The effect of the DNA metabolism of the recipient cell on the fate of T-DNA translocated by Agrobacterium

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Chapter 1
General introduction

Agrobacterium tumefaciens T-DNA processing, transfer and integration in the eukaryotic genome; mechanisms and variables

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1 Introduction

Agrobacterium tumefaciens is a Gram-negative phytopathogen that is the causal agent of crown gall disease in a broad range of dicotyledonous plants. Crown gall disease is characterized by the formation of tumors at sites where plant tissue has been infected by *Agrobacterium*. This ground-dwelling bacterium has drawn a lot of attention during the past three decades because of its ability to stably integrate a discrete fragment of a large tumor-inducing (Ti) plasmid, into the genome of host cells. The *Agrobacterium* mediated transformation (AMT) of plants has become the technology of choice to introduce or alter genetic traits of plants for plant biotechnologists.

AMT could be put to use to meet some of the needs that are expected to become more prevalent in a future with a growing world population. To give a few examples of potential applications of AMT worth looking forward to: The development of drought resistant plants that can be grown at localities currently unavailable for agriculture, the development of crops offering higher yields of their edible parts or the development of pest resistant plants that could effectively reduce the amount of pesticides that are used now.

A number of research groups have successfully applied AMT to several non-plant organisms ranging from yeast [1, 2], filamentous fungi [3] to sea urchin [4]. *Agrobacterium* apparently is able to adhere sufficiently strong to a broad range of organisms to allow for AMT, provided that the *Agrobacterium* virulence machinery is triggered. AMT of yeast cells has proven to be a very convenient tool to assay the relevance of several host factors for T-DNA integration. Knock-out mutant yeast lines can be easily obtained and transformant-screening after AMT only takes a few days. However, the transformation efficiencies that can be achieved in yeast are relatively low compared to those following AMT of plant cells, especially when regarding the integration of T-DNA without sequence homology to the recipient cells genome. The search for variables that can increase the efficiency of T-DNA integration via the NHEJ pathway during AMT of yeast could provide a deeper insight in the rate limiting steps during AMT.

The process of AMT can be divided into three main parts. Firstly, there is a preparative phase which sets on with the expression of virulence genes, the processing of the transfer-DNA (T-DNA) region located on the Ti plasmid, leading to the formation of a single stranded T-DNA-protein conjugate (the T-strand) and the attachment of *Agrobacterium tumefaciens* to the host cells. Secondly, there is an intrusive phase here the transfer of the T-strand and effector proteins into the host cell via a type IV secretion system (T4SS) takes place and the successive nuclear targeting of the T-strand. The intrusive phase is finalized with the integration of the T-strand in the host cells genome. Thirdly, there is the submissive phase where transcription of the genes located on the integrated T-strand takes place leading to opine synthesis and enhanced cell proliferation. In this introduction the focus will be mostly on the preparative and intrusive phase.

1.1 *Agrobacterium* mediated transformation: the preparative phase

The *Agrobacterium tumefaciens* Ti-plasmid

Agrobacterium strains that are able to cause crown gall disease all carry a circa 200 kb Ti plasmid that is required for virulence [5, 6]. On the Ti plasmid, a specific region is present that is transferred and integrated into the genome of host cells: the transfer-DNA (T-DNA) [7, 8]. The T-DNA is flanked by two imperfect direct 25 bp repeats: the T-DNA *left border* (LB) and *right border* (RB) [9]. The genes required for T-DNA transfer are not located on the T-DNA itself but are transcribed from the *vir* region located on the Ti plasmid. The virulence genes located on the *vir* region are involved in T-DNA processing, the T4SS mediated transport of T-strands and also act as accessory proteins in both the *Agrobacterium* and host cells.

The T-DNA contains genes that are involved in the synthesis of opines, a diverse group of amino acid derivatives that *Agrobacterium tumefaciens* utilizes as carbon and nitrogen source [10]. Although these opine synthesis genes are transferred from a bacterial plasmid, they are equipped with eukaryotic expression signals [7]. The *Agrobacterium* Ti plasmids are classified depending on the type of opine synthesis genes they transfer [11]. Among others, there are nopaline and octopine type Ti plasmids [12]. Besides genes for opine biosynthesis, the T-DNA also carries genes involved in the production of the phytohormones auxin and cytokinin which induce the tumor genesis that is characteristic for crown gall disease. The formation of these tumorous outgrowths enable a further increase of the opine production.

