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Review

A guide to binary vectors and strategies for targeted genome modification in fungi using *Agrobacterium tumefaciens*-mediated transformation

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ABSTRACT

Agrobacterium tumefaciens-mediated transformation (ATMT) of fungi has become a common technique for the study of a wide variety of different fungal species over the past 12 years. The discovery that the host range of *A. tumefaciens* could be extended to include fungi provided an efficient transformation tool for species in which it was previously impossible to conduct molecular genetics experiments. ATMT experiments can be divided into three groups: i) Forward genetics (i.e., random mutagenesis), ii) Reverse genetics (i.e., targeted genome modification and random integration) and iii) the introduction of reporter genes (e.g., GFP, RFP and GUS) that allow in situ monitoring of the fungus. The use of ATMT for forward genetics experiments has primarily included classic random insertional inactivation strategies to obtain loss-of-function mutants. For reverse genetics experiments, ATMT has been used to introduce targeted genome modifications (e.g., disruptions, replacements, overexpression and complementation) and to generate random integrations for complementation, heterologous expression, expression of transcriptional and translational fusion reporters and RNAi-mediated down-regulation of gene expression. This review summarizes the technical advances within the field from 1998 to the summer of 2011, focusing on the development of binary vectors that are compatible with fungal transformation (over 180 general vectors) and methods for constructing binary vectors for targeted integration of T-DNA into fungal genomes.

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1. Introduction

The ability to introduce foreign DNA into a fungus is a pillar of modern fungal genetics. The availability of an efficient transformation system enables experiments such as random mutagenesis, the introduction of reporter genes and targeted modification of the genome. These are valuable tools for the functional characterization of genes and the analysis of fungal biology. Over the last 30 years, several transformation techniques have been developed, such as electroporation (electropermeabilization), polyethylene glycol (PEG)-mediated transformation, biolistics (particle bombardment) and, most recently, *Agrobacterium tumefaciens*-mediated transformation (ATMT). Electroporation and PEG-mediated transformation systems typically depend on protoplasts (spheroblasts), which are cells that have had their cell walls enzymatically removed. However, the ability to regenerate cell walls varies among fungal species, and this has limited the application of these otherwise very successful techniques. Biolistics and ATMT, on the other hand, allow for transformation of intact cells and tissues, making them ideal for non-model fungi. However, biolistics often results in multiple or tandem insertion events, while such events are seldom observed in ATMT.

Twelve years have passed since the first reported use of ATMT for the introduction of DNA into a fungus, and the technique has now successfully been applied in over 125 different fungal species, including members of the ascomycetes, basidiomycetes, zygomycetes, oomycetes and glomeromycetes groups (see Supplementary file 1). Michielse and coworkers reviewed the field in 2005 with a focus on the experimental parameters that affect transformation frequency, which include co-cultivation conditions (e.g., temperature, duration, ratio between donor and accepter organism), the use of particular marker genes for selection and the use of particular promoters for driving the expression of selection markers (Michielse et al., 2005b). Recent publications have not significantly advanced this work, and, for the most part, they have supported the summaries presented by Michielse and coworkers. However, the availability of an overwhelming number of different binary vectors for fungal experiments and the existence of multiple strategies for the construction of vectors for targeted gene replacement has created a situation best described as chaotic. This unfortunately means that many researchers who use ATMT may no longer keep track of the available vectors and the strengths and weaknesses of different vectors and experimental setups. This review provides up-to-date information that will allow researchers to make informed decisions about which vectors and experimental strategies they should use in their research.

2. Agrobacterium tumefaciens as a pathogen

Members of the *Agrobacterium* genus are common soil-dwelling bacteria. The vast majority of *Agrobacterium* species survive as saprophytes; however, several are pathogenic and cause neoplastic diseases in plants, such as crown gall disease (*A. tumefaciens*) and hairy root disease (*Agrobacterium rhizogenes*). These diseases involve the inter-kingdom transfer of DNA from the infecting bacterium to the host plant via conjugation, resulting in the development of opine-synthesizing tumors in the host plant (Pitzschke and Hirt, 2010). The infection process has been particularly well described for *A. tumefaciens* (*At*) (updated name: *Rhizobium radiobacter*), which is capable of infecting over 140 dicot species (De Cleene and De Ley, 1976). A detailed description of the disease progression and the *At* virulence machinery is beyond the scope of this review, and several highly detailed reviews are available on the subject (Citovsky et al., 2007). However, a brief description of the infection process is provided here: The transfer DNA (T-DNA) is located on a >200-kb tumor-inducing (Ti) plasmid, which also includes genes encoding the required transfer mechanism (virulence genes). The T-DNA is delimited by 25-bp directional imperfect repeat sequences, known as the left and right border (LB and RB, respectively). Prior to transfer, the T-DNA region is released from the Ti plasmid by endonucleases, which introduce site- and strand-specific single-stranded breaks into the LB and RB sequences. Once T-DNA has entered the host cell it is targeted to the nucleus and integrates into the host genome, ensuring stable replication.

ATMT of fungi is believed to proceed via a T-DNA transfer mechanism similar to that described for plants; however, induction of the bacterial virulence systems in fungal interactions requires an exogenous supply of phenolic inducer compounds (Bundock and Hooykaas, 1996).

3. Taming a fierce pathogen for use in the laboratory

The natural ability of *At* to transfer portions of its DNA to its plant host has been successfully used by the plant research community

since 1977, allowing for random mutagenesis and heterologous expression (Schell and Van Montagu, 1977).

Initially, the use of ATMT for the genetic modification of plants was hampered by the lack of unique restriction enzyme sites in the T-DNA region and the large size of the Ti plasmid. Modifications had to be introduced into the T-DNA region via techniques involving in vivo double homologous recombination between the large Ti plasmid and the introduced engineered plasmids (Gelvin, 2003). However, in 1983 Hoekema et al., exploiting the *trans*-acting nature of the virulence machinery, developed a two-component vector system consisting of a Ti plasmid lacking a T-DNA region (vir helper plasmid/disarmed Ti-plasmid: pLBA4404) and a small shuttle vector containing a T-DNA region (binary vector: pPZP). The T-DNA of the binary vector contained only a multiple cloning site (MCS) surrounded by RB and LB (Hoekema et al., 1983). The small, MCS-containing shuttle vector allowed for easier manipulation of the T-DNA region using standard molecular biology techniques in *Escherichia coli* and the finished vector could subsequently be transformed into and replicated in *At*. Separating the T-DNA region from the Ti plasmid eliminated the need to modify the unwieldy Ti plasmid in standard experiments, meaning that it simply could be maintained in *At*. Introduction of the modified binary vector (containing T-DNA) into an *At* strain carrying the vir helper plasmid was sufficient to recreate a functional virulence system with the same efficiency as the natural system. An equally successful system (pBIN19) was presented by Bevan the following year (Bevan, 1984). The host range of *At* has continually expanded, and, with the advent of artificial induction of the *At* virulence system using compounds such as acetosyringone, it is now a transformation system that can be easily controlled in the laboratory.

Currently, the mutated Ti (vir helper) plasmids are seldom modified, and a wide range of different *At* strains carrying various vir helper plasmids are available, such as LBA4404 (Hoekema et al., 1983), EHA101 (Hood et al., 1986), GV3101 (Koncz and Shell, 1986), AGL1 (Lazo et al., 1991) and EHA105 (Hood et al., 1993), all of which have been used for ATMT of fungi.

4. ATMT of fungi

For two decades, the use of ATMT was restricted to the plant kingdom. Then, in 1995, Bundock and coworkers reported the successful transformation of *Saccharomyces cerevisiae* using ATMT (Bundock et al., 1995), a result that was confirmed by Piers et al. and Risseeuw et al. in 1996 (Piers et al., 1996; Risseeuw et al., 1996). These initial studies were driven by a desire to develop a model system for analyzing the molecular mechanisms responsible for the integration of T-DNA into plant genomes. Analysis of the resulting transformants revealed the fate of the T-DNA inside the recipient organism and confirmed both episomal replication (via cyclization of T-DNA) and integration into the genome via the non-homologous end joining (NHEJ) or homologous recombination (HR) pathway (Bundock et al., 1995; Piers et al., 1996).

An analysis of T-DNA integration events showed that the NHEJ pathway (also known as illegitimate recombination) was dependent on microhomology between the T-DNA ends and the genomic locus. Furthermore, integration was often accompanied by truncation of the LB and RB sequences (particularly the LB) or the introduction of filler DNA sequences linking the T-DNA to the genome sequence (Bundock and Hooykaas, 1996). This has been found to be the case in other fungal species as well (Choi et al., 2007; Ji et al., 2010; Li et al., 2007). In addition to their work on integration via NHEJ, Bundock et al. also analyzed integration via HR, demonstrating that integration of DNA fragments with homologous recombination sequences (HRS) was approximately 100 times more efficient when using ATMT than with contemporary transformation protocols for yeast, such as electroporation (Bundock et al., 1995). The technical significance of this finding was largely ignored by the yeast research community, as efficient transformation and gene targeting systems were already established and offered a sufficiently high HR frequency to allow for gene replacement.

In 1998, de Groot and coworkers extent the host range of *At* to include other fungal species, thus providing a superior alternative to the prevailing protoplast transformation technique (de Groot et al., 1998). Their groundbreaking paper described the successful ATMT of six different ascomycetes and a single basidiomycete. Compared to protoplast-based transformation, the advantages of ATMT include independence from protoplasts, which are often time-consuming and laborious to prepare; increased transformation efficiency; and a higher frequency of single integration events. De Groot and coworkers further showed that ATMT also is compatible with targeted gene replacement in *Aspergillus awamori* which corroborated the results of Bundock et al. for yeast, showing that ATMT yielded a higher gene targeting frequency than that obtained with other transformation techniques (Gouka et al., 1999). The higher targeting frequency has later been ascribed to protection of the introduced DNA and/or the single-stranded nature of the T-DNA that induces the HR system (Michielse et al., 2005a). The advances made by de Groot and coworkers paved the way for molecular biological work in a wide range of fungal species for which no transformation tools had previously been available (Wang et al., 2010a).

