International Journal of Multidisciplinary Research and Development
www.allsubjectjournal.com
ISSN Online: 2349-4182 ISSN Print: 2349-5979
Received: 27-07-2025, Accepted: 27-08-2025, Published: 11-09-2025
Volume 12, Issue 9, 2025, Page No. 35-42

Virulence factor in Agrobacterium tumefaciens Biovar I

Raghad Nawaf Gergees[1], Abdel-Raheem MA[2]
[1] Department of Biology, College of Education for Pure Sciences, University of Mosul, Iraq
[2] Pests & Plant Protection, Department of Agricultural and Biological Research Institute, National Research Centre, Cairo, Egypt

Abstract
The ability of *Agrobacterium* to transform plants and other organisms is under highly regulated genetic control. Two Virulence (Vir) proteins, VirA and VirG, function as a two-component regulatory system to sense particular phenolic compounds synthesized by wounded plant tissues. Induction by these phenolic compounds, in the presence of certain neutral or acid sugars, results in activation of other *vir* genes, leading to the processing of T-DNA from the Ti-plasmid and transfer of T-DNA to recipient host cells. Many plants, and most nonplant, species do not provide sufficient quantities of the correct phenolic compounds to permit efficient *Agrobacterium*-mediated genetic transformation to occur. In order to transform these species, phenolic inducing compounds must be added to *agrobacteria* before and/or during cocultivation of recipient cells with the bacteria.
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, and expression of transcriptional and translational fusion reporters and RNAi-mediated down-regulation of gene expression.

Keywords: Agrobacterium tumefaciens mediated transformation, virulence genes, ATMT, Fungi, Vector construction

Introduction

Agrobacterium tumefaciens genetically transforms plants and other organisms by transferring a portion of a large Ti (tumor inducing)-plasmid, the T (transferred)-DNA, from the bacterium to the host. Proteins required for processing of the T-DNA from the Ti-plasmid, export of the T-DNA from the bacterium, and "guiding" the T-DNA to the nucleus once inside the eukaryotic host cell are encoded by genes residing in the virulence *(vir)* region of the Ti-plasmid. Many transformation-associated events that occur within the bacterium are under tight genetic regulation. These events are directed by proteins encoded by the *"vir* regulon", a group of genes that respond to phenolic molecules synthesized by wounded (and therefore susceptible) plant cells. The "sensor" for the vir-inducing phenolics is the VirA protein, a member of a two-component genetic regulatory system [1, 2]. VirA localizes to the bacterial periplasmic membrane. In the presence of specific phenolic molecules, and in the presence of specific neutral or acidic sugars that interact with the ChvE protein, VirA autophosphorylates and subsequently transfers the phosphate to an inactive "response regulator" protein, VirG. Phosphorylation "activates" VirG, which then binds to "vir box" sequences within the promoters of *vir* regulon genes, stimulating their transcription [3, 5]. 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. 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. 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 [6].

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. 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 [7, 8]. 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 [9].

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.

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. 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 or in an intermediate vector, which require a subsequent cloning of the replacement cassette into a binary vector [10, 12].

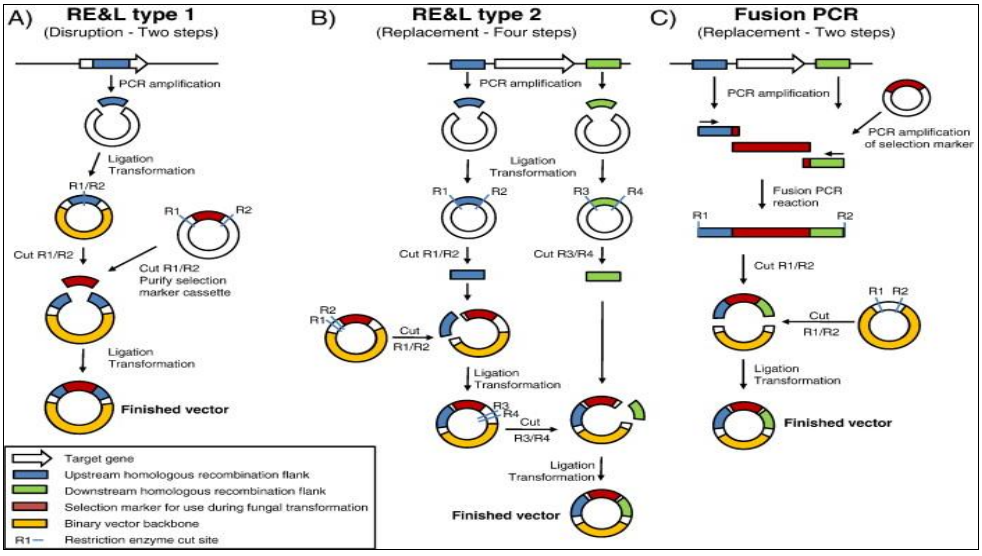

Fig 1: Construction of disruption and replacement vectors using Restriction enzyme- and ligase-dependent cloning and Fusion PCR.

