Involvement of host and bacterial factors in Agrobacterium-mediated transformation

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Chapter 3
Detection of extrachromosomal circular T-DNA formed during *Agrobacterium*-mediated transformation

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Abstract

Agrobacterium tumefaciens is able to transfer a segment of its Ti-plasmid, called transferred DNA (T-DNA), to plant cells and under laboratory conditions also to other eukaryotes such as yeast and fungi. Inside the host cell the T-DNA integrates into the chromosomal DNA. Alternatively, the T-DNA can be circularized and form (sometimes complex) extrachromosomal structures. In this chapter, we adopted an approach developed for plants to recover circularized extrachromosomal T-DNA structures from transformed yeast and plants. With this approach we isolated circularized T-DNA from wild type and *ada2Δ* mutant yeast transformants and from the plant *Nicotiana benthamiana*. Sequence analysis showed that the isolated T-circles have very different structures, some containing a precise fusion of the left and right border repeat sequences, whereas others contain filler DNA originating either from the binary vector or from the host genome. Our data also suggest that the frequency of T-circle formation is higher in the *ada2Δ* deletion mutant than in wild type yeast cells.

Introduction

Agrobacterium tumefaciens can induce tumor formation in plants by transferring a segment of its tumor-inducing plasmid (Ti-plasmid) to the plant cell. Tumorigenic DNA within the transferred DNA (T-DNA) can be substituted by foreign DNA without perturbing its ability to integrate into the plant genome. Under laboratory conditions, other eukaryotes such as yeast and fungi can also be transformed. Therefore, *Agrobacterium*-mediated transformation (AMT) is widely used to create transgenic plants and fungi (for review see: Nester et al., 1984; Gelvin, 2003; Tzfira and Citovsky, 2006; Păcurar et al., 2011; Christie and Gordon, 2014; Gelvin, 2017; Hooykaas et al., 2018).

The process of T-DNA integration has been widely studied and several alternative mechanisms have been proposed including T-strand invasion and double-strand T-DNA ligation into the host genome. Generally the integration is not precise and clean, leading to complex junction patterns between T-DNA and the plant genome. Deletions of host DNA at the integration sites are common and insertion is often accompanied by the insertion of "filler DNA" from neighbor sequences or from distant areas (reviewed in Gelvin, 2017). At the integration sites often multiple copies are present, commonly in an inverted repeat structure. Besides, double-stranded non-integrated T-DNA molecules were occasionally found in transformed plants (Offringa et al, 1990; Narasimhulu et al., 1996). Some of these might serve as intermediates for integration and eventually result in the formation of various T-DNA structures. Alternatively, they are only temporarily present in a linear (concatemeric) form or a circular form (T-circle).

T-circles were first recovered from bacteria after activation of the virulence genes. They were formed by precise DNA recombination of nicked border repeats and rescued by using λ *in vitro* packaging followed by plasmid rescue (Koukolilova-Nicola et al., 1985). At the time, before the discovery of the T-strand, they were thought to be transfer intermediates. Subsequently, a virus-based system was used to recover T-circles after transfer by replacing the T-DNA genes by a virus genome (Bakkeren et al., 1989). Rescued virus had a structure compatible with non-precise end-joining of a T-strand, with loss of few nucleotides from the LB and insertion of 1-14 nt filler from unknown origin. Based on these ideas, Singer et al. developed a plasmid-recue approach to recover extrachromosomal T-circles from *Agrobacterium* infected plants (Singer et al., 2012). By sequence analysis, it was shown that the junctions between the ends of the T-DNA included T-DNA border fusions, T-DNA truncations, binary plasmid sequences, and filler DNA sequences, suggesting that they were formed by a process resembling T-DNA integration.

In yeast extrachromosomal T-circles could be stably maintained, when they contained a replication unit (Bundock et al., 1995; Soltani, 2009; Rolloos et al, 2014; Ohmine et al., 2016). For instance, T-DNAs containing the 2μ replication origin exhibited high transformation efficiencies and were present as plasmids generated by the fusion of left and right border (Bundock et al., 1995). To date there is no evidence to support the idea that T-circles can further integrate into the host genome in nature even though T-circle formation may share a similar mechanism as T-DNA integration. Moreover, T-DNAs containing two directly repeated *lox* sites were reported to circularize and integrate through Cre/*lox*-mediated single cross over in the plant genome at a genomic *lox* site, but no evidence for similar Cre/*lox*-mediated

integration was found when using a T-DNA with a single *lox* site, whereby circularization would be dependent on border fusion (Vergunst and Hooykaas, 1998).

