CRISPR/Cas-induced targeted mutagenesis with Agrobacterium mediated protein delivery

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Chapter 1

General Introduction

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General introduction

In the first part of this chapter I will review the developments in the field of genome editing. Specifically the DNA repair mechanisms involved, the tools available and the applications of genome editing in plants. As I have used *Agrobacterium tumefaciens* for delivery, the second part of this chapter will focus on the plant pathogen *Agrobacterium*, describing the mechanisms underlying the transformation of plants by *Agrobacterium* and its biotechnological applications.

Part 1

Genetic engineering and genome editing

Controlled integration of transgenes and controlled genetic replacement of endogenous genes by transgenes based on homologous recombination, also known as gene targeting, is an important tool in biotechnology because it can accelerate the rate of functional gene analysis and guarantees a save introduction of novel traits. This controlled manipulation of genomes is based on the introduction of DNA that contains homology with the chromosomal locus allowing for DNA recombination. In lower eukaryotes such as yeast, integration of DNA occurs predominantly via homologous recombination and therefore the recombinatorial events required for gene-targeting were first studied in the budding yeast *Saccharomyces cerevisiae* [1,2]. In higher eukaryotes this kind of integration also occurs, albeit at low frequencies compared to random integration. The first molecular evidence for gene-targeting in animal cells was provided by the laboratories of Smithies [3] and Capecchi [4,5] in experiments that generated loss-of-function mutations in embryonic mouse stem cells.

In plant cells the first report of gene-targeting appeared in 1988. A DNA repair template transferred to tobacco protoplasts was shown to recombine with an integrated defective copy of a resistance gene leading to integration of a restored gene [6]. In 1989 this was followed by a report showing that delivery of a repair construct via *Agrobacterium*, a bacterium commonly used to transform plants, in the form of a T-DNA could be used for gene targeting leading to the correction of a defective gene at its original locus in the genome [7]. However, to harness the power of homology-directed recombination for gene insertion or gene repair in plants the naturally low HDR frequencies ranging between 10^{-4} and 10^{-6} needed to be enhanced [7–13].

Early experiments showed that DNA-damaging agents stimulated the exchange between sister chromatids [14], but the most compelling evidence that breaks in the DNA enhanced recombination came from studies that showed that a single double-strand break (DSB) in the genome dramatically increases the local frequency of recombination. In these studies a fragment encompassing the recognition site for a specific DNA endonuclease and a defective resistance gene was first inserted into the genome. Recombination with a homologous donor DNA increased several orders of magnitude when the cognate DNA endonuclease was expressed in the recipient cells [15,16].

Initially only naturally occurring site specific nucleases (SSNs) were available for this purpose. During the last decade, however, artificial site specific endonucleases with a customizable DNA recognition and cleavage site were developed that can now be employed for targeted modification of almost any genetic information in the genomes of organisms. These SSNs can also be used for targeted mutagenesis. When SSNs are expressed and used to

induce DSBs, the DNA repair machinery of the cell will seal the break, but when the nuclease is persistently present the DNA will be broken again leading to a cycle of break-repair until imprecise repair leads to a loss of the nuclease restriction site. In this way mutations can be selected at the nuclease target site. If these mutations occur in coding sequences genes can be knocked out making it easier to study the function of the mutated gene. Mutations created in non-coding regions can be used for instance to disrupt binding sites of pathogen produced transcription factors that promote disease [17]. Using SSNs even entire gene clusters can be removed by inducing DNA breaks at opposing ends of the gene cluster [18–20] or for the knockout of multiple (redundant), non-allelic homologous genes at the same time.

Double-strand break repair

The genome is subject to many agents, both exogenous and endogenous leading to DNA damage, that, if unrepaired, may lead to mutation or gross chromosomal rearrangements. Such damage includes base lesions, DNA single-strand breaks (SSBs) and DSBs. Repairing the damage is vital to maintain an organisms genomic integrity and stability and therefore multiple DNA repair pathways have evolved. Exploiting such DNA repair mechanisms of DSBs, and to a lesser extent those involved in SSB repair and base excision repair (BER), underlies genome editing. Two distinct pathways can be used for the repair of DSBs; non-homologous end joining (NHEJ) and homologous recombination (HR). Which repair pathway is used for the repair of DSBs differs greatly between organisms and the cell cycle phase at which the repair of a DSB occurs. Repair via NHEJ operates throughout the cell cycle but predominantly in the G1 phase [21], whereas repair via HR is restricted to the S and G2 phases when sister chromatids are available as a repair template.

NHEJ repairs DSBs by religating the broken ends irrespective of sequence homology and can be precise, but may also result in small deletions or insertions disrupting the genetic information [22]. The first step in NHEJ is the binding of the heterodimeric Ku complex to both ends of a DSB. This complex is composed of a 70kDa and a 80kDa subunit, named Ku70 and Ku80, respectively [23]. After the binding of Ku to a DSB, in mammalian cells DNA-PKcs is recruited to the end of the DNA break [24,25]. DNA-PKcs, however, is not present in plants and fungi. NHEJ is completed by ligation of the DNA ends; this rejoining is carried out by a complex of DNA ligase IV, XRCC4 and the XRCC4-like factor [26]. In plants and yeast orthologues of these C-NHEJ components have been identified [27–31]. Although several bacterial strains have been identified with a NHEJ-like DNA repair mechanism, the repair of DSBs in bacteria is predominately based on repair by HR [32,33].

In the absence of canonical factors involved in Ku-dependent NHEJ, back-up pathways are responsible for residual end joining of DSBs [34–37]. Repair via back-up NHEJ starts with the resection of the ends producing ssDNA ends that can anneal at microhomology regions. The ends may be linked at these microhomology regions, whereafter non-homologous tails are removed. The remaining gaps are filled by a specific DNA polymerase and re-ligated by DNA ligase I [38]. In these back-up pathways microhomologies sequences (5-25 basepairs) are frequently used for the repair of the DSB resulting in deletions [39] and therefore this pathway has also been called microhomology mediated end-joining (MMEJ) [40]. Several factors involved in repair via MMEJ have been identified: PARP1/2, MRN, CtIP, Ligase3, XRCC1 and DNA polymerase θ (Pol θ). The exact molecular mechanism behind MMEJ is poorly understood, but PARP1, the MRN complex and Pol θ have been implicated as key

players [41–43]. Recently, it has been shown that theta mediated end joining (TMEJ) is the dominant pathway for repair in Ku-deficient cells and the occurrence of microhomologies at the break site which is the result of the ability of Pol θ to mediate joining of two resected 3' ends harboring DNA sequence microhomology [42,43].

Homologous recombination is a DNA repair mechanism that uses DNA homology to direct DNA repair. These homologous sequences required for repair by HR are preferentially found on sister chromatids, but other naturally occurring homologous sequences or an artificially introduced repair template may sometimes be used instead. Repair via HR can be divided into several subpathways, classical double-strand break repair (DSBR), synthesis-dependent strand annealing (SDSