioides	*hph*	Conidia		de Groot et al. (1998)
C. lagenarium	*hph*	Conidia		Tsuji et al. (2003)
C. trifolii	*hph*	Conidia		Takahara et al. (2004)
Coniothyrium minitans	*hph*	Germinated conidia		Rogers et al. (2004); Li et al. (2005)
Cryphonectria parasitica	*hph*	Conidia		Park and Kim (2004)
Fusarium circinatum	*hph*	Conidia		Covert et al. (2001)
F. culmorum	*hph*	Conidia		Skov and Giese at 22nd FGC, 2003
F. oxysporum	*hph*, *nptII*	Conidia	Targeted gene disruption of a kinase (*SNF*) in combination with the two-marker gene technique	Mullins et al. (2001); Mullins and Kang (2001); Khang et al. (2005); M. Rep, personal communication
F. venenatum	*hph*	Conidia, freeze-dried mycelium		de Groot et al. (1998)
Glarea lozoyensis	*hph*	Mycelium	Targeted gene disruption of polyketide synthase (*PKS1*)	Zhang et al. (2003)
Helminthosporium turcicum	*hph*	Conidia	Liquid co-cultivation	Degefu and Hanif (2003)
Histoplasma capsulatum	*hph*, *Hc ura5*	Yeast-like cells, germinated conidia		Sullivan et al. (2002)
Kluyveromyces lactis	*Sc ura3*	Yeast cells	Targeted gene disruption of N-(5′-phosphoribosyl)anthranilate isomerase (*TRP1*) and a centromere-associated factor gene (*SKP1*)	Bundock et al. (1999)
Leptosphaeria maculans	*hph*	Conidia	Targeted gene disruption of ABC transporter (*LmABCt4*), histidine kinase gene (*LmHK1*) and peptide synthetase (*sirP*) in combination with the two-marker gene technique	Gardiner and Howlett (2004); Gardiner et al. (2005)

Table 1 Continued

Fungal species	Selection marker	Starting material	Remarks	References
Magnaporthe grisea	*hph*, *nptII*	Conidia	Targeted gene disruption of a class II hydrophobin (*MHP1*) in combination with the two-marker gene technique	Rho et al. (2001); Khang et al. (2005)
Metarhizium anisopliae var. *acridum*	beta-tubulin	Conidia		M. Furlaneto, personal communication
Monascus purpureus	*hph*	Conidia		Campoy et al. (2003)
Mycosphaerella fijiensis	*hph*	Mycelium		Donzelli et al. at 22nd FGC, 2003
M. graminicola	*hph*	Protoplasts, yeast-like cells	Targeted gene disruption of ABC transporter genes (*MgAtr1*, *MgAtr2*)	Zwiers and de Waard (2001)
Neurospora crassa	*hph*	Conidia		de Groot et al. (1998)
Ophiostoma floccosum	*hph*	Yeast-like cells	RNA interference of a polyketide synthase gene (*PKS*)	C. Breuil, personal communication
O. piceae	*hph*	Yeast-like cells		Tanguay and Breuil (2003)
O. piliferum	*hph*	Yeast-like cells	Targeted gene disruption of subtilase *albin1*	Hoffman and Breuil (2004)
Paecilomyces fumosoroseus	*hph*	Conidia		M. Furlaneto, personal communication
Paracoccidioides brasiliensis	*hph*	Yeast-like cells		Leal et al. (2004)
Saccharomyces cerevisiae	*trp1*, *Sc ura3*, *aphI*	Yeast cells	Autonomous replication of the T-DNA	Bundock et al. (1995;) Piers et al. (1996); Bundock et al. (2002)
Trichoderma asperellum	*hph*, *BLE*	Conidia		Cardoza et al. at ECFG7, 2004
T. atroviride	*hph*	Conidia	Reduction background growth by overlay method, targeted gene disruption of MAP kinase (*tmk1*) and alpha subunit heterotrimeric G protein (*tga3*)	Cardoza et al. at ECFG7, 2004; Zeilinger (2003)
T. hazianum	*hph*	Mycelium		Quoc-Khanh et al. at 21st FGC, 2001
T. longibrachiatum	*hph*, *BLE*			Cardoza et al. at ECFG7, 2004
T. reesei	*hph*	Conidia		de Groot et al. (1998)
Venturia inaequalis	*hph*	Mycelium	RNA interference of a melanin biosynthesis gene (*THN*). Improved transformation after blending mycelium	Fitzgerald et al. (2003); Fitzgerald et al. (2004);K. Plummer, personal communication
Verticillium dahliae	*hph*	Conidia	Targeted gene disruption of trypsin protease (*VTP1*)	Dobinson et al. (2003)
V. fungicola	*hph*	Conidia	Targeted gene disruption of a beta-1,6-glucanase gene (*VfGlu1*)	Amey et al. (2002); Amey et al. (2003)
Basidiomycetes				
Agaricus bisporus	*hph*	Germinating conidia, mycelium, and fruiting body tissue	Vacuum infiltration used for fruiting body tissue	Chen et al. (2000); de Groot et al. (1998); Mikosch et al. (2001)
Cryptococcus neoformans	*NAT*	Yeast-like cells		Idnurm et al. (2004)
Hebeloma cylindrosporum	*hph*, *Sh BLE*	Mycelium		Combier et al. (2003); Pardo et al. (2002)
Hypholoma sublateritium	*hph*	Conidia		Godio et al. (2004)
Omphalotus olearius	*hph*	Mycelium		Voss et al. at ECFG6, 2002
Paxillus involutus	*Sh BLE*	Mycelium		Pardo et al. (2002)
Phaffia rhodozyma	*hph*	?		E. Gudina, R.P. Godio, and J.F. Martin, personal communication
Suillus bovinus	*hph*, *Sh BLE*	Mycelium		Hanif et al. (2002); Pardo et al. (2002)
Zygomycetes				
Blakeslea trispora	*hph*	Conidia	Unstable phenotye	Heinekamp et al. at ECFG7, 2004

Mucor circinelloides	*Mc leu*	Protoplasts		J. Gomez-Mateo, C.B. Michielse, and V. Garre, personal communication
M. michei	*aphI*	Germinated conidia	Unstable phenotype	Monfort et al. (2003)
Rhizopus oryzae	*Rn pyr4*	Protoplasts	Stable phenotype, genomic integration confirmed	Michielse et al. (2004c)
Oomycetes				
Pythium ultimum var. *sporangiiferum*	*npt11*	Germinated conidia		Vijn and Govers (2003)
Phytophthora infestans	*npt11*	Germinated conidia		Vijn and Govers (2003)
P. palmivora	*npt11, hph*	Germinated conidia, mycelium		Vijn and Govers (2003); Quoc-Khanh et al. at 22nd FGC, 2003

hph Escherichia coli hygromycin resistance gene, *BLE* phleomycin resistance gene, *Aa pyrG Aspergillus awamori* orotidine-5′-phosphate decarboxylase (allows selection of uracil prototrophs), *Ao pyrG A. oryzae* orotidine-5′-phosphate decarboxylase (allows selection of uracil prototrophs), *Hc ura5 Histoplasma capsulatum* orotate phosphoribosyltransferase gene (allows selection of uracil prototrophs), *Sh BLE Streptoalloteicus hindustanus* phleomicin resistance gene, *Sc ura3 Saccharomyces cerevisiae ura3* gene (allows selection of uracil prototrophs), *AphI E. coli* transposon Tn903 [conferring resistance to kanamycin analogue G-418 (geneticin) sulfate], *NAT* nourseothricin acetyltransferase (conferring resistance to nourseothricin), *nptII* neomycin phosphotransferase (conferring resistance to geneticin), *Rn pyr4 R. niveus* orotidine-5′-phosphate decarboxylase gene (allows selection of uridine prototrophs), *trp1 S. cerevisiae* N-C 5′ phosphoribosyl-anthranilate isomerase gene (allows selection of tryptophan prototrophs). *ECFG* European Conference on Fungal Genetics, *FGC* Fungal Genetic Conference

Formation, transfer, and integration of the T-DNA into its host

Phenolic compounds, such as acetosyringone, are used to induce the *vir* genes that encode the T-DNA transfer machinery of *A. tumefaciens* (Fig. 2). A two-component regulatory system composed of the virulence proteins VirA and VirG is activated upon recognition of acetosyringone. The chromosomally encoded protein, ChvE, interacts with the VirA protein to further enhance levels of *vir* induction in the presence of specific monosaccharides (Cangelosi et al. 1990). VirA, an inner membrane protein, senses acetosyringone and responds by autophosphorylation. Subsequently, the phosphorylated VirA transfers its phosphoryl group to VirG. The activated VirG, which has DNA-binding properties, then acts as a transcriptional activator of itself and other virulence genes located in the virulence region.

For the generation of a single-stranded DNA copy of the T-DNA, the form in which the T-DNA is transferred to the recipient, the gene products of the *virC* and *virD* operons are needed. The VirD2 protein, assisted by VirD1, makes a precise nick in the bottom strand of each of the border repeats. It is thought that DNA synthesis starts at the free 3′ OH of the right border nick and terminates at the left border repeat, leading to strand displacement and the release of a single-stranded T-DNA. The formation of the T-strand is stimulated by a 25-bp ''overdrive'' sequence located near the right border repeat (van Haaren et al. 1987; Veluthambi et al. 1988). VirC1 can bind this ''overdrive'' and thereby stimulates T-strand production (Toro et al. 1988). The VirD2 protein stays covalently attached to the 5′ end of the T-strand.

The next step in T-DNA transfer is piloting the T-strand through the bacterial membrane and cell wall via a type IV secretion mechanism. The virulence proteins VirB1-11 and VirD4 are involved in this process. The VirB proteins form a transport pore and a structure on the surface called the T-pilus, which is composed of T-pilin, a processed form of VirB2 (reviewed by Christie 1997; Kado 2000). The inner membrane VirD4 protein belongs to a family of so-called coupling proteins, which mediate interaction between the T-strand and the VirB complex. In addition to the VirD2/T-strand complex, the virulence proteins VirE2, VirE3, and VirF are also exported via the type IV secretion system (Regensburg-Tuink and Hooykaas 1993; Schrammeijer et al. 2003; Vergunst et al. 2000, 2003). VirE2 is a single-stranded DNA-binding protein and is thought to coat the T-strand in the host to protect it against nucleases and to keep the T-strand in an unfolded state to facilitate transport through the nuclear pore (Citovsky et al. 1989).