The formation of T-circles could be beneficial for *Agrobacterium* because the transformed plant cells would already start generating opines and tumor producing hormones before or even without the need of genomic integration of the T-DNA. Although probably less prone to integration, circular T-DNA molecules are probably more stable than linear molecules and may thus be important for transient expression and intermediates for further T-DNA integration. In this chapter, we adapted the approach developed by Singer et al. to recover T-circles from yeast and plants. To this end, we constructed a new T-DNA vector containing a selection marker and replication unit for *E.coli*, but not for yeast and plant cells. We compared the recovery of T-circles from wildtype yeast and *ada2Δ* mutant, and determined the structures of them in comparison to those from plants.

Methods

Bacterial cells and growth conditions

Agrobacterium tumefaciens strain LBA1100 and its derivatives used in this chapter are listed in Table 1. All the plasmids were transferred into *Agrobacterium* by electroporation (den Dulk-Ras and Hooykaas, 1995). *A. tumefaciens* strains were grown and cultured at 29°C in LC medium (10 g/L tryptone, 5 g/L yeast extract and 8 g/L NaCl) containing the appropriate antibiotics such as carbenicillin (100 μg/mL), kanamycin (100 μg/mL) or rifampicin (20 μg/mL) when required. *Escherichia coli* DH10B was used for plasmid amplification and grown on LC plates with carbenicillin (100 μg/mL) and kanamycin (100 μg/mL) as required.

Yeast strains and growth conditions

S. cerevisiae strains used in this study can be found in Table 1. Yeast was grown at 30°C in yeast extract-peptone-dextrose (YPD) medium supplemented when required with the appropriate antibiotic hygromycin (200 μg/mL).

Plasmid constructions

The plasmids used in this chapter are listed in Table 2. All newly constructed plasmids were checked by restriction digestion and sequence analysis. All primes used are presented in Table 3.

For detection of T-circles, the binary vector pSDM4708 was constructed as follows. A T-DNA fragment containing the KAN.MX cassette was amplified by PCR using pSDM8000 as a template and pSDM8000-Fw/Rev as primers and then cloned into plasmid pBBR6 after digestion with *Not*I and *Apa*I to form plasmid pSDM4710. Then pUC9 was linearized with *SalI* and ligated into *SalI* digested pSDM4710, generating plasmid pSDM4711. The linearized pUC9 with ampicillin resistance gene replaced the KAN.MX cassette in the T-DNA. Subsequently, the bacterial kanamycin resistance gene (Km^R) was amplified by PCR on the vector pCAMBIA1390 using primers Km-Fw/Rev thereby introducing *Not*I and *Apa*I sites at the ends and inserted into plasmid pSDM4711 after digestion with *NotI* and *ApaI* to remove the pBBR6 vector part, producing plasmid pSDM4712. Finally, a fragment containing the pVS1 replication origin of replication needed for maintenance in *Agrobacterium* was amplified from vector pCAMBIA1390 using the primers pVS1-Fw/Rev and cloned into the *ApaI* site of pSDM4712, resulting in plasmid pSDM4708 (Figure 1A).

Agroinfiltration

Seeds of *Nicotiana benthamiana* were germinated and grown in controlled climate chambers at 24°C with a 16h light/8h dark photoperiod with 75% humidity for three weeks before infiltration. *A. tumefaciens* strains were cultured overnight at 29°C. After dilution to A_{600} of around 0.8 in 10 ml of induction medium (IM) containing 200 μM acetosyringone (AS), the cultures were grown for three hours at 28°C. Subsequently, the cultures were transferred into a blunt-tipped plastic 10 ml syringe (Nissho NIPRO Europe N.V.) and injected into the leaves of the *N. benthamiana* line. This was done by applying the tip of the syringe to the lower surface of the leaves and injecting with gentle pressure. Young leaves of three weeks old plants were used for agroinfiltration. After 2 days, the lower side of the injected leaf was harvested for DNA isolation (Wroblewski et al., 2005).