5. Binary vectors for fungal transformation

The last 12 years of research have seen the development of over 180 different binary vectors that are compatible with ATMT of fungi, many of which can be used for specialized applications or used as a basis for the construction of new vectors. The lack of a centralized infrastructure for the sharing of knowledge on binary vectors for fungal transformation has resulted in redundancy, and highly similar vectors have been constructed repeatedly. The developed vectors have all been derived from plant transformation vectors (Table S1). The pCAMBIA vector series based on the pPZP series has been the most common starting material, followed by the original pPZP series, pBIN19, pGreen, pAg1, pCB301 and pBI121. The backbones of these binary vectors have typically not been subjected to modification, whereas the T-DNA region is continuously being modified to be compatible with expression and functionality in the targeted fungal species (Table S2–S8). However, in a few cases, such as for pAg1, the backbone has been trimmed by removing nonessential structures from the pBIN19 backbone (Zhang et al., 2003). Supplementary file 3 contains a description of 200 binary vectors that have been used for transformation of fungi.

5.1. Selection marker cassettes for ascomycetes, basidiomycetes and zygomycetes

The use of a suitable selection marker system is essential for successful transformation. The system should allow for the elimination of non-transformed cells, while ensuring a high level of resistance in recipient transformants.

The most widely used selection marker system in fungi, regardless of the transformation technique, is the dominant, *E. coli* antibiotic resistance gene hygromycin phosphotransferase (*hph* or *hpt*), which confers resistance to hygromycin B (Gritz and Davies, 1983; Kaster et al., 1983). The *hph* cassette found in most binary vectors for fungal transformation has been obtained from the pAN7-1 vector (Punt et al., 1987) and includes a constitutive *Aspergillus nidulans* (*An*) glyceraldehyde dehydrogenase (*gpdA*) promoter and anthranilate synthase (*trpC*) terminator sequence. This resistance cassette has been successfully used with ascomycetes, but it has had limited success in basidiomycetes due to its incompatibility with the promoter and mRNA structures, as shown in several studies of the basidiomycete *Schizophyllum commune* (Lugones et al., 1999; Scholtmeijer et al., 2001).

To solve this problem and to obtain promoters of various strengths, several studies have analyzed a wide range of different basidiomycetes promoters, mostly from *Agricus bisporus* (*Ab*) and *Coprinopsis cinerea* (*Cc*) (Burns et al., 2006; McClelland et al., 2005). The most widely used promoters in basidiomycete vectors have been the *AbPgpdII* and *CcPact1* promoters, though many plasmids use alternative fungal promoters or even the *Cauliflower mosaic virus 35S* (CaMV35S) promoter normally used in plant experiments (Sharma et al., 2006). For transformation of zygomycetes, the pAN7-1 *hph* resistance cassette has proven to be functional in some species, but it typically has a lower efficiency than resistance cassettes driven by zygomycete promoters, such as the *Mortierella alpina Histone H4* promoter (Wei et al., 2010). Several successful protocols for the transformation of oomycetes have been reported using the *Bremia lactucae* heat shock protein 70 (*hsp70*) promoter for driving expression of a geneticin resistance marker (Vijn and Govers, 2003). For glomeromycetes, such as *Glomus intraradices*, only two ATMT studies have been reported; these studies used the pAN7-1 *hph* resistance cassette to produce transient transformants (Helber and Requena, 2008).

The availability of multiple dominant selection markers is essential for performing more advanced molecular biological techniques, such as random mutagenesis followed by complementation, and for experiments combining overexpression and deletions or multiple successive deletions. Currently a range of different antibiotic resistance genes has been shown to work in different fungal species (Table 1). The efficiency of these selection systems varies greatly from fungus to fungus, and it is therefore highly recommended to test the efficiency of the antibiotic before commencing molecular biology work that relies on a particular selection system, as exemplified by (Sharma and Kuhad, 2010).

In addition to the dominant antibiotic selection marker genes, systems relying on nutrition auxotrophy can be used for selection, including those based on uracil (*Ura3*), pyrimidines (*pyrG*) or tryptophan (*trp1*). These systems are typically efficient and cheap, but they require that corresponding auxotroph mutant strains are available or can be generated, a situation that makes them less useful when attempting to transform a novel fungal species for the first time. Recently, Wang and coworkers reported the use of temperature as an effective selection regime for the basidiomycete *Volvariella volvacea*. They used the ability of *afp* (antifreeze protein from budworms) to protect transformed fungal cells from damage caused by exposure to 0 °C for 30 min, a treatment that kills wild type cells (Wang et al., 2008).

5.2. Binary vectors for forward genetics

Initially, ATMT was used primarily for the generation of random insertional mutant libraries that could be screened for phenotypic effects (forward genetics). The finding that ATMT typically leads to single insertion events makes the technique ideal for this type of experiment, as it increases the likelihood that the observed phenotype is the result of a single mutation. In addition, the integration of the T-DNA provides a molecular tag that can be used for identification of the mutated locus, which is typically achieved using techniques such as Thermal Asymmetric InterLaced PCR (TAIL-PCR) (Mullins et al., 2001), plasmid rescue (Michielse et al., 2004), Vectorette PCR (Gupta and Chattoo, 2007) or Y-shaped Adaptor Dependent Extension PCR (YADE-PCR) (Fang et al., 2010). A large number of binary vectors are available for these types of experiments in asco-, basidio-, zygo- and oomycetes (see Table S3).

Table 1
The number of vectors available for particular selection markers.

Selection marker	Number of vectors	Application range (μg/ml)			
		Asco-	Basidio-	Zygo-	Oo-
hph (Hygromycin B)	115	20–600	25–300	50–200	–
bar/*Nat* (phosphinothricin)	23	25–750	100	–	–
Ble (Bleomycin/Phleomycin)	12	15–100	5–200	–	–
aphI/*NPTII*/*Neo* (Geneticin/Neomycin)	11	20–800	100	–	5
ilv1 (Sulfonylurea)	4	4–100	–	–	–
sdhR (Carboxin)	3	–	0.2	–	–
β-*tub* (Benomyl)	2	1.5–100	–	–	–
Zeo (Zeomycin)	1	150	–	–	–

The accumulating data on T-DNA integration sites from various random mutagenesis experiments and integration studies, including studies evaluating 135, 672, 741 and 2026 *Magnaporthe grisea* mutants (Betts et al., 2007; Choi et al., 2007; Jeon et al., 2007; Li et al., 2007), 118 *Leptosphaeria maculans* mutants (Blaise et al., 2007), 204 *Ustilago maydis* mutants (Ji et al., 2010), 70 *Penicillium marneffei* mutants (Zhang et al., 2008) and 86 *Laccaria bicolor* mutants (Kemppainen et al., 2008), have revealed that T-DNA integration is not entirely random; rather, it displays a bias for promoters and the start of coding sequences. This bias is best explained by the higher AT content and increased flexibility of the DNA combined with unwinding of the DNA during transcription making these regions more susceptible to double strand breaks, which is a prerequisite for integration via the NHEJ pathway (Choi et al., 2007).

The vast majority of forward genetics experiments have depended on inactivating mutations in which the random integration of the T-DNA leads to disruption of a coding sequence in the genome. In fact, only a single study, conducted by Chen and coworkers working with the basidiomycete *Antrodia cinnamonea*, has used an activation approach in which the T-DNA includes a *cis*-acting transcription enhancer sequence, four CaMv35S enhancer elements, such that integration in the vicinity of a gene would increase its transcription level (Chen et al., 2009).

The identified T-DNA integration site bias can be considered an advantage for both activation and inactivation experiments, as it increases the likelihood of obtaining transformants that are affected in a coding sequence or promoter region of potential interest. However, it also reduces the likelihood of obtaining neutral transformants where T-DNA integration does not affect the function of the organism.

5.3. Binary vectors for complementation and overexpression (ectopic integration)

In many forward genetics experiments, complementation is used to prove that the observed phenotype is caused by the identified mutation (i.e., the T-DNA integration site) and not by an unmapped mutation. These experiments can either rely on the gene's endogenous promoter or a heterologous promoter. The same is true for experiments aimed at expressing additional copies of an endogenous gene or heterologous genes from ectopic loci in the genome. Binary vectors allowing for expression via both heterologous and endogenous promoters are available (see Table S4). Three of the four available overexpression vectors feature an *An gpdA* promoter, ensuring a high level of constitutive expression (Punt et al., 1992). For the fourth vector, expression depends on the promoter from the *M. griesea* hydrophobin gene 1 (*Mpg1*), which allows for a high expression level when the fungus is growing *in planta* (Beckerman and Ebbole, 1996; Talbot et al., 1993).

Complementation experiments aimed at restoring the normal phenotype in a mutant strain, even when relying on the natural promoter of the gene under investigation, seldom achieve complete rescue of the wild type trait. This situation has been ascribed to differences in expression patterns resulting from differences in chromatin structure and epigenetic factors between the endogenous locus and the ectopic integration site (Rep et al., 2004).

5.4. Introduction of visual marker genes

Studies of phytopathogenic and endophytic fungi often rely on microscopy to track the spread of the fungus in the host plant tissues, a

task that can be simplified by tagging the fungus with a visible marker, such as green fluorescent protein (GFP), red fluorescent protein (RFP), beta-glucuronidase (GUS) or uroporphyrinogen-III methyltransferases (*cob*) (Lan et al., 2008).