The T-strand is targeted to the nucleus by a nuclear localization signal, which is present in the C-terminal half of VirD2. Once inside the nucleus, the T-DNA stably integrates into the genome. The precise mechanism of T-DNA integration is not known, but host

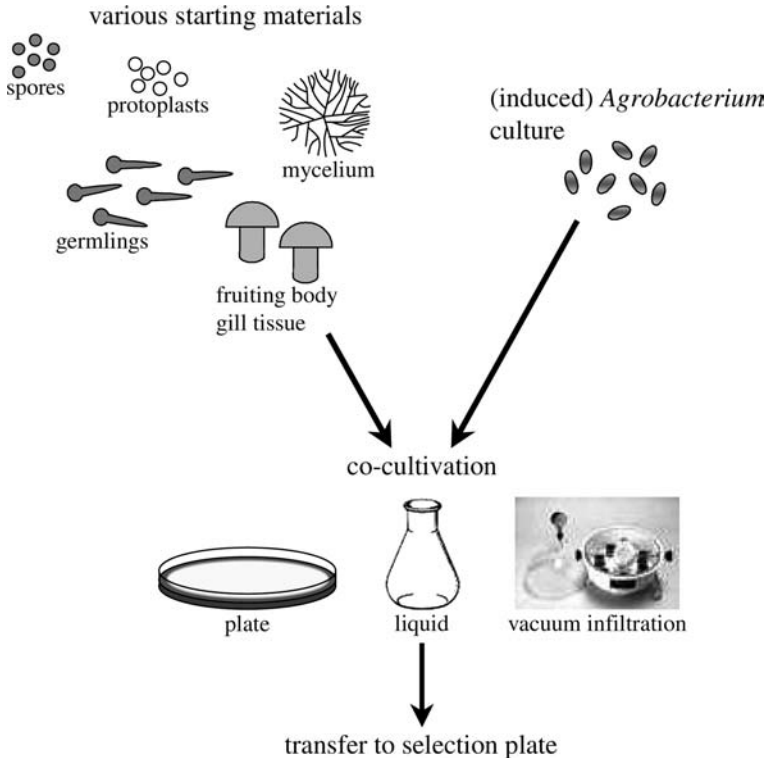

Fig. 1 Schematic overview of the experimental set-up of *Agrobacterium*-mediated transformation of fungi

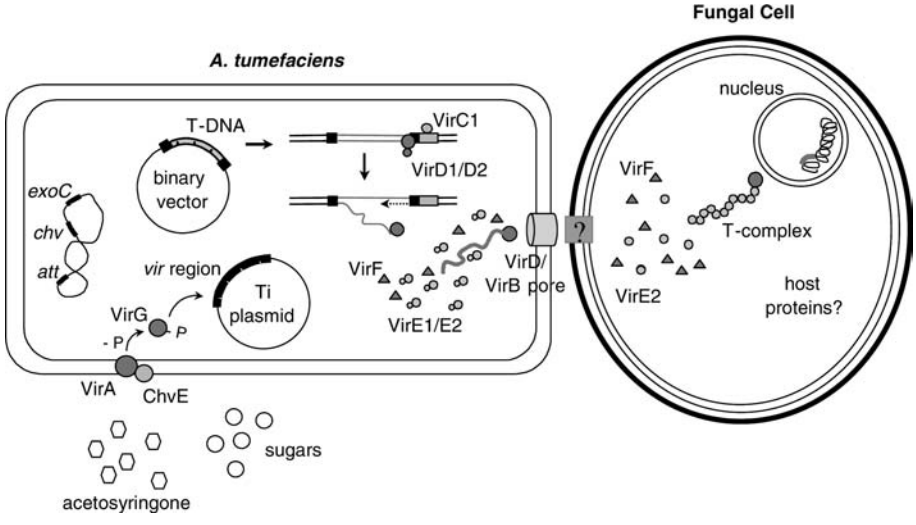

Fig. 2 Schematic overview of the *A. tumefaciens* T-DNA transfer system

proteins are thought to play a dominant role in this process (Bundock et al. 1995; Gelvin 2000; Tzfira and Citovsky 2002; van Attikum et al. 2001).

Transformation of fungi by *Agrobacterium* requires a functional T-DNA synthesis and transfer machinery

Based on the requirement of acetosyringone to obtain transformants and on the structure of the integrated T-DNA, it was inferred that the transformation of fungi by *A. tumefaciens* is mediated by the virulence system (de Groot et al. 1998). Indeed, experiments with *Agrobacterium* mutants which were inactivated in one of the key components required for the induction of the T-DNA transfer system, and the subsequent generation and transport of the T-strand, completely abolished or severely reduced the number of transformants formed both for *S. cerevisiae* and *Aspergillus awamori* (Bundock et al. 1995; Michielse et al. 2004b; Piers et al. 1996). This indicated that the T-DNA transfer mechanism of *A. tumefaciens* plays an important role in the generation of fungal transformants. However, some differences were seen between the AMT of plants and fungi. Between plants, yeasts, and fungi, a different requirement was observed for the virulence protein VirE2 (Michielse

et al. 2004b), which is transported to the host during the infection process and subsequently plays an important role in the protection of the T-DNA against nucleases. Inactivation of the virulence protein VirE2 led to a severely attenuated virulence of *A. tumefaciens* on plants (Dombek and Ream 1997; Stachel and Nester 1986), but only reduced the number of transformants in *S. cerevisiae* and *A. awamori* to 10% and 63%, respectively (Bundock et al. 1995; Michielse et al. 2004b). In addition, the plant host range factors, VirF, VirH, and VirE3, necessary for the infection of certain plant species, were dispensable for the transformation of *A. awamori* (Michielse et al. 2004b). Furthermore, *Agrobacterium* strains with mutations in the chromosomal genes *chvA*, *chvB*, or *exoC* were equally as efficient in the transformation of *S. cerevisiae* as the wild-type *Agrobacterium* strain (Piers et al. 1996). These chromosomal genes are involved in synthesis and export of β-1,2-glucan and are required for attachment to and subsequent transformation of plant cells. The result of AMT of *S. cerevisiae* with these *Agrobacterium* mutants indicated that β-1,2-glucan is not necessary for AMT of *S. cerevisiae*. Inactivation of *cel1*, a gene involved in the generation of cellulose and also necessary for attachment and virulence on plants (Matthysse 1983, 1987), led to a seven-fold increase in the transformation frequency, compared with the wild-type *Agrobacterium* strain, in AMT of *Aspergillus awamori* (AHA van Dijk and CB Michielse, unpublished data). This suggests that the presence of cellulose fibrils under the circumstances used to transform *A. awamori* might have a negative influence on the attachment of *A. tumefaciens* to the fungal host and thus reduces the efficiency of T-DNA transfer.

The role of host proteins during AMT

Host proteins that play a role during the transformation process have recently been identified in plants. For example, there are some plant proteins which interact with virulence proteins translocating into the plant cell (reviewed by Gelvin 2000, 2003; Tzfira and Citovsky 2002). One of these proteins is the VirD2-interacting protein karyopherin α (Ballas and Citovsky 1997), which is a conserved component of the protein nuclear transport pathway in eukaryotes. Also, specific plant proteins which interact with virulence proteins have been identified, e.g. VirE2 interacting proteins 1 and 2 (VIP1, VIP2; Tzfira and Citovsky 2002). Over-expression of VIP1 in tobacco plants resulted in an increased susceptibility to *Agrobacterium* infection and enhanced transient T-DNA gene expression (Tzfira et al. 2002). Another potential way to increase transformation levels in AMT of fungi might be the addition of purine synthesis inhibitors during transformation. The addition of purine synthesis inhibitors during plant transformation resulted in an increase in the transient expression of the T-DNA (Roberts et al. 2003). The AMT of *S. cerevisiae* mutants deficient in purine biosynthesis also seemed more efficient than that of the wild type (Roberts et al. 2003). It was also shown in *S. cerevisiae* that autonomous replication of the T-DNA (by addition of the autonomous replicating elements 2μ or telomere sequences in combination with ARS1 on the T-DNA) enhanced the transformation efficiency, compared with a T-DNA which lacked these elements and needed to integrate into the genome in order to produce transformants (Bundock et al. 1995; Roberts et al. 2003).

Factors influencing AMT efficiency in fungi

The development of AMT protocols for various fungi indicated a number of parameters which affect the transformation frequency. These parameters include the nature of the fungal starting material, the *Agrobacterium* strain and fungal isolate used, and the selection of co-cultivation conditions. It seems that each fungus and/or isolate requires its own optimal conditions to obtain optimal transformation frequency. The following sections give a summary of the different parameters determining the efficiency of AMT of fungi.

Fungal starting material

One of the advantages of AMT is that various starting materials can be used for transformation. The use of protoplasts, spores, mycelium, and fruiting body tissue all resulted in successful transformation. In several studies, it has been shown that protoplasts and intact cells can be transformed with equal efficiency by *A. tumefaciens* (Abuodeh et al. 2000; de Groot et al. 1998; Zwiers and De Waard 2001). Unfortunately, this is not always the case. For instance, for the transformation of the zygomycetes *Rhizopus oryzae* and *Mucor circinelloides*, transformants were only obtained when protoplasts were used as starting material. No transformants were obtained when spores or germinated spores were used (J. Gomez-Mateo, C.B. Michielse, and V. Garre, unpublished data; Michielse et al. 2004c). In most studies, spores or germinated spores are used as the starting material (Table 1). It has been shown that germination of spores is necessary for the transformation of *Coccidioides immitis* and that a prolonged germination has a positive influence on the transformation frequency (Abuodeh et al. 2000). However, for other species the opposite has been found (Campoy et al. 2003; Meyer et al. 2003). Also, in the case of the basidiomycete *Agaricus bisporus*, the choice of the starting material greatly determines the transformation efficiency. Although germinated conidia can be used (de Groot et al. 1998; Mikosch et al. 2001), higher efficiencies were obtained with vegetative and fruiting body mycelia as starting material (Chen et al. 2000; Mikosch et al. 2001). Even the different tissues of the fruiting body were shown to vary in their susceptibility to AMT (Chen et al.

2000). Another factor which influences the transformation frequency is the age of the starting material. Older cultures of *Blastomyces dermatitidis* were less efficiently transformed with *A. tumefaciens* (Sullivan et al. 2002). We found for *A. awamori* and *A. niger* that usage of ageing conidiospores stored for prolonged time at 4°C resulted in a decreased transformation frequency, most probably due to a reduced viablility or less efficient germination of the spores, resulting in less spores/germlings during co-cultivation which are susceptible for T-DNA uptake (C.B. Michielse, unpublished data).

