Agrobacterium-Mediated Transformation of Yeast and Fungi

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Contents

Abstract Two decades ago, it was discovered that the well-known plant vector *Agrobacterium tumefaciens* can also transform yeasts and fungi when these microorganisms are co-cultivated on a solid substrate in the presence of a phenolic inducer such as acetosyringone. It is important that the medium has a low pH (5–6) and that the temperature is kept at room temperature (20–25 °C) during co-cultivation. Nowadays, *Agrobacterium*-mediated transformation (AMT) is the method of choice for the transformation of many fungal species; as the method is simple, the transformation efficiencies are much higher than with other methods,

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and AMT leads to single-copy integration much more frequently than do other methods. Integration of T-DNA in fungi occurs by non-homologous end-joining (NHEJ), but also targeted integration of the T-DNA by homologous recombination (HR) is possible. In contrast to AMT of plants, which relies on the assistance of a number of translocated virulence (effector) proteins, none of these (VirE2, VirE3, VirD5, VirF) are necessary for AMT of yeast or fungi. This is in line with the idea that some of these proteins help to overcome plant defense. Importantly, it also showed that VirE2 is not necessary for the transport of the T-strand into the nucleus. The yeast *Saccharomyces cerevisiae* is a fast-growing organism with a relatively simple genome with reduced genetic redundancy. This yeast species has therefore been used to unravel basic molecular processes in eukaryotic cells as well as to elucidate the function of virulence factors of pathogenic microorganisms acting in plants or animals. Translocation of *Agrobacterium* virulence proteins into yeast was recently visualized in real time by confocal microscopy. In addition, the yeast 2-hybrid system, one of many tools that have been developed for use in this yeast, was used to identify plant and yeast proteins interacting with the translocated *Agrobacterium* virulence proteins. Dedicated mutant libraries, containing for each gene a mutant with a precise deletion, have been used to unravel the mode of action of some of the *Agrobacterium* virulence proteins. Yeast deletion mutant collections were also helpful in identifying host factors promoting or inhibiting AMT, including factors involved in T-DNA integration. Thus, the homologous recombination (HR) factor Rad52 was found to be essential for targeted integration of T-DNA by HR in yeast. Proteins mediating double-strand break (DSB) repair by end-joining (Ku70, Ku80, Lig4) turned out to be essential for non-homologous integration. Inactivation of any one of the genes encoding these end-joining factors in other yeasts and fungi was employed to reduce or totally eliminate non-homologous integration and promote efficient targeted integration at the homologous locus by HR. In plants, however, their inactivation did not prevent non-homologous integration, indicating that T-DNA is captured by different DNA repair pathways in plants and fungi.

1 Introduction

Agrobacterium tumefaciens causes crown gall disease on many dicotyledonous plant species and some gymnosperms (De Cleene and De Ley 1976). Below we present a short introduction to the molecular mechanisms underlying this disease, but for more details about *Agrobacterium* biology and literature references, see the following reviews: Nester et al. 1984; Winans 1991; Zhu et al. 2000; Tzfira et al. 2000; Gelvin 2003, 2010; and the other chapters in this volume.

Crown galls consist of cells that have been transformed into tumor cells by the transfer of an oncogenic piece of DNA, transferred DNA or T-DNA, from the bacterium. T-DNA is a segment of DNA that is naturally present in a large Ti plasmid in *Agrobacterium*. It contains a number of oncogenes (*onc*-genes) that

encode enzymes involved in the production of plant growth regulators. Transfer of T-DNA to plant cells leads to their uncontrolled growth and thus to tumor formation. None of the T-DNA genes is involved in T-DNA transfer. Rather, a set of genes (the virulence genes), which are located elsewhere in the Ti plasmid, are needed for the mobilization of T-DNA into plant cells. These *vir* genes act *in trans* to process and transfer T-DNA, which is surrounded by direct repeat (border repeat) sequences of 24 bp. This has led to the development of the binary vector system consisting of an *Agrobacterium* strain containing a Ti plasmid from which the T-DNA has been removed (helper strain) and a separate cloning vector containing a plant selection marker between 24 bp border repeats into which genes of interest can be cloned (binary vector). Nowadays, *Agrobacterium* is often the preferred vector for plant transformation in plant biotechnology and plant research. This is because of the ease of handling, the use of plant tissues as targets for transformation rather than protoplasts, and the relatively low cost associated with the use of *Agrobacterium* as a vector in comparison to other methods requiring expensive equipment such as an electroporator or a particle gun.

The virulence (*vir)* genes are activated in an acidic environment (pH 5–6) when the bacteria sense the presence of phenolic compounds such as acetosyringone, which are released from wounded plant cells. The VirA chemoreceptor becomes activated by autophosphorylation when the proper inducing conditions are met. Subsequently, VirA activates the transcriptional activator VirG by phosphorylation, which then mediates transcription of the other *vir* genes. These include the *vir*B operon (with 11 genes) and the *virD* operon (with 4 or 5 genes; intact *virD3* is absent in some Ti plasmids), which are essential for transformation. The *virB* operon encodes a type four secretion system (T4SS) which is the nanomachine for delivery of T-DNA and a number of virulence effector proteins into host cells. The *virD* operon encodes the VirD2 relaxase and its associated protein VirD1, which initiate T-DNA transfer by nicking the border repeats. This leads to release of single-stranded DNA copies of the T-DNA (T-strands) that are translocated into plant cells. The VirC1 and VirC2 proteins are accessory factors which enhance nicking of the border repeats by VirD2 and thus potentiate transformation. The VirD4 protein is a coupling protein which forms the interface between the relaxase and the T4SS. Some other virulence proteins do not act in the bacterium, but are translocated by the T4SS into the host cells, where they assist in transformation. VirE2 protein is especially important, as plant transformation occurs with a 1000–10,000-fold lower efficiency in its absence. The VirE2 protein encodes a single-stranded DNA binding protein that is thought to coat the T-strand in the plant cell and thus protects it against nucleases. VirE2 may also assist in the delivery of the T-strand into the nucleus. The VirD2 protein, which remains covalently attached to the T-strand during the nicking reaction, contains a nuclear localization sequence, which is essential for nuclear delivery. Besides VirE2, effector proteins transferred by *A. tumefaciens* into host cells by the T4SS include VirE3, VirF, and VirD5. Their localization in plant cells and more recently also in yeast cells (Fig. 1) has been determined, but the functions of these proteins are still not fully understood.

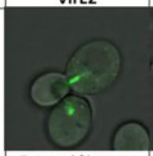
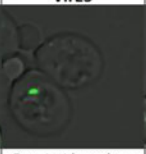
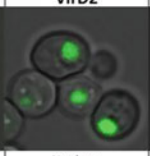
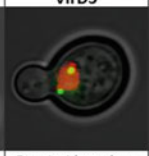
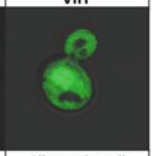
	VirE2	VirE3	VirD2	VirD5	VirF
Localization after ectopic expression					
	Dots and filaments outside nucleus	Dots inside nucleus and near spindle pole bodies	Nucleus	Dots inside nucleus	All over the cell
Possible function	ssDNA binding protein	Transcriptional enhancer	Relaxase; guides T-strand through T4SS	Disruption of kinetochore-microtubule interaction	F-box protein involved in protein degradation
Essential for *S. cerevisiae* transformation?	No	No	Yes	No	No

Fig. 1 Visualization of the *Agrobacterium* virulence proteins fused to GFP (green color) expressed from a yeast promoter in yeast cells

In plants, exogenous DNA integrates with high efficiency by non-homologous recombination. This is also the case for T-DNA, whether it contains homology with the plant genome or not. The ends of T-DNA are reasonably well protected during integration, with sometimes full preservation of the right border end and with usually only a small truncation of the left border end. Integration may be accompanied by the formation of small deletions in the host genome at the integration site. Data regarding the preferred integration site are likely to be biased by the demand for expression of T-DNA in the cell lines that are selected as T-DNA transformants. In the transformants obtained, however, integration seems to be random, and therefore, T-DNA integration can and has been successfully used as a mutagen leading to (T-DNA) tagged mutations.

Although tumors are not formed on monocots, infection with *Agrobacterium* can still lead to transformation of these plants, which include important food crops such as the cereals (Hooykaas-van Slogteren et al. 1984; Ishida et al. 1996). This prompted us to determine whether other organisms could be transformed by *Agrobacterium* as well. In view of the resemblance of AMT to bacterial conjugation, we first tested and found evidence that the *Agrobacterium* virulence system could mobilize plasmids to other bacteria (Beijersbergen et al. 1992). Some twenty years ago, we could show that *Agrobacterium* can also transform the yeast *S. cerevisiae* (Bundock et al. 1995) and fungi (De Groot et al. 1998) under laboratory conditions. Since then, *Agrobacterium*-mediated transformation (AMT) of many other yeasts and fungi has been demonstrated (reviewed in: Michielse et al. 2005a; Soltani et al. 2008). The ability of *Agrobacterium* to transform the yeast *S. cerevisiae* offered the possibility to use the many experimental tools available for this organism to study the transformation process in detail. In this chapter, we will review the molecular mechanisms underlying AMT of yeast and fungi and compare the requirements with those for AMT of plants. We shall not, however, discuss

detailed practical requirements of AMT for yeast and fungi as these have already been discussed in earlier reviews (Michielse et al. 2005a, 2008; Soltani et al. 2008). Instead, we rather focus on the more recent discoveries using yeast as a model to study *Agrobacterium* virulence effectors.