***Agrobacterium* mediated transformation of yeast**

AMT efficiency was determined as described by Bundock et al. (1995) with some modifications. Firstly, *S. cerevisiae* strains and *Agrobacterium* strains were grown overnight at 30°C and 28°C respectively, under continuous shaking and with the appropriate antibiotic selection. The following day, the *Agrobacterium* culture was centrifuged, the cells were washed with induction medium (IM) and re-suspended to an A_{620} of 0.25 in IM with added glucose (10 mM), acetosyringone (AS, 0.2 mM) and appropriate antibiotics, and incubated for another 6 hours at 28°C. Meanwhile, yeast cultures were diluted to an A_{620} of 0.1 and incubated again in liquid YPD for 6 hours. Then, yeast cultures were centrifuged and cells were washed with IM and re-suspended in 0.5 ml of IM to a final A_{620} of 0.4 - 0.6 and mixed vigorously with an equal volume of Agrobacterium cells. Subsequently, 100 μl of the mixture were pipetted onto sterile nitrocellulose filters laid on IM plates supplemented with histidine, leucine and methionine. Once the filters were dry, plates were incubated at 21 °C for 3 days, after which DNA was isolated from the mixture

DNA isolation, recovery of T-circle, sequencing and analysis

For DNA isolation from plants, leaves were harvested and disrupted to powders under liquid N_2 in a Tissuelyser (Retch, Haan, Germany). Total DNA was extracted according to the CTAB method (De Pater et al., 2009). DNA isolation from yeast was performed following standard rapid isolation protocol (Sambrook and Russell, 2006). Isolated DNA was used for the transformation of *E.coli* strain DH10B with selection for carbenicillin and kanamycin resistance. Plasmid DNA was isolated from Cb^R/Km^S colonies.

For analysis of the extrachromosomal structures the junctions between the LB and RB sequences were amplified via PCR with the primers pA1/pA2 or pA3/pA4. Then, PCR products were purified by agarose gel electrophoresis and sequenced by Macrogen (Amsterdam, the Netherlands). Primers were ordered from Sigma-Aldrich.