Adhesion to host cells

For pathogenic bacteria, adhesion to host cells is a necessary step for the initiation and maintenance of an infection and is also an important determinant for host specificity. Adherence to the cell surface of the host organism is mediated by a specialized group of proteins, the adhesins. For some human pathogens, the adhesins together with their cognate host receptors are well characterized, for example for the human gastric pathogen *Helicobacter pylori* [13, 14]. The importance of host binding for virulence and host specificity of plant pathogens has also become evident [15-17]. *Agrobacterium* attaches efficiently to both plant cells as well as a variety of abiotic surfaces [18]. Possibly, the aspecific binding to a wide range of surfaces allows *Agrobacterium* to transform so many different cell types.

In Gram-negative bacteria, fimbriae often function as adhesins. However, in many cases it is only a minor subunit at the tip of the fimbriae that is acting as the actual adhesin. Also non-fimbrial adhesins are known to occur in Gram-negative bacteria. *Agrobacterium* strains lacking a Ti plasmid, hence without any T4SS constituents, still confer some capacity to bind tobacco tissue culture cells although strongly reduced [19].

VirB2 is known as the major constituent of the T4SS pilus, while VirB5 is a minor constituent located at its very tip. Both proteins are considered candidates to act as adhesins [17]. VirB2 has been shown to bind to several proteins expressed from an *Arabidopsis thaliana* cDNA library in yeast: BTI1, BTI2, which are currently of unknown function and the membrane associated ATPase AtRAB8. Additionally, *Arabidopsis* with silenced BTI and AtRAB8 expression are less susceptible to transformation by *Agrobacterium* [16].

Although these findings are promising, whether the genes encoded by the Ti plasmid are required for host cell adherence is not clear yet. In a pioneering research a reduced adherence of *Agrobacterium* cells to cultured plant tissue cells was found for *Agrobacterium* cells cured from the Ti plasmid [19] however in another study determinants for adherence were predicted to reside on the chromosomal DNA of *Agrobacterium* [20]. Until now, a direct link between any of the potential *Agrobacterium* adhesins and host cell binding has not yet been established. Performing a reliable binding assay to validate candidate adhesins will be very challenging considering the high rate of aspecific binding that can be expected and the likeliness of redundancy among the *Agrobacterium* proteins that facilitate the attachment to its host cells.

Induction of virulence genes

Agrobacterium tumefaciens carries over 20 virulence genes on the Ti plasmid that are involved in the many steps that ultimately lead to tumor formation. The currently described genes required for T-DNA processing and transfer are all located on the *vir* region of the Ti plasmid, grouped in a varying number of operons. *Agrobacterium tumefaciens* utilizes a two component signal transduction pathway to regulate the expression of the virulence genes. This transduction pathway consists of the response regulator VirG and the transmembrane receptor kinase VirA. VirA is responsive to phenolic compounds like acetosyringone that are released by damaged plant cells (Figure 1A, 1B) [21-23].

There are also a number of sugars like D-(+)-Glucose, D-(+)-Galactose and L-(+)-Arabinose that act synergistically with the phenolic compounds in the induction of *Agrobacterium vir*-genes. The presence of these sugars is sensed by the chromosomally encoded periplasmic protein ChvE that acts in concert with VirA (Figure 1B) [24]. The cytoplasmic domain of VirA is prone to autophosphorylation and also has been shown to phosphorylate the transcriptional activator VirG (Figure 1C) [25, 26]. VirG in turn activates the virulence genes present on the *vir* region (Figure 1D) [27, 28].