2 Yeasts and Fungi Transformed by *Agrobacterium*

In the early 1990s, it was discovered that the yeast *S. cerevisiae* can be transformed by *Agrobacterium* during co-cultivation on plates with *vir*-induction medium (Bundock et al. 1995; Piers et al. 1996). A few years later, the transformation of mycelium-forming fungi, including *Aspergillus awamori*, *Colletotrichum gloeosporioides*, *Fusarium venenatum*, *Neurospora crassa*, *Trichoderma reesei*, and the edible white button mushroom *Agaricus bisporus*, was reported (De Groot et al. 1998). Since then, *Agrobacterium*-mediated transformation (AMT) has been demonstrated for numerous other yeasts and fungi (reviewed in: Michielse et al. 2005a; Soltani et al. 2008), including species from the phyla Ascomycota, Basidiomycota, Glomeromycota, and Zygomycota. These encompass fungi important for industry (De Groot et al. 1998), plant and animal pathogens (Almeida et al. 2007; Betts et al. 2007; Blaise et al. 2007; Bourras et al. 2012; Jeon et al. 2007; Nemecek et al. 2006; Zhang et al. 2015), and fungal species living in a symbiosis with plants (mycorrhiza) or algae (lichens) (Murata et al. 2006; Pardo et al. 2002; Park et al. 2013). Edible species such as the mushrooms *A. bisporus* (de Groot et al. 1998; Mikosch et al. 2001), *Flammulina velutipes*, *Grifola frondosa*, and *Hypsizygus marmoreus* (Hatoh et al. 2013; Zhang et al. 2014), *Pleurotus eryngii* (Wang et al. 2016), *Tricholoma matsutake* (Murata et al. 2006), and the truffle *Tuber borchii* (Grimaldi et al. 2005) have also been transformed by AMT.

For fungi, AMT has several advantages over conventional transformation methods. First, the use of protoplasts, required in most other methods of transformation, can mostly be omitted in AMT (de Groot et al. 1998). The isolation of protoplasts is not only laborious, but also depends on the quality of the cell wall-degrading enzymes, which are not always commercially available (Gardiner and Howlett 2004; Rolland et al. 2003). It is therefore a great advantage that intact yeast cells, germinating conidia, or even vegetative and fruiting body mycelia can often be used as starting material for AMT. Secondly, some fungal species including *Agaricus bisporus*, *Armillaria mellea*, *Calonectria morganii*, *Ceratocystis resinifera*, and *Helminthosporium turcicum*, that could not be stably transformed by any of the traditional methods, could be transformed by *Agrobacterium* (de Groot et al. 1998; Loppnau et al. 2004; Malonek and Meinhardt 2001; Degefu and Hanif, 2003; Baumgartner et al. 2010). Sometimes, however, setting up a system for AMT can meet with difficulties, and AMT was not successful for the species *Sclerotinia sclerotiorum* (Rolland et al. 2003) and the black yeast *Knufia petricola* (Noack-Schönmann et al. 2014). Thirdly, AMT is preferred in many fungal species because it leads to less complex DNA integration patterns than does polyethylene

glycol (PEG) transformation or electroporation and also results in a much higher frequency of single-copy events (Betts et al. 2007; Blaise et al. 2007; Campoy et al. 2003; Kilaru et al. 2009; Meyer et al. 2003; Rogers et al. 2004; Sugui et al. 2005; Tanguay and Breuil 2003).

The binary vectors used for AMT of yeast and fungi have a selectable marker between the T-DNA borders, just like those used for plants. As with plants, antibiotic resistance genes and herbicide resistance genes have been used as selection markers in yeast and fungi (Frandsen 2011). It is important that these markers are controlled by a promoter active in the host organism. In some fungi, the Cauliflower Mosaic Virus 35S promoter, which is frequently used for plant transformation, can be employed to drive expression of the selectable marker (Mullins et al. 2001). However, an endogenous promoter or a promoter from a related fungus confers better growth in the selection medium (Mullins et al. 2001; White and Chen 2006). Also, sometimes a 5′ intron is required for sufficient expression (Kilaru et al. 2009). Genes such as *URA3*, *TRP1*, and *LEU2* have also been used as selection markers, especially for the transformation of auxotrophic mutants of the yeast *S. cerevisiae* (Bundock et al. 1995; Piers et al. 1996).

Various *Agrobacterium* helper strains have been used for the transformation of yeast and fungi. The most popular are the strains AGL1, EHA105, LBA1100, LBA1126, and LBA4404. Systematic comparisons of different strains in relation to transformation frequencies have not been published, but the use of *Agrobacterium* strains containing helpers derived from the supervirulent pTiBo542 plasmid (AGL1, EHA105) or with mutations leading to higher virulence gene expression (LBA1126) often compared favorably in their transformation performance (Campoy et al. 2003; Park and Kim 2004; Piers et al. 1996). The introduction of a construct carrying the *virG* mutant gene coding for the constitutively active VirGN54D transcriptional activator can also sometimes considerably improve transformation efficiency (Betts et al. 2007).

Transformation efficiency is influenced by many variables, with each fungus requiring slightly different conditions to obtain an optimal transformation frequency. Factors affecting the transformation efficiency include: the starting material (mycelium, conidiospores, fruiting bodies, protoplasts); the ratio between *Agrobacterium* and recipient cells; the length of the co-cultivation period, whereby a longer period generally yields more transformants, but these are usually more difficult to select from the co-cultivation mixtures; concentration of the inducer acetosyringone; a temperature of between 20 and 25 °C is usually optimal, but cold-adapted fungus *Pseudogymnoascus destructans* is only transformed by *Agrobacterium* at temperatures between 15 and 18 °C (Zhang et al. 2015); pH between 5.0 and 5.3, and the choice of the solid support (nitrocellulose, cellophane, Hybond, etc.) (Almeida et al. 2007; Betts et al. 2007; Flowers and Vaillancourt 2005; Leclerque et al. 2004; Tsuji et al. 2003; White and Chen 2006; Yousefi-Pour et al. 2013; Zhang et al. 2014). The optimal pH (usually between pH 5.0 and 5.3) also depends on the *Agrobacterium* strain used, as the pH requirements for optimal *vir* gene induction are slightly different for different *Agrobacterium* strains (Turk et al. 1991). It has been reported that AMT of *S. cerevisiae* mutants deficient in

purine biosynthesis was more efficient than that of the wild-type (Roberts et al. 2003; Soltani 2009) and that addition of purine synthesis inhibitors during AMT of tobacco resulted in increased transformation (Roberts et al. 2003). However, it was subsequently found that the effects of nucleobase auxotrophies and purine synthesis inhibitors are rather species- or even strain-specific. For instance, adenine auxotrophy did not increase transformation in *Cryptococcus neoformans* and *C. gattii*, but uracil auxotrophy gave larger numbers of transformants in some strains (McClelland et al. 2005). Addition of a purine synthesis inhibitor even led to a strongly decreased transformation frequency in the fungus *Paracoccidioides brasiliensis* (Almeida et al. 2007).

3 T-DNA

3.1 T-DNA Structure in Yeast and Fungi

The T-strand is converted into a dsDNA molecule upon arrival in the host nucleus. Whether this occurs before or during the integration process is still uncertain, although data indicate that both may be possible (Chilton and Que 2003; Tzfira et al. 2003; Van Kregten et al. 2016). In *S. cerevisiae*, AMT occurred only at a low frequency when T-DNA lacked homology with the yeast genome. Integrants revealed that, as in plants, the T-DNA ends were relatively well preserved during integration in the yeast genome, sometimes ending exactly at the site where the Ti plasmid DNA had been nicked by VirD2 (Bundock and Hooykaas 1996; Bundock et al. 2002). Small genomic deletions were often found at the T-DNA insertion sites, and filler sequences may be present, which was similarly seen in other yeasts and fungi (Choi et al. 2007; Li et al. 2007; Meng et al. 2007). Sometimes, as in plants, T-DNA integration in fungi is accompanied by gross genomic rearrangements such as large deletions, inversions, or translocations (Choi et al. 2007; Li et al. 2007; Michielse et al. 2009). Remarkably, whereas *S. cerevisiae* T-DNA transformants only have a single copy of the (non-homologous) T-DNA inserted in the genome (Bundock et al. 2002), the integration of multiple copies of T-DNA has been reported for other yeasts and fungi. These may be present in an inverted or direct repeat at one locus or located at a few different loci in the genome (Betts et al. 2007; Campoy et al. 2003; Combier et al. 2003; de Groot et al. 1998; Degefu and Hanif 2003; Flowers and Vaillancourt 2005; Li et al. 2007; Meng et al. 2007; Malonek and Meinhardt 2001; Michielse et al. 2004a, 2009; Mullins et al. 2001; Park et al. 2013; Rho et al. 2001; Sullivan et al. 2002; Tanguay and Breuil 2003; Tsuji et al. 2003). Whether single copy or multi-copy integration, predominates may also depend on the transformation conditions, i.e., the type of tissue transformed, the presence of inducer in the pre-culture medium, duration of the co-cultivation, and the ratio of the numbers of *Agrobacterium*:fungus cells used (Combier et al. 2003; Mikosch et al. 2001; Rho et al. 2001; Sullivan et al. 2002). As mentioned above, T-DNA integration appears to occur at fairly random positions in the plant genome. Without provided homology to