Agrobacterium and host strain

Various *Agrobacterium* strains have been used for the transformation of fungi and oomycetes, e.g. LBA4404, EHA105, and LBA1100. Systematic comparisons of these different strains in relation to transformation frequencies have not been performed, making it difficult to say which strain is the best to use. Three studies showed that the usage of *Agrobacterium* strains derived from the supervirulent A281 strain (high level of *vir* gene expression) resulted in higher transformation frequencies in *S. cerevisiae*, *Monascus purpureus*, and the Oomycete *Phytophthora infestans*, compared with AMT using *Agrobacterium* strain LBA1100 (Campoy et al. 2003; Piers et al. 1996; Vijn and Govers 2003). The introduction of a ternary plasmid carrying the *virG* mutant gene coding for the constitutive active VirGN54D protein into *Agrobacterium* strain LBA1100 resulted in a considerable improvement in the transformation efficiency of *P. infestans* by this strain (Vijn and Govers 2003). In another study, it was also found that the supervirulent *Agrobacterium* A281 strain and its derivative (AGL-1) were more efficient in transferring T-DNA to *Cryphonectria parasitica* than the *Agrobacterium* LBA4404 strain (Park et al. 2004). Although at the moment it is not possible to point out which *Agrobacterium* strains are most suitable for AMT of fungi, it is clear that the choice of *Agrobacterium* strain can have an effect on transformation frequency.

In several studies, the transformation frequency obtained varied between different isolates of the same fungal species (Covert et al. 2001; Fitzgerald et al. 2003; Sullivan et al. 2002). Minor differences in, for example, cell wall or growth characteristics could explain the observed difference.

Acetosyringone concentration

In most studies, the addition of acetosyringone (AS) during the *Agrobacterium* co-cultivation period is required for transformation, indicating that induction of the *vir* genes is necessary for T-DNA transfer. The addition of AS during the pre-culture of *Agrobacterium* does not seem to be an absolute requirement for transformation, although omission of AS in the *Agrobacterium* pre-culture led to a lower transformation frequency in *Beauveria bassiana*, *Fusarium oxysporum*, and *Magnaporthe grisea* and a delay in the formation of the transformants (Leclerque et al. 2003; Mullins et al. 2001; Rho et al. 2001). No difference in the transformation frequency and formation of transformants was observed when AS was omitted from preculture in the case of *Hebeloma cylindrosporum* and *Colletotrichum trifolii* (Combier et al. 2003; Takahara et al. 2004). Leclerque et al. (2003) also investigated the effect of AS concentration during *Agrobacterium* pre-culture and during co-cultivation. It was found that increasing the concentration of AS up to 500 μM increased the number of transformants and that high transformation frequencies were only obtained when sufficient AS was present, not only in the co-cultivation plates but also during the *Agrobacterium* pre-culture.

Co-cultivation conditions

Other important factors determining the efficiency of AMT relate to the co-cultivation conditions. These include the ratio between *A. tumefaciens* and fungal recipient, length of the co-cultivation period, temperature, pH, and choice of filters. Increasing the amount of *A. tumefaciens* cells or fungal recipient cells in the co-cultivation mixture (changing the ratio) led in most cases to an increase in the transformation frequency. However, for both components, there is a limitation in the concentration which can be used. The addition of too many *A. tumefaciens* cells can result in a decrease in transformation efficiency (Meyer et al. 2003; C.B. Michielse, unpublished data), probably due to nutritional or space limitations. Excessive growth of *A. tumefaciens* during co-cultivation also makes it difficult to kill the bacteria after transformation. In contrast, the addition of too many fungal recipients can result in too much fungal growth during co-cultivation, which makes the subsequent isolation of single transformants difficult (Covert et al. 2001). Several studies have shown that each fungus has an optimal combination of co-cultivation period and temperature to obtain a maximum number of transformants (Combier et al. 2003; Gardiner and Howlett 2004; Meyer et al. 2003; Michielse et al. 2004b; Mullins et al. 2001; Rho et al. 2001; Rolland et al. 2003). The growth rate of the hosts and differences in their susceptibility to *A. tumefaciens* might account for these differences.

The co-cultivation temperature has also been shown to have an influence on transformation efficiency. The temperatures tested range from 20°C to 37°C; and temperatures between 22°C and 25°C are found to be optimal (Combier et al. 2003; Gardiner and Howlett 2004; Michielse et al. 2004b; Rolland et al. 2003). This is consistent with observations made in AMT of plants, where similar temperatures were found to be optimal for T-DNA transfer, compared with temperatures of 20°C or 28°C (Dillen et al. 2002; Salas et al. 2002). This

correlates with the idea that at higher temperatures (>28°C) the T-DNA transfer machinery of *A. tumefaciens* does not function properly (Fullner and Nester 1996). We studied the combined effect of co-cultivation time and temperature in *A. awamori* and found that increasing the temperature stepwise from 20°C to 28°C during a short co-cultivation period (24 h) led to an increased transformation frequency. However, this temperature effect was less pronounced at prolonged incubation periods. A prolonged incubation period (48 h, 72 h) at a temperature of 20°C or 28°C led to irreproducible results and, at 28°C, also led to increased fungal background growth during co-cultivation (Michielse et al. 2004b).

The effect of pH during co-cultivation on the transformation frequency was tested in AMT of *C. trifolii* and *C. lagenarium* (Takahara et al. 2004; Tsuji et al. 2003). It was found that the optimal pH, leading to the highest transformation frequency, is between 5.0 and 5.3.

Different filters, such as nitrocellulose, Hybond, filter paper, cellophane sheets, and polyvinylidene difluoride, have been used successfully in the co-cultivation step. It was found that filter paper, Hybond N, or Hybond N^+ resulted in equally high transformation rates in AMT of *A. awamori*. The use of Hybond C and nitrocellulose filters led to lower transformation efficiencies (C.B. Michielse, unpublished data). For the Oomycete *P. infestans*, it was reported that no transformants were found after incubation on nitrocellulose, but incubation on Nytran or Hybond N^+ resulted in transformants with Hybond N^+ being two- to three-fold better (Vijn and Govers 2003).

Fungal strains recalcitrant for AMT

Although AMT of fungi is usually successful, there are also cases where AMT is less successful or fails to produce transformants. Rolland et al. (2003) reported that AMT of *Sclerotinia sclerotiorum* did not result in transformants, even after varying the various parameters described above. In our own hands, the transformation frequency obtained for AMT of *A. niger* was low and not reproducible. Several parameters, such as co-cultivation conditions, *Agrobacterium* strains, *A. tumefaciens*:conidia ratio, host strain, and selection marker were tested. Unfortunately, this did not result in a reproducible and efficient transformation protocol (C.B. Michielse, unpublished data). The number of transformants obtained with an *Aspergillus oryzae pyrG* or dominant hygromycin selection marker varied between 0 and 40 transformants per 10^6 spores. The AMT of *A. niger* is also hampered due to the high background growth of *A. niger* during transformation. In other cases, methods to reduce background growth have been described, such as using an overlay technique (Zeilinger 2003) or harvesting fungal and bacterial cells after co-cultivation and subsequently plating this mixture directly onto selective medium (Leclerque et al. 2003).

Although our knowledge on AMT of fungi is increasing, we do not fully understand the entire T-DNA transfer and integration process or the experimental parameters involved in *Agrobacterium*-mediated fungal transformation, which results in the different susceptibilities of the various fungal hosts in AMT. The continuing study of the *A. tumefaciens* T-DNA transfer system and the development of new *Agrobacterium*-mediated fungal transformation protocols will contribute to our understanding of the AMT transfer process and might also provide more insight into the parameters required for successful AMT of recalcitrant fungi.

Agrobacterium as a tool for mutagenesis

Targeted mutagenesis

One of the major goals of functional genomics is to assess the function of putative proteins encoded by the predicted open reading frames (ORF) in the genome sequence. A direct approach to determine gene function is to specifically disrupt the gene of interest using targeted mutagenesis (gene disruption or gene replacement). Specific gene targeting in *S. cerevisiae* is highly efficient and small homologous flanks (50 bp) can efficiently target the gene of interest (Bahler et al. 1998; Wach et al. 1994). This led to the development of PCR-based gene-disruption cassettes where the selectable marker was amplified by PCR with primers that contained a 50-bp extension corresponding to the gene of interest (Baudin et al. 1993). Based on this PCR-based homologous recombination method, a complete set of *S. cerevisiae* deletion mutants has been constructed (Winzeler et al. 1999). However, for fungi, large-scale disruption of the predicted genes is seriously limited by the large homologous flanks needed to obtain a reasonable homologous recombination (HR) frequency (Asch and Kinsey 1990; Hynes 1996).

The efficiency of HR is mainly determined by the dominant pathway of the host to repair double-stranded DNA breaks (Schaefer 2001). However, other parameters, such as the length of the homologous DNA flanks, the G/C content of these flanks, the transcriptional status of the targeted gene, and the chromatin structure determined by the location of the targeted gene on the chromosome, have also been shown to influence HR efficiency (Baudin et al. 1993; Bird and Bradshaw 1997; Gray and Honigberg 2001; Hua et al. 1997; Nelson et al. 2003; Versaw and Metzenberg 1996). Several methods have been developed to increase gene-targeting efficiency and/or identify those transformants which have undergone recombination in fungi. They include the generation of gene replacement cassettes by the subcloning of genomic fragments, PCR, or in vivo recombination in *E. coli* or *S. cerevisiae* (reviewed by Wendland 2003). Also, the addition of a second selectable marker to the

gene disruption cassette (double-marker enrichment technique) or the use of the split-marker technique enriches or increases gene-targeting events (reviewed by Wendland 2003).