The largest toolset is available for the ascomycetes, for which there are vectors with various combinations of selection and visual marker cassettes (GFP, DsRed, GUS and cob) (Table S5). Fewer marker combinations (DsRed, GFP and cob) have been developed for basidiomycetes and only GUS and GFP have been tested in ATMT experiments for the study of oomycetes and zygomycetes, respectively. The use of GFP in basidiomycetes has been challenging due to incompatibility with the ascomycete promoters that are normally used. However, the fusion of GFP to the 5′ end of the *pgd* gene, including the first intron, from *Phanerochaete chrysosporium* has resulted in a functional system (Burns et al., 2005).

Experiments aimed at introducing a visual marker cassette have typically relied on random integration into the fungal genome. However, the reported T-DNA integration site bias increases the risk that the introduced reporter cassette will disrupt a gene and thereby affect the fitness of the fungus. This situation makes it imperative to map the T-DNA integration locus and to phenotypically characterize the reporter-containing strain in detail.

5.5. Binary vectors for targeted gene replacement and overexpression of endogenous genes

The finding that ATMT improves gene targeting efficiency has prompted the development of nineteen different generic binary vectors for performing targeted genome modifications, and these vectors provide a wide range of different selection marker and promoter combinations (see Table S6). A general feature of these vectors is that they allow for the introduction of homologous recombination sequences (HRS) on either side of the selection marker gene in the T-DNA region of the vectors. A setup that allows for targeted integration of the T-DNA by double HR with the target genome. In addition to these specialized binary vectors, there are general vectors (containing only LB-MCS-RB) that are typically used in experiments in which the targeting construct is first assembled in a standard subcloning vector and then moved into the binary vector (Table S2). Relatively recent progress has involved the development of general vectors that allow for in locus/in situ overexpression of endogenous genes by targeted insertion of a constitutive promoter in front of the coding sequence of interest (Frandsen et al., 2008). These strategies simplify the analysis of large genes by eliminating the need for the cloning of the entire coding sequence during construction of the overexpression vectors.

Complementation experiments relying on random ectopic integration of the gene of interest are often problematic, as discussed above. However, several vectors have recently been developed for *Fusarium* sp. that enable expression from a predetermined locus in the genome (*tet* in *Fusarium asiaticum* (Xu et al., 2010) and *PKS12* in *Fusarium graminearum*)(Sørensen et al., submitted for publication). These vectors provide a way to construct nearly isogenic strains and eliminate the risk of unintentional disruption of uncharacterized genes. In the case of *F. graminearum PKS12,* the system also features red/white color selection of correct transformants based on a loss of the red mycelium pigment aurofusarin, which is synthesized by FgPKS12.

6. Gene targeting efficiency

6.1. Factors affecting the homologous recombination frequency

The introduction of T-DNA into fungal cells can have multiple outcomes, including partial or complete degradation of the T-DNA, autosomal replication of the T-DNA or integration of the T-DNA into the genome (van Attikum and Hooykaas, 2003). Integration relies on two competing DNA repair mechanisms, NHEJ and HR (reviewed by (Shrivastav et al., 2008)). Integration via the HR pathway depends on the presence of identical sequences in the exogenous DNA and the genome, and integration proceeds via crossover events, similar to the process that is responsible for meiotic recombination. The HR pathway is often only active in the S/G2 phases of the cell cycle, thus limiting the number of cells that are amendable to targeted integration of T-DNA (Takata et al., 1998). Among the 350 published ATMT studies, gene replacement frequencies as low as 0.04% for *Blastomyces dermatitidis* (Gauthier et al., 2010) have been reported, and only a few studies have seen frequencies that come close to what is reported for *S. cerevisiae*, the highest being 78% in *F. graminearum* (Frandsen et al., 2011) and 76% in *Fusarium oxysporum* (Duyvesteijn et al., 2005).

The low frequency of HR in many fungal species has put focus on the factors that affect the HR efficiency in ATMT experiments, and the results have been highly similar to those found for other transformation techniques. Several studies have shown that the length of the HRS affects the frequency of HR, regardless of the transformation technique used (Maier et al., 2005). However, factors such as the distance between the HRSs in the genome, the local chromatin structure and the transcriptional state of the targeted gene have also been found to affect the gene targeting efficiency (Ma et al., 2009).

6.2. Improving gene targeting efficiency

The most significant solution to the problem of low gene-targeting efficiency has been the generation of mutants with defects in the NHEJ system (*ku70, ku80* or *lig4* mutants). These NHEJ mutations force the T-DNA to integrate via the HR system (van Attikum et al., 2001). Several phenotypic effects, such as telomere shortening and genomic instability, have been reported for NHEJ mutants (Fisher and Zakian, 2005), and these effects can reduce the fitness of the resulting mutant strains and may complicate downstream phenotypic analysis. However, none of the published ATMT-dependent studies relying on NHEJ mutants have addressed this problem. Reconstruction of the NHEJ mutation after completion of the targeted genome modification can be achieved by using a transient NHEJ mutant or via sexual backcrossing to the wild type (Nielsen et al., 2008).

Another approach for increasing the gene targeting frequency in fungi has been to flank the gene targeting cassette with a negative selection marker that is lost if the DNA integrates via the HR pathway but can be preserved if the DNA integrates via the NHEJ pathway. This strategy allows for counter selection of transformants carrying ectopic copies of the introduced DNA, which enriches the obtained transformation pool with the desired gene replacement transformants. Gardiner and Howlett, working with *L. maculans,* were the first to use this strategy in combination with ATMT, and they relied on a vector in which the targeting cassette was surrounded by copies of the Herpes simplex virus thymidine kinase (*hv-tk*) gene (Gardiner and Howlett, 2004). The presence of *hv-tk* allows for negative selection using trifluorothymidine or fluorodeoxyuridine, which are converted into toxic compounds by the hv-tk enzyme, thereby killing transformants that possess this gene. This strategy allowed for the easy elimination of two thirds of the transformants, however, further analysis of the remaining transformants revealed a very high level of false positives (19 out of 20). Similar results were reported by Khang et al. and were attributed to the truncation of the T-DNA ends upon ectopic integration, which results in partial or complete loss of the negative selection marker (Khang et al., 2005). Though the use of both positive and negative selection reduces the required screening work, the gain can be considered marginal due to the high rate of false positives.

In addition to the use of NHEJ-deficient strains and negative selection marker systems, the split-marker strategy described by Wang and coworkers, which will be described later, is a promising

technique for increasing the gene targeting efficiency in ATMT experiments.

7. Construction of binary vectors for targeted genome modifications

7.1. General strategies for constructing binary vectors for targeted gene replacement

The following section describes the various strategies that have been used for the construction of binary vectors for targeted genome modifications. These modifications include targeted disruptions (e.g., insertional disruption of the coding sequence), deletions (e.g., replacement/removal of the coding sequence) and in locus overexpression (e.g., promoter exchange or insertion). The various techniques are compared in Table 2. The techniques discussed for the construction of vectors for targeted gene replacement using ATMT also apply for other transformation methods.

7.2. Restriction enzyme- and ligase-dependent cloning

The most commonly used technology for the construction of targeted binary vectors has been classic restriction enzyme- and ligase-dependent cloning (RE&L), which relies on either naturally occurring restriction enzyme sites in the HRSs or on restriction sites that are introduced during PCR amplification. Four fundamental RE&L strategies exist: 1) insertion of the selection marker into the center of a cloned target sequence, creating two HRSs, 2) sequential insertion of the HRSs on either side of the selection marker gene in a vector, 3) assembly of the construct via a single four-fragment ligation reaction and 4) insertion of a single HRS into a Direct Repeat Recombination-mediated Gene Targeting (DRGT) compatible vector.

7.2.1. Type 1

For strategies that introduce the selection marker gene into a target sequence, a fragment of the target gene is first cloned into a binary vector via RE&L. The cloned targeting fragment is then digested at a naturally occurring unique restriction site in the sequence to allow for insertion of a selection marker gene in the center of the targeted gene (Fig. 1A), resulting in a disruption vector (Cousin et al., 2006). Alternatively, the cloned target sequence is digested with two enzymes with natural unique sites to allow for replacement of a central region of the target gene with a selection marker gene, resulting in a deletion vector. This is either performed directly in the binary vector (Gourgues et al., 2004) or in an intermediate vector, which require a subsequent cloning of the replacement cassette into a binary vector (Hoffman and Breuil, 2004).

7.2.2. Type 2

Strategies that depend on the introduction of HRSs into binary vectors that already contain a selection marker gene typically also rely on the introduction of unique restriction sites into the ends of the HRSs (Fig. 1B). Here, the HRSs are amplified with primers that contain 5′ tails that introduce unique restriction enzyme sites into