the genomic DNA, the same is observed in yeasts and fungi, although there may sometimes be some bias toward intergenic/regulatory regions, possibly because selection of transformants requires expression of the selection marker on the T-DNA (Blaise et al. 2007; Bourras et al. 2012; Choi et al. 2007; Li et al. 2007; Meng et al. 2007; Walton et al. 2005). By integration, T-DNA can inactivate the gene at the insertion site, resulting in an insertion mutation. Therefore, when efficient, AMT has been used to create collections of T-DNA insertion mutants useful to identify tagged mutations both in plants (Krysan et al. 1999) and in fungi, including the human pathogens *Cryptococcus neoformans* (Walton et al. 2005) and *Blastomyces dermatitidis* (Nemecek et al. 2006), and the phytopathogens *Fusarium oxysporum* (Michielse et al. 2009), *Leptosphaeria maculans* (Bourras et al. 2012), and the rice blast fungus *Magnaporthe oryzae* (Betts et al. 2007; Jeon et al. 2007). In plants, mutant phenotypes seen are not always due to T-DNA insertion, but may be due to other genetic or epigenetic changes brought about by the transformation procedure. This was also observed in T-DNA mutant collections in fungi: From a low percentage up to about a third to half of the mutant phenotypes were not due to a T-DNA disruption of a particular gene, depending on the species involved (Blaise et al. 2007; Idnurm et al. 2004: Walton et al. 2005). This lack of linkage between the T-DNA disruption and phenotype is, however, not unique to AMT, but has also been observed in transformants obtained in other ways (Mullins et al. 2001).

3.2 Integration of T-DNA by Homologous Recombination

In contrast to plants and many fungi, the yeast *S. cerevisiae* integrates exogenous DNA preferentially by homologous recombination (HR). This turned out to be the case also for T-DNA: T-DNAs embracing a segment homologous to the yeast genome gave 100–1000-fold higher transformation frequencies than did T-DNAs lacking such homology (Bundock et al. 1995; Bundock and Hooykaas 1996). This is not the case in plants, where T-DNAs with and without homology are integrated with similar efficiency and almost exclusively by non-homologous recombination (Offringa et al. 1990). This showed that the host cell largely determines the fate of T-DNA. When replacement vectors were used, which harbor a T-DNA where a selectable marker is surrounded by sequences homologous to an endogenous genomic sequence of the recipient host cell, not only HR-directed replacement events, but also HR-directed insertions of the complete T-DNA were found, both in about equal frequency (Bundock et al. 1995; van Attikum and Hooykaas 2003). In the case of insertion of the complete T-DNA, the transferred T-DNA must have formed a circular molecule before integration. DNA sequencing revealed that the circles had formed by a precise fusion of the parts of the right and left border repeats that entered the host as part of the T-strand, reconstituting a complete (mixed) border repeat (Bundock et al. 1995). This can be ascribed to the strand transferase activity of the VirD2 protein, which is the reversal of its nicking activity (Pansegrau et al. 1993). T-circle formation has also been observed in plants, but

here circles had deletions of the ends and were also sometimes accompanied by insertions of filler DNA, thus more resembling non-homologous T-DNA integration (Singer et al. 2012). With T-DNA insertion vectors (with the border repeats not at the outside flanks of the homologous sequences, but with the homology disrupted internally by the border repeats), evidence for integration by gap-repair was obtained, although also both insertion and replacement events were seen (Risseeuw et al. 1996). Integration of T-DNA by HR was fully dependent on the action of the *RAD52* gene (van Attikum and Hooykaas 2003).

In other yeasts such as *Kluyveromyces lactis* and fungi, the frequency of AMT is similar whether the vector contains homology or not. When homology is present in the vector, integration may preferentially occur by homologous recombination (Amey et al. 2003; Lee and Bostock 2006; Michielse et al. 2005a; Sugui et al. 2005; Yu et al. 2015; Zhang et al. 2003; Zwiers and de Waard 2001) or still by non-homologous recombination (Gardiner and Howlett 2004; Loppnau et al. 2004; McClelland et al. 2005), depending on the species. This may also depend on the length of homology that is offered by the vector, with only longer segments of homology promoting integration by HR in some species (Michielse et al. 2005a; Zhang et al. 2003). In contrast to *S. cerevisiae*, where the insertion events were all insertions of a single copy of the re-circularized T-DNA, in other yeasts and fungi such as *K. lactis* and the brown rot pathogen *Monilinia fructicola*, multiple copies of T-DNA had sometimes integrated in tandem at the homologous locus in the chromosome (Bundock et al. 1999; Lee and Bostock 2006).

3.3 Extrachromosomal T-DNA

High transformation frequencies were seen in yeast when a T-DNA was transferred that could be maintained as a plasmid or mini-chromosome without the need for integration into any of the chromosomes. This was accomplished in several ways. Firstly, by the addition of the replication unit of the yeast 2μ plasmid to T-DNA: Such T-DNAs were stably maintained as circular autonomous plasmids, which had the transferred parts of the left and right border repeat fused back to a complete (mixed) border repeat (Bundock et al. 1995); secondly, by the inclusion of a chromosomal autonomous replicating sequence (ARS) in T-DNA (Piers et al. 1996; Rolloos et al. 2014; Ohmine et al. 2016). When such T-DNA also contained telomeric repeats adjacent to both border repeats, it could be maintained as an unstable mini-chromosome (Piers et al. 1996). In the absence of telomeric repeats, the transfer frequency of T-DNA dropped 500-fold and only colonies with a chromosomally integrated T-DNA were obtained (Piers et al. 1996). Yeast is unique in having very small chromosomal centromeres (CENs), and addition of such a CEN in addition to an ARS to T-DNA resulted in T-DNAs that were transferred at high frequency and were stably maintained as a single-copy plasmid (Rolloos et al. 2014; Ohmine et al. 2016). It is remarkable that the homologous repair protein Rad52, but not the crucial end-joining factor yKu70, turned out to be

important for T-circle formation: In the *rad52* mutant, transformation was reduced by 75% (Rolloos et al. 2014; Ohmine et al. 2016). When in a control experiment an identical linearized T-DNA vector was introduced by lithium acetate transformation, a similar transformation frequency was seen in both the wild-type yeast and the *rad52* mutant. Taken together, these results suggest that concatemers of T-strands were formed upon AMT by a process of strand-transfer catalyzed by VirD2, for which such activity has been demonstrated in in vitro experiments (Pansegrau et al. 1993). These concatemers could subsequently be resolved into T-circles by HR. Only at most 25% of the T-circles would then be formed immediately from single T-strands by the VirD2 strand transferase activity linking its left and right border parts. This would lead to stable transformants in the absence of HR (Rolloos et al. 2014). In a similar series of experiments, a T-DNA vector was used that, in addition to ARS and CEN sequences, also had telomeric repeats at both ends of T-DNA. After transfer by AMT, this T-DNA could be maintained in yeast either as a linear mini-chromosome or as a T-circle. In the *rad52* mutant, the transfer was strongly reduced, in line with what was described above. Interestingly in the remaining transformants only T-circles, but no linear mini-chromosomes, were found, suggesting that *RAD52* is needed for conversion of the introduced T-strands into a mini-chromosome (Ohmine et al. 2016).

4 Role of Virulence Proteins in AMT of Yeast and Fungi

Based on the requirement of acetosyringone to obtain transformants, and on the structure of integrated T-DNA, it was inferred that transformation of yeasts and fungi by *A. tumefaciens* was mediated by the virulence system (Bundock et al. 1995; de Groot et al. 1998; Piers et al. 1996). Indeed, mutation of *Agrobacterium* genes in one of the key components required for the induction of the T-DNA transfer system (VirA, VirG), or the subsequent generation (VirD1, VirD2) and transport of the T-strand (VirD4, VirB1-11), completely abolished the ability to transform either the yeast *S. cerevisiae* or the fungus *A. awamori.* These results showed that the *virA*, *virB*, *virD*, and *virG* genes that are essential for plant transformation are likewise needed for transformation of yeast (Bundock et al. 1995; Piers et al. 1996) and the fungus *A. awamori* (Michielse et al. 2004b). The products of the *virC* genes, VirC1 and VirC2, are DNA-binding proteins which enhance the nicking reaction of VirD2 at the border repeats and thus the formation of the T-strands (Atmakuri et al. 2007). In plants, *virC* mutants are attenuated in virulence. Similarly, a tenfold reduced transformation is seen with *virC* mutants in yeast and fungi. Fungal transformants in this case were characterized by the presence of complex T-DNA structures containing multicopy and truncated T-DNAs and vector backbone sequences (Michielse et al. 2004b). This is in line with reduced T-DNA border processing in the *virC* mutants, and thus reduced T-DNA transfer and increased left border skipping. That AMT occurs by a similar process in plants, yeasts, and fungi is also in line with the observation that similar pH and temperature conditions are required for both

plant and fungal transformation. A low pH of 5–6 is required for the induction of the *vir* genes, whereas temperatures of around 22 °C are optimal for the T-DNA transfer machinery (Fullner and Nester 1996). The *Agrobacterium* virulence system has also been used to transfer into yeast a modified CloDF13 plasmid, in which a yeast selectable marker and the replication unit of the yeast 2μ plasmid had been inserted. As this CloDF13 plasmid encoded its own relaxase and its own coupling protein and had its own nick site, transfer did not require any of the Agrobacterium *virD* genes, but only relied on expression of the *virB* encoded transport system (Escudero et al. 2003).