However, the existing methods to generate gene-disruption cassettes with large homologous flanks does not guarantee efficient HR if the introduction of these disruption cassettes into the host is a bottleneck. The ease of AMT and the increased transformation frequencies obtained for the various fungi transformed with AMT might make this method a useful tool in targeted mutagenesis experiments. Another important aspect is that AMT promotes HR. In the yeast *Kluyveromyces lactis*, it was found that introducing the gene-disruption cassette by AMT resulted in a ten- to 71-fold increase in the gene-targeting frequency, compared with introducing the gene-replacement cassettes by electroporation (Bundock et al. 1999). Also, introducing gene-replacement cassettes by AMT into *A. awamori* led to increased frequencies, compared with the frequencies obtained after introducing the gene-replacement cassettes by $CaCl_2$/polyethylene glycol-mediated transformation. For two genes investigated, orotidine-5′-monophosphate decarboxylase (*pyrG*) and glucosamine/fructose-6-phosphate aminotransferase (*gfaA*), a three- and six-fold increase in the gene-targeting frequency was found (Michielse et al. 2005). Furthermore, it was shown that AMT allowed the use of shorter DNA flanks to obtain a reasonable gene-targeting frequency (Michielse et al. 2005). AMT has also been used successfully to disrupt genes involved in various processes, such as cell wall biosynthesis, melanin synthesis, and pathogenicity with high efficiency in e.g. *Coccidioides posadasii*, *Fusarium oxysporum*, *Glarea lozoyensis*, *Mycosphaerella graminicola*, *Trichoderma atroviride*, *Verticillium dahliae*, and *V. fungicola* (see Table 1). The gene-targeting efficiencies obtained ranged from 14% to 75%, indicating the potential of AMT to accomplish gene-targeting in fungi, which is usually more difficult to achieve with conventional transformation methods.

A potential explanation for the high homologous recombination frequencies found might be that *A. tumefaciens* delivers its DNA to the host as a single-stranded DNA–protein complex, rather than a double-stranded DNA molecule, as in the case when using other transformation methods. The single-stranded nature of the transferred DNA might promote HR. It has been shown in *Streptomyces* species and in *S. cerevisiae* that single-stranded DNA is a preferred substrate for integration and homologous recombination, respectively (Hilleman et al. 1991; Simon and Moore 1987). In addition to the single-stranded nature of the T-strand, a role for the co-introduced VirD2 and VirE2 proteins in promoting homologous recombination cannot be ruled out, as the binding of these proteins to the single-stranded DNA has been shown to protect the DNA against nucleases and to target the DNA–protein complex to the nucleus (Christie et al. 1988; Durrenberger et al. 1989; Rossi et al. 1993, 1996). The presence of the VirD2 protein may also have a beneficial effect on the integration event, as it is thought to play a role in DNA repair (Bako et al. 2003).

Targeted gene disruption with AMT has been combined with the double-marker enrichment technique in *A. awamori* and *Leptosphaeria maculans* (Gardiner and Howlett 2004; Michielse et al. 2005). In this strategy, a second selectable marker is added to the gene disruption cassette, to distinguish between homologous and non-homologous recombination. Upon HR, the second selection marker is lost whereas, after integration by non-homologous recombination, transformants carrying both selectable markers are obtained. In *A. awamori*, the addition of the dominant *A. nidulans amdS* selection marker to the gene replacement cassette led to two-fold enrichment in putative gene replacement transformants (Michielse et al. 2005). Addition of the negative selectable marker thymidine kinase as a second selection marker to the gene disruption cassette in AMT of *L. maculans* led to a homologous recombination frequency of 17%. For this fungus, the combination of the double-marker enrichment technique and AMT proved to be essential for obtaining gene disruption mutants (Gardiner and Howlett 2004). The thymidine kinase gene was also successfully used as a negative selection marker in AMT targeted gene-disruption experiments with *Magnaporthe grisea* and *Fusarium oxysporum* (Khang et al. 2005). It should be noted however that, although a negative selection marker is used, false-positive gene disruption transformants are also found at a low frequency. It seems that the percentage of false-positive gene disruption transformants is higher when the negative selection marker is placed at the left T-DNA border repeat (Khang et al. 2005; Michielse et al. 2005), putatively due to T-DNA truncation, which is more profound at the left T-DNA border than at the right border. This should be considered when designing vectors with a negative selection marker for gene-disruption purposes.

Perspectives of targeted mutagenesis using AMT

Higher homologous recombination frequencies may still be obtained if AMT is combined with transposon-arrayed gene knockouts (TAGKO). Gene disruption using TAGKO has been shown to result in high HR frequencies in *M. graminicola* and *M. grisea* and has the potential to be used on a large scale (Adachi et al. 2002; Hamer et al. 2001). To combine the TAGKO method with AMT, the cosmid or BAC libraries used as mutational targets in TAGKO need to be converted into binary vectors suitable for AMT. To avoid the generation of new genomic libraries in binary vectors, it is possible to convert the existing libraries into a binary vector, using the in vivo recombination method in *E. coli* as described by Takken et al. (2004), thereby combining the advantages of AMT and TAGKO in targeted mutagenesis. The conversion method described by

Takken et al. (2004), can also be used to convert a cosmid carrying a gene-replacement cassette [generated by in vivo recombination between a PCR-based gene-replacement cassette and a cosmid, as described by Chaveroche et al. (2000)] into a binary vector.

It has been shown that T-DNA integration in the yeast *S. cerevisiae* is mediated by the non-homologous end-joining (NHEJ) machinery (van Attikum et al. 2001). In the absence of NHEJ proteins, such as Ku70 or Ku80, integration can only occur by homologous recombination by proteins such as Rad52 (van Attikum and Hooykaas 2003). Inactivation of Ku70 or Ku80 can thus be used to promote integration by homologous recombination. Recently, it was shown that this is indeed the case for the yeast *K. lactis* (Kooistra et al. 2004) and the filamentous fungus *Neurospora crassa* (Ninomiya et al. 2004). Disruption of Ku70 or Ku80 led to integration by HR in 97% of the *K. lactis* transformants and 100% of the *S. cerevisiae* and *N. crassa* transformants analyzed, indicating that such mutants will also be very useful for gene-targeting experiments in other fungi. Once the target gene has been deleted, the wild-type *ku70* or *ku80* genes might be restored by crossings or complementation studies to allow analysis of the disruption strain in an otherwise wild-type background. We are convinced that this strategy, in combination with AMT, will be very valuable for future gene-deletion studies in fungi.

An alternative to gene replacement and gene disruption for studying gene function is RNA interference (RNAi), which has been successfully used to down-regulate genes in filamentous fungi (Kadotani et al. 2003; Liu et al. 2002). Genome-wide RNAi has been shown to be a rapid approach for obtaining knock-down phenotypes in *Caenorhabditis elegans* (Kamath and Ahringer 2003; Piano et al. 2000; Simmer et al. 2003). RNAi has also been used successfully in combination with AMT in plants (Chuang and Meyerowitz 2000; Kumagai and Kouchi 2003). Recently, the use of RNAi in combination with AMT in the fungus *Venturia inaequalis* and in *Ophiostoma floccosum* was shown to be successful (Fitzgerald et al. 2004; C. Breuil, personal communication).

In summary, the high homologous recombination frequency obtained in fungi using AMT technology could provide a good basis for the development of large-scale gene-disruption approaches, either alone or in combination with existing methods, such as inhibition of NHEJ, the double-marker enrichment technique, or TAGKO. Alternatively, for fungi in which homologous recombinations are rare, AMT using constructs producing RNAi might be a good alternative to generate knock-down or knock-out transformants.

Insertional mutagenesis

Insertional mutagenesis can be used to saturate the genome with mutations. In collections of such mutants, deficiencies in specific processes can be sought to identify the mutants of interest. The subsequent identification of the tagged genes and verification that the observed phenotype is the result of the tagged gene may reveal its function. A requirement for insertional mutagenesis is that it should be easy to generate a large number of transformants. After screening for transformants with the desired phenotype, it should also be easy to subsequently re-isolate the tagged gene. Furthermore, the element used for insertional mutagenesis should integrate at random into the host genome in a non-sequence-specific manner, remain intact, and should be present as a single-copy or in low-copy numbers to simplify downstream analysis. As AMT and the integration substrate, the T-DNA, fulfil these requirements, AMT is a standard tool for insertional mutagenesis in plants (Jeon et al. 2000; Koncz et al. 1989, 1992; Krysan et al. 1999; Pereira 2000), has been used in *S. cerevisiae* (Bundock et al. 2002) for the same purpose, and is currently being used to construct mutant libraries for several fungi (see below).

Gene inactivation can simply be achieved using a T-DNA that consists of only a selection marker. Upon integration into the coding region of a gene, T-DNA can potentially block transcription. Adding certain elements onto the T-DNA allows use of the T-DNA for other purposes in addition to gene activation, such as enhancer- or promoter-tagging, or gene activation by activation-tagging.

Factors influencing the number of T-DNA integrations per transformant

In applying AMT to generate mutant libraries, single-copy T-DNA integration is preferred, to ensure the link between an observed phenotype and one single alteration of the genome. Although AMT leads to mostly multi-locus and/or multi-copy integration in plants, it mainly results in single-copy T-DNA integration in yeasts and fungi (Abuodeh et al. 2000; Amey et al. 2002; Bundock et al. 2002; Campoy et al. 2003; Combier et al. 2003; Covert et al. 2001; de Groot et al. 1998; Degefu and Hanif 2003; Fitzgerald et al. 2003; Hanif et al. 2002; Leclerque et al. 2003; Malonek and Meinhardt 2001; Michielse et al. 2004a; Mullins et al. 2001; Rho et al. 2001; Sullivan et al. 2002; Tanguay and Breuil 2003; Zwiers and De Waard 2001). Also, the T-DNA ends are relatively well preserved during integration into the yeast or fungal genome, sometimes ending exactly at the site where the Ti plasmid DNA was nicked by VirD2 (Bundock and Hooykaas 1996). In some of these studies, it was shown that several parameters which have an influence on the transformation frequency (see Insertional mutagenesis, above) also have an influence on the T-DNA copy number. Mikosch et al. (2001) reported that the use of germinated spores led to predominantly multi-copy transformants, whereas the use of mycelium led predominantly to single-copy transformants (Mikosch et al. 2001). The addition of AS to the

Agrobacterium pre-culture has been reported to result in either a decrease or an increase in single-copy T-DNA integration, for reasons unknown (Combier et al. 2003; Mullins et al. 2001; Rho et al. 2001). Prolongation of the co-cultivation period has been shown to lead to multiple T-DNA integrations in *M. grisea* (Rho et al. 2001). However, the length of the co-cultivation period had no influence on T-DNA copy number in *F. oxysporum* (Mullins et al. 2001). Finally, increasing the *A. tumefaciens* concentration during co-cultivation resulted in an increased number of T-DNA copies in *Beauveria dermatiditis* (Sullivan et al. 2002) and similarly in *Suillus bovinus* (Hanif et al. 2002). However, the *A. tumefaciens* concentration had no effect on the T-DNA copy number in *F. oxysporum* (Mullins et al. 2001). Thus, single-copy events may be directed by the addition or omission of AS prior to co-cultivation, by changing the *A. tumefaciens*:fungal recipient ratio, or by using a different kind of starting material.