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.

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 the ends, allowing for sequential cloning of the HRSs into the binary vector at either site in the selection marker gene. Alternatively, the replacement construct is assembled in an intermediate vector and then moved into a binary vector [13, 14].

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 [15].

Type 4

Direct Repeat Recombination-mediated Gene Targeting (DRGT) 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. [16] 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.

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. 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 [17, 18]

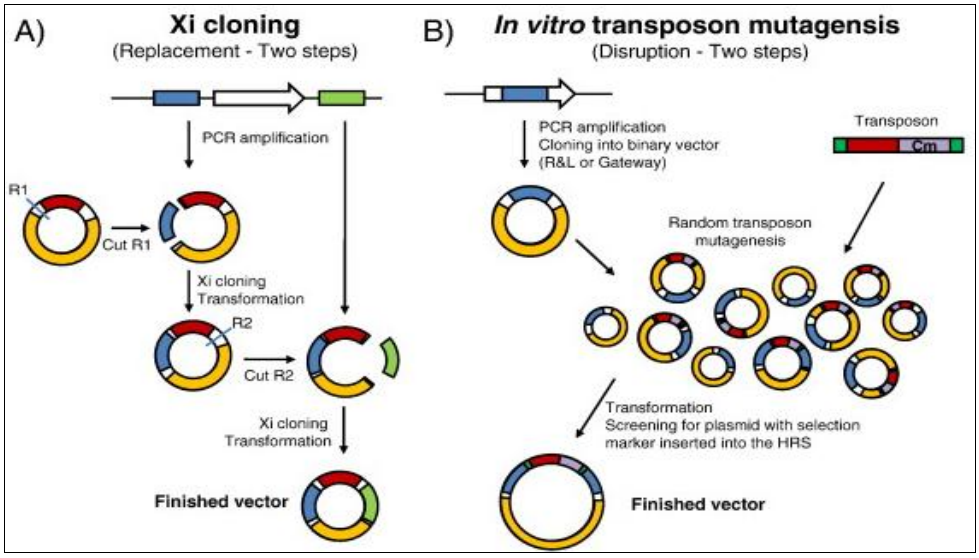

Fig 2: Construction of replacement vectors using Xi and In vitro transposon mutagenesis.

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. 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.

Fusion PCR

The use of three-way Fusion PCR, also known as "overlap extension PCR" 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 [19, 20]. Alternatively, the fusion product is cloned directly into the binary vector relying on introduced unique terminal restriction enzyme sites or blunt-end cloning (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. [21] 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 [21]. 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.

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

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 for non-binary vectors, suggesting that the system can be further optimized [22].

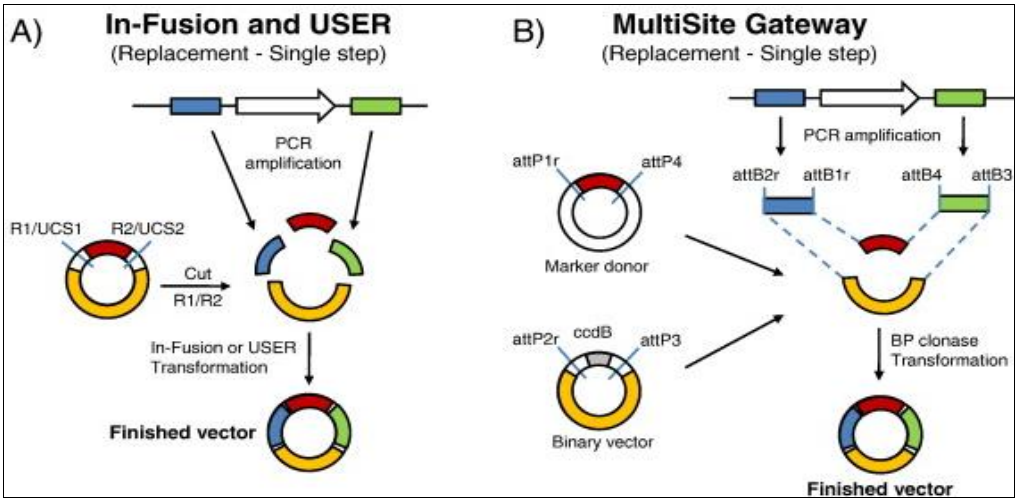

Fig 3: Construction of replacement vectors using MultiSite Gateway, In-Fusion and USER cloning.