Some important differences between the AMT requirements of plants and fungi were noticed regarding the effector proteins that are delivered by *Agrobacterium* into host cells concomitantly with T-DNA. As a single-strand DNA-binding protein, VirE2 is thought to play an important role in the protection of the T-strand from nucleases in the host cell. In the absence of VirE2, there is almost no transformation of most plants (Rossi et al. 1996). Transformation of yeast and fungi by *virE2* mutants still occurs, but at 2–10-fold reduced frequency (Bundock et al. 1995; Michielse et al. 2004a). The *A. awamori* transformants obtained after transformation with a *virE2* mutant had more pronounced left border truncations (Michielse et al. 2004b), indicating that VirE2 in fungi, as in plants, may help to protect the T-strand against nucleases. The translocated effector proteins VirE3, VirF, and VirD5, necessary for optimal infection of plants, were both alone and in combination dispensable for transformation of yeast (Bundock et al. 1995; our unpublished results) and *A. awamori* (Michielse et al. 2004a).

5 Use of Yeast to Study the *Agrobacterium* Virulence System

5.1 Visualization of Effector Protein Translocation

Although the absence of the translocated effector proteins does not prevent AMT of yeasts or fungi, these proteins are still translocated efficiently into yeast and, by inference, into fungal cells. To study protein transfer from *Agrobacterium* to yeast, the Cre recombinase reporter assay for translocation (CRAfT) was used (Vergunst et al. 2000). To this end, fusions between the Cre recombinase and Vir proteins were expressed in *Agrobacterium*. Transfer of the Cre–Vir fusion proteins from *Agrobacterium* to yeast was subsequently monitored by the selectable excision of a floxed *URA3* marker gene (i.e., *URA3* surrounded by *lox*-sequences in a direct repeat) from the yeast genome by the Cre recombinase. In this way, the translocation of the VirE2, VirE3, and VirF proteins into yeast cells was demonstrated (Schrammeijer et al. 2003). More recently, the translocation of VirE2 protein into yeast cells could be visualized by using bimolecular fluorescence complementation (BiFC) and split GFP strategies (Li et al. 2014; Sakalis et al. 2014). To this end, *Agrobacterium* strains expressing VirE2 tagged with one part of a fluorescent protein

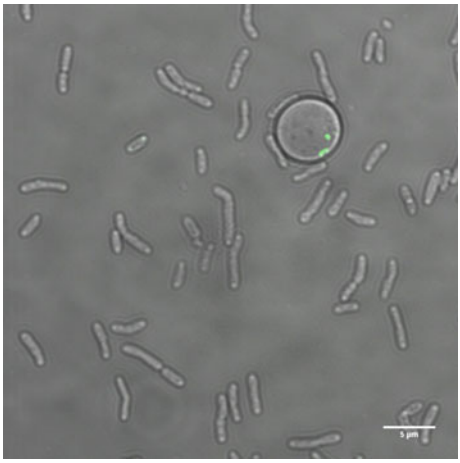


Fig. 2 Co-cultivation of *Agrobacterium* (rods) and yeast (the globular cell). Green color shows translocation of VirE2 from *Agrobacterium* into a yeast cell and is visualized by a split GFP approach

were co-cultivated with yeast cells expressing the complementary part, either fused to VirE2 (for BiFC) or not (Split GFP). Fluorescent dots and filaments were visible in recipient cells 20–25 h after the start of the co-cultivation, indicative of VirE2 protein translocation (Fig. 2). Under optimal conditions, a fluorescent signal was sometimes seen already 8 h after the start of co-cultivation. Evidence was obtained that the fluorescent filaments co-localized with microtubules, as they disappeared after treatment with benomyl (Sakalis et al. 2014). Interestingly, Salman et al. (2005) showed that "animalized VirE2" is able to move along microtubules in Xenopus cells. Formation of these fluorescent structures in the yeast cell was independent of T-DNA transfer. By a similar strategy, the translocation of the other Vir effector proteins (VirE3, VirF, VirD2, and VirD5) could be followed in real time (Sakalis 2013; Roushan, Hooykaas and van Heusden, unpublished).

5.2 *Functional Analysis of Translocated Effector Proteins in Yeast*

The yeast 2-hybrid system has invariably been the starting point for the identification of plant interaction partners of *Agrobacterium* virulence proteins. In this way, it was found that VirD2 protein could interact with a set of plant cyclophilins (peptidyl-prolyl *cis-trans* isomerases), which also function as chaperones (Deng et al. 1998). In addition, VirD2 interacted with importin α/karyopherin α via its C-terminal nuclear localization sequence (NLS), which is necessary for nuclear import (Ballas and Citovsky 1997). Bhattacharjee et al. (2008) showed that both VirD2 and VirE2 could interact in yeast with multiple *Arabidopsis* importin α isoforms. By means of a yeast 2-hybrid screen, two interactors were also identified for VirE2, which were called VIP1 and VIP2 (Tzfira et al. 2000). Both VIP1 (Lacroix and Citovsky 2013) and VIP2 (Anand et al. 2007) act as transcription

factors in plant cells. It was proposed that VIP1, by binding to VirE2 molecules coating the T-strand, plays an important role in the transport of the T-complex into the nucleus (Tzfira et al. 2001). However, recently it was found that *vip1* mutants of *Arabidopsis thaliana* are equally well transformed as the wild-type, indicating that VIP1 is not essential for transformation of plants (Shi et al. 2014). Nevertheless, both the VIP1 and the VIP2 protein may assist in transformation by mediating binding of the T-complex to the chromatin (Lacroix et al. 2008). Regarding targeting the T-complex to chromatin, VirD2 may also play a role here as it can bind to the core histone proteins in yeast after entry into the yeast cell during AMT (Wolterink-van Loo et al. 2015).

Arabidopsis Skp1-like ASK proteins were identified by a 2-hybrid screen as interactors of VirF (Schrammeijer et al. 2001). The Skp1-like proteins are essential components of SCF-complexes, which have an important role in eukaryotic cells in the ubiquitination and proteolytic degradation of specific target proteins, which often need to be phosphorylated first (Skowyra et al. 1997). The Skp1-like subunit connects the Cul1 scaffold of the SCF-complex to the F-box subunit which specifies the target proteins to be degraded. After the discovery of the plant Skp1-like proteins as interactors of VirF, the VirF protein sequence was inspected for the presence of an F-box, and indeed such an F-box turned out to be present and essential for the biological function of VirF (Schrammeijer et al. 2001). The hunt for target proteins, which are degraded in the host plant upon the introduction of VirF, has not yet been completed. Initially, it was discovered that VirF can interact with the defense transcription factor VIP1, one of the interactors of VirE2 (Tzfira et al. 2004). Yeast cells expressing GFP-VIP1 lost fluorescence when VirF was expressed. Moreover, yeast cells expressing GFP-VirE2 lost fluorescence when both VIP1 and VirF were co-expressed, suggesting that VirE2 may be degraded under the direction of VirF in the presence of VIP1 (Tzfira et al. 2004). It was hypothesized that VirF may have an important function in the transformation process by the degradation of the VirE2 coat that may be formed on the T-strand in the host cell and which eventually could be inhibitory to T-DNA integration (Tzfira et al. 2004). The VirF protein is important for transformation of plants of the Solanaceae family such as tobacco and tomato, but not of many other plants (Hooykaas et al. 1984). These latter plants may have a host F-box protein which can compensate for the absence of *virF* in the bacterium. A host gene encoding such an F-box protein was identified in *A. thaliana*, and called *VBF* (Zaltsman et al. 2010).

By a yeast 2-hybrid screen, three *Arabidopsis* interactors were identified with VirE3 as a bait. The first was importin α/karyopherin α, with which VirE3 interacts through its NLSs to gain entry into the nucleus (Garcia-Rodriguez et al. 2006). Secondly, the Csn5 subunit of the COP9 signalosome was identified as an interactor. Thirdly, VirE3 also interacts with the host TFIIB-like protein pBrp (Garcia-Rodriguez et al. 2006). While Brp is normally located at the outside of the plastids, it moves to the nucleus in the presence of VirE3, and together with VirE3 it activates the transcription of a set of host genes (Niu et al. 2015). These induced genes include *VBF* (Niu et al. 2015), which was already known to be induced during transformation (Zaltsman et al. 2010). This result explained why *virE3 virF*

double mutants are much more attenuated in virulence on some host plants that are single mutants (Garcia-Rodriguez et al. 2006; Niu et al. 2015).

Transformation of yeast and fungi can occur at high frequencies even in the absence of VirF and/or VirE3 (Bundock et al. 1995; Michielse et al. 2004a; our unpublished results). Just as in plants, as mentioned above, the yeast and fungal genomes may also encode an F-box protein which can compensate for the absence of VirF in AMT. However, mutation of all the individual yeast genes encoding F-box proteins (except for the few essential genes) did not lead in any case to a reduced AMT (Niu 2013). Also, no reduced accumulation of VIP1 or VirE2 was observed in yeast in the presence of VirF in our laboratory, and whereas we observed an interaction of VirF with the *Arabidopsis* Skp1-like proteins, we did not observe a clear interaction with the yeast Skp1 protein (Niu 2013). This may be due to strain differences, but further work is needed to clarify the biological role of VirF. Using a yeast 2-hybrid screen with the VirF protein lacking the F-box as a bait, several new putative interactors have been identified recently (Garcia-Cano et al. 2015, 2018). These include the trihelix-domain transcription factors VFP3 and VFP5 and the GLABROUS1 enhancer/binding protein-like transcription factor VFP4, involved in activation of the defense response. Targeted degradation of these new transcription factors under control of VirF may help to suppress plant defense and increase transformation (Garcia-Cano et al. 2015, 2018). Nevertheless, it is clear that none of the translocated effector proteins VirE2, VirE3, and VirF plays an essential role in the transformation of yeasts and fungi. Therefore, it is likely that these proteins have a plant-specific function, such as in the suppression of plant defense, rather than in a process invariably linked to the transformation process such as uncoating of the T-strand.