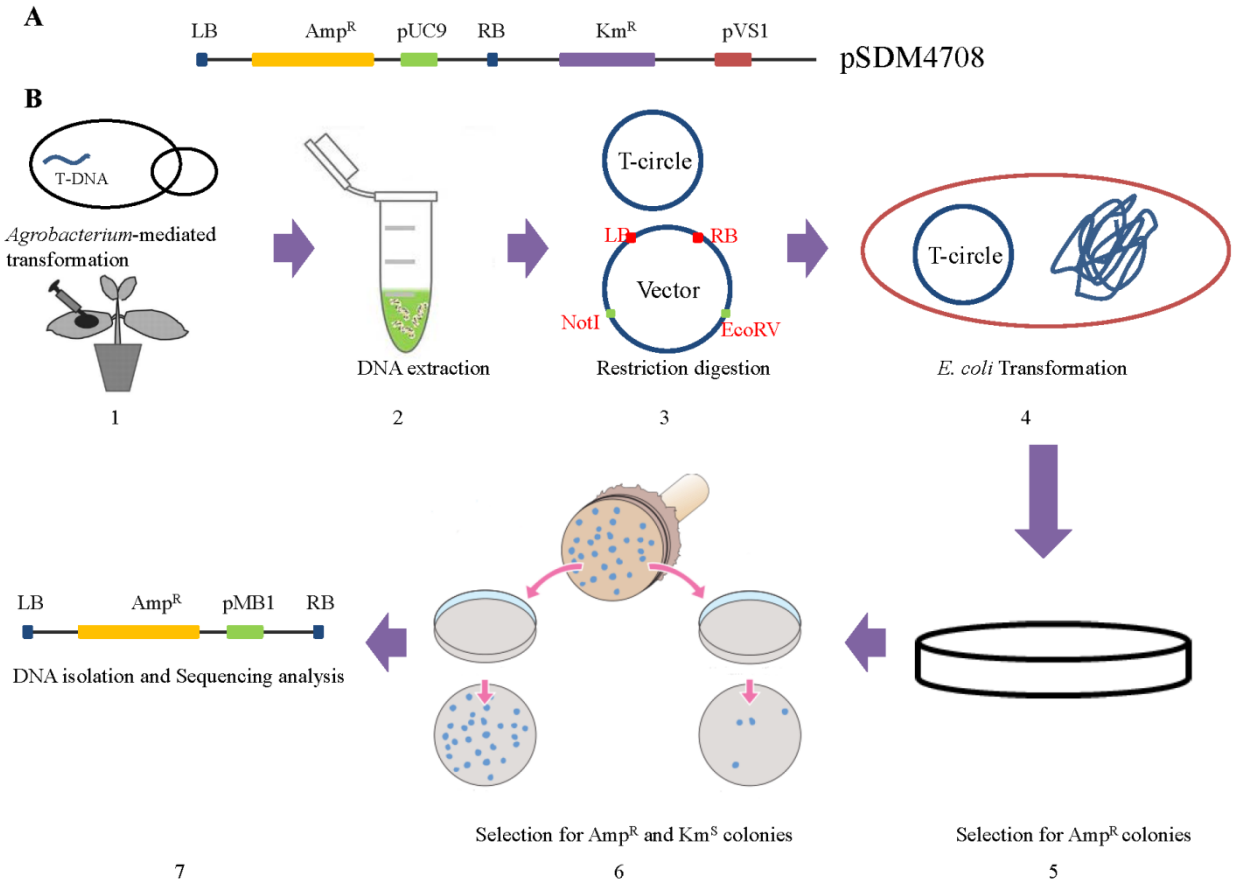
Results

A plasmid-rescue method to capture T-circles from plant and yeast cells

We were interested in capturing T-circles for which we found genetic evidence in Chapter 2 in the yeast *ada2Δ* mutant. As no T-circles had previously been recovered from transformed yeast without prior selection of transformants, we first tested the recovery of T-circles from plant cells. In order to study circularization of T-DNA we modified the plasmid-rescue technique described previously (Singer et al., 2009; Rolloos et al., 2014). To this end, we constructed plasmid pSDM4708 containing T-DNA with the ampicillin resistance marker and the MP1 origin of replication (Figure 1A). Upon circularization of the T-DNA in the host cell a plasmid is formed with an origin of replication for *E. coli* and an ampicillin resistance marker allowing amplification and selection in *E. coli*. This construct was introduced in *Agrobacterium* strain LBA1100 (lacking T-DNA). The T-DNA construct was introduced into plant leaves through agroinfiltration and into yeast cells by co-cultivation using the AMT protocols mentioned in Chapter 2. At 2 days post agroinfiltration or co-cultivation, total DNA was isolated from the mixture of *Agrobacterium* and infected host cells, including the T-DNA binary vectors from *A. tumefaciens* itself. To remove the original T-DNA binary vector pSDM4708, the isolated DNA was digested with *EcoRV* which cuts the plasmid in the backbone, but not in the T-DNA (Figure 1B). The digested DNA was used to transform *E. coli* and bacteria containing circularized T-DNA (T-circles) were selected on plates containing ampicillin.

In our experiments using the wild type yeast strain BY4741 we isolated only a very low number of ampicillin resistant, but kanamycin sensitive (backbone marker) *E. coli* colonies (less than 10 per experiment). As the *ada2Δ* mutant gives a higher transformation frequency (Chapter 2) we also isolated T-circles after AMT of this mutant. This resulted in the isolation of more than 30 ampicillin resistant *E. coli* colonies per experiment, whereby the proportion of kanamycin sensitive was 10 out of 80. In addition, the same approach was applied to transform the plant *N. benthamiana*, which was used successfully in previous research (Singer et al., 2012). Also from the plant we were able to obtain several ampicillin resistant kanamycin sensitive *E. coli* colonies for further analysis. The number of colonies obtained from DNA isolated from plants was much higher than the number of colonies obtained from DNA isolated from yeast. Among those resistant to ampicillin, kanamycin sensitive colonies occupied around 17% (16 out of 96). As there is an alternative possibility that T-circles are generated inside *A. tumefaciens* instead of in the host cell, we also used *A. tumefaciens* strains LBA1143 and LBA1144 carrying pSDM4708, in which *virB4* and *virB7*, respectively, are mutated and thus fail to form a T4SS apparatus. After AMT of the *ada2Δ* mutant with either of these *virBΔ* mutant strains, we did not obtain any ampicillin resistant kanamycin sensitive *E. coli* colonies, suggesting that T-circle formation occurs inside the host cell after transfer rather than in *A. tumefaciens* prior to transformation, in line with previous results showing that T-DNA transfer is required for T-circle formation (Singer et al., 2012).