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. 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.

USER Friendly cloning

The Uracil-Specific Excision Reagent (USER) cloning technology allows for single-step construction of targeted binary vectors. 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 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 an uracil-DNA glycosylase and the DNA glycosylase-lyase Endo VII (USER-enzyme mix), 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). [18, 23].

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 cloningin vitro transposon mutagenesis or Fusion PCR. [24] 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. 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 [25].

Split-marker

The split-marker technique, also known as bipartite gene targeting, has been used extensively in combination with protoplast-based transformation systems. 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 [26]. 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. 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. The resulting amplicons were then cloned, via RE&L, into binary vectors that were used for ATMT [27].

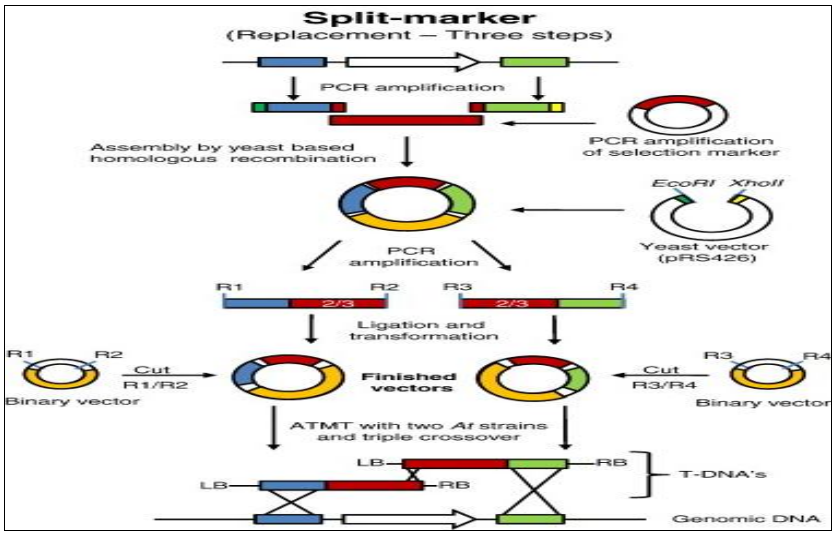

Fig 4: Multistep construction of vectors for split-marker gene replacement.

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.

Agrobacterium virulence factors induce the expression of host DNA

The plant genomic response to *Agrobacterium* infection by measuring gamma H2AX levels, which reflect the levels of double-strand DNA breaks (DSBs), and by characterizing transcription of three major DNA repair marker genes *NAC82*, *KU70*, and *AGO2*. *Agrobacterium* infection does not significantly affect global levels of gamma-H2AX. The authors used western blotting and custom-generated antibodies against the gamma-H2AX mark to measure DNA damage levels. Note that in this approach, the development of the autoradiography may vary between experiments, depending on the global level of gamma-H2AX observed; thus, the intensity of the band for each treatment should be compared to the corresponding mock infiltration control in the same experiment. Figure 5A shows that, in positive control experiments, a strong increase in gamma-H2AX was observed following infiltration of the tobacco tissues with bleomycin (top panel, lanes Bl), a glycopeptide that causes DSBs [28, 29]. This DNA damage occurred already after 6 h and the levels of the gamma-H2AX mark remained relatively stable for up to 72 h of treatment. In contrast, only background amounts of gamma-H2AX were detected after mock infiltration at any of the tested time points (Fig. 5A, top panel, lanes Mo); Coomassie blue staining confirmed equal loading of all samples (Fig. 5A, bottom panel).