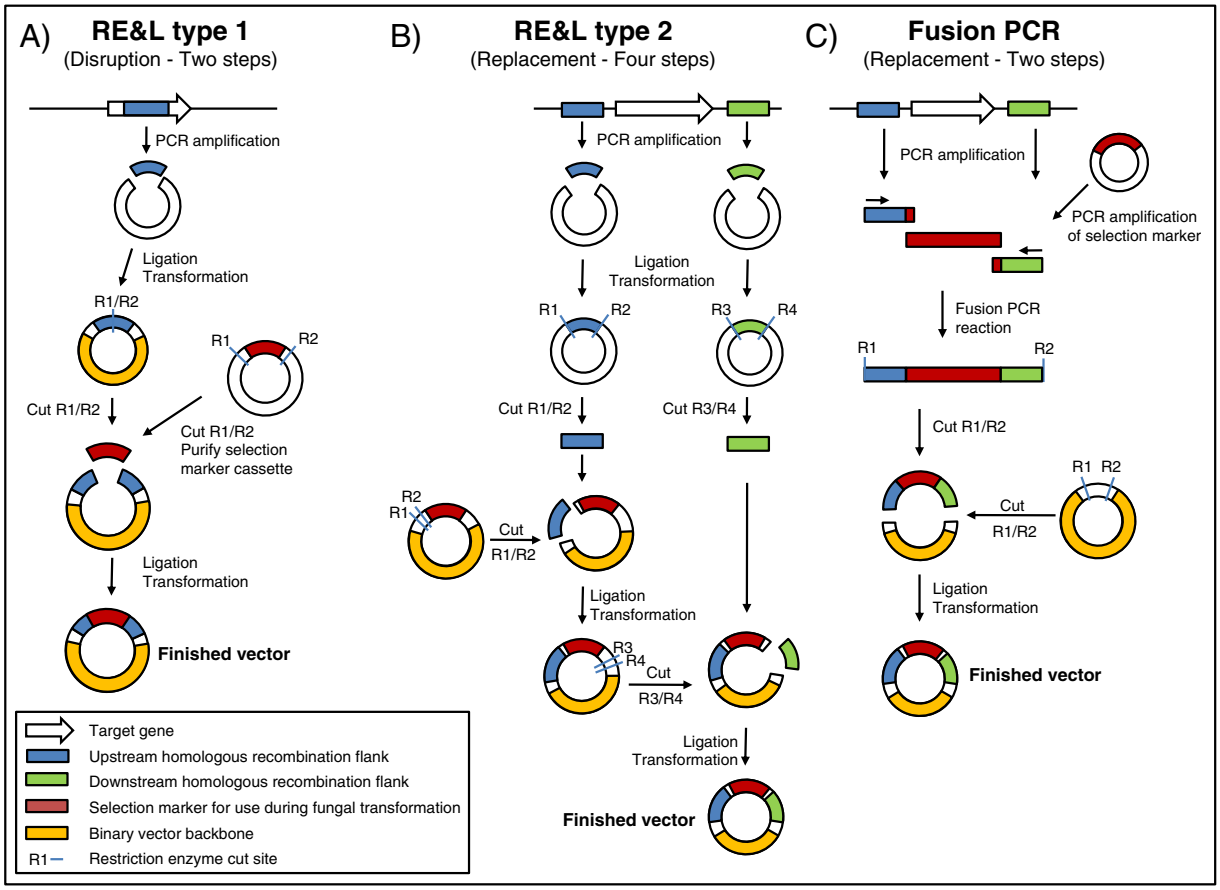

Fig. 1. Construction of disruption and replacement vectors using Restriction enzyme- and ligase-dependent cloning and Fusion PCR. Restriction enzyme- and ligase-dependent cloning strategies for constructing binary vectors for targeted gene A) disruption and B) replacement. The disruption strategy depends on naturally occurring restriction enzyme cut sites in the relevant HRSs. The replacement strategy depends on the presence of four unique restriction enzyme cut sites, which typically are introduced into the ends of HRSs during the initial PCR reactions. C) Construction of replacement vectors using Fusion PCR. The two HRSs are amplified with primers containing overhangs that allow for subsequent annealing with the ends of a selection marker fragment. The three fragments are fused by cross-fragment priming via a single PCR reaction.

the ends, allowing for sequential cloning of the HRSs into the binary vector at either site in the selection marker gene (Kellner et al., 2005). Alternatively, the replacement construct is assembled in an intermediate vector and then moved into a binary vector (Sugui et al., 2005).

7.2.3. Type 3

In a four-fragment ligation reaction, the two HRSs are ligated to the selection marker gene and the vector backbone in a single cloning reaction. Though this setup is appealing because it offers single-step construction of vectors, the efficiency of the four-fragment ligation reaction is typically very low, and only a few studies have relied on this technique (Moon et al., 2008).

7.2.4. Type 4

Direct Repeat Recombination-mediated Gene Targeting (DRGT) (Ushimaru et al., 2010) relies on the RE&L cloning of a single HRS between a selection marker gene and a partial direct repeat of the selection marker. Integration of the disruption construct into the genome relies on two HR events, the first between the repeated part of the selection marker gene, resulting in formation of a circular T-DNA, and the second between the single HRS and the genomic target locus. The resulting locus contains directional repeats of the HRS separated by a functional marker gene, a highly unstable situation that allows for loop-out of the marker, recreating the wild type genotype, if the selection pressure is removed.

RE&L strategies commonly depend on the presence of unique restriction sites in the HRSs that are compatible with unique sites in the used binary vector. This requirement imposes serious limitations on which HRSs can be used, thereby reducing the control over which modifications can be introduced into the fungal genome. The RE&L strategies require a complicated design process that is unique for the individual targeting vector being constructed. In addition, the RE&L strategies typically also rely on subcloning of the target sequence or HRSs, meaning that between three and five cloning steps are required for construction of a single vector. The advantage of the technique is its compatibility with all existing binary vector systems, as long as unique restriction enzymes can be identified.

7.3. Xi cloning

The Xi cloning technique relies on bacterial in vivo homologous recombination for the directional assembly of DNA fragments with identical sequences at their ends (Liang et al., 2005). The strategy entails digestion of the binary vector with a unique-cutting restriction enzyme and amplification of the first HRS to be cloned using primers that introduce 30-bp 5′ overhangs that are identical to the sequences surrounding the restriction sites in the recipient vector (Fig. 2A). Transformation of *E. coli* with the amplified DNA fragment and digested vector allows for directional assembly of the fragments via the endogenous DNA repair machinery. The intermediary vector is then digested with a second unique-cutting enzyme and fused with the other required HRS, as described for the first HRS, resulting in a replacement cassette. The low efficiency of the cloning technique means that targeting vectors have to be constructed via sequential introduction of the two HRSs into the binary vector (Frandsen et al., 2006).

This technique is independent of restriction enzyme digestion of the HRSs, lifting many of the limitations of the classic RE&L strategy. Therefore, the design process is subjected to fewer restrictions and gives a greater level of operational freedom to modify the fungal genome compared to RE&L strategies. It should be noted, however, that the cloning of the second HRS still requires that the first HRS does not contain the restriction site needed for the second round of cloning. The technique is compatible with all binary vectors that contain a unique restriction site on either site of the selection marker gene; enzymes resulting in either blunt or sticky-ends are equally efficient. In addition, it eliminates the need for sub-cloning of the HRSs and relies on very few materials, making it superior to standard RE&L strategies.

7.4. In vitro transposon mutagenesis

The in vitro transposon mutagenesis approach, initially used by Zwiers and De Waard, is the next most popular approach for the construction of targeted binary vectors (Zwiers and De Waard, 2001). The strategy relies on the GPS Mutagenesis system (discontinued by

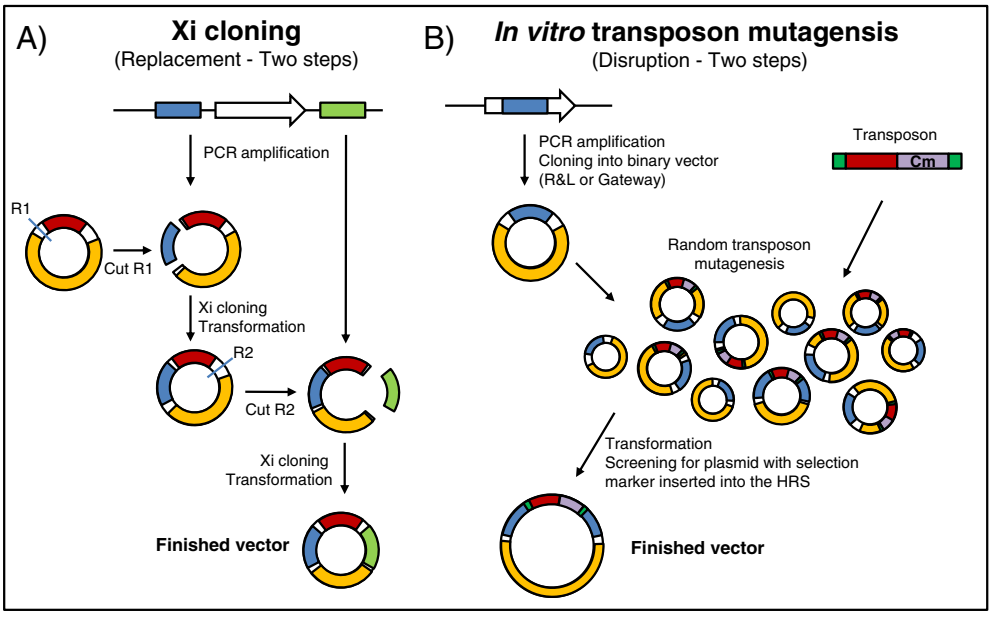

Fig. 2. Construction of replacement vectors using Xi and In vitro transposon mutagenesis. A) Construction of replacement vector via Xi cloning. Restriction enzymes resulting in either blunt or sticky ends can be used for strategy. The only requirements are that the restriction enzymes only cut once in the vector backbone and that the second enzyme does not cut in the HRS that was introduced in the first round. B) Construction of binary vectors for targeted gene disruption via in vitro transposon mutagenesis. The transposon encodes a fungal selection marker and a bacterial selection marker, the latter of which enables the selection of mutated binary vectors.

New England Biolabs in 2009) for random introduction of a selection marker cassette into the fungal targeting sequence, resulting in the formation of two HRSs surrounding the marker gene (disruption construct). The selection marker cassette is located in a Tn7 transposon element. The technique depends on two cloning steps. First, a region of the targeted gene is RE&L-cloned into a binary vector. The purified plasmid is then mixed with Tn7 containing plasmid (pGPS3hyg) and treated with TnsA/TnsB/TnsC* transposase, which catalyzes the transposition reaction in vitro. The mutated plasmids are transformed into *E. coli*, and the resulting transformants are screened via colony hybridization or PCR for plasmids containing the transposon. Selected plasmids are typically sequenced to determine the precise integration site and the modifications that will be introduced into the fungal genome (Fig. 2B). The low efficiency of the transposition reaction typically means that only 5% of the obtained transformants contain a plasmid harboring a transposon (Amey et al., 2003). This problem was solved by the introduction of an additional bacterial selection marker into the Tn7 donor plasmid (pGPS3HygKan) allowing for selection of transposon-containing binary plasmids (Mehrabi et al., 2006). A system compatible with Gateway cloning is also available that allows for RE&L-independent cloning of the fungal targeting sequence prior to the transposon mutagenesis reaction (Gardiner et al., 2004).