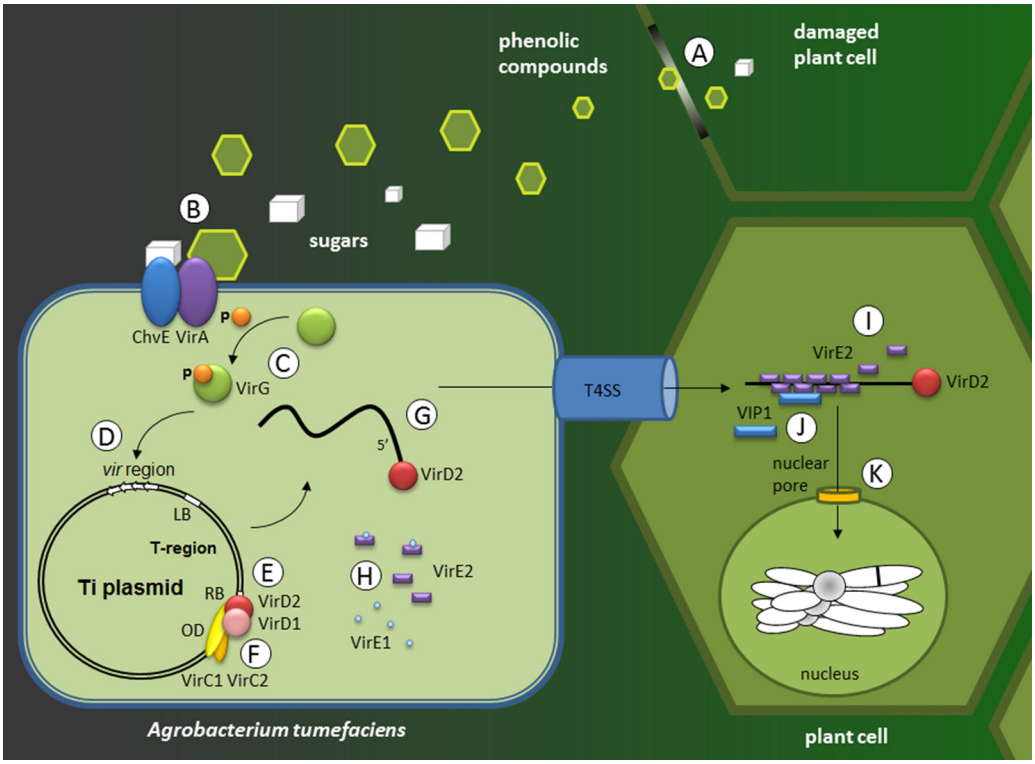

Figure 1. Schematic overview of Agrobacterium mediated transformation (AMT) of plant cells. **(A)** Damaged plant cells release phenolic compounds and sugars. **(B)** Phenolic compounds, like acetosyringone, are recognized by the transmembrane receptor kinase VirA. ChvE senses the presence of sugars. **(C)** VirA phosphorylates VirG as a response to sugars or phenolic compounds released by plant cells. **(D)** VirG in turn activates the virulence genes present on the *vir* region of the Ti plasmid. **(E)** The combined activity of VirD1 and VirD2 induces a nick in the T-DNA RB and LB. **(F)** VirC1 enhances the assembly of the T-DNA relaxosome by interacting with VirD1, VirD2 and VirC2. **(G)** A T-strand, is released whereby virD2 remains covalently attached to the 5'-end. **(H)** VirE1 competes with the T-strand for binding to VirE2. This enables VirE2 to be translocated separately from the T-strand. **(I)** In the plant cell the T-strand forms a complex with VirE2 which protects the T-strand from degradation by plant nucleases. **(J)** VIP1 binds via VirE2 to the T- complex and facilitates nuclear targeting. **(K)** The T-complex enters the nucleus via the nuclear pores.

T-DNA processing

T-DNA processing involves *virD2, virD1, virC2* and *virC1*. VirD2 induces a single strand break (nick) between the 22nd and 23rd base of the border sequences and remains covalently attached via a tyrosine residue to the 5'-end of the T-strand that is eventually released. The transesterification reaction carried out by VirD2 is typical for enzymes belonging to the bacterial relaxase family [29, 30]. Relaxases are involved in the initiation and termination of conjugal transfer of DNA by catalyzing a nicking reaction within their cognate origin of transfer (oriT) using supercoiled DNA as substrate and Mg^{2+} as a cofactor [17] . A typical relaxase has one active site equipped with one or two tyrosyl groups [31].

When the relaxase has formed a complex with the oriT, the free hydroxyl of the active site tyrosine is correctly positioned to perform a nucleophilic attack on the phosphate-deoxyribose backbone of the DNA [18]. The cleaving reaction results in a new phosphodiester bond between the 5'-end of the cleaved DNA strand and the tyrosyl group of the relaxase (Figure 2) [19, 20]. For *Agrobacterium* relaxase VirD2 it has been confirmed by in vitro studies showing that it catalyzes both the site specific nicking reaction and the strand transfer of single stranded DNA (ssDNA) border substrate [22].