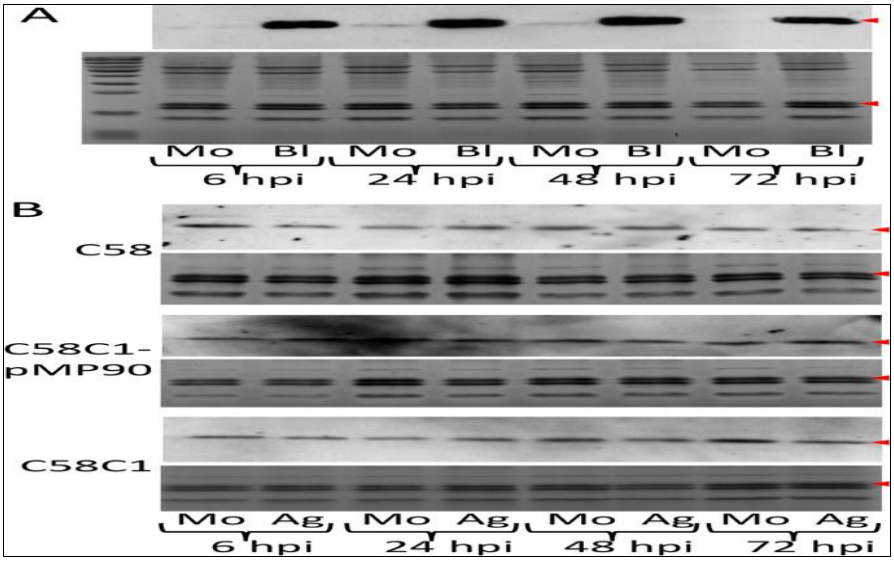

Fig 5: Western blot analysis of the gamma-H2AX content in tobacco leaves infiltrated with bleomycin and different *Agrobacterium* strains.

(A) Infiltration with bleomycin. Mo, mock infiltration; Bl, bleomycin infiltration. (B) Infiltration with *Agrobacterium* strains C58, C58-C1, and C58C1 pMP90Mo, mock infiltration; Ag, *Agrobacterium* infiltration. Tissues were analyzed at 6, 24, 48, and 72 hpi. For each experiment, a western blot and its corresponding Coomassie blue staining are shown in the top and bottom panels, respectively; arrows indicate the electrophoretic mobility of gamma-H2AX. Full-length/uncropped gels and blots are shown in Supplementary Information.