A competing Tn5-based system (EZ::TN system, Epicentre Technologies) offers similar possibilities but relies on a purified transposon element and the Tn5 transposase. The system was first used for the construction of binary vectors by Ospina-Giraldo and coworkers, relying on a transposon containing an *AnPtrpC*::*hph* fungal selection marker (pSK597) (Ospina-Giraldo et al., 2003). Later, a transposon containing a similar fungal selection marker and a chloramphenicol selection marker gene was developed, allowing for the selection of transposon-containing vectors (Dobinson et al., 2004).

The experimental design process is simple compared to RE&L strategies, as it only depends on unique restriction sites at the ends of the fungal targeting sequence or Gateway recombination sequences for the initial cloning into the binary vector. However, the random nature of the in vitro transposon step rules out a rationally designed targeting and, to some extent, limits which modifications can be introduced into the fungal genome. This requires the user to sequence multiple plasmids to identify one with the desired HRS size and disruption site.

7.5. Fusion PCR

The use of three-way Fusion PCR, also known as "overlap extension PCR" (Ho et al., 1989), for the assembly of the HRSs and the selection marker gene offers complete control over which alterations are introduced into the fungal genome. The technique typically consists of four PCR reactions and one or two cloning steps. First, the HRSs are individually amplified using PCR with primer sets that introduce a restriction enzyme site at one end of the amplicon and a short sequence that is identical to one end of the selection marker cassette at the other end. The selection marker cassette is amplified by PCR, mixed with the two HRS amplicons and subjected to Fusion PCR using the two terminal primers, resulting in cross-fragment annealing/priming and joining of the three fragments into one. The fused construct is subsequently subcloned into an intermediate vector (using TA or blunt-end cloning) and then moved into a binary vector via RE&L (Michielse et al., 2005a). Alternatively, the fusion product is cloned directly into the binary vector relying on introduced unique terminal restriction enzyme sites (Idnurm et al., 2007) or blunt-end cloning (Li et al., 2010) (Fig. 1C). The fusion PCR reaction step can sometimes be complicated by the presence of repetitive DNA sequences in the HRSs, making it difficult to obtain high levels of the desired fused PCR amplicon.

The approaches described above, except for the blunt-end approach developed by Li et al., all depend on the presence of unique restriction enzyme sites at the ends of the fused amplicon, which limits which HRSs can be used for vector construction. This limitation is avoided in the setup described by Li et al. because they are able to clone the fusion product directly into the binary vector without restriction enzyme digestion of the replacement construct. This makes the strategy insert independent and greatly simplifies the experimental design because the 5′ overhangs of the terminal primers are identical for all constructs (Li et al., 2010). However, the fact that the selection marker cassette is amplified by PCR means that the entire replacement cassette has to be sequenced to rule out PCR-introduced mutations that may render the selection marker gene dysfunctional. Although this is less of an issue for protoplast transformation, which uses a diverse pool of PCR amplicons, full sequencing of the cassette is essential in ATMT, as a single DNA molecule is the progenitor of all plasmids used for fungal transformation.

7.6. In-Fusion cloning (single-stranded exonuclease-based cloning)

Single-stranded exonuclease-based cloning techniques, such as In-Fusion and CloneEZ, allow for seamless fusion of multiple PCR amplicons and vector DNA fragments in a single cloning reaction, as shown by Zu et al. for non-binary vectors (Zhu et al., 2007). The technology depends on the 3′ to 5′ single-stranded exonuclease activity of the In-Fusion enzyme (Clontech) or CloneEZ enzyme (GenScript) for generating complementary 15-bp single stranded 5′ overhangs on the mixed DNA fragments (Clontech, 2008).

Our laboratory has recently used this technology for single-step construction of vectors for targeted gene replacement in *F. graminearum* (Frandsen et al., 2011). The vectors were constructed by restriction enzyme digestion of the binary vector, resulting in two blunt-end fragments (a vector backbone and a selection marker cassette). The two HRSs were PCR amplified with primers introducing 15-bp 5′ overhangs to the ends of the HRSs, which are identical to the sequences surrounding the two restriction enzyme sites used in the recipient vector. Treatment of the four DNA fragments with single-strand exonuclease resulted in the formation of unique 5′ complementary overhangs, ensuring directional assembly of the four fragments (Fig. 3A). The resulting chimeric DNA molecules were then transformed into *E. coli* and covalently joined by the endogenous DNA repair system. In our experiments, we typically found a four-fragment assembly efficiency of 10–20%, which is significantly less than the 50–100% assembly efficiency reported by Zhu (Zhu et al., 2007) for non-binary vectors, suggesting that the system can be further optimized.

This vector construction strategy is highly versatile and is compatible with all available binary vectors that contain a unique restriction enzyme site on either side of the selection marker gene. In addition, the independence of the restriction enzyme digestion of the HRSs and seamless fusion of the DNA fragments offers complete freedom over which HRSs are used for vector construction and which modifications are introduced into the fungal genome. The experimental design is insert independent, allowing for easy automation of the design process, as required for large-scale projects.

7.7. USER Friendly cloning

The Uracil-Specific Excision Reagent (USER) cloning technology allows for single-step construction of targeted binary vectors (Frandsen et al., 2008). The technique depends on special USER cloning sites (UCSs) in the recipient binary vector, which are placed on either side of the selection marker gene (Fig. 3A). Each of the UCSs contains recognition sites for a standard restriction enzyme (*PacI*), two sites for a nicking endonuclease (*Nt.BbvCI*) and four variable nucleotide positions. Digestion results in the formation of unique 9-bp 3′ overhangs that

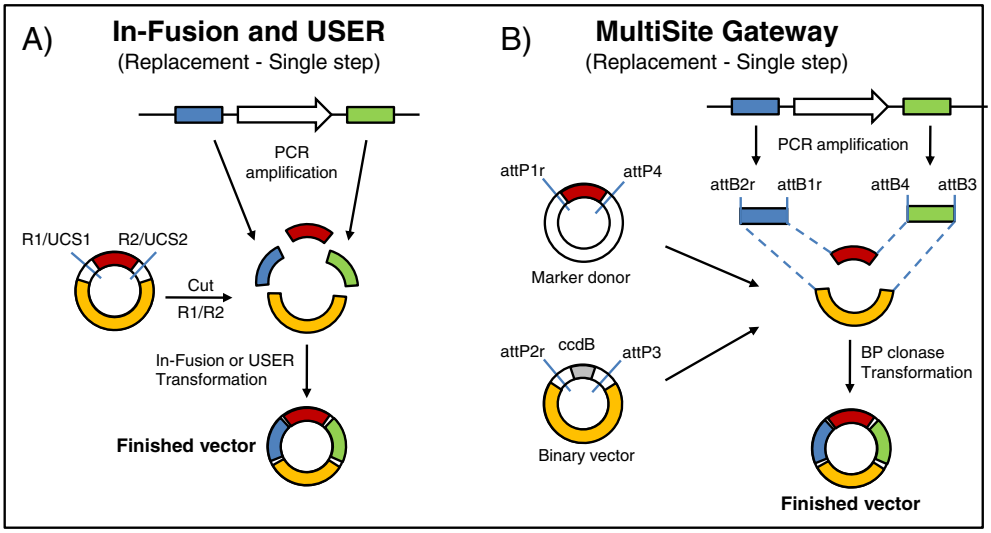

Fig. 3. Construction of replacement vectors using MultiSite Gateway, In-Fusion and USER cloning. A) Single step four fragment assembly of replacement vector via multifragment In-Fusion (exonuclease digestion) or USER cloning. In-Fusion cloning dependent on 15 bp 5′ overhangs on the PCR amplicons, identical to the sequences surrounding the use restriction enzyme sites. USER cloning dependent on nicking and restriction enzyme digestion of the USER cloning sites (UCS) and treatment of the PCR amplicons with USER enzyme mix. B) Construction of replacement vector using MultiSite Gateway technology. Single-step directional assembly is ensured by four unique BP clonase recombination sites: *att*B in the two PCR amplicons and *att*P in the binary vector backbone and selection marker cassette.

can be used for directional cloning. The HRSs are amplified by PCR using primers that introduce 5′ overhangs, each containing a 2-deoxyuridine and a sequence that is complementary to the sticky ends generated in the UCSs of the recipient vector. The amplified HRSs are treated with a uracil-DNA glycosylase and the DNA glycosylase-lyase Endo VII (USER-enzyme mix) (New England Biolabs, 2008), resulting in excision of the 2-deoxyuridines, thereby introducing single-strand breaks and forming 9-bp 3′ overhangs that are compatible with those found in the vector. The compatible 3′ overhangs on the vector fragments and the HRSs allow for directional assembly of the four fragments into a chimeric molecule, which is stable enough to survive transformation into *E. coli* and become covalently joined by the DNA repair system. The four-fragment assembly efficiency is typically 85% or higher, and it reduces the required screening work compared to the In-Fusion/CloneEZ cloning technique.

The USER system, similar to In-Fusion, offers single-step construction of targeted binary vectors, easy experimental design (insert independent) and total freedom over which sequences are used as HRSs. Though the technique is dependent on the presence of UCSs in the recipient vector, which reduces the versatility, the higher cloning efficiency makes it more attractive for large-scale knockout projects. Currently, vectors are available for targeted gene replacement (pRF-HU2), in locus overexpression (pRF-HU2E), ectopic overexpression (pRF-HUE) and general cloning into T-DNA (pRF-HU) (Frandsen et al., 2008). The technique also allows for single-step construction of complex vectors by relying on the USER Fusion approach described by (Geu-Flores et al., 2007).