The function of VirD5 is still largely unknown. An interaction between VirD5 and VirF was shown by bimolecular fluorescence complementation; further results indicated that VirD5 may protect VirF from proteolytic degradation (Magori and Citovsky 2011). On the other hand, it has been reported that VirD5 is a nuclear competitor of VBF for binding to VIP1 to stabilize VIP1 and VirE2 (Wang et al. 2014). In order to study the function of VirD5, a gene construct that would express VirD5 constitutively in plants was introduced by AMT, but no stable transformants were obtained. Subsequently, a construct was introduced into plants in which VirD5 was expressed from an inducible promoter; this time, transformants were obtained. Induction of VirD5, however, led to an inhibition of growth and death of the seedlings. Stable expression in yeast was also not possible, and therefore, it was not possible to screen for interactors in a yeast 2-hybrid screen. When *virD5* was present behind the inducible *GAL1* promoter no growth occurred on galactose induction medium, but growth was normal in glucose repression medium (Zhang et al. 2017; our unpublished results). Transient expression of a GFP-VirD5 fusion in yeast revealed that VirD5 was present at a few specific sites, (seen as fluorescent dots) in the nucleus (Fig. 1). A yeast deletion library, consisting of about 5000 mutants, was transformed with the p*GAL1*-VirD5 construct to find mutant strains with deletions of genes that normally mediate the toxicity of VirD5; these strains would now survive VirD5 induction by galactose. Three mutants were found that could grow in the

presence of galactose. Two of these were defective in inducing the expression of VirD5 by having deletions in the *GAL3* and *GAL4* genes, respectively. The third one lacked the *SPT4* gene. Interestingly, Spt4 protein had a subcellular localization similar to that of VirD5, showing a limited number of specific dots in the nucleus that represented the centromeres/kinetochores (Crotti and Basrai 2004). Indeed, VirD5 was similarly found to co-localize with proteins such as Ndc10 that form part of the kinetochores, and this localization of VirD5 was dependent on the presence of the Spt4 protein (Zhang et al. 2017). We found that most cells expressing VirD5 displayed a large elongated bud and failed to segregate their chromosomes equally to daughter cells at anaphase. As a consequence, many cells became aneuploid. VirD5 expressing cells also showed a more than tenfold higher loss of mini-chromosomes than did control cells. Subsequently, VirD5 was found by BiFC to interact at the centromeres/kinetochores with the essential mitotic regulatory Ipl1/Aurora kinase (Zhang 2016). This serine/threonine protein kinase plays an essential role in the sensing and correction of erroneous kinetochore–microtubule attachments during mitosis. It phosphorylates key substrates involved in the kinetochore–spindle binding and contributes to the activation of the spindle checkpoint (Biggins et al. 1999). Both loss and overexpression of the Ipl1/Aurora kinases lead to chromosome mis-segregation and aneuploidy in yeast cells. In in vitro experiments, VirD5 stimulated the kinase activity of the Ipl1/Aurora kinase (Zhang 2016). The results obtained with VirD5 in yeast were corroborated in plants: VirD5 was found to interact with the three plant Aurora kinases and to cause chromosome mis-segregation in plant cells (Zhang 2016; Zhang et al. 2017). These results suggest that stimulation of the Aurora kinase by VirD5 leads to a temporary spindle checkpoint, allowing T-DNA more time for integration. Otherwise, aneuploidy is a hallmark of tumor cells and may contribute to crown gall tumor formation.

6 Host Factors

6.1 The Role of Host Proteins During Agrobacterium-Mediated Transformation

By screening large mutant collections for mutants with reduced and increased AMT, many genes affecting AMT have been identified in the model plant *A. thaliana* (Zhu et al. 2003). These include genes involved in chromatin structure and remodeling, cytoskeletal functions, and cell wall structure. As the requirements for AMT may be somewhat different for yeasts and fungi than for plants, Soltani (2009) screened a genome-wide yeast deletion collection for mutants with reduced or enhanced AMT using both a replacement T-DNA vector integrating by HR and a T-DNA vector that could autonomously replicate in yeast by a 2μ replication unit. As in plants, many different host factors were found to affect AMT. Most striking was that deletion strains lacking components of SAGA, SLIK, ADA, and NuA4

histone acetyltransferase complexes including *EAF7*, *GCN5*, *NGG,1* and *YAF9* had a strongly enhanced AMT efficiency, whereas strains lacking components of histone deacetylase complexes such as *HDA2*, *HDA3,* and *HST4* had a strongly diminished AMT efficiency (Soltani et al. 2009). The Yaf9 protein also forms part of the SWR1 chromatin-remodeling complex, and mutation of any of the other components, such as *ARP6* and *EAF6* increases AMT to some extent (Luo et al. 2015; Soltani 2009; Soltani et al. 2009). However, mutation of *ARP6* also leads to disrupted microtubule structures, and this suggests that binding of the T-strand or virulence proteins such as VirE2 to the microtubules may in fact be inhibitory for transformation (Luo et al. 2015). A role of chromatin-related genes in plants has also been reported (Crane and Gelvin 2007).

Genes involved in HR such as *RAD52* were also important for AMT by these two types of T-DNA vector (van Attikum and Hooykaas 2003; Rolloos et al. 2014; Soltani et al. 2009). A yeast deletion collection was screened for factors negatively affecting AMT by a T-DNA vector that contained not only an ARS and CEN sequence, but also telomeric repeats near both border repeats (Ohmine et al. 2016). In this screen reduced AMT was seen again in the *rad52* mutant, but also in mutants with deletions of *SRS2*, encoding a DNA helicase, the cell wall regulator *SMI1*, and the membrane sterol synthesis scaffold gene *ERG28*. The lower efficiency of the *erg28* mutant was probably due to less growth inhibition of the mutant yeast as compared to the wild-type by the presence of *Agrobacterium*, resulting in lower AMT frequencies per recipient (Ohmine et al. 2016). How *SMI1* affects AMT is not yet clear, but virulence protein translocation as measured by the CRAfT assay is also severely affected in this mutant (Ohmine et al. 2016).

6.2 Role of Host DNA Repair Factors in Non-homologous T-DNA Integration

In *S. cerevisiae,* the integration of exogenous DNA by homologous recombination is very efficient, in contrast to integration by non-homologous end-joining. However, in plants and certain fungi, the insertion of exogenous DNA mainly occurs by non-homologous recombination, even when the DNA fragment has extensive sequence homology to the host chromosome (Offringa et al. 1990; Loppnau et al. 2004; Gardiner and Howlett 2004; McClelland et al. 2005). This is not a peculiarity of T-DNA, but is also the case when DNA is introduced by other means. In fact, delivery of a single-stranded DNA molecule protected by virulence proteins, as is the case with AMT, may be beneficial for integration by HR. Introduction of a gene disruption construct by AMT into *K. lactis* gave a large increase in targeted integration as compared to delivery of an identical construct by electroporation (Bundock et al. 1999). A similar increase in gene targeting frequency was seen in *A. awamori* when delivery of a disruption cassette was done by AMT in comparison with PEG transformation; this also allowed the use of shorter

DNA flanking sequences to obtain an adequate gene targeting frequency (Michielse et al. 2005b). In the taxol-producing fungus *Pestalotiopsis microspore*, AMT gave highly efficient gene targeting, whereas the same construct introduced by PEG transformation did not integrate at all, but rather formed unstable extrachromosomal DNAs with telomeric repeats (Yu et al. 2015). For the application of AMT in biotechnology, it is of great importance to improve the efficiency of integration via homologous recombination over non-homologous recombination, as this would not only favor the targeted integration of transgenes at desired safe havens in the genome, but would ultimately also allow the directed modification of any endogenous gene at its locus in the genome. Our goal was therefore to find the genes encoding the proteins involved in non-homologous integration with the aim to inactivate these to obtain host cells that would exclusively integrate transgenes by HR. Using the yeast *S. cerevisiae* as a model we found that the proteins essential for the repair of DSBs in genomic DNA by non-homologous end-joining (NHEJ) (i.e. yKu70, yKu80, Lig4) are essential for non-homologous T-DNA integration (van Attikum et al. 2001; van Attikum and Hooykaas 2003). Binding of the Ku70-Ku80 heterodimer to the ends of a DSB protects these and attracts other proteins such as Nej1 and Lif1 to the DNA ends, eventually leading to ligation of the ends by Ligase 4 (Critchlow and Jackson 1998; Lewis and Resnick 2000). In yeast, the MRX complex, consisting of Mre11-Rad50-Xrs2, also plays a role in end-joining. In the *mre11*, *rad50*, and *xrs2* mutants non-homologous T-DNA integration was 20–50-fold reduced. Residual T-DNA integration in these mutants occurred predominantly at the (sub)telomeric repeats or at the rDNA repeats (van Attikum et al. 2001). Mutations in *RAD51* or *RAD52* did not negatively affect non-homologous T-DNA integration; in the *rad52,* mutant integration was in fact twofold enhanced (van Attikum et al. 2001). In *S. cerevisiae,* the Rad52 and yKu70 proteins play a critical role in determining whether T-DNA is integrated via HR or via NHEJ; when both are inactivated, no T-DNA integration occurs at all (van Attikum and Hooykaas 2003). Histone modifiers and ATP-dependent chromatin-remodeling complexes are recruited to sites of DNA damage (reviewed by Hauer and Gasser 2018; Smeenk and van Attikum 2013) and are necessary for optimal repair. The presence or absence of specific components of such complexes is therefore likely to play an important role in T-DNA integration and may explain the effects of such mutations on T-DNA integration.