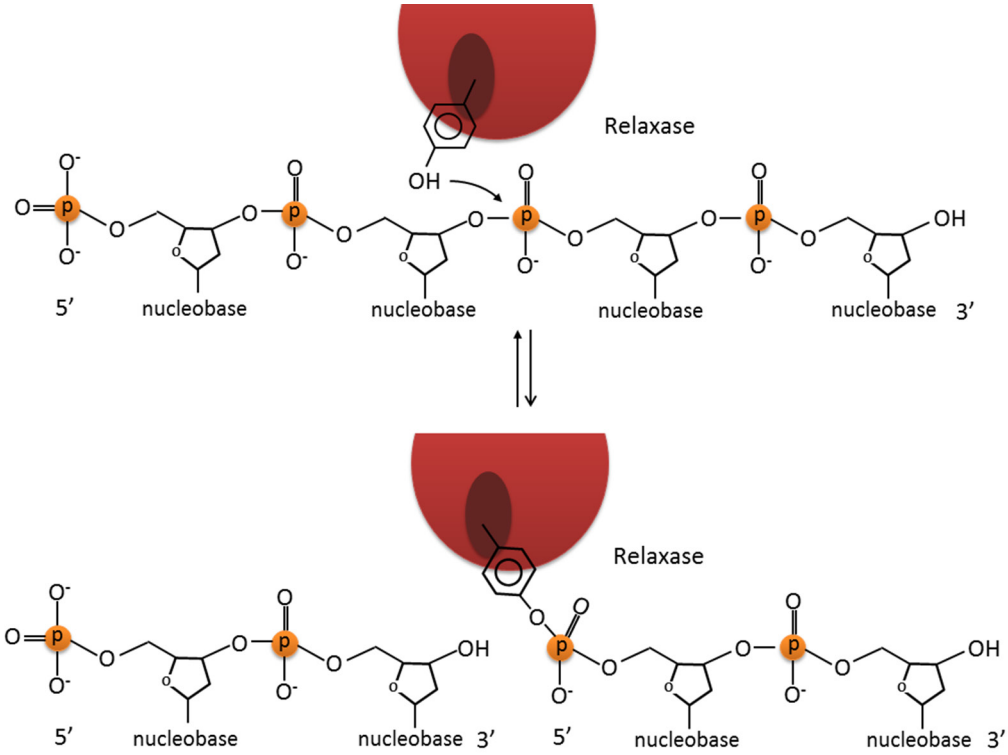

Figure 2. Schematic of a typical nicking reaction catalyzed by a relaxase on a ssDNA substrate. The active site tyrosine of the relaxase performs a nucleophilic attack on the phosphate group of the DNA backbone resulting in a phosphodiester bond between the tyrosyl group and the 5'-end of the nicked DNA strand.

In vitro experiments showed that the combined activity of VirD2 and VirD1 is required to induce a nick at the RB of double stranded plasmid DNA (Figure 1E) [32]. These experiments also revealed that purified VirD2 and VirD1 could process a superhelical plasmid DNA substrate but not linearized DNA or plasmid DNA that was brought into a relaxed conformation by treatment with topoisomerase I. Likely, after the first nicking reaction the upper and lower strand of the T-DNA are separated to enable the releases of a T-strand. The melting of the dsDNA during T-DNA processing is governed by either displacement synthesis or by helicase activity [33]. Displacement synthesis would involve complementary strand synthesis where a DNA polymerase elongates the 3'-end hydroxyl terminus of the nick, thereby simultaneously separating the T-DNA strands towards the LB sequence.

Displacement synthesis is well described as part of the rolling circle replication of plasmids [34]. Helicases unwind dsDNA using the hydrolysis of nucleoside 5'-triphosphates as an energy source while translocating along the DNA strand. To date, there is no candidate helicase described for *Agrobacterium* that acts on T-DNA after RB nicking. Although many relaxases are known to contain a helicase domain, both VirD2 and VirD1 do not have the A and B motifs of the Walker Box ATP binding site that are conserved among all known members of the helicase families [35].