7.8. Gateway (recombination-based) cloning

The Gateway technology (Invitrogen) depends on the action of lambda recombinases (clonases) to catalyze recombination between specific sequences (*att* sites). Four Gateway recombination-based cloning systems are available for the construction of targeted binary vectors used in fungi. Three of these techniques are multistep processes that, in addition to Gateway cloning, depends on RE&L cloning (Saitoh et al., 2008), in vitro transposon mutagenesis (Gardiner et al., 2004) or Fusion PCR (Khang et al., 2005; Tucker et al., 2010).

Recently, Paz and co-workers published a true Gateway strategy termed “One Step Construction of *Agrobacterium*-Recombination-ready-plasmids” (OSCAR) that allows for single step four fragment fusion by MultiSite Gateway cloning (Paz et al., 2011). The system relies on four unique *attB* recombination sites, each 27 or 28 bp long. The two HRSs are amplified by PCR with primers introducing *att*B2r-*att*B1r and *att*B4-*att*B3 terminal recombination sites, respectively. The amplicons are then combined with a Marker vector, carrying an *att*P1r-*hygR*-*aat*P4 cassette (pA-Hyg-OSCAR) and a Gateway destination plasmid carrying the binary vector backbone and an *att*P2r-*ccdB*-*att*P3 cassette (pOSCAR), via a single BP clonase catalyzed reaction (Fig. 3B). The resulting binary plasmids pose the selection marker gene surrounded by the two HRSs, while the *ccdB* killer gene is lost in the recombination reaction. The presence of the *ccdB* killer gene in the recipient plasmid should ensure that only correctly assembled plasmids should yield viable transformants, however the system pose assembly efficiencies of 37 to 88%.

The multisite Gateway technique allows for insert independent design of deletion vectors combined with single-step assembly, as In-Fusion and USER cloning. Currently only marker plasmids for transformation of ascomycetes are available, however, the system can easily be extended to other fungal groups.

7.9. Split-marker

The split-marker technique, also known as bipartite gene targeting, has been used extensively in combination with protoplast-based transformation systems (Fairhead et al., 1996). The technique relies on the construction of two DNA fragments, each containing two thirds of the selection marker gene combined with one of the required HRSs. Integration of the two fragments into the fungal genome depends on a triple crossover reaction between the genomic target and the overlapping parts of the selection marker cassette. The technique has been found to increase the HR integration frequency compared to systems relying on a single DNA fragment (Jeong et al., 2007).

Recently, Wang and coworkers showed that the split-marker strategy is also compatible with ATMT by co-cultivating the target fungus (*Grosmannia clavigera*) with two different *A. tumefaciens*

strains, each carrying a binary plasmid with either the up- or downstream HRS combined with two thirds of the selection marker (Wang et al., 2010b). The number of transformants obtained using the split-marker approach with two separate T-DNA transfers was approximately 20 times lower than the number obtained using a contiguous version of the construct. However, the split-marker approach increased the gene targeting frequency from 46% to 74%. The lower number of transformants obtained with the split-marker construct is best explained by its dependence on two separate T-DNA transfer events and its requirement of a triple crossover event for integration into the genome to produce a functional selection marker gene. The vector construction process used by Wang and coworkers for proving the ATMT/split-marker concept relied on a multistep construction approach (Fig. 4). The two HRSs and the selection marker gene were initially fused using yeast-based recombination cloning, as described by Colot, followed by PCR amplification of the required overlapping DNA fragments (Colot et al., 2006). The resulting amplicons were then cloned, via RE&L, into binary vectors that were used for ATMT.

7.10. Summary of vector construction strategies

The majority of the published studies depending on ATMT for targeted gene replacement in fungi have relied on classic vector construction strategies that offer limited control over which genome modifications are introduced into the fungal genome, and these approaches typically require multiple experimental steps (Table 2). The use of techniques such as In-Fusion, USER and Multisite Gateway cloning can greatly simplify the experimental design and offer complete control over the modifications that are introduced into the fungal genome. The fact that they depend on a single experimental step makes them highly attractive for projects aimed at producing large numbers of targeted gene replacement mutants. The In-Fusion technique offers great versatility due to its compatibility with all existing binary vectors that contain two unique restriction enzyme sites; however, the technique is associated with a relative low cloning efficiency. USER cloning and MultiSite Gateway, on the other hand, offers highly efficient, four-fragment cloning, but are less versatile because they dependent on special structures in the recipient binary vector. For large-scale projects, the greater efficiency of the USER and Gateway systems should make the development of new vectors cost effective if the available vectors do not meet the requirements of a particular project. Smaller projects and single experiments

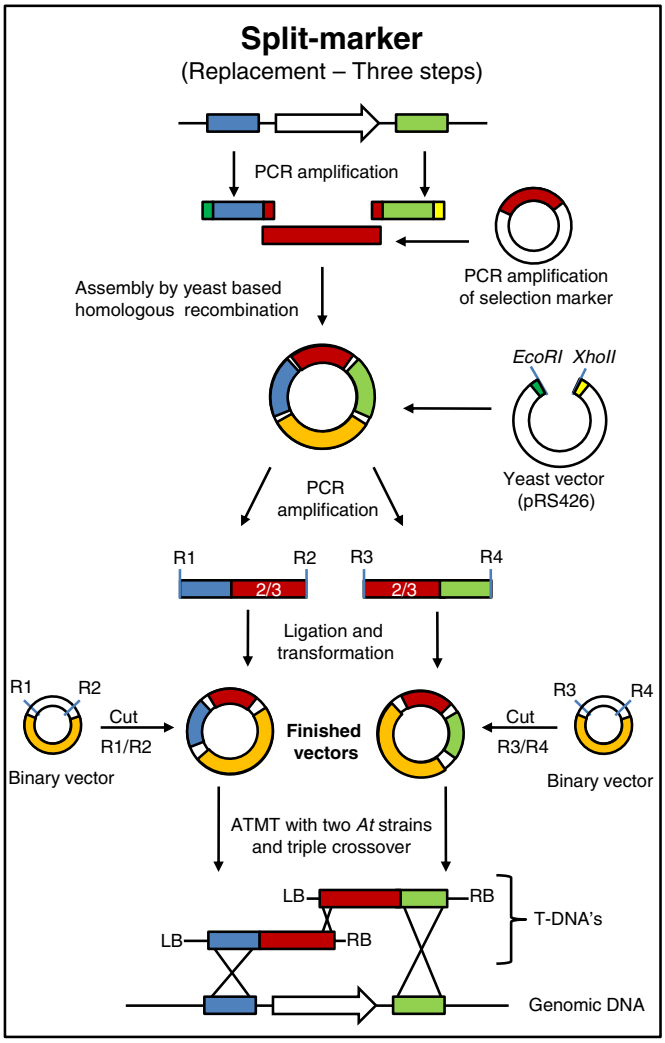

Fig. 4. Multistep construction of vectors for split-marker gene replacement. The ATMT split-marker (bipartite) transformation strategy using a triple-crossover event between two different T-DNA molecules and the genomic target locus. The vector construction strategy relies on yeast-based fusion of HRSs and a selection marker, PCR amplification of a bipartite fragment and RE&L cloning into a binary vector.

Table 2
Cloning systems. Comparison of the cloning systems used in the construction of binary vectors for targeted gene replacement in fungi. Abbreviations: UCS = USER cloning site, RE = unique restriction enzyme sites, *att*P = BP clonase recognition sites.

	Standardized experimental design	Number of cloning steps	Structures in vector	Structures in HRSs	Level of control over the genetic modification	Reuse of HRS for multiple constructs	Complex construct in a single step	Number of available vectors	Articles describing the use of the technique for construction of binary targeted gene replacement vectors
RE&L	No	2–5	2–4 RE	3–4 RE	Low	Limited	No	High	Zhang et al., 2003
In vitro transposition	No	2	2 RE	2 RE	None	None	No	Low	Zwiers et al., 2007; Dobinson et al., 2004; Mehrabi et al., 2006
Fusion PCR	No	1–2	2 RE	2 RE	Moderate	Moderate	No	High	Li et al., 2010
Gateway	No	3	2–4 RE	2–4 RE	Moderate	None	No	Low	Gardiner et al., 2004 Saitoh et al. 2008 Tucker et al. 2010
Split-marker (current version)	No	3	2 RE	2 RE	Moderate	None	No	Low	Wang et al., 2010a, 2010b
Split-marker (optimal setup)	Yes	2	UCS	None	High	High	No	None	(Theoretical)
MultiSite Gateway	Yes	1	4 *att*P	None	High	High	Yes	Low	(Paz et al., 2011)
Xi-cloning	Yes	2	2 RE	None	High	Limited	No	High	Frandsen et al., 2006
In-Fusion	Yes	1	2 RE	None	High	Limited	Yes	High	(Frandsen et al., 2011)
USER	Yes	1	UCS	None	High	High	Yes	Low	Frandsen et al., 2008

should probably use the In-Fusion technique if special vectors are needed.

7.11. Targeted overexpression (in locus overexpression)

The above described vector construction strategies all aim to disrupt the function of the targeted gene. However, an increasing number of studies aim to overexpress the target gene, by targeted integration of an exogenous promoter in front of the endogenous copy of the gene. To allow for generally applicable cloning strategies, my associates and I constructed a USER cloning compatible binary vector (pRF-HU2E) for in locus overexpression experiments in ascomycetes (Table S6). The vector allow for simultaneous directional cloning of the two required HRSs, the promoter region and the start of the ORF into the vector, resulting in fusion of an *AnPgpdA* promoter with the partial ORF. In this setup, the start of the ORF acts as the downstream HRS (Frandsen et al., 2008).