In line with the finding that the factors involved in DSB repair by NHEJ are involved in T-DNA integration, T-DNA can be captured at a unique DSB in the plant genome made by the homing endonuclease I-*Sce*I (Salomon and Puchta 1998). Even without selection for this event, integration at such DSB occurred with a frequency of 1–2% of the transformants (Chilton and Que 2003; Tzfira et al. 2003). In our laboratory, we have studied the integration of *Agrobacterium* T-DNA at a single chromosomal DSB created by the HO endonuclease at the *MAT* locus in the yeast genome (van Attikum 2003). We found T-DNA insertions at the DSB with a 1% frequency, when we expressed the HO endonuclease in yeast cells during AMT. We did not obtain any transformants when we performed such experiments with the *yKu70* mutant, showing that yKu70 is essential for T-DNA integration by

NHEJ at a preformed DSB. Conversely, in similar co-cultivation experiments with the *rad52* mutant expressing the HO endonuclease, the percentage of T-DNA insertions at the DSB increased to 16%. Therefore, T-DNA integration at the DSB is likely suppressed by the presence of the Rad52 protein (van Attikum 2003).

Inactivation of Ku70 or Ku80 prevents non-homologous integration and can thus be used to obtain transformants that have integrated the transgenes by homologous recombination (Hooykaas et al. 2000; van Attikum et al. 2001). This idea has been used to promote gene targeting in many different yeasts and fungi, including the yeast *K. lactis* (Kooistra et al. 2004) and the filamentous fungus *Neurospora crassa* (Ninomiya et al. 2004). Gene targeting can be enhanced by the introduction of a DSB in the target locus. We found that the signal for transport by the T4SS of the *Agrobacterium* virulence system lies in the 30 C-terminal amino acids of translocated virulence proteins. Coupling of this transport signal to the C-terminus of heterologous proteins enables their mobilization from *Agrobacterium* to eukaryotic target cells (Vergunst et al. 2005). In this way, recombinases such as Cre and nucleases such as the homing endonuclease I-*Sce*I can be transferred by *Agrobacterium* into host cells together with the T-DNA vector (Vergunst et al. 2000; van Kregten et al. 2009). Translocation of I-*Sce*I together with a gene-targeting vector leads to an increased frequency of gene targeting in yeast if there is a nuclease recognition site at the target locus (Rolloos et al. 2015).

7 Conclusions

AMT has become a widely used tool for transformation of various fungi because AMT has important advantages over other transformation methods. The transformation protocols are relatively simple, frequencies of transformation are relatively high, single-copy integration events are more numerous than with other methods, and gene targeting is sometimes achieved much more easily with AMT. Non-homologous T-DNA integration at random positions in the genome of fungi has made *Agrobacterium* a useful tool for mutagenesis and gene tagging in fungi. Conversely, for biotechnology purposes targeted integration to a safe haven in the genome is preferred, thus guaranteeing stability of the transgene and preventing unwanted mutagenesis. This can easily be realized in the yeast *S. cerevisiae* but is much less efficient or virtually impossible in other yeasts and fungi. Identification of the genes involved in DSB repair by NHEJ as the key factors that control (T-)DNA integration by non-homologous integration in *S. cerevisiae* (van Attikum et al. 2001) enabled the development of strains with strongly improved gene targeting (e.g., Kooistra et al. 2004; Ninomiya et al. 2004). Such targeting is potentiated by inactivating or disrupting one of the key players of NHEJ (i.e. Ku70, Ku80 or Lig4).

The presence of natural T-DNAs has been discovered in the genome of some plant species, including the edible sweet potato (Kyndt et al. 2015). The ability of *Agrobacterium* to transfer T-DNA to yeasts and fungi may also occur in nature, at sites where these microorganisms are living in close proximity and where AMT may

become possible when phenolic *vir* inducers are abundantly secreted by wounded plant cells. Such "natural" transformation has been observed in in vitro experiments, whereby *Agrobacterium* and the fungus *Verticillium albo-atrum* were co-cultivated on plant tissues (potato and carrot slices, tobacco leaves and stems) in the absence of acetosyringone (Knight et al. 2010). Where horizontal transfer of T-DNA may have contributed to the evolution of plant species (Kyndt et al. 2015), it is not unreasonable to suspect that AMT may have likewise contributed to the evolution of some fungi.

The use of the yeast *S. cerevisiae* as a model for the studies of AMT has not only led to the development of an efficient new method for the transformation of various fungi, but also generated new insight in the process of AMT itself. Yeast cells are transparent and do not contain large amounts of endogenous fluorescent compounds, such as chlorophyll and are, therefore, very suitable for microscopical analysis. These attributes enabled the development of a system based on split GFP by which virulence protein translocation into host cells could be visualized in real time; the results of which subsequently could be applied in plants (Li et al. 2014; Sakalis et al. 2014). The finding that AMT of yeast and fungi is still efficient in the absence of the translocated virulence proteins VirD5, VirE3, and VirF indicated that these proteins are not essential for the transformation process itself, but rather have plant-specific functions. The translocated VirE2 protein is slightly different as some reduction in the transformation of yeasts and fungi was seen. Nevertheless, we conclude that even in the absence of VirE2, the T-strand can reach the fungal nucleus and integrate into the genome. As expected, the NLS of VirD2 is essential for AMT and is probably the main factor essential for nuclear targeting. As to (non-homologous) T-DNA integration, studies in yeasts and fungi revealed that this occurred (almost) exclusively by NHEJ. Whereas T-DNA integration is abolished in fungal NHEJ-mutants, *A. thaliana* mutants in *AtKU70*, *AtKU80*, and *AtLIG4* are still efficiently transformed by AMT (Park et al. 2015). Recently, it was found that T-DNA integration in plants occurs through a process of alternative end-joining, which is independent of Ku70, Ku80, and Lig4, but which is mediated by the polymerase PolQ, which is conserved in animals and plants, but not present in yeasts and fungi (van Kregten et al. 2016). This underscored that (T)-DNA integration is determined by the host cell and the DNA recombination enzymes available in the host cell, and thus may occur by entirely different means in different host cells. We conclude that the yeast *S.cerevisiae* is an excellent model organism to start the study of AMT and virulence gene function, but that one should never forget to put the findings to the test in fungi and plants, for which AMT is a preferred method of transformation.

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References

Almeida AJ, Carmona JAC, Cunha C et al (2007) Towards a molecular genetic system for the pathogenic fungus *Paracoccidioides brasilensis*. Fungal Genet Biol 44:1387–1398

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

Anand A, Krichevsky A, Schornack S et al (2007) *Arabidopsis* VIRE2 INTERACTING PROTEIN2 is required for *Agrobacterium* T-DNA integration in plants. Plant Cell 19:1695–1708

Atmakuri K, Cascales E, Burton OT et al (2007) *Agrobacterium* ParA/MinD-like VirC1 spatially coordinates early conjugative DNA transfer reactions. EMBO J 26:2540–2551

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

Baumgartner K, Fujiyoshi P, Foster GD, Bailey AM (2010) *Agrobacterium tumefaciens*-mediated transformation for investigation of somatic recombination in the fungal pathogen *Armillaria mellea*. Appl Environ Microbiol 76:7990–7996

Betts MF, Tucker SL, Galadima N et al (2007) Development of a high throughput transformation system for insertional mutagenesis in *Magnaporthe oryzae*. Fungal Genet Biol 44:1035–1049

Beijersbergen A, Dulk-Ras AD, Schilperoort RA, Hooykaas PJ (1992) Conjugative transfer by the virulence system of *Agrobacterium tumefaciens*. Nature 256:1324–1327

Bhattacharjee S, Lee LY, Oltmanns H, Cao H, Veena Cuperus J, Gelvin SB (2008) IMPa-4, an Arabidopsis importin α isoform, is preferentially involved in Agrobacterium-mediated plant transformation. Plant Cell 20:2661–2680

Biggins S, Severin FF, Bhalla N et al (1999) The conserved protein kinase Ipl1 regulates microtubule binding to kinetochores in budding yeast. Genes Devel 13:532–544

Blaise F, Rémy E, Meyer M et al (2007) A critical assessment of *Agrobacterium tumefaciens*-mediated transformation as a tool for pathogenicity gene discovery in the phytopathogenic fungus *Leptosphaeria maculans*. Fungal Genet Biol 44:123–138

Bourras S, Meyer M, Grandaubert J et al (2012) Incidence of genome structure, DNA asymmetry, and cell physiology on T-DNA integration in chromosomes of the phytopathogenic fungus *Leptosphaeria maculans*. G3 (Bethesda) 2:891–904