References

1. Gelvin S.B. Agrobacterium and plant genes involved in T-DNA transfer and integration. Annual Review of Plant Physiology and Plant Molecular Biology,2000:51:223–256.
2. Winans SC. Two-way chemical signaling in Agrobacterium-plant interactions. Microbiological Reviews,1992:56:12–31.
3. Cangelosi GA, Ankenbauer RG, Nester EW. Sugars induce the Agrobacterium virulence genes through a periplasmic binding protein and a transmembrane signal protein. Proceedings of the National Academy of Sciences of the United States of America,1990:87:6708–6712.
4. Jin S, Roitsch T, Ankenbauer RG, Gordon MP, Nester EW. The VirA protein of Agrobacterium tumefaciens is autophosphorylated and is essential for vir gene regulation. Journal of Bacteriology,1990:172:525–530.
5. Pazour GJ, Das A. Characterization of the VirG binding site of Agrobacterium tumefaciens. Nucleic Acids Research,1990:18:6909–6913.
6. Michielse CB, Hooykaas PJJ, van den Hondel CAMJ, Ram AFJ. Agrobacterium-mediated transformation as a tool for functional genomics in fungi. Current Genetics,2005:48:1–17.
7. Pitzschke A, Hirt H. New insights into an old story Agrobacterium-induced tumour formation in plants by plant transformation. EMBO Journal,2010:29:1021–1032.
8. De Cleene H, De Ley J. The host range of crown gall. Botanical Review,1976:42:389–466.
9. Bundock P, Hooykaas PJJ. Integration of Agrobacterium tumefaciens T-DNA in the Saccharomyces cerevisiae genome by illegitimate recombination. Proceedings of the National Academy of Sciences of the United States of America,1996:93:15272–15275.
10. Cousin A, Mehrabi R, Guilleroux M, Dufresne M, van der Lee T, Waalwijk C, *et al*. The MAP kinase-encoding gene MgFus3 of the non-appressorium phytopathogen Mycosphaerella graminicola is required for penetration and in vitro pycnidia formation. Molecular Plant Pathology,2006:7:269–278.
11. Gourgues M, Brunet-Simon A, Lebrun MH, Levis C. The tetraspanin BcPls1 is required for appressorium-mediated penetration of Botrytis cinerea into host plant leaves. Molecular Microbiology,2004:51:619–629.
12. Hoffman B, Breuil C. Disruption of the subtilase gene, albin1, in Ophiostoma piliferum. Applied and Environmental Microbiology,2004:70:3898–3903.
13. Kellner EM, Orsborn KI, Siegel EM, Mandel MA, Orbach MJ, Galgiani JN. Coccidioides posadasii contains a single 1,3-beta-glucan synthase gene that appears to be essential for growth. Eukaryotic Cell,2005:4:111–120.
14. Sugui JA, Chang YC, Kwon-Chung KJ. Agrobacterium tumefaciens-mediated transformation of Aspergillus fumigatus an efficient tool for insertional mutagenesis and targeted gene disruption. Applied and Environmental Microbiology,2005:71:1798–1802.
15. Moon YS, Donzelli BGG, Krasnoff SB, McLane H, Griggs MH, Cooke P, *et al*. Agrobacterium-mediated disruption of a nonribosomal peptide synthetase gene in the invertebrate pathogen Metarhizium anisopliae reveals a peptide spore factor. Applied and Environmental Microbiology,2008:74:4366–4380.
16. Ushimaru T, Terada H, Tsuboi K, Kogou Y, Sakaguchi A, Tsuji G, *et al*. Development of an efficient gene targeting system in Colletotrichum higginsianum using a non-homologous end-joining mutant and Agrobacterium tumefaciens-mediated gene transfer. Molecular Genetics and Genomics,2010:284:357–371.
17. Liang X, Teng A, Chen S, Xia D, Felgner PL. Rapid and enzymeless cloning of nucleic acid fragments. United States Patent,2005:6,936,470 B2.
18. Frandsen RJN, Andersson JA, Kristensen MB, Giese H. Efficient four fragment cloning for the construction of vectors for targeted gene replacement in filamentous fungi. BMC Molecular Biology,2008:9:1–10.
19. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene,1989:77:51–59.
20. Michielse CB, Arentshorst M, Ram AFJ, van den Hondel C.A.M.J. Agrobacterium-mediated transformation leads to improved gene replacement efficiency in Aspergillus awamori. Fungal Genetics and Biology,2005:42:9–19.
21. Li L, Shao Y, Li Q, Yang S, Chen F. Identification of Mga1, a G-protein α-subunit gene involved in regulating citrinin and pigment production in Monascus ruber M7. FEMS Microbiology Letters,2010:308:108–114.
22. Zhu B, Cai G, Hall EO, Freeman GJ. In-fusion assembly seamless engineering of multidomain fusion proteins, modular vectors, and mutations. Biotechniques,2007:43:354–359.
23. New England Biolabs. Instruction manual: USER TM Friendly Cloning Kit - A Novel Tool for Cloning PCR Products by Uracil Excision. Version 1.3. New England Biolabs, 2008.
24. Tucker SL, Besi MI, Galhano R, Franceschetti M, Goetz S, Lenhert S, *et al*. Common genetic pathways regulate organ-specific infection-related development in the rice blast fungus. Plant Cell,2010:22:953–972.
25. Paz Z, Garćıa-Pedrajas MD, Andrews DL, Klosterman SJ, Baeza-Montáñez L, Gold SE. One step construction of Agrobacterium-recombination-ready-plasmids OSCAR, an efficient and robust tool for ATMT based gene deletion construction in fungi. Fungal Genetics and Biology,2011:48:677–685.
26. Jeon J, Park SY, Chi MH, Choi J, Park J, Rho HS, *et al*. Genome-wide functional analysis of pathogenicity genes in the rice blast fungus. Nature Genetics,2007:39:561–565.
27. Wang Y, DiGuistini S, Wang TCT, Bohlmann J, Breuil C. Agrobacterium-mediated gene disruption using split-marker in Grosmannia clavigera, a mountain pine beetle associated pathogen. Current Genetics,2010:56:297–307.
28. Menke M, Chen I, Angelis KJ, Schubert I. DNA damage and repair in Arabidopsis thaliana as measured by the comet assay after treatment with different classes of genotoxins. Mutation Research,2001:493:87–93. https://doi.org/10.1016/s1383-5718(01)00165-6.
29. Kim JH, Ryu TH, Lee SS, Lee S, Chung BY. Ionizing radiation manifesting DNA damage response in plants: an overview of DNA damage signaling and repair mechanisms in plants. Plant Science,2019:278:44–53. https://doi.org/10.1016/j.plantsci.2018.10.013.