8. General strategies for constructing binary vectors for complementation and heterologous expression

Reintroduction of genes to reverse a mutant phenotype has been used in many random mutagenesis studies to prove that the observed phenotype is attributable to the identified T-DNA integration site. For this two experimental strategies are available: classic random ectopic integration or integration into a predetermined locus. Similar to the trends for the construction of targeted gene replacement vectors, the majority of complementation studies have relied on custom vector construction strategies. However, a few generic systems have been developed based on RE&L (Fang et al., 2005), USER Friendly cloning (Frandsen et al., 2008) or Gateway cloning (Hilty et al., 2008).

8.1. Ectopic expression from a random locus

The majority of complementation studies have been performed in ascomycetes. These studies have mostly relied on PCR amplification of the complementing gene with its endogenous regulatory sequence and subsequently, RE&L or CloneEZ-based cloning into a wide range of binary vectors (Table S4) (Sesma and Osbourn, 2004). Alternative strategies have included blunt-end or TA subcloning of the PCR amplicon into pGEM-T Easy (Hoi et al., 2007) or TOPO (Gardiner et al., 2005) vectors, followed by an RE&L-dependent transfer of the gene into a binary vector. Several studies have also relied on RE&L cloning of the complementing gene from cosmid libraries into a binary vector (Gout et al., 2006). In basidiomycetes, only a few studies have been completed, and these have relied on RE&L vector construction (Godio and Martin, 2009) or TOPO subcloning (Idnurm et al., 2009). As an alternative to the use of the endogenous promoter for the complementing genes, several studies in ascomycetes have used the *AnPgpdA* promoter to complement the gene of interest via overexpression. The vectors have been constructed via RE&L (Klimes et al., 2008), pGEM-T Easy subcloning vector construction (Fang et al., 2005) or direct binary vector construction by USER cloning (Frandsen et al., 2008).

8.2. Complementation using binary BAC vectors

A classic strategy for identification of a mutated gene responsible for an observed phenotype has been complementation using large DNA fragments such as Bacterial Artificial Chromosomes (BAC) that are randomly integrated. This strategy is also compatible with ATMT, but it requires that the BAC is converted into a binary BAC (BIBAC) by the introduction of the LB and RB sequences and a fungal selection marker. The large size of BACs (up to 350 kb) makes it impossible to rely on standard RE&L cloning strategies; however, Takken and coworkers have developed a system that allows for easy conversion via *E. coli*-based homologous recombination. The available BAC libraries for fungal species have typically been created in vectors containing both a bacterial and a fungal selection marker gene. The conversion strategy depends on a linear DNA fragment (termed a REC vector) containing an inverted T-DNA region/*At* vector backbone (LB-KanR-ori-RB) flanked by HRSs that are identical to the 5′ and 3′ parts of the bacterial selection marker gene found in the targeted BAC vector. In vivo recombination between the REC vector and the BAC results in replacement of the BAC resistance cassette with the inverted T-DNA region. Recombination events can be detected by the loss of the targeted bacterial selection marker and the gain of the alternative marker (Fig. 5) (Takken et al., 2004). Using this conversion strategy, Takken et al. were able to introduce 1–75 kb into the ascomycetes *F. oxysporum* and *A. awamori* and later by Ali and Bakkeren in the basidiomycete *Ustilago hordei* (Ali and Bakkeren, 2010).

8.3. Ectopic expression from a predetermined locus

Expression from a predetermined locus allows for the construction of near-isogenic strains, eliminating the risk of accidental gene disruption and variation in expression strength due to locus effects. Two generally applicable binary vector construction strategies are available that are both specific to *Fusarium* sp. Xu et al. relied on blunt-end cloning to introduce the entire chitin synthase gene (*CHS1*), including its regulatory sequences, into pHAU-Neo-Tet, a vector containing targeting sequences for the *PLS1* (tetraspanin) locus in *F. asiaticum* (Xu et al., 2010). My associates and I have developed vectors that allow for targeted integration into the genome

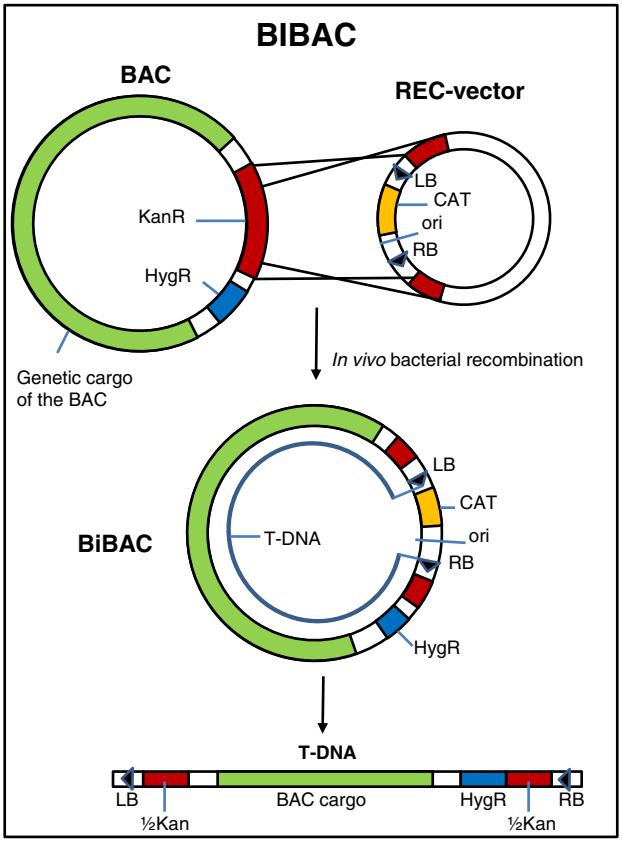

Fig. 5. Construction of BIBAC vectors. A system for converting Bacterial Artificial Chromosomes (BACs) into a binary vector compatible with ATMT. The REC vector contains two HRSs for recombination with the selection marker gene in the BAC backbone. In addition, the vector contains a new selection marker gene (CAT) and the RB and LB sequences. Recombination between the BAC and the REC vectors results in the formation of a BIBAC, in which the insert from the BAC is placed between the RB and LB, converting it to T-DNA.

of *F. graminearum*; the genes to be overexpressed are introduced in these vectors (pRF-HUEA and pTJ-GUEA) behind the *A. nidulans gpdA* promoter using USER cloning, and both vectors target the *PKS12* locus (responsible for mycelium pigmentation) (Sørensen et al., submitted for publication). In our case, the construction of these vectors was prompted in part by the need for near-isogenic strains and by data suggesting that ectopic integration via ATMT in the *F. graminearum* wild type strain occurs very infrequently (unpublished data).

9. Strategies for construction of binary vectors for translational and transcriptional fusion reporters

Key information can be gained by studying the expression pattern of a gene. However, techniques relying on PCR, DNA microarrays and sequencing typically require large samples, making it impossible to obtain cell-specific data in complex tissues. The use of transcriptional fusion reporter constructs, where the expression of a visual reporter (GFP/RFP or GUS) is controlled by the promoter of the gene of interest, can provide expression data at the single-cell level. Though these types of experiments only rely on the cloning of a single DNA fragment (the promoter), just one generic binary vector for ascomycetes has been reported (Table S7). Sakaguchi et al. reported the development of a GFP-containing binary vector (pBI-HglyGFP) that allows for fusion of promoter sequences with GFP via RE&L cloning into an *EcoRI* site located in front of the GFP coding region (Sakaguchi et al., 2008). Reliance on a single restriction enzyme means that the cloning reaction is not directional and is limited to promoter elements that do not contain *EcoRI* sites. Both of these problems can be solved using alternative cloning techniques, such as Xi or In-Fusion cloning.

Other projects have used custom vector construction strategies, such as three-fragment ligation with GFP (Fan et al., 2005), multi-step RE&L with β-glucuronidase (GUS) (Rolland et al., 2009) and multi-step RE&L with GFP as a reporter gene (Fang et al., 2010).

The construction of translational fusion proteins represents an important tool for the functional characterization of genes by providing a means to tag proteins with fluorescent markers and to visualize their subcellular location. The majority of reported ATMT studies have depended on custom vector construction strategies, relying on fusion PCR (Elliott and Howlett, 2006), multi-step RE&L (Idnurm et al., 2007) and three point ligation (Fujihara et al., 2010) (Jonkers and Rep, 2009). Recently, Tucker et al. used MultiSite Gateway cloning to construct both amino and carboxy translational fusions of mCherry and the exportin-5/Msn5p ortholog (EXP5) from *Magnaporthe oryzae*. The vectors were constructed in two steps: first, the required DNA fragments were cloned into Gateway entry vectors (pDONR P4-P1R and pDONR P2R-P3), and then they were assembled into the binary vector (pSUR-GFP-MS) by MultiSite Gateway recombination (Tucker et al., 2010). This system is generic in nature and only requires that primers be designed for amplification of two gene-specific DNA fragments for each construct (N-terminal: promoter plus ORF-terminator; C-terminal: promoter-ORF plus terminator), to which 5′ Gateway recombination sequences must be added.

The USER fusion cloning technique described by Geu-Flores et al. (2007) could potentially be used to enable single-step construction of both transcriptional and translational fusion. Currently, only binary vectors for transcriptional and translational fusions in ascomycetes are available.

10. ATMT for the integration of RNAi constructs for the analysis of essential genes

In fungi, where the optimization of the ATMT process has not significantly increased the gene replacement frequency, RNA interference/silencing (RNAi) has emerged as a fruitful alternative. Random integration of *trans*-acting silencing constructs allows for the knockdown of the target gene via posttranscriptional silencing. The technique also allows for the analysis of essential genes in situations where removal of the target gene would result in nonviable transformants.