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

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

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

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

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

Chilton MD, Que Q (2003) Targeted integration of T-DNA into the tobacco genome at double-stranded breaks: new insights on the mechanism of T-DNA integration. Plant Physiol 133:956–965

Choi J, Park J, Jeon J et al (2007) Genome-wide analysis of T-DNA integration into the chromosomes of *Magnaporthe oryzae*. Mol Microbiol 66:371–382

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

Crane YM, Gelvin SB (2007) RNAi-mediated gene silencing reveals involvement of *Arabidopsis* chromatin-related genes in *Agrobacterium*-mediated root transformation. Proc Natl Acad Sci U S A 104:15156–15161

Critchlow SE, Jackson SP (1998) DNA end-joining: from yeast to man. Trends Biochem Sci 23:394–398

Crotti LB, Basrai MA (2004) Functional roles for evolutionarily conserved Spt4p at centromeres and heterochromatin in *Saccharomyces cerevisiae*. EMBO J 23:1804–1814

De Cleene M, De Ley J (1976) The host range of crown gall. Botan Rev 42:389–466

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

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

Deng W, Chen L, Wood DW et al (1998) *Agrobacterium* VirD2 protein interacts with plant host cyclophilins. Proc Natl Acad Sci U S A 95:7040–7045

Escudero J, den Dulk-Ras H, Regensburg-Tuïnk TJG, Hooykaas PJJ (2003) VirD4-independent transformation by CloDF13 evidences an unknown factor required for the genetic colonization of plants via *Agrobacterium*. Mol Microbiol 47:891–901

Flowers JL, Vaillancourt LJ (2005) Parameters affecting the efficiency of *Agrobacterium tumefaciens*-mediated transformation of *Colletotrichum graminicola*. Curr Genet 15:1–9

Frandsen RJ (2011) A guide to binary vectors and strategies for targeted genome modification in fungi using *Agrobacterium tumefaciens*-mediated transformation. J Microbiol Methods 87:247–262

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

García-Cano E, Magori S, Sun Q et al (2015) Interaction of *Arabidopsis* trihelix-domain transcription factors VFP3 and VFP5 with *Agrobacterium* virulence protein VirF. PLoS ONE 10:e0142128

García-Cano E, Hak H, Magori S et al (2018) The *Agrobacterium* F-box protein effector VirF destabilizes the *Arabidopsis* GLABROUS1 enhancer/binding protein-like transcription factor VFP4, a transcriptional activator of defense response genes. Mol Plant Microbe Interact 31:576–586

Garcia-Rodriguez FM, Schrammeijer B, Hooykaas PJJ (2006) The *Agrobacterium* VirE3 effector protein: a potential plant transcriptional activator. Nucleic Acids Res 34:6496–6504

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

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

Gelvin SB (2010) Plant proteins involved in *Agrobacterium*-mediated genetic transformation. Annu Rev Phytopathol 48:45–68

Grimaldi B, de Raaf MA, Filetici P et al (2005) *Agrobacterium*-mediated gene transfer and enhanced green fluorescent protein visualization in the mycorrhizal ascomycete *Tuber borchii*: a first step towards truffle genetics. Curr Genet 48:69–74

Hatoh K, Izumitsu K, Morita A et al (2013) Transformation of the mushroom species *Hypsizigus marmoreus*, *Flammulina velutipes*, and *Grifola frondosa* by an *Agrobacterium*-mediated method using a universal transformation plasmid. Mycoscience 54:8–12

Hauer MH, Gasser SM (2018) Chromatin and nucleosome dynamics in DNA damage and repair. Genes Devel 31:2204–2221

Hooykaas PJJ, van Attikum H, Bundock P (2000) Nucleic acid integration in eukaryotes. EP00204693.6

Hooykaas PJ, Hofker M, den Dulk-Ras H, Schilperoort RA (1984) A comparison of virulence determinants in an octopine Ti plasmid, a nopaline Ti plasmid, and an Ri plasmid by complementation analysis of *Agrobacterium tumefaciens* mutants. Plasmid 11:195–205

Hooykaas-van Slogteren GMS, Hooykaas PJJ, Schilperoort RA (1984) Expression of Ti plasmid genes in monocotyledonous plants infected with *Agrobacterium tumefaciens*. Nature 311:763–764

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

Ishida Y, Saito H, Ohta S et al (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nat Biotech 14:745–750

Jeon J, Park SY, Chi MH et al (2007) Genome-wide functional analysis of pathogenicity genes in the rice blast fungus. Nature Genet 39:561–565

Kilaru S, Collins CM, Hartley AJ et al (2009) Establishing molecular tools for genetic manipulation of the pleuromutilin-producing fungus *Clitopilus passeckerianus*. Appl Environ Microbiol 75:7196–7204

Knight CJ, Bailey AM, Foster GD (2010) Investigating *Agrobacterium*-mediated transformation of *Verticillium albo-atrum* on plant surfaces. PLoS ONE 5:e13684

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

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

Kyndt T, Quispe D, Zhai H et al (2015) The genome of cultivated sweet potato contains *Agrobacterium* T-DNAs with expressed genes: An example of a naturally transgenic food crop. Proc Natl Acad Sci U S A 112:5844–5849

Lacroix B, Citovsky V (2013) Characterization of VIP1 activity as a transcriptional regulator *in vitro* and *in planta*. Sci Rep 3:2440

Leclerque A, Wan H, Abschütz A et al (2004) *Agrobacterium*-mediated insertional mutagenesis (AIM) of the entomopathogenic fungus *Beauveria bassiana*. Curr Genet 45:111–119

Lee MH, Bostock RM (2006) *Agrobacterium* T-DNA-mediated integration and gene replacement in the brown rot pathogen *Monilinia fructicola*. Curr Genet 49:309–322

Lewis LK, Resnick MA (2000) Tying up loose ends: nonhomologous end-joining in *Saccharomyces cerevisiae*. Mutat Res 451:71–89

Li G, Zhou Z, Liu G et al (2007) Characterization of T-DNA insertion patterns in the genome of rice blast fungus *Magnaporthe oryzae*. Curr Genet 51:233–243

Li X, Yang Q, Tu H et al (2014) Direct visualization of *Agrobacterium*-delivered VirE2 in recipient cells. Plant J 77:487–495

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

Luo Y, Chen Z, Zhu D et al (2015) Yeast actin-related protein ARP6 negatively regulates *Agrobacterium*-mediated transformation of yeast cell. BioMed Res Int article ID 275092. http://dx.doi.org/10.1155/2015/275092

Magori S, Citovsky V (2011) *Agrobacterium* counteracts host-induced degradation of its effector F-box protein. Sci Signal 4(195):ra69

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

McClelland CM, Chang YC, Kwon-Chung KJ (2005) High frequency transformation of *Cryptococcus neoformans* and *Cryptococcus gattii* by *Agrobacterium tumefaciens*. Fungal Genet Biol 42:904–913

Meng Y, Patel G, Heist M et al (2007) A systematic analysis of T-DNA insertion events in *Magnaporthe oryzae*. Fungal Genet Biol 44:1050–1064

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

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

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

Michielse CB, Hooykaas PJ, van den Hondel CAMJJ, Ram AFJ (2005a) *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. Curr Genet 48:1–17

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

Michielse CB, Hooykaas PJ, van den Hondel CA, Ram AF (2008) *Agrobacterium*-mediated transformation of the filamentous fungus *Aspergillus awamori*. Nat Protocols 3:1671–1678

Michielse CB, van Wijk R, Reijnen L et al (2009) Insight into the molecular requirements for pathogenicity of *Fusarium oxysporum* f. sp. lycopersici through large-scale insertional mutagenesis. Genome Biol 10:R4

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

Mullins ED, Chen X, Romaine P at al. (2001) *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: an efficient tool for insertional mutagenesis and gene transfer. Phytopathol 91:173–180

Murata H, Sunagawa M, Igasaki T, Shishido K (2006) *Agrobacterium*-mediated transformation of the ectomycorrhizal basidiomycete *Tricholoma matsutake* that produces commercially valuable fruit bodies, matsutake. Mycoscience 47:228–231

Nemecek JC, Wüthrich M, Klein BS (2006) Global control of dimorphism and virulence in fungi. Science 312:583–588

Nester EW, Gordon MP, Amasino RM, Yanofsky MF (1984) Crown gall: a molecular and physiological analysis. Annu Rev Plant Physiol 35:387–413

Ninomiya Y, Suzuki K, Ishii C, Inoue H (2004) Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining. Proc Natl Acad Sci U S A 101:12248–12253

Niu X (2013) Functional analysis of Agrobacterium virulence genes. Ph.D. thesis. Leiden University, Leiden, The Netherlands

Niu X, Zhou M, Henkel CV et al (2015) The *Agrobacterium tumefaciens* virulence protein VirE3 is a transcriptional activator of the F-box gene VBF. Plant J 84:914–924

Noack-Schonmann S, Bus T, Banasiak R et al. (2014) Genetic transformation of *Knufia petricola* A95—a model organism for biofilm-material interactions AMB Express 4:80

Offringa R, de Groot MJA, Haagsman HJ et al (1990) Extrachromosomal homologous recombination and gene targeting in plant cells after *Agrobacterium* mediated transformation. EMBO J 9:3077–3084

Ohmine Y, Satoh Y, Kiyokawa K et al (2016) DNA repair genes RAD52 and SRS2, a cell wall synthesis regulator gene SMI1, and the membrane sterol synthesis scaffold gene ERG28 are important in efficient *Agrobacterium*-mediated yeast transformation with chromosomal T-DNA. BMC Microbiol 16:58