Figure 1. Constructs and experimental procedure for the isolation of T-circles. A, schematic diagram of the T-DNA construct pSDM4708 used throughout the experiments. B, illustration of the experimental procedure: 1, *A. tumefaciens* carrying the binary plasmid pSDM4708 was injected into the leaves of *N. benthamiana* or co-culvitated with yeast cells; 2, extraction of DNA from the agroinfiltrated leaves or from cells of the Agrobacterium-yeast co-cultivation mixture (3 days after infiltration/cocultivation); 3, digestion with restriction enzymes *NotI* and *EcoRV* to remove the original pSDM4708; 4, transformation of *E. coli* competent cells with digested DNA; 5, selection of *E. coli* transformants resistant to ampicillin; 6, further selection of *E. coli* transformants sensitive to kanamycin; 7, isolation of plasmid-like DNA from each independent colony and analysis by DNA sequencing. Amp, ampicillin; Km, kanamycin.



Details of T-DNA junctions recovered from host cells

To characterize the T-circles, the LB-RB junctions were analyzed by DNA sequencing of either PCR products obtained by using two pairs of primers (pA1/A2 or pA3/A4) or by direct sequencing the isolated T-circles. In total, sixteen T-circles isolated from plant *N. benthamiana* and ten T-circles isolated from the yeast *ada2Δ* mutant were analyzed. The structures of six representatives (T-1 to T-6) are shown in Figure 2. The majority of the T-circles isolated from yeast (7 out of 10) had the same structure as T-1. The other three T-circles have a different structure (T5 and two times T6). On the other hand, of the T-circles isolated from plants, only

1 out of 16 had the same structure as T1. The majority (13 out of 16) of plant derived T-circles had a structure like T-3 and two had a different structure (T-2 and T-4).