In the octopine type Ti plasmids, VirC1 binds to *overdrive* (OD) a DNA sequence located upstream of the T-DNA RB [36, 37]. VirC1 enhances the assembly of the T-DNA relaxosome by interacting with VirD1, VirD2 and VirC2 (Figure 1F) and is involved in the polar localization of the T-DNA relaxosome hence priming the T-DNA for secretion by a T4SS (Figure 1G) [38]. Recently, the X-ray crystal structure of the DNA binding domain of VirC2 was elucidated. This domain contained a ribbon-helix-helix fold (RHH fold) that strongly resembles the dimeric RHH folds that are found in bacterial DNA binding proteins like the Arc repressor and CopG. VirC2 however is exceptional within the RHH family because it binds dsDNA with a pseudo-2-fold symmetric RHH fold in a single polypeptide chain instead of forming a symmetric dimer of RHH motifs [39]. It has been suggested that VirC2 facilitates T-DNA processing by VirD1 and VirD2 by destabilization of the border regions [39].

1.2 *Agrobacterium* mediated transformation: the intrusive phase

Translocation of the T-strand and effector proteins

T4SSs are highly complex cell-envelope spanning transport channels that are considered to be ancestrally related to conjugal transfer mechanisms [40]. The T4SS is unique among the prokaryotic secretion systems because it can transfer both DNA and effector proteins into its cognate recipient cells. Effector proteins are proteins produced by prokaryotic pathogens which are transported to their cognate host cells where they interact with host factors in order to modulate their functions thereby facilitating or enhancing the virulence of the pathogen. T4SSs that translocate effector proteins have been the focus of much research because many human pathogens like *Helicobacter pylori* and *Bordetella pertussis* utilize a T4SS to translocate effector proteins to their host cells that have great impact on the disease outcome.

The Ti plasmid of *Agrobacterium* encodes for two structurally similar T4SSs: one for the conjugal transfer of the Ti plasmid itself and an additional T4SS for T-strand transfer to susceptible host cells. The onset of conjugal transfer of Ti plasmids is regulated on both the protein and the genetic level by two environmental signals: on the protein level the inactive monomeric form of the transcriptional activator TraR is dimerized to its active form by interacting with the acyl-homoserine lactone quorum-sensing signal termed *Agrobacterium* autoinducer. When dimeric, TraR activates the *tra* regulon thereby initiating conjugal transfer.

On the genetic level the expression of *traR* is regulated by the presence of a subset of opines that are excreted by the crown gall tumors developed by transformed plant cells [41]. Ti plasmid conjugation thus only occurs in the direct vicinity of plant tumors. The onset of T-strand and effector protein translocation by the second T4SS is differently regulated. Genes encoding the components of the latter T4SS are located within the *vir* region and are acetosyringone-inducible. In the *vir* region, eleven *virB* genes, *virB1-virB11*, together with *virD4*, form the constituents of the T4SS. The genes encoding VirB1-VirB11 are tightly organized in the *virB* operon [42]. VirD4 is encoded within the *virD* operon and is regarded the coupling protein, forming the interface between substrates which are to be transported and the actual pilus itself [43, 44]. The hydrolyzation of ATP by VirD4 and VirB11 ATP-binding subunits triggers VirB10 to undergo a conformational change [45]. This structural change is a prerequisite for DNA substrate passage though the T4SS making the ATPase activity of VirD4 and VirB11 an analogy to the passing of silver coins to Charon, the ferryman of the underworld, in Greek mythology [46].

In addition to the T-strand, with VirD2 covalently attached to the 5'-end, *Agrobacterium* translocates the proteins VirE2, VirE3, VirD5 and VirF to plant cells [47, 48]. Proteins known to be transferred have translocation signals present at or near their C terminus, consisting of clusters of positively charged amino acids which seem to mark these proteins a substrate for the VirB/VirD4 complex [47]. These translocation signals proved to be a portable trait, allowing the *Agrobacterium* mediated translocation of other proteins via the T4SS by making translational fusions with these proteins and *Agrobacterium* translocation signals [47-49].