Identification of DNA sequences flanking T-DNA insertions

As mentioned above, single T-DNA integration is preferred to simplify the subsequent isolation of the tagged gene. Several methods have been described to isolate the chromosomal DNA flanking the T-DNA, e.g. plasmid rescue and PCR-based methods (Balzergue et al. 2001; Cottage et al. 2001; Gibson and Sommerville 1992; Jones and Winistorfer 1993; Liu et al. 1995; Ochman et al. 1988; Singer and Burke 2003). Plasmid rescue has been successfully used to isolate the T-DNA and flanking chromosomal sequences of various fungi (Bundock et al. 2002; Bundock and Hooykaas 1996; de Groot et al. 1998; Leclerque et al. 2003; Michielse et al. 2004a). Plasmid rescue is very efficient and in most cases the flanking DNA sequences of both T-DNA borders can be obtained. This technique requires an origin of replication and a selection marker to be present on the T-DNA which are both active in *E. coli*. Alternative approaches are mostly based on PCR, e.g. inverse-PCR, vectorette-PCR, and TAIL-PCR can be powerful for rapid and efficient recovery of flanking DNA sequences. These methods have been used to isolate flanking fungal chromosomal DNA (Combier et al. 2003; Mullins et al. 2001; Rho et al. 2001; Rolland et al. 2003; van Attikum et al. 2001) and can be applied to a large number of transformants. TAIL-PCR has already been used as a tool for the identification of integration sites in various plant mutant libraries in a high-throughput manner (Singer and Burke 2003). A success rate of 90–100% was obtained during the isolation of T-DNA borders and flanking fungal chromosomal DNA by TAIL-PCR (Mullins et al. 2001; Rho et al. 2001; Rolland et al. 2003). Further analysis of the isolated flanking fungal chromosomal DNA and T-DNA borders revealed that, upon T-DNA integration, truncation of both the right and left borders occurred (Bundock and Hooykaas 1996; de Groot et al. 1998, Michielse et al. 2004a), but to a much lesser extend compared with plants (Gheysen et al. 1991; Matsumoto et al. 1990). Furthermore, no extensive homology between the T-DNA and integration site or among the integration sites themselves was found, indicating random and non-sequence specific T-DNA integration (Bundock and Hooykaas 1996; Combier et al. 2003; de Groot et al. 1998; Leclerque et al. 2003; Michielse et al. 2004a; Mullins et al. 2001; Rho et al. 2001; Rolland et al. 2003).

Distribution of T-DNA insertions in the genome

Mapping of T-DNA insertions at the chromosomal level in a large collection of *S. cerevisiae* transformants confirmed the observed non-sequence specific and random T-DNA integration behavior (Bundock et al. 2002). The T-DNA integrations were located in upstream elements (24%), ORFs (26%), downstream elements (6%), and intergenic regions (41%; Bundock et al. 2002). In plants, conflicting statements about the randomness of T-DNA integration have been made. Analysis of large pools of plant transformants indicated that T-DNA integration is non-sequence-specific and occurs at random throughout the genome (reviewed by Azpiroz-Leehan and Feldmann 1997; Koncz et al. 1992). Rosso et al. (2003) reported a bias towards T-DNA integration in intergenic regions in *A. thaliana*. In addition, Bakarat et al. (2000) reported differences in patterns of T-DNA integration between *A. thaliana* and rice, which were correlated with the differences in gene distribution between these two species. It was shown that T-DNA integrates at random in the *A. thaliana* genome, but integration in the rice genome occurs predominantly in gene-rich and transcriptionally active regions (Bakarat et al. 2000). Further analysis of T-DNA integration sites in various plant mutant libraries should reveal whether T-DNA integration really is random, as is presumed. However, it should be kept in mind that isolation of T-DNA insertion mutants automatically selects for integration into sites where the T-DNA is expressed.

Currently, large-scale insertional mutagenesis by AMT is being carried out in fungi such as *Cryptococcus neoformans* (Idnurm et al. 2004), *Botrytis cinerea* (P. Tudzynski, personal communication), *Hebeloma cylindrosporum* (G. Gay, personal communication) *Coniothyrium minitans* (Rogers et al. 2004), *M. grisea* (S. Tucker, personal communication) and *F. oxysporum* (M. Rep, personal communication). This work has led to the identification of e.g. sporulation-, pathogenicity-, pigmentation-, and antibiotic-deficient mutants (Idnurm et al. 2004; Li et al. 2005; Tsuji et al. 2003). Using *Agrobacterium*-mediated insertional mutagenesis, a laccase gene (*LAC1*), a gene encoding a voltage-gated chloride channel (*CLC1*; which appears to be involved in pigmentation), and a gene involved in controlling light responses (*BWC2*) were identified (Idnurm and Heitman 2005; Idnurm et al. 2004). These examples indicate that

AMT of fungi can be used to identify and isolate mutants with a desired phenotype and subsequently elucidate gene functions.

Characterization of a *C. neoformans* mutant library revealed that not all mutant phenotypes were correlated with a T-DNA insertion. In the mutant collection, about 50–66% of the mutant phenotypes seem to be due to a T-DNA insertion (Idnurm and Heitman 2005; J. Heitman, personal communication). As it is currently not known how often the mutant phenotype is actually caused by a T-DNA integration event, it is advisable to perform segregation tests to determine whether the mutant phenotype co-segragates with the selection marker. Insertional mutagenesis in *F. circinatum* revealed that the majority of the isolated pathogenicity mutants lost their female fertility, which made it difficult to determine whether the mutation of interest co-segregated with the T-DNA insertion (S. Covert, personal communication).

Chromosomal rearrangements have been observed in a small number of plant transformants which were generated by AMT (Castle et al. 1993; Nacry et al. 1998; Ohba et al. 1995; Takano et al. 1997). Analysis of the occurrence of chromosomal rearrangements has been carried out in *B. cinerea*. Only one of the 30 mutants showed chromosomal rearrangements, indicating that in this fungus chromosomal rearrangements are not of major importance (P. Tudzynski, personal communication).

Thus, high transformation rates in combination with non-sequence-specific and apparently random T-DNA integration could make AMT an attractive tool for performing insertional mutagenesis in fungi. Preliminary results are very promising and could warrant AMT as an additional tool or even the preferred tool to replace existing mutational methods for carrying out high-throughput functional genomics.

Conclusions

In the decade since its first use, AMT has proven itself as an important tool for the transformation of a diverse array of yeasts, fungi, and oomycetes. Fungi in various stages of development can be used in AMT and the transformation itself is easy to perform. Relevant parameters for AMT of fungi have been identified, which might help to optimize this system for organisms more recalcitrant to AMT. In addition, AMT is becoming a promising tool for targeted mutagenesis due to the high homologous recombination rates observed and, in combination with the existing methods to generate gene disruption/replacement cassettes, it could provide a method to perform large-scale targeted mutagenesis. Furthermore, it seems that AMT can be efficiently used for insertional mutagenesis. It is easy to generate a large number of mutants and evidence is accumulating which shows that T-DNA integrates into the host genome in a non-sequence-specific manner and at random. As it is relatively easy to isolate tagged genes, AMT is currently used to carry out large-scale insertional mutagenesis experiments for a number of different fungi. Data obtained from these studies will validate the true potential of T-DNA as a tool for insertional mutagenesis; and preliminary results are very promising. All of the advantages described for AMT should position this method as a key instrument to further develop fungal genomics.

Acknowledgements We thank R. Cardoza, M. Challen, B. Donzelli, S. Gutierrez, T. Heinekamp, H. Khanh, Y. Lee, C. Rogers, J. Skov, J. Kan, and K. Welzel for their consent to use their data that were presented at the 21st and 22nd FGC (Asilomar, USA), at ECFG6 (Pisa, Italy), and/or at ECFG7 (Copenhagen, Denmark). We thank V. Garre, G. Gay, J. Gomez-Mateo, S. Covert, M. Rep, M. Furlaneto, K. Plummer, S. Kang, A. Idnurm, D. Gardiner, J.F. Martin, and P. Tudzynski for sharing their unpublished data and discussion. We also thank Patricia vanKuyk and Jaap Visser for critically reading this manuscript.

References

Abuodeh RO, Orbach MJ, Mandel MA, Das A, Galgiani JN (2000) Genetic transformation of *Coccidioides immitis* facilitated by *Agrobacterium tumefaciens*. J Infect Dis 181:2106–2110

Adachi K, Nelson GH, Peoples KA, Frank SA, Montenegro-Chamorro MV, DeZwaan TM, Ramamurthy L, Shuster JR, Hamer L, Tanzer MM (2002) Efficient gene identification and targeted gene disruption in the wheat blotch fungus *Mycosphaerella graminicola* using TAGKO. Curr Genet 42:123–127

Amey RC, Athey-Pollard A, Burns C, Mills PR, Bailey A, Foster GD (2002) PEG-mediated and *Agrobacterium*-mediated transformation in the mycopathogen *Verticillium fungicola*. Mycol Res 106:4–11

Amey RC, Mills PR, Bailey A, Foster GD (2003) Investigating the role of a *Verticillium fungicola* beta-1,6-glucanase during infection of *Agaricus bisporus* using targeted gene disruption. Fungal Genet Biol 39:264–275

Asch DK, Kinsey JA (1990) Relationship of vector insert size to homologous integration during transformation of *Neurospora crassa* with the cloned *am* (GDH) gene. Mol Gen Genet 221:37–43

Attikum H van, Hooykaas PJ (2003) Genetic requirements for the targeted integration of *Agrobacterium* T-DNA in *Saccharomyces cerevisiae*. Nucleic Acid Res 31:826–832

Attikum H van, Bundock P, Hooykaas PJ (2001) Non-homologous end-joining proteins are required for *Agrobacterium* T-DNA integration. EMBO J 20:6550–6558

Azpiroz-Leehan R, Feldmann KA (1997) T-DNA insertion mutagenesis in *Arabidopsis*: going back and forth. Trends Genet 13:152–156

Bader GD, Heilbut A, Andrews B, Tyers M, Hughes T, Boone C (2003) Functional genomics and proteomics: charting a multidimensional map of the yeast cell. Trends Cell Biol 13:344–356

Bahler J, Wu JQ, Longtine MS, Shah NG, McKenzie A, Steever AB, Wach A, Philippsen P, Pringle JR (1998) Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. Yeast 14:943–951