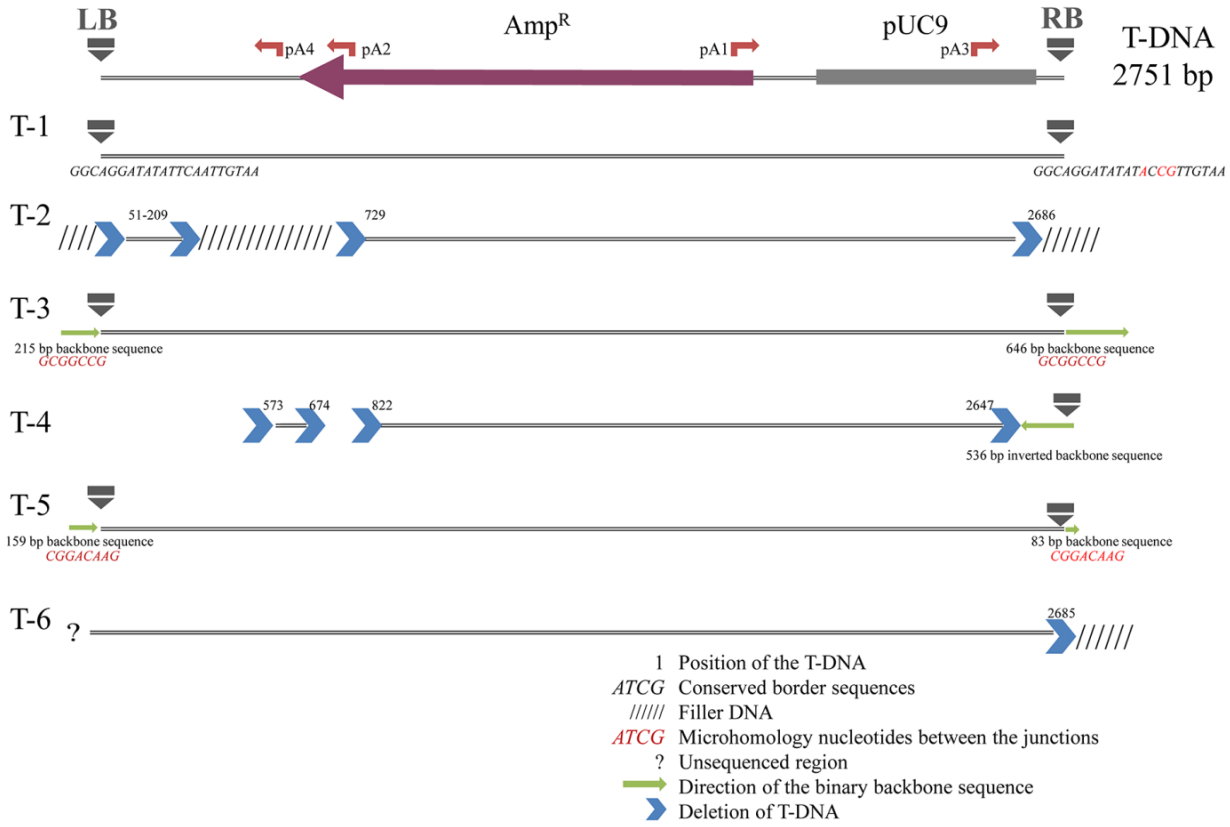


Figure 2. Schematic illustration of the T-circles T-1 to T-6 based on DNA sequencing. T-1 to T-6 are shown in a linear form, opened at the position of the junction, and aligned to the complete construct-DNA of plasmid pSDM4708. The annotations represent different events identified by sequence analysis.

In T-1, a T-circle isolated from yeast, a precise border fusion was found, compatible with a reversion of the nicking reaction of relaxase VirD2. This could immediately lead to circularization (monomeric reaction) or first lead to concatemerization (dimer or multimer formation), after which HR is necessary for conversion in a T-circle as suggested by Rolloos et al., 2014. A similar structure of T-circle was also identified in infected plant cells at a much lower frequency (1 out of 16).

The structure of T-2, isolated from plant, is complex. Instead of a RB-LB fusion two pieces of filler DNA were found with T-DNA sequences in between. It contained a patchwork of filler sequences ("scrambled") interrupted by short T-DNA sequences (159 bp). Both border sequences were lost. At the LB side two fragments (50 bp and 520 bp) were missing and from the RB side of T-DNA, a 66-bp fragment including right border sequence disappeared. All these filler sequences start with short sequences homologous with parts of the associated *vir*-helper plasmid in *Agrobacterium* (79 bp, 49 bp and 63 bp). Previous studies have already shown that transfer of *Agrobacterium* chromosomal DNA fragments is possible (Ulker et al., 2008). By

tracing the sources of filler sequence found at T-DNA integration sites in plants, around 1.1% of them turned out to be homologous with *Agrobacterium* genome sequences (Kleinboelting et al., 2015). Taken together, we hypothesize that some DNA fragments could be translocated into plant cells independent of T-DNA and these fragments may further serve as templates for microhomology-mediated end-joining.

In T-3, isolated from plant, two stretches of backbone sequences adjacent to either side of the T-DNA were present at the junction. The length of the stretch of backbone sequences at the LB side is 215 bp and that of the RB side is 646 bp. They may have been fused due to 7 bp of microhomologous sequences (GCGGCCGC) at the end of them. Such "read-through" binary backbone sequence fragments were reported before and probably caused by improper "border skipping" of the VirD2 endonuclease (Kononov et al., 1997). They are most likely translocated into host cells as single strand DNA, then converted to double strand molecules and finally ligated with microhomology end-joining. A similar structure was present in 13 out of 16 T-circles isolated from *N. benthamiana*.

In T-4, isolated from plant, at the LB side two large fragments including the border sequence are missing. The larger deletion (572 bp) is at the LB and the other deletion corresponds to nucleotides 675-821 of the original T-DNA. The RB side is incomplete and is replaced by an 536 bp inverted backbone fragment from inside to outside of T-DNA. The RB is still intact, while, the orientation is reverse, thus indicating a possible RB-RB junction formed between two T-DNA repeats. This distinct structure may be formed when two T-strands were delivered into plant cells independently, then converted to dsDNA and eventually linked to form a T-circle. Similarly, a repeat feature is frequently found at the T-DNA integration sites of plant cells after AMT (Krizkova and Hrouda, 1998) and also supported by the co-integration of different T-DNAs from different binary vectors at the same site following co-transformation (De Neve et al., 1997).

With respect to the T-circles recovered from yeast, most of them were generated by the "perfect" fusions of LB and RB (T-1). An example of an imprecise T-circle is represented as T-5. Two short binary read-through backbone sequences adjacent to either repeat border (159 bp at the LB side and 83 bp at the RB side) were linked together, possibly because of the microhomology at the end of them as in the T-3 structure.