Pansegrau W, Schoumacher F, Hohn B, Lanka E (1993) Site-specific cleavage and joining of single-stranded DNA by VirD2 protein of *Agrobacterium tumefaciens* Ti plasmids: analogy to bacterial conjugation. Proc Natl Acad Sci U S A 90:11538–11542

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

Park SM, Kim DK (2004) Transformation of a filamentous fungus *Cryphonectria parasitica* using *Agrobacterium tumefaciens*. Biotechnol Bioprocess Eng 9:217–222

Park SY, Jeong MH, Wang HY et al (2013) *Agrobacterium tumefaciens*-mediated transformation of the lichen fungus, *Umbilicaria muehlenbergii*. PLoS ONE 8:e83896

Park SY, Vaghchhipawala Z, Vasudevan B et al (2015) *Agrobacterium* T-DNA integration into the plant genome can occur without the activity of key non-homologous end-joining proteins. Plant J 81:934–946

Piers KL, Heath JD, Liang X et al (1996) *Agrobacterium tumefaciens*-mediated transformation of yeast. Proc Natl Acad Sci U S A 93:1613–1618

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

Risseeuw E, Franke-van Dijk ME, Hooykaas PJ (1996) Integration of an insertion-type transferred DNA vector from *Agrobacterium tumefaciens* into the *Saccharomyces cerevisiae* genome by gap repair. Mol Cell Biol 16:5924–5932

Roberts RL, Metz M, Monks DE et al (2003) Purine synthesis and increased *Agrobacterium tumefaciens* transformation of yeast and plants. Proc Natl Acad Sci U S A 100:6634–6639

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

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

Rolloos M, Dohmen MHC, Hooykaas PJJ, van der Zaal BJ (2014) Involvement of Rad52 in T-circle formation during *Agrobacterium tumefaciens*-mediated transformation of *Saccharomyces cerevisiae*. Mol Microbiol 91:1240–1251

Rolloos M, Hooykaas PJJ, van der Zaal BJ (2015) Enhanced targeted integration mediated by translocated I-*Sce*I during the *Agrobacterium* mediated transformation of yeast. Sci Rep 5:8345

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

Sakalis PA, van Heusden GPH, Hooykaas PJJ (2014) Visualization of VirE2 protein translocation by the *Agrobacterium* type IV secretion system into host cells. Microbiol Open 3:104–117

Salomon S, Puchta H (1998) Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells. EMBO J 17:6086–6095

Schrammeijer B, Risseeuw E, Pansegrau W et al (2001) Interaction of the virulence protein VirF of *Agrobacterium tumefaciens* with plant homologs of the yeast Skp1 protein. Curr Biol 11:258–262

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

Shi Y, Lee LY, Gelvin SB (2014) Is VIP1 important for *Agrobacterium*-mediated transformation? Plant J 79:848–860

Singer K, Shiboleth YM, Li J, Tzfira T (2012) Formation of complex extrachromosomal T-DNA structures in *Agrobacterium tumefaciens*-infected plants. Plant Physiol 160:511–522

Skowyra D, Craig KL, Tyers M et al (1997) F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell 91:209–219

Smeenk G, van Attikum H (2013) The chromatin response to DNA breaks: leaving a mark on genome integrity. Annu Rev Biochem 82:55–80

Soltani J (2009) Host genes involved in *Agrobacterium*-mediated transformation. Ph.D. thesis. Leiden University, Leiden, The Netherlands

Soltani J, van Heusden GPH, Hooykaas PJJ (2008) *Agrobacterium*-mediated transformation of non-plant organisms. In: Tzfira T, Citovsky V (eds) *Agrobacterium*: from biology to biotechnology, pp 649–675. Springer Press, New York

Soltani J, van Heusden GPH, Hooykaas PJJ (2009) Deletion of host histone acetyltransferases and deacetylases strongly affects *Agrobacterium*-mediated transformation of *Saccharomyces cerevisiae*. FEMS Microbiol Lett 298:228–233

Sugui JA, Chang YC, Kwon-Chung KJ (2005) *Agrobacterium tumefaciens*-mediated transformation of *Aspergillus fumigatus*: an efficient tool for insertional mutagenesis and targeted gene disruption. Appl Environ Microbiol 71:1798–1802

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

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

Tsuji G, Fujii S, Fujihara N et al (2003) *Agrobacterium tumefaciens*-mediated transformation for random insertional mutagenesis in *Colletotrichum lagenarium*. J Gen Plant Pathol 69:230–239

Turk SC, Melchers LS, den Dulk-Ras H et al (1991) Environmental conditions differentially affect vir gene induction in different *Agrobacterium* strains. Role of the VirA sensor protein. Plant Mol Biol 16:1051–1059

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

Tzfira T, Vaidya M, Citovsky V (2001) VIP1, an *Arabidopsis* protein that interacts with *Agrobacterium* VirE2, is involved in VirE2 nuclear import and *Agrobacterium* infectivity. EMBO J 20:3596–3607

Tzfira T, Frankman LR, Vaidya M, Citovsky V (2003) Site-specific integration of *Agrobacterium tumefaciens* T-DNA via double-stranded intermediates. Plant Physiol 133:1011–1023

Tzfira T, Vaidya M, Citovsky V (2004) Involvement of targeted proteolysis in plant genetic transformation by *Agrobacterium*. Nature 431:87–92

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

van Attikum H (2003) Genetic requirements for the integration of *Agrobacterium* T-DNA in the eukaryotic genome. PhD thesis. Leiden University, Leiden, The Netherlands

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

van Kregten M, Lindhout BI, Hooykaas PJJ, van der Zaal BJ (2009) *Agrobacterium*-mediated T-DNA transfer and integration by minimal VirD2 consisting of the relaxase domain and a type IV secretion system translocation signal. Mol Plant-Micr Int 22:1356–1365

van Kregten M, de Pater S, Romeijn R, van Schendel R, Hooykaas PJJ, Tijsterman M (2016) T-DNA integration in plants results from polymerase-θ-mediated DNA repair. Nature Plants 2:16164

Vergunst AC, Schrammeijer B, Den Dulk A et al (2000) VirB/D4-dependent protein translocation from *Agrobacterium* into plant cells. Science 290:979–982

Vergunst AC, van Lier MCM, den Dulk-Ras A et al (2005) Positive charge is an important feature of the C-terminal teransport signal of the VirB/D4-translocated proteins of *Agrobacterium*. Proc Natl Acad Sci U S A 102:832–837

Walton FJ, Idnurm A, Heitman J (2005) Novel gene functions required for melanization of the human pathogen *Cryptococcus neoformans*. Mol Microbiol 57:1381–1396

Wang Y, Peng W, Zhou X, Huang F, Shao L, Luo M (2014) The putative *Agrobacterium* transcriptional activator-like virulence protein VirD5 may target T-complex to prevent the degradation of coat proteins in the plant cell nucleus. New Phytol 203:1266–1281

Wang Y, Wang R, Mao WJ et al (2016) A simple and efficient transformation system for the edible mushroom *Pleurotus eryngii*. Mycoscience 57:356–360

White D, Chen W (2006) Genetic transformation of *Ascochyta rabiei* using *Agrobacterium*-mediated transformation. Curr Genet 49:272–280

Winans SC (1991) An *Agrobacterium* two-component regulatory system for the detection of chemicals released from plant wounds. Mol Microbiol 5:2345–2350

Wolterink-van Loo S, Ayala AAE, Hooykaas PJJ, van Heusden GPH (2015) Interaction of the *Agrobacterium tumefaciens* virulence protein VirD2 with histones. Microbiology 161:401–410

Yousefi-Pour HM, Soltani J, Nazeri S (2013) A survey on optimization of *Agrobacterium*-mediated genetic transformation of the fungus *Colletotrichum gloeosporioides*. J Cell Mol Res 5:35–41

Yu X, Huo L, Liu H et al (2015) Melanin is required for the formation of the multi-cellular conidia in the endophytic fungus *Pestalotiopsis microspora*. Microbiol Res 179:1–11

Zaltsman A, Krichevsky A, Loyter A, Citovsky V (2010) *Agrobacterium* induces expression of a host F-box protein required for tumorigenicity. Cell Host Microbe 7:197–209

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

Zhang J, Shi L, Chen H et al (2014) An efficient *Agrobacterium*-mediated transformation method for the edible mushroom *Hypsizygus marmoreus*. Microbiol Res 169:741–748

Zhang T, Ren P, Chaturvedi V et al (2015) Development of an *Agrobacterium*-mediated transformation system for the cold-adapted fungi *Pseudogymnoascus destructans* and *P. pannorum*. Fungal Genet Biol 81:73–81

Zhang X (2016) Functional analysis of *Agrobacterium tumefaciens* virulence protein VirD5. Ph.D. thesis. Leiden University, Leiden, The Netherlands

Zhang X, van Heusden GPH, Hooykaas PJJ (2017) Virulence protein VirD5 of *Agrobacterium tumefaciens* binds to kinetochores in host cells via an interaction with Spt4. Proc Natl Acad Sci U S A 114:10238–10243

Zhu J, Oger PM, Schrammeijer B et al (2000) The bases of crown gall tumorigenesis. J Bacteriol 182:3885–3895

Zhu Y, Nam J, Humara JM et al (2003) Identification of *Arabidopsis rat* mutants. Plant Physiol 132:494–505

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