Nuclear transfer of the T-strand

In *Arabidopsis thaliana* it has been shown that the importin IMPα-4 is involved in AMT [50]. Importin α recognizes proteins that are targeted for nuclear import by their NLS and consequently binds to Importin β. Importin β targets the complex to the nuclear pore by binding to nuclear pore proteins (Figure 1K) [51]. After the T-complex is assembled in the plant cytosol, it is generally accepted that VirD2 and VirE2 play a part in the nuclear import of the T-complex. VirD2 has a monopartite nuclear localization signal (NLS) located in its N-terminal part and a bipartite NLS near its C-terminus. Both these NLSs allow nuclear import of reporter genes in plant cells, and also in other eukaryotes like animal and yeast cells [52-56]. However, for the nuclear import of the T-complex only the C-terminal bipartite NLS proved to be indispensable [52, 53]. VirE2 binds to ssDNA without a preference for a specific sequence [57].

It is generally assumed that in the recipient cell VirE2 covers the entire length of the T-strand forming the T-complex although to date no *in vivo* data has been published supporting this. VirE2 associated with the T-strand is thought to offer the enclosed single stranded DNA protection against degradation by plant nucleases (Figure 1I) [58-60]. Despite the affinity of VirE2 for ssDNA it has been shown that VirE2 enters the plant cells independent of the T-strand [61].

This phenomenon was better understood once the crystal structure of the chaperone-like protein VirE1 in complex with VirE2 became available; VirE1 forms a soluble heterodimer with VirE2 in the *Agrobacterium* cells, thus avoiding premature VirE2 binding to the T-strand preventing its translocation (Figure 1H) [62].

Because of this interaction, the nuclear import machinery of the host cell is manipulated to facilitate the nuclear import of the T-complex. VIP1 is a transcription factor that is targeted for nuclear import by the mitogen-activated kinase (MAPK) MPK3 that is part of the defense mechanism by which plants usually fight off pathogens. In that manner, the plant defense pathway is hijacked to facilitate AMT [64]. This strategy for the nuclear targeting of the T-complex does not apply to the AMT of non-plant organisms; VirE2 requires co-expression with VIP1 for optimal nuclear uptake of VirE2 in both yeast cells and mammalian cells, neither of which possess any VIP homologues themselves [65]. Nevertheless, AMT of yeast and mammalian cells is possible without the expression of VirE2 in *Agrobacterium*. It therefore seems that a host factor or another virulence protein can facilitate the nuclear targeting of the T-strand, or alternatively, that the VirD2 moiety of the T-strand already suffices for a basic level of nuclear import. There is experimental evidence suggesting that the VirE3 protein from *Agrobacterium* can mimic the "carrier" function of VIP1, thereby adding to the spectrum of potential host organisms [66].

Characteristics of the integration site

Several research groups have recovered integrated T-strands from transformed plants to determine if there is a preference for specific genomic loci for T-DNA integration. Several publications described a preference for T-strands to integrate in the transcriptionally active regions of the chromatin [67, 68]. A more recent genome wide analysis of T-DNA integration sites in *Arabidopsis* plants, that were grown under nonselective conditions after AMT, revealed a randomized integration pattern with respect to transcriptionally active or non-active regions of the genome [69]. Nevertheless, when looking at the details, the integration of T-DNA is not always completely random; sequencing of the genomic DNA flanking the integrated T-DNA revealed that there is often a region of micro-homology between the T-strand LB and the integration site [70, 71]. These findings support an early model for single stranded T-DNA integration where the free 3'-end of the incoming T-strand scans the genomic DNA for micro-homologies to anneal to one of the DNA strands of the recipient cell's genome [72]. An additional model for T-DNA integration has been coined more recently that describes the integration process as the integration of a dsDNA molecule, derived from the T-strand, that integrates in double stranded breaks (DSBs) present in the genome of the host cell [73].

Mechanisms behind the formation of aberrant T-DNA integrations

The integration of T-strand does not always result in the presence of a perfect copy of the original T-DNA region in the recipient cell genome; several categories of aberrant T-strand derived structures were found to be integrated in the recipient cell's genome. These aberrations can offer more insight into some of the peculiarities of AMT and possibly will provide leads for the further improvement of the currently used transformation protocols. Deviant T-strand inserts that are frequently observed are vector backbone fragments. A common cause for the integration of T-DNA containing vector DNA is LB skipping [74-76]. The addition of extra LB repeats [77], the preservation of the natural inner and outer sequence on the binary vector [78] and the usage of a nopaline instead of an octopine type LB sequence have been postulated as ways of increasing the efficiency of left border T-strand termination [78]. Alternatively, the transfer of vector DNA can also result from a combination of VirD2 mediated LB nicking combined with RB skipping [79, 80].