In T-6, isolated from yeast, small fragments close to both the LB and RB are missing and are replaced by large filler sequences with strong homology with yeast chromosomal DNA. However, unfortunately, we were not able to sequence the internal region of the fragment, possibly due to the formation of secondary structures. Unlike filler sequences in T-2, they were identified to share homology with host genomic DNA, such as with the *BUD3* locus in chromosome III and the *ECM38* locus in chromosome XII from yeast in two junctions. The relationship between them has not been identified and seems to be obtained by random integration. These patterns of filler sequence are often seen at the T-DNA integration sites in *Agrobacterium*-infected plants as well and may indicate that a random DNA template can potentially be used utilizing microhomology by DNA polymerase during end-joining or integration.

Discussion

It has been shown that the AMT efficiency of yeast is higher, when the T-DNA contains an autonomous replication origin, than when the T-DNA lacks such a replication origin and needs to be integrated into one of the chromosomes for stable maintenance (Bundock et al., 1995; Soltani, 2009). However, maintenance of T-DNAs with an autonomous replication origin requires circularization. Circles were formed by precise joining of the RB and LB ends, possibly mediated by VirD2 as a reversal of the nicking reaction. This reaction could immediately lead to circularization (monomeric reaction) or lead to concatemer formation first (dimeric and multimeric reaction), which can be resolved by the homologous recombination pathway (Soltani, 2009; Rolloos et al., 2014; Ohmine et al., 2016). Non-integrated T-DNA "free" molecules (T-circles) were detected in transformed plants as well in early stages after transformation (Singer et al., 2012). These seem to be formed by non-homologous end joining of the ends. To our knowledge, the formation and (temporary) presence of extrachromosomal T-circles without host replication origin in yeast has not been reported as yet. Here we obtained evidence for the presence of such T-circles without any replication origin after AMT of yeast cells.

The complex extrachromosomal T-DNA structures identified in this research contain various formats including perfect fusions of LB and RB, binary plasmid sequences, filler host DNA sequences and T-DNA truncations (Figure 2). These observations are consistent with previous results from similar experiments with plants (Singer et al., 2012). The ligation patterns present in T-circles mentioned above are thought to resemble the parts of repaired DNA double-strand breaks sites, in other words, it is likely that the formation of extrachromosomal T-circles share the same DNA repair mechanisms with the integration of T-DNA into host genome. Recently, the successful transfer of a T-DNA plasmid to *E.coli* has been reported (Ohmine et al., 2018). They co-cultivated *Agrobacterium* containing a modified T-DNA plasmid harboring a RB but not a LB with *E.coli* or yeast. The modified plasmid would be nicked by VirD2, subsequently guided into the recipient cells as single-strand and eventually circularized and maintained as plasmid. In bacteria, the AMT efficiency was affected by RecA which plays a crucial role in DNA HR repair pathway. It is in line with the observation that T-DNA circularization efficiency relies on Rad52 and Rad51 in yeast (Rolloos et al., 2014; Ohmine et al., 2016). Compared to wild type yeast cells, the efficiency was reduced around 70-75% when using *rad52Δ* deletion mutant as recipient cells, while only a slight difference was seen in the y*ku70Δ* yeast strains. In yeast, Rad51 is a homologous protein of RecA and works together with Rad52 in the HR pathway, whereas KU70 is responsible for NHEJ. Results indicated that the process of T-DNA circularization largely depends on the HR pathway in yeast and bacteria, which is the most important DSB repair pathway in these microorganisms. In contrast, end-joining is the dominant DSB repair pathway in plants and the identified patterns of T-DNA junctions resemble those of end-joining events. It would be interesting to investigate the frequency of T-circle formation in plant end-joining mutants. Recently, the DNA polymerase theta (*Pol θ*) was unraveled to mediate random T-DNA integration in plants, whose role is indispensable and essential (van Kregten et al., 2016). It mediates the 3' end capture of T-DNA at genomic breaks for which only one or few bps of micro-homology are required, thus also explaining the frequent presence of filler DNA sequences at the integration sites. As the junctions present in T-circles obtained from plants also contain microhomology and filler

sequences, their formation may have been mediated by *Pol θ*. The analysis of T-circles may thus lead to new insights in the mechanism of T-DNA integration in host cells.