Bakarat A, Gallois P, Raynal M, Mestre-Ortega D, Sallaud C, Guiderdoni E, Delseny M, Bernardi G (2000) The distribution of T-DNA in the genomes of transgenic *Arabidopsis* and rice. FEBS Lett 471:161–164

Bako L, Umeda M, Tiburcio AF, Schell J, Koncz C (2003) The VirD2 pilot protein of *Agrobacterium*-transferred DNA interacts with the TATA box-binding protein and a nuclear protein kinase in plants. Proc Natl Acad Sci USA 100:10108–10113

Ballas N, Citovsky V (1997) Nuclear localization signal binding protein from *Arabidopsis* mediates nuclear import of *Agrobacterium* VirD2 protein. Proc Natl Acad Sci USA 94:10723–10728

Balzergue S, Dubreucq B, Chauvin S, Le Clainche I, Le Boulaire F, de Rose R, Samson F, Biaudet V, Lecharny A, Cruaud C, Weissenbach J, Caboche M, Lepiniec L (2001) Improved PCR-walking for large-scale isolation of plant T-DNA borders. Biotechniques 30:496–498, 502, 504

Baudin A, Ozier-Kalogeropoulos O, Denouel A, LaCroute F, Cullin C (1993) A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. Nucleic Acids Res 21:3329–3330

Bird D, Bradshaw R (1997) Gene targeting is locus dependent in the filamentous fungus *Aspergillus nidulans*. Mol Gen Genet 255:219–225

Bolker M, Bohnert HU, Braun KH, Gorl J, Kahmann R (1995) Tagged pathogenicity genes in *Ustilago maydis* by restriction enzyme-mediated integration (REMI). Mol Gen Genet 248:547–552

Brandhorst TT, Rooney PJ, Sullivan TD, Klein B (2002) Molecular genetic analysis of *Blastomyces dermatitidis* reveals new insights about pathogenic mechanisms. Int J Med Microbiol 292:363–371

Brown JS, Holden DW (1998) Insertional mutagenesis of pathogenic fungi. Curr Opin Microbiol 1:390–394

Bundock P, Hooykaas PJ (1996) Integration of *Agrobacterium tumefaciens* T-DNA in the *Saccharomyces cerevisiae* genome by illegitimate recombination. Proc Natl Acad Sci USA 93:15272–15275

Bundock P, Dulk-Ras A, Beijersbergen A, Hooykaas PJ (1995) Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. EMBO J 14:3206–3214

Bundock P, Mroczek K, Winkler AA, Steensma HY, Hooykaas PJ (1999) T-DNA from *Agrobacterium tumefaciens* as an efficient tool for gene targeting in *Kluyveromyces lactis*. Mol Gen Genet 261:115–121

Bundock P, van Attikum H, Dulk-Ras A, Hooykaas PJ (2002) Insertional mutagenesis in yeasts using T-DNA from *Agrobacterium tumefaciens*. Yeast 19:529–536

Campoy S, Perez F, Martin JF, Gutierrez S, Liras P (2003) Stable transformants of the azaphilone pigment-producing *Monascus purpureus* obtained by protoplast transformation and *Agrobacterium*-mediated DNA transfer. Curr Genet 43:447–452

Cangelosi GA, Ankenbauer RG, Nester EW (1990) Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. Proc Natl Acad Sci USA 87:6708–6712

Castle LA, Errampalli D, Atherton TL, Franzmann LH, Yoon ES, Meinke DW (1993) Genetic and molecular characterization of embryonic mutants identified following seed transformation in *Arabidopsis*. Mol Gen Genet 241:504–514

Chaveroche MK, Ghigo JM, d'Enfert C (2000) A rapid method for efficient gene replacement in the filamentous fungus *Aspergillus nidulans*. Nucleic Acids Res 28:E97

Chen X, Stone M, Schlagnhaufer C, Romaine CP (2000) A fruiting body tissue method for efficient *Agrobacterium*-mediated transformation of *Agaricus bisporus*. Appl Environ Microbiol 66:4510–4513

Christie PJ (1997) *Agrobacterium tumefaciens* T-complex transport apparatus: a paradigm for a new family of multifunctional transporters in eubacteria. J Bacteriol 179:3085–3094

Christie PJ, Ward JE, Winans SC, Nester EW (1988) The *Agrobacterium tumefaciens virE2* gene product is a single-stranded-DNA-binding protein that associates with T-DNA. J Bacteriol 170:2659–2667

Chuang CF, Meyerowitz EM (2000) Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 97:4985–4990

Citovsky V, Wong ML, Zambryski P (1989) Cooperative interaction of *Agrobacterium* VirE2 protein with single-stranded DNA: implications for the T-DNA transfer process. Proc Natl Acad Sci USA 86:1193–1197

Combier JP, Melayah D, Raffier C, Gay G, Marmeisse R (2003) *Agrobacterium tumefaciens*-mediated transformation as a tool for insertional mutagenesis in the symbiotic ectomycorrhizal fungus *Hebeloma cylindrosporum*. FEMS Microbiol Lett 220:141–148

Cottage A, Yang A, Maunders H, de Lacy RC, Ramsay NA (2001) Identification of DNA sequences flanking T-DNA insertions by PCR-walking. Plant Mol Biol Rep 19:321–327

Covert SF, Kapoor P, Lee M, Briley A, Nairn CJ (2001) *Agrobacterium*-mediated transformation of *Fusarium circinatum*. Mycol Res 105:259–264

Daboussi MJ, Capy P (2003) Transposable elements in filamentous fungi. Annu Rev Microbiol 57:275–299

Degefu Y, Hanif M (2003) *Agrobacterium tumefaciens*-mediated transformation of *Helminthosporium turcicum*, the maize leaf-blight fungus. Arch Microbiol 180:279–284

Dillen W, DeClercq J, Kapila J, Zambre M, Montagu M van, Angenon G (2002) The effect of temperature on *Agrobacterium tumefaciens*-mediated gene transfer to plants. Plant J 12:1459–1463

Dobinson KF, Grant SJ, Kang S (2003) Cloning and targeted disruption, via *Agrobacterium tumefaciens*-mediated transformation, of a trypsin protease gene from the vascular wilt fungus *Verticillium dahliae*. Curr Genet 45:104–110

Dombek P, Ream W (1997) Functional domains of *Agrobacterium tumefaciens* single-stranded DNA-binding protein VirE2. J Bacteriol 179:1165–1173

dos-Reis MC, Pelegrinelli Fungaro MH, Duarte RTD, Furlaneto L, Furnaleto MC (2004) *Agrobacterium tumefaciens*-mediated genetic transformation of the entomopathogenic fungus *Beauveria bassiana*. J Microbiol Methods 58:197–202

Durrenberger F, Crameri A, Hohn B, Koukolikova-Nicola Z (1989) Covalently bound VirD2 protein of *Agrobacterium tumefaciens* protects the T-DNA from exonucleolytic degradation. Proc Natl Acad Sci USA 86:9154–9158

Fang W, Zhang Y, Yang X, Zheng X, Duan H, Li Y, Pei Y (2004) *Agrobacterium tumefaciens*-mediated transformation of *Beauveria bassiana* using an herbicide resistance gene as a selection marker. J Invertebr Pathol 85:18–24

Firon A, Villalba F, Beffa R, d'Enfert C (2003) Identification of essential genes in the human fungal pathogen *Aspergillus fumigatus* by transposon mutagenesis. Eukaryot Cell 2:247–255

Fitzgerald AM, Mudge AM, Gleave AP, Plummer KM (2003) *Agrobacterium* and PEG-mediated transformation of the phytopathogen *Venturia inaequalis*. Mycol Res 107:803–810

Fitzgerald A, Van Kan JA, Plummer KM (2004) Simultaneous silencing of multiple genes in the apple scab fungus, *Venturia inaequalis*, by expression of RNA with chimeric inverted repeats. Fungal Genet Biol 41:963–971

Fullner KJ, Nester EW (1996) Temperature affects the T-DNA transfer machinery of *Agrobacterium tumefaciens*. J Bacteriol 178:1498–1504

Gardiner DM, Howlett BJ (2004) Negative selection using thymidine kinase increases the efficiency of recovery of transformants with targeted genes in the filamentous fungus *Leptosphaeria maculans*. Curr Genet 45:249–255

Gardiner DM, Cozijnsen AJ, Wilson LM, Soledade M, Pedras C, Howlett BJ (2005) The sirodesmin biosynthetic gene cluster of the plant pathogenic fungus *Leptosphaeria maculans*. Mol Microbiol 53:1307–1318

Gelvin SB (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Annu Rev Plant Physiol Plant Mol Biol 51:223–256

Gelvin SB (2003) *Agrobacterium*-mediated plant transformation: the biology behind the 'gene-jockeying tool'. Microbiol Mol Biol Rev 67:16–37

Gheysen G, Villarroel R, van Montagu M (1991) Illegitimate recombination in plants: a model for T-DNA integration. Genes Dev 5:287–297

Gibson SI, Sommerville C (1992) In: Koncz C, Chua N-H, Schell J (eds) Methods in *Arabidopsis* research. World Scientific, Singapore, pp 119–143

Godio RP, Fouces R, Gudina EJ, Martin JF (2004) *Agrobacterium tumefaciens*-mediated transformation of the antitumor clavaric acid-producing basidiomycete *Hypholoma sublateritium*. Curr Genet 46:287–294

Gouka RJ, Gerk C, Hooykaas PJ, Bundock P, Musters W, Verrips CT, Groot MJ de (1999) Transformation of *Aspergillus awamori* by *Agrobacterium tumefaciens*-mediated homologous recombination. Nat Biotechnol 17:598–601

Gray M, Honigberg SM (2001) Effect of chromosomal locus, GC content and length of homology on PCR-mediated targeted gene replacement in *Saccharomyces*. Nucleic Acids Res 29:5156–5162

Groot MJ de, Bundock P, Hooykaas PJ, Beijersbergen AG (1998) *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nat Biotechnol 16:839–842

Haaren MJ van, Sedee NJ, Schilperoort RA, Hooykaas PJ (1987) Overdrive is a T-region transfer enhancer which stimulates T-strand production in *Agrobacterium tumefaciens*. Nucleic Acids Res 15:8983–8997