RNA silencing via ATMT has been achieved in both ascomycetes and basidiomycetes through the expression of either antisense RNA (Godio et al., 2007) or hairpin structures (hsRNA) (Fitzgerald et al., 2004). Several binary vectors are available for silencing experiments (Table S8). For hsRNA constructs two strategies have been used. One relies on a part of the targeted gene sequence acting as a spacer, which is achieved by making either the sense or antisense sequence longer than the other and then fusing them via RE&L into a binary vector (Gong et al., 2007). In the second strategy, the recipient vector contains two multiple cloning sites surrounding a spacer region (typically an intron), allowing for the sequential RE&L cloning of two identical sequences in opposite directions, either directly into a binary vector (Wang et al., 2009). Fox et al. have developed a binary vector compatible with Gateway cloning that allows for single-step assembly of hsRNA constructs for ascomycetes (Fig. 6). The system is based on the pHellsgate 8 vector (Helliwell and Waterhouse, 2003) and consists of a binary vector (pHYGGS) containing inverted *att*R1-*att*R2 repeats spaced by an intron. A fragment of the fungal gene to be silenced is PCR amplified using *att*B1- and *att*B2-tailed primers and cloned into a Gateway entry vector (pDONR207) with BP clonase. The arrangement of the two Gateway recombination sites (*att*R1-2 and *att*R2-1) in the pHYGGS vector allows for the simultaneous introduction of two copies of the cloned sequence in the 'sense' and 'anti-sense' orientations using LR clonase (Fox et al., 2008).

For construction of shRNA cassettes to be used in basidiomycetes, the pSILBA system is likely to be the most applicable. The system allow for the sequential RE&L cloning of the two parts of the hairpin structure into the two MCSs of pSILBAγ. The silencing construct is subsequently moved into the binary pHg vector using RE&L cloning (Kemppainen and Pardo, 2010).

11. Perspectives and suggested improvements

11.1. The optimal vector construction setup

The ability to perform targeted genome modifications is becoming more important with the rapid accumulation of fungal genome sequence data and the increasing demand for the functional characterization of novel genes. The ideal targeted genome modification pipeline should include automated experimental design (e.g., primer design), efficient strain construction (e.g., vector construction, fungal transformation and gene targeting), efficient screening for correct transformants and easy validation of the generated mutant strain. For many fungal species, ATMT is the only option for the introduction of genetic modifications. However, until recently, the vector construction stage in most ATMT strategies has been an experimental bottleneck because it depends on custom-designed cloning strategies and time-consuming, multi-step cloning strategies. The development of efficient *E. coli*-based cloning techniques for binary vector construction allows for high-throughput vector construction, and, in addition, it offers researchers complete control over which genomic modifications are introduced into the fungal genome. Vector construction strategies using techniques such as In-Fusion, USER and MultiSite Gateway based cloning already offer standardized experimental design, insert-independent cloning and highly efficient single-step vector construction.

In addition to these existing systems, the ATMT split-marker strategy used by Wang and coworkers also has the potential for simplifying vector construction and improving the gene targeting efficiency in many fungal species (Wang et al., 2010b). The vector construction process can be greatly simplified by designing two binary vectors, each containing two thirds of the required selection marker gene

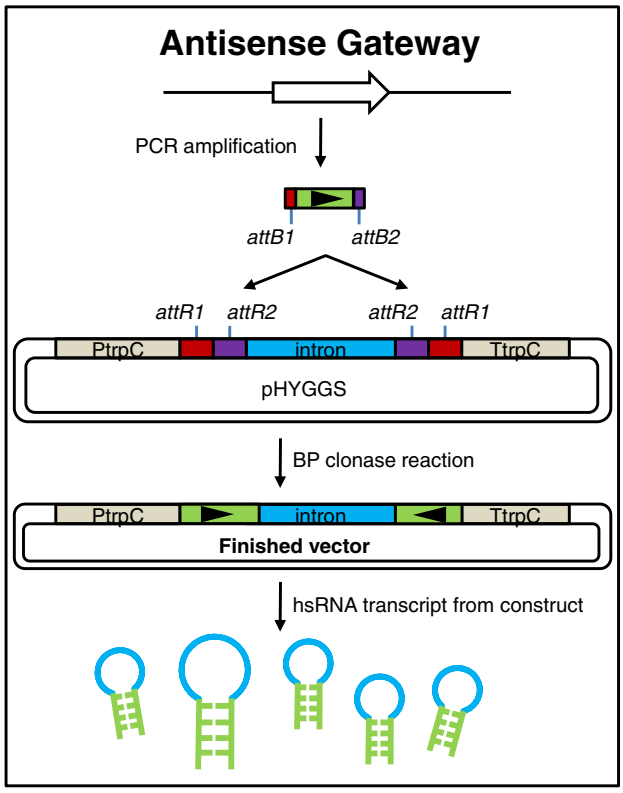

Fig. 6. Construction of hsRNA vectors using Gateway cloning. A system for single-step construction of a vector for gene silencing by production of hairpin structures (hsRNA). PCR amplification of part of the coding sequence of the target gene introduces *att*B1 and *att*B2 sites into the ends of the amplicon. The introduced recombinase sites allow for subsequent cloning into the two *att*R1/R2 sites of the pHYGGS vector. The resulting vector contains two copies of the PCR amplicon pointing in opposite directions and separated by an intron. Expression of the silencing construct is ensured by the constitutive trpC promoter and the transcript forms a hairpin.

and a USER cloning site to allow efficient and parallel cloning of the two HRSs into the two vectors (Fig. 7). Implementation of this setup would make the split-marker technique competitive with the previously described In-Fusion, USER and MultiSite Gateway cloning setups with respect to speed. However, the dependence on two plasmids increases the overall number of experimental steps (vector verification and introduction into *At*). The improvement in the gene replacement frequency obtained with the split-marker strategy is negligible in the case of *G. clavigera* based on the required screening work; however, the improvement might be more significant for other fungal species that have a lower gene targeting frequency.

11.2. Strategies for identifying and verifying correctly targeted integration

Confidence in the fidelity of targeted genome modifications is essential in order to draw biological conclusions. Currently, no common standard for verification of mutants has been agreed upon, and journals and reviewers often have very different standards for the level of validation needed for the publication of a mutant. However, it is essential that published genetically modified strains can be trusted.

Targeted gene replacement strains can possess a wide range of different problems, as described above, luckily the genotype of any genetically modified strain can be determined using a combination of four diagnostic PCR reactions and a single Southern blot analysis, as described in Frandsen et al., 2011.

11.3. Future improvements to ATMT vectors

The phenotypic impact that the introduction of a selection marker gene potentially has on the resulting mutant strain is an increasing concern for many, especially in fields such as plant pathology or studies dealing with ecological questions. However, there is no doubt that constitutive expression of a heterologous marker gene (e.g., a nutritional marker or an antibiotic resistance gene) is bound to have an effect on the fitness of the fungus, which is why the question instead should be how big this impact is. The presence of antibiotic resistance markers is also a concern for industrial applications based on the growing demand for marker-free strains. Currently, no binary vectors are available that allow for the removal of the introduced selection marker gene. Such a system would also be beneficial for basic research by allowing for marker recycling, thereby reducing the need for the development of multiple different vectors each carrying unique selection markers. For other transformation techniques, several vector systems are available that are based on the Cre/lox recombination system or direct repeats combined with the use of a negative selection marker (such as *ura3* or *amdS*) that allows for the efficient removal of the selection marker by loop-out events.

11.4. Concluding remarks

A wide range of different binary vectors that are compatible with fungal transformation have been developed over the past decade. Unfortunately, there is extensive redundancy in the collective vector pool, most likely due to the lack of a suitable resource for information sharing. In addition, only a fraction of the constructed vectors have been deposited in strain collections, making them difficult to obtain. It is my hope that the fungal research community will find a way to resolve these problems in the near future.

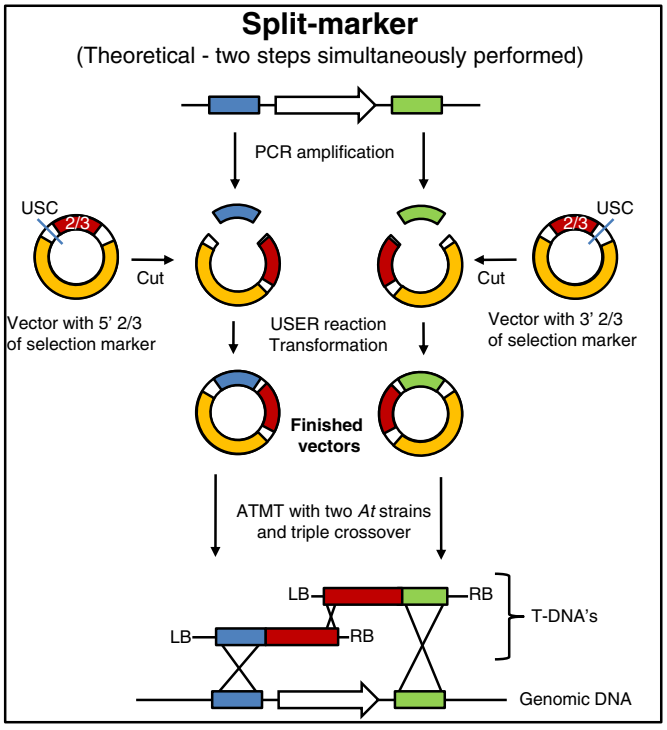

Fig. 7. Single step construction of vectors for split-marker based gene replacement. Optimized strategy for construction of vectors compatible with ATMT split-marker (bipartial). The vector construction strategy used by Wang to prove the concept (see main text) included multiple experimental steps. The vector construction strategy shown represents the optimal theoretical setup, and the cloning steps could depend on cloning techniques such as USER, In-Fusion, Ligase Independent Cloning (LIC) or Gateway.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.mimet.2011.09.004.

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