VirD2 may be involved in the formation of T-circles because of its ability to catalyze a reverse transesterification reaction between the free ends of the T-strand and reverse the nicking reaction (Pansegrau et al., 1993). This could explain the precise fusion of the border repeat sequences seen in yeast T-circles and in exceptional plant T-circles. However, if the formation of T-circles is mediated by VirD2 without any help from host factors, T-circles could be generated in *Agrobacterium* cells as well. This hypothesis is not consistent with our finding that no T-circles were detected from donor *Agrobacterium* strains LBA1143 and LBA1144, lacking the T4SS. It confirms the notion that the circularization of T-circles occurs in the host cells and not in *Agrobacterium* and that T-DNA transfer to the host is required (Singer et al., 2012). Considering the high frequency of precise fusions of the two borders occurring in the host (yeast), further research on the involvement of VirD2 in T-DNA integration is needed.

As described in Chapter 2, a relatively large number of T-DNAs were maintained extrachromosomally, thus not integrated into the yeast genome, especially in the *ada2Δ* deletion mutant. Analogously, the frequency with which T-circles were obtained, varied in different plant species. In tobacco *N. tabacum*, the frequency of T-circles among the rescued plasmids ranged from 5% to 10%, while it increased to more than 30% in *N. benthamiana* (Singer et al., 2012). It can be proposed that host factors play an important role in the circularization of T-DNAs. In yeast, *ADA2* may be involved in either the fusion of the border repeats or the maintenance of T-circles. Further research is needed to find out how *ADA2* is involved.

Table 1. Yeast and Agrobacterium strains used in this study

Yeast strain	**Genotype**	**Source/Reference**
BY4741	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Brachmann et al., 1998
BY4741 *ada2Δ*	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ada2::hphMX4*	Chapter 2
***Agrobacterium* strain**	**Description**	**Source/Reference**
LBA1100	*Agrobacterium* strain C58 containing pTiB6Δ(*ΔT-DNA, Δocc, Δtra*), Rif, Spc	Beijersbergen et al., 1992
LBA1143	LBA1100 *virB4Δ*, Rif, Spc, Cb	Beijersbergen et al., 1992
LBA1144	LBA1100 *virB7Δ*, Rif, Spc, Cb	Beijersbergen et al., 1992
LBA1100-pSDM4708	LBA1100 with plasmid pSDM4708	This study
LBA1143-pSDM4708	LBA1143 with plasmid pSDM4708	This study
LBA1144-pSDM4708	LBA1144 with plasmid pSDM4708	This study

Table 2. All the plasmids involved in this study

Plasmid	**Specifications**	**Source/Reference**
pUC9	Cloning vector with ampicillin-resistance gene	Vieiria and Messing, 1982
pCAMBIA1390	*Agrobacterium* binary vector with kanamycin-resistance gene	Cambia labs
pSDM8000	*Agrobacterium* binary vector with KanMX selectable marker	van Attikum et al., 2001
pSDM4708	pSDM4712 with pVS1 replication origin	This study
pSDM4710	pBBR6 with T-DNA harboring kanamycin-resistance gene	This study
pSDM4711	T-DNA harboring ampicillin-resistance gene and pUC9 replication origin	This study
pSDM4712	pSDM4711 with kanamycin-resistance gene out of T-DNA region	This study

Table 3. Primers used in this study

Primer name	Sequence (5'-3')	Restriction enzyme
pSDM8000-Fw	AAAGCGGCCGCATCATGAGCGCCAAGCTCAA	*NotI*
pSDM8000-Rev	AAAGGGCCCGGCGCCAGATCTGAGCTTT	*ApaI*
Km-Fw	AAAGCGGCCGCTTGATCCGGCAAACAAACCAC	*NotI*
Km-Rev	AAAGGGCCCCCGCGGTTTCAAAATCGGCT	*ApaI*
pVS1-Fw	AAAGGGCCCTGCTATAGTGCAGTCGGCTT	*ApaI*
pVS1-Rev	AAAGGGCCCACAAGCTGTGACCGTCTCCG	*ApaI*
pA1	ATCGCTGAGATAGGTGCCTCA	
pA2	GATGCTGAAGATCAGTTGGG	
pA3	GAGCGCAACGCAATTAATGTGAGTTA	
pA4	ATATGGTGCACTCTCAGTACAATC	

Restriction sites are underlined.

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