Other aberrant T-strand insertions that are commonly found are T-DNA repeats, present either as inverted or as direct repeats. In plants that were subjected to AMT with two separate *Agrobacterium* strains, more than 50% of the transformed cells contained integrated DNA sequences that can be regarded as fusions of independently transferred T-strand complexes [81]. These data suggest that such T-DNA repeats are forged *in planta* and not in the *Agrobacterium* cells [82]. In the case that a second incoming T-strand successively anneals with its 3'-end to the first T-strand in a similar fashion, it seems plausible that complementary DNA strands are synthesized during repair replication after the T-strands are connected. Another route leading to the formation of complex T-DNA loci was proposed by another group after analyzing several fused T-DNA inserts where the T-strands seemed to be fused together by an illegitimate recombination followed by integration as paired double stranded T-strands [83].

The involvement of host DNA repair mechanisms in T-DNA integration in plants and yeast

It is tempting to speculate that strand-transferase activity of VirD2 covalently bound to the incoming T-strand plays part in the genomic integration of T-DNA. However, sufficient evidence has been collected that contradicted the direct involvement of VirD2 in T-DNA integration [84-86]. Instead, the DNA repair pathways of the recipient cell involved in the repair of DSBs turned out to be a determining factor in genomic T-DNA integration. DSBs of genomic DNA in eukaryotic organisms are repaired by two conserved major pathways, homologous recombination (HR) and non-homologous end joining (NHEJ). For HR the sister chromatids are used as a template to restore DSBs while during NHEJ the broken ends are directly ligated to each other without a necessity for sequence homology. Although contradicting reports have been published regarding this subject, it is generally assumed that the NHEJ protein Ku80 is involved in

T-DNA integration in plants [87-89]. It is likely that there are several pathways present in plants that facilitate T-DNA integration [90]. T-DNA integration in *Arabidopsis* is also aided by Mre11 that is a part of the MRN complex which among other cellular processes like meiosis and telomere maintenance is part of both the HR and NHEJ DNA repair pathways [91].

For *Saccharomyces cerevisiae*, it was shown that NHEJ components Ku70, Rad50, Mre11, Xrs2, Lig4 and Sir4 are required for the chromosomal integration of non-homologous T-strands [92]. This study also predicted an alternative pathway for T-DNA integration at the telomeres and in an rDNA region in *rad50Δ*, *mre11Δ* and *xrs2Δ* strains associated with drastic chromosomal rearrangements. Strikingly, telomeres and rDNA have in common that, despite not forming hetero chromatin-like structures, these regions are not accessible for RNA polymerase and various recombination proteins [93,94]. It would be interesting to know if the silencing pathway for telomeric DNA and rRNA is involved in this alternative integration mechanism for example by using a *sir2Δ* that is deficient in DNA silencing [95,96]. In an earlier publication, where lithium acetate/single-stranded carrier DNA/PEG transformation [97] was applied instead of AMT to introduce DNA into yeast cells, similar observations are described for the illegitimate genomic integration of dsDNA of a *rad50Δ* yeast strain [98]. Here the deletion of *rad50* led to 47% of the inserts to integrate into repetitive sequences like telomeres, rDNA regions and Ty elements compared to 11% of the inserts analyzed in WT. In addition to this, also chromosomal deletions and translocation events were observed at the integration sites. For the efficient integration of T-DNA homologous to the yeast genome, the HR DNA repair pathway proteins Rad51 and Rad52 are required. *Rad51Δ* and *rad52Δ* yeast strains retained only 9 to 10% of the integration efficiency observed after AMT of WT strains while the deletion of genes specifically involved in NHEJ, *ku70*, *lig4*, *rad50*, *mre11* and *xrs2* had no significant impact on the transformation efficiency [99]. These results taken together showcase a key role for either the NHEJ or HR DNA repair pathway for T-DNA integration in yeast, depending on the absence or presence of regions of homology to the yeast genome on the T-strand.