Hamer L, Adachi K, Montenegro-Chamorro MV, Tanzer MM, Mahanty SK, Lo C, Tarpey RW, Skalchunes AR, Heiniger RW, Frank SA, Darveaux BA, Lampe DJ, Slater TM, Ramamurthy L, DeZwaan TM, Nelson GH, Shuster JR, Woessner J, Hamer JE (2001) Gene discovery and gene function assignment in filamentous fungi. Proc Natl Acad Sci USA 98:5110–5115

Hanif M, Pardo AG, Gorfer M, Raudaskoski M (2002) T-DNA transfer and integration in the ectomycorrhizal fungus *Suillus bovinus* using hygromycin B as a selectable marker. Curr Genet 41:183–188

Hilleman D, Puhler A, Wohlleben W (1991) Gene disruption and gene replacement in *Streptomyces* via single-stranded DNA transformation of integration vectors. Nucleic Acids Res 19:727–731

Hoekema A, Hirsch PR, Hooykaas PJ, Schilperoort RA (1983) A binary vector strategy based on separation of *vir*-region and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303:179–180

Hoffman B, Breuil C (2004) Disruption of the subtilase gene, *albin1*, in *Ophiostoma piliferum*. Appl Environ Microbiol 70:3898–3903

Hooykaas PJ, Beijersbergen AG (1994) The virulence system of *Agrobacterium tumefaciens*. Annu Rev Phytopathol 32:157–179

Hua SB, Qiu M, Chan E, Zhu L, Luo Y (1997) Minimum length of sequence homology required for in vivo cloning by homologous recombination in yeast. Plasmid 38:91–96

Hua-Van A, Langin T, Daboussi MJ (2002) Aberrant transposition of a Tc1-mariner element, impala, in the fungus *Fusarium oxysporum*. Mol Genet Genomics 267:79–87

Hynes MJ (1996) Genetic transformation of filamentous fungi. J Genet 75:297–311

Idnurm A, Heitman J (2005) Light controls growth and development via a conserved pathway in the fungal kingdom. PLoS Biol 3:e95

Idnurm A, Reedy JL, Nussbaum JC, Heitman J (2004) *Cryptococcus neoformans* virulence gene discovery through insertional mutagenesis. Eukaryot Cell 3:420–429

Jeon J-S, Lee S, Jung K-H, Jun S-H, Jeong D-H, Lee J, Kim C, Jang S, Lee S, Yang K, Nam J, An K, Han M-J, Sung R-J, Choi H-S, Yu J-H, Choi J-H, Cho S-Y, Cha S-S, Kim S-I, An G (2000) T-DNA insertional mutagenesis for functional genomics in rice. Plant J 22:561–570

Jones DH, Winistorfer SC (1993) Genome walking with 2-kb to 4-kb steps using panhandle PCR. PCR Methods Appl 2:197–203

Kado CI (2000) The role of the T-pilus in horizontal gene transfer and tumorigenesis. Curr Opin Microbiol 3:643–648

Kadotani N, Nakayashiki H, Tosa Y, Mayama S (2003) RNA silencing in the phytopathogenic fungus *Magnaporthe oryzae*. Mol Plant Microbe Interact 16:769–776

Kahmann R, Basse C (1999) Restriction enzyme mediated integration (REMI) and its impact on the isolation of pathogenicity genes in fungi attacking plants. Eur J Plant Pathol 105:221–229

Kamath RS, Ahringer J (2003) Genome-wide RNAi screening in *Caenorhabditis elegans*. Methods 30:313–321

Kellner EM, Orsborn KI, Siegel EM, Mandel MA, Orbach MJ, Galgiani JN (2005) *Coccidiodes posadasii* contains a single 1,3-beta-glucan synthase gene that appears to be essential for growth. Eukaryot Cell 4:111–120

Kempken F, Kuck U (1996) Transposons in filamentous fungi—facts and perspectives. Bioassays 20:652–659

Khang CH, Park S-Y, Lee Y-H, Kang S (2005) A dual selection based, targeted gene replacement tool for *Magnaporthe grisea* and *Fusarium oxysporum*. Fungal Genet Biol (in press)

Koncz C, Martini N, Mayerhofer R, Koncz-Kalman Z, Korber H, Redei GP, Schell J (1989) High-frequency T-DNA-mediated gene tagging in plants. Proc Natl Acad Sci USA 86:8467–8471

Koncz C, Nemeth K, Redei GP, Schell J (1992) T-DNA insertional mutagenesis in *Arabidopsis*. Plant Mol Biol 20:963–976

Kooistra R, Hooykaas PJ, Steensma HY (2004) Efficient gene targeting in *Kluyveromyces lactis*. Yeast 21:781–792

Krysan PJ, Young JC, Sussman MR (1999) T-DNA as an insertional mutagen in *Arabidopsis*. Plant Cell 11:2283–2290

Kumagai H, Kouchi H (2003) Gene silencing by expression of hairpin RNA in *Lotus japonicus* roots and root nodules. Mol Plant Microbe Interact 16:663–668

Ladendorf O, Brachmann A, Kamper J (2003) Heterologous transposition in *Ustilago maydis*. Mol Genet Genomics 269:395–405

Leal CV, Montes BA, Mesa AC, Rua AL, Corredor M, Restrepo A, McEwen JG (2004) *Agrobacterium tumefaciens*-mediated transformation of *Paracoccidioides brasiliensis*. Med Mycol 42:391–395

Leclerque A, Wan H, Abschutz A, Chen S, Mitina GV, Zimmermann G, Schairer HU (2003) *Agrobacterium*-mediated insertional mutagenesis (AIM) of the entomopathogenic fungus *Beauveria bassiana*. Curr Genet 45:111–119

Li M, Gong X, Zheng J, Jiang D, Fu Y, Hou M (2005) Transformation of *Coniothyrium minitans*, a parasite of *Sclerotinia sclerotiorum*, with *Agrobacterium tumefaciens*. FEMS Microbiol Lett 243:323–329

Li Destri Nicosia MG, Brocard-Masson C, Demais S, Hua VA, Daboussi MJ, Scazzocchio C (2001) Heterologous transposition in *Aspergillus nidulans*. Mol Microbiol 39:1330–1344

Linnemannstons P, Vob T, Hedden P, Gaskin P, Tudzynski B (1999) Deletions in the gibberellin biosynthesis gene cluster of *Gibberella fujikuroi* by restriction enzyme-mediated integration and conventional transformation-mediated mutagenesis. Appl Environ Microbiol 65:2558–2564

Liu YG, Mitsukawa N, Oosumi T, Whittier RF (1995) Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J 8:457–463

Liu H, Cottrel TR, Pierini LM, Goldman WE, Doering TL (2002) RNA interference in the pathogenic fungus *Cryptococcus neoformans*. Genetics 160:463–470

Loppnau P, Tanguay P, Breuil C (2004) Isolation and disruption of the melanin pathway polyketide synthase gene of the softwood deep stain fungus *Ceratocystis resinifera*. Fungal Genet Biol 41:33–41

Lu S, Lyngholm L, Yang G, Bronson C, Yoder OC, Turgen BG (1994) Tagged mutations at the *Tox1* locus of *Cochliobolus heterostrophus* by restriction enzyme-mediated integration. Proc Natl Acad Sci USA 91:12649–12653

Maier FJ, Schafer W (1999) Mutagenesis via insertional- or restriction enzyme-mediated-integration (REMI) as a tool to tag pathogenicity related genes in plant pathogenic fungi. Biol Chem 380:855–864

Malonek S, Meinhardt F (2001) *Agrobacterium tumefaciens*-mediated genetic transformation of the phytopathogenic ascomycete *Calonectria morganii*. Curr Genet 40:152–155

Mannhaupt G, Montrone C, Haase D, Mewes HW, Aign V, Hoheisel JD, Fartmann B, Nyakatura G, Kempken F, Maier J, Schulte U (2003) What's in the genome of a filamentous fungus? Analysis of the *Neurospora* genome sequence. Nucleic Acids Res 31:1944–1954

Matsumoto S, Ito Y, Hosoi T, Takahashi Y, Machida Y (1990) Integration of *Agrobacterium* T-DNA into a tobacco chromosome: possible involvement of DNA homology between T-DNA and plant DNA. Mol Gen Genet 224:309–316

Matthysse AG (1983) Role of bacterial cellulose fibrils in *Agrobacterium tumefaciens* infection. J Bacteriol 154:906–915

Matthysse AG (1987) Characterization of nonattaching mutants of *Agrobacterium tumefaciens*. J Bacteriol 169:313–323

Meyer V, Mueller D, Strowig T, Stahl U (2003) Comparison of different transformation methods for *Aspergillus giganteus*. Curr Genet 43:371–377

Michielse CB, Salim K, Ragas P, Ram AFJ, Kudla B, Jarry B, Punt PJ, Hondel CAMJJ van den (2004c) Development of a system for integrative and stable transformation of the zygomycete *Rhizopus oryzae* by *Agrobacterium*-mediated DNA transfer. Mol Gen Genomics 271:499–510

Michielse CB, Ram AFJ, Hooykaas PJJ, Hondel CAMJJ van den (2004a) *Agrobacterium*-mediated transformation of *Aspergillus awamori* in the absence of full length VirD2, VirC2 or VirE2 leads to insertion of aberrant T-DNA structures. J Bacteriol 186:2038–2045

Michielse CB, Ram AFJ, Hooykaas PJJ, Hondel CAMJJ van den (2004b) Role of bacterial virulence proteins in *Agrobacterium*-mediated transformation of *Aspergillus awamori*. Fungal Genet Biol 45:571–578

Michielse CB, Arentshorst M, Ram AFJ, Hondel CAMJJ van den (2005) *Agrobacterium*-mediated transformation leads to improved gene replacement efficiency in *Aspergillus awamori*. Fungal Genet Biol 42:9–19

Mikosch TS, Lavrijssen B, Sonnenberg AS, Griensven LJ van (2001) Transformation of the cultivated mushroom *Agaricus bisporus* (Lange) using T-DNA from *Agrobacterium tumefaciens*. Curr Genet 39:35–39

Monfort A, Cordero L, Maicas S, Polaina J (2003) Transformation of *Mucor miehei* results in plasmid deletion and phenotypic instability. FEMS Microbiol Lett 224:101–106

Mullins ED, Kang S (2001) Transformation: a tool for studying fungal pathogens of plants. Cell Mol Life Sci 58:2043–2052

Mullins ED, Chen X, Romaine P, Raina R, Geiser DM, Kang S (2001) *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: an efficient tool for insertional mutagenesis and gene transfer. Phytopathology 91:173–180