Interactions between *Agrobacterium* effector proteins and the host factors

While it is relatively easy to assay T-DNA integrations by determining their final positions in the genome of a recipient cell, it is much more challenging to define those factors within these cells that mediate the integration. In a pioneering study, Mysore and coworkers described a screening where they analyzed an *Arabidopsis* mutant line that is recalcitrant to *Agrobacterium* mediated root transformation but still has the ability to transiently express beta-glucuronidase from the T-strand [100] This mutant carried a mutation in the core histone protein H2A-1, a member of the 13-gene H2A family in *Arabidopsis*. Interestingly, expression of H2A-1 is induced upon infection with a

transfer competent *Agrobacterium* [101]. These findings make H2A-1 a likely candidate to form the main docking station for the T-complex thereby priming T-DNA integration. However there is probably considerable redundancy in T-complex binding histones in *Arabidopsis* as the overexpression of many of the other core histones leads to an elevation in transformation efficiency [102].

As described above, in *Arabidopsis* the VirE2 protein of the cytosolic T-complex interacts with the transcriptional activator VIP1 which then facilitates nuclear import of the T-complex [63]. VIP1 and VirE2 are good candidates to mediate the association of the T-strand and the host cell's chromatin. Indeed it is reported that VIP1 associates with the chromatin component H2A, probably as a homomultimer [103]. In addition to this, it was shown that the region of *VIP1* that enables homomultimerization is required for tumorigenesis but not for the transient transformation of *Arabidopsis* roots [103]. These findings suggest that VIP1 is not only involved in the nuclear targeting of the T-strand but also in the association of the T-complex with the plant chromatin. In line with such a model, it was shown that purified VirE2 is not able to form a complex with nucleosomes isolated from cauliflower florets unless VIP1 acted as a molecular link between VirE2 and the cauliflower nucleosomes [104]. For T-DNA integration to occur it is generally assumed that at a certain point the T-complex must be disassembled by removal of the proteins associated with the T-strand. A recent article reports that synthetic T-complex disassembly can be mediated by Skp1/Cullin/F-box protein VBF pathway present in the recipient plant cells [105]. Earlier reports suggested the involvement of VirF in the disassembly of the T-complex [106]. VirF is dispensable for the AMT of yeast [1] but aids in the AMT of some plant species [107-109].

1.3 Outline of this thesis

For almost two decades yeast has been used as a model to study the prerequisites for T-DNA integration and proved to be a very decent working horse. AMT of yeast cells enabled swift transformant screening and offered high convenience when many different mutant backgrounds are required for cocultivation experiments. The AMT of yeast is however not without limitations. AMT of yeast typically yields low transformation efficiencies compared to the AMT of plant cells, especially when NHEJ is required for the integration of the T-strand. Also the variation between experimental replicas is often considerable. **Chapter two** describes the first steps that have been taken to construct an all-in-yeast system enabling T-DNA processing and T-DNA integration in a single yeast cell to address these issues.

A big step forward in plant biotechnology would be the generation of genetically enhanced crops without leaving any genetic marks that are not directly required for the intended plant phenotype. To achieve this, new tools are required that enable the editing of the host cell's genome but without the requirement of T-DNA integration. For this, the expression of transgenes or DNA modifying proteins from circular non-integrative T-DNA derivatives (T-DNA circles) would be an interesting option. **Chapter three** describes a search for the prerequisites of T-DNA circle formation using yeast as a model, focusing mainly on the involvement of the host cell's DNA repair pathways. This chapter provides a model that explains the formation of T-DNA circles and the role of the HR protein rad52 therein.

The natural mechanism behind AMT of plants results in the random integration of T-DNA. More ideally, for plant biotechnological purposes, the T-strand would integrate into a predetermined locus, preferentially via the plant's HR mechanism to ensure highly accurate integration events. In spite of this, in plants T-DNA integration occurs predominantly via the NHEJ DNA repair pathway. **Chapter four** describes the effects of DSB induction on the efficiency of T-DNA integration via the HR pathway in yeast and the role the nucleosome occupancy of the target locus has on DSB induction.

To conclude the research chapters, **Chapter five** will provide an overview of the new insights that can be derived from this thesis and additionally highlight some future prospects.

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