Nacry P, Camilleri C, Courtial B, Caboche M, Bouchez D (1998) Major chromosomal rearrangements induced by T-DNA transformation in *Arabidopsis*. Genetics 149:641–650

Nelson RT, Pryor BA, Lodge JK (2003) Sequence length required for homologous recombination in *Cryptococcus neoformans*. Fungal Genet Biol 38:1–9

Ninomiya Y, Suzuki K, Ishii C, Inoue H (2004) Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining. Proc Natl Acad Sci USA 101:12248–11253

Ochman H, Gerber AS, Hartl DL (1988) Genetic applications of an inverse polymerase chain reaction. Genetics 120:621–623

Ohba T, Yoshioka Y, Machida C, Machida Y (1995) DNA rearrangements associated with the integration of T-DNA in tobacco: an example for multiple duplications of DNA around the integration target. Plant J 7:157–164

Pardo AG, Hanif M, Raudaskoski M, Gorfer M (2002) Genetic transformation of ectomycorrhizal fungi mediated by *Agrobacterium tumefaciens*. Mycol Res 106:132–137

Park S-M, Kim D-K (2004) Transformation of a filamentous fungus *Cryphonectria parasitica* using *Agrobacterium tumefaciens*. Biotechnol Bioprocess Eng 9:217–222

Pereira A (2000) A transgenic perspective on plant functional genomics. Transgenic Res 9:245–260

Piano F, Schetter AJ, Mangone M, Stein L, Kemphues KJ (2000) RNAi analysis of genes expressed in the ovary of *Caenorhabditis elegans*. Curr Biol 10:1619–1622

Piers KL, Heath JD, Liang X, Stephens KM, Nester EW (1996) *Agrobacterium tumefaciens*-mediated transformation of yeast. Proc Natl Acad Sci USA 93:1613–1618

Regensburg-Tuink AJ, Hooykaas PJ (1993) Transgenic *N. glauca* plants expressing bacterial virulence gene *virF* are converted into hosts for nopaline strains of *A. tumefaciens*. Nature 36369–36371

Rho HS, Kang S, Lee YH (2001) *Agrobacterium tumefaciens*-mediated transformation of the plant pathogenic fungus, *Magnaporthe grisea*. Mol Cells 12:407–411

Roberts RL, Metz M, Monks DE, Mullaney ML, Hall T, Nester EW (2003) Purine synthesis and increased *Agrobacterium tumefaciens* transformation of yeast and plants. Proc Natl Acad Sci USA 100:6634–6639

Rogers CW, Challen MP, Green, JR, Whipps JM (2004) Use of REMI and *Agrobacterium*-mediated transformation to identify pathogenicity mutants of the biocontrol fungus, *Coniothyrium minitans*. FEMS Microbiol Lett 241:207–214

Rolland S, Jobic C, Fevre M, Bruel C (2003) *Agrobacterium*-mediated transformation of *Botrytis cinerea*, simple purification of monokaryotic transformants and rapid conidia-based identification of the transfer-DNA host genomic DNA flanking sequences. Curr Genet 44:164–171

Rossi L, Hohn B, Tinland B (1993) The VirD2 protein of *Agrobacterium tumefaciens* carries nuclear localization signals important for transfer of T-DNA to plant. Mol Gen Genet 239:345–353

Rossi L, Hohn B, Tinland B (1996) Integration of complete transferred DNA units is dependent on the activity of virulence E2 protein of *Agrobacterium tumefaciens*. Proc Natl Acad Sci USA 93:126–130

Rosso MG, Li Y, Strizhov N, Reiss B, Dekker K, Weisshaar B (2003) An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. Plant Mol Biol 53:247–259

Salas MG, Park SH, Srivatanakul M, Smith RH (2002) Temperature influence on stable T-DNA integration in plant cells. Plant Cell Rep 2001:701–705

Schaefer DG (2001) Gene targeting in *Physcomitrella patens*. Curr Opin Plant Biol 4:143–150

Schrammeijer B, Dulk-Ras A, Vergunst AC, Jurado JE, Hooykaas PJ (2003) Analysis of Vir protein translocation from *Agrobacterium tumefaciens* using *Saccharomyces cerevisiae* as a model: evidence for transport of a novel effector protein VirE3. Nucleic Acids Res 31:860–868

Simmer F, Moorman C, Linden AM van der, Kuijk E, Berghe PV van den, Kamath R, Fraser AG, Ahringer J, Plasterk RH (2003) Genome-wide RNAi of *C. elegans* using the hypersensitive rrf-3 strain reveals novel gene functions. PLoS Biol 1:e12

Simon JR, Moore PD (1987) Homologous recombination between single-stranded DNA and chromosomal genes in *Saccharomyces cerevisiae*. Mol Cell Biol 7:2329–2334

Sims AH, Robson GD, Hoyle DC, Oliver SG, Turner G, Prade RA, Russell HH, Dunn-Coleman NS, Gent ME (2004) Use of expressed sequence tag analysis and cDNA microarrays of the filamentous fungus *Aspergillus nidulans*. Fungal Genet Biol 41:199–212

Singer T, Burke E (2003) High-throughput TAIL-PCR as a tool to identify DNA flanking insertions. Methods Mol Biol 236:241–272

Stachel SE, Nester EW (1986) The genetic and transcriptional organization of the *vir* region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. EMBO J 5:1445–1454

Stafford HA (2000) Crown gall disease and *Agrobacterium tumefaciens*: a study of the history, present knowledge, missing information, and impact on molecular genetics. Bot Rev 66:101–118

Sullivan TD, Rooney PJ, Klein BS (2002) *Agrobacterium tumefaciens* integrates transfer DNA into single chromosomal sites of dimorphic fungi and yields homokaryotic progeny from multinucleate yeast. Eukaryot Cell 1:895–905

Sweigard JA, Carroll AM, Farrall L, Chumley FG, Valent B (1998) *Magnaporthe grisea* pathogenicity genes obtained through insertional mutagenesis. Mol Plant Microbe Interact 11:404–412

Takahara H, Tsuji G, Kubo Y, Yamamoto M, Toyoda K, Inagaki Y, Ichinose Y, Shiraishi T (2004) *Agrobacterium tumefaciens*-mediated transformation as a tool for random mutagenesis of *Colletotrichum trifolii*. J Gen Plant Pathol 70:93–96

Takano M, Egawa H, Ikeda JE, Wakasa K (1997) The structures of integration sites in transgenic rice. Plant J 11:353–361

Takken FL, Wijk R van, Michielse CB, Houterman PM, Ram AF, Cornelissen BJ (2004) A one-step method to convert vectors into binary vectors suited for *Agrobacterium*-mediated transformation. Curr Genet 45:242–248

Tanguay P, Breuil C (2003) Transforming the sapstaining fungus *Ophiostoma piceae* with *Agrobacterium tumefaciens*. Can J Microbiol 49:301–304

Toro N, Datta A, Yanofsky M, Nester E (1988) Role of the overdrive sequence in T-DNA border cleavage in *Agrobacterium*. Proc Natl Acad Sci USA 85:8558–8562

Tsuji G, Fujii S, Fujihara N, Hirose C, Tsuge S, Shiraishi T, Kubo Y (2003) *Agrobacterium tumefaciens*-mediated transformation for random insertional mutagenesis in *Colletotrichum lagenarium*. J Gen Plant Pathol 69:230–239

Tzfira T, Citovsky V (2002) Partners-in-infection: host proteins involved in the transformation of plant cells by *Agrobacterium*. Trends Cell Biol 12:121–129

Tzfira T, Rhee Y, Chen MH, Kunik T, Citovsky V (2000) Nucleic acid transport in plant-microbe interactions: the molecules that walk through the walls. Annu Rev Microbiol 54:187–219

Tzfira T, Vaidya M, Citovsky V (2002) Increasing plant susceptibility to *Agrobacterium* infection by over-expression of the *Arabidopsis* nuclear protein VIP1. Proc Natl Acad Sci USA 99:10435–10440

Veluthambi K, Ream W, Gelvin SB (1988) Virulence genes, borders, and overdrive generate single-stranded T-DNA molecules from the A6 Ti plasmid of *Agrobacterium tumefaciens*. J Bacteriol 170:1523–1532

Vergunst AC, Schrammeijer B, Den Dulk A, Vlaam CMT de, Regensburg-Tuink AJ, Hooykaas PJ (2000) VirB/D4-dependent protein translocation from *Agrobacterium* into plant cells. Science 290:979–982

Vergunst AC, Lier MC van, Dulk-Ras A, Hooykaas PJ (2003) Recognition of the *Agrobacterium tumefaciens* VirE2 translocation signal by the VirB/D4 transport system does not require VirE1. Plant Physiol 133:978–988

Versaw WK, Metzenberg RL (1996) Activator-independent gene expression in *Neurospora crassa*. Genetics 142:417–423

Vijn I, Govers F (2003) *Agrobacterium tumefaciens* mediated transformation of the oomycete plant pathogen *Phytophthora infestans*. Mol Plant Pathol 4:459–467

Wach A, Brachat A, Pohlmann R, Philippsen P (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast 10:1793–1808

Wendland J (2003) PCR-based methods facilitate targeted gene manipulations and cloning procedures. Curr Genet 44:115–123

Winzeler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, Andre B, Bangham R, Benito R, Boeke JD, Bussey H, Chu AM, Connelly C, Davis K, Dietrich F, Dow SW, El Bakkoury M, Foury F, Friend SH, Gentalen E, Giaever G, Hegemann JH, Jones T, Laub M, Liao H, Davis RW (1999) Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science 285:901–906

Zeilinger S (2003) Gene disruption in *Trichoderma atroviride* via *Agrobacterium*-mediated transformation. Curr Genet 45:54–60

Zhang A, Lu P, Dahl-Roshak AM, Paress PS, Kennedy S, Tkacz JS, An Z (2003) Efficient disruption of a polyketide synthase gene (*pks1*) required for melanin synthesis through *Agrobacterium*-mediated transformation of *Glarea lozoyensis*. Mol Genet Genomics 268:645–655

Zhu J, Oger PM, Schrammeijer B, Hooykaas PJ, Farrand SK, Winans SC (2000) The bases of crown gall tumorigenesis. J Bacteriol 182:3885–3895

Zupan J, Muth TR, Draper O, Zambryski P (2000) The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. Plant J 23:11–28

Zwiers LH, De Waard MA (2001) Efficient *Agrobacterium tumefaciens*-mediated gene disruption in the phytopathogen *Mycosphaerella graminicola*. Curr Genet 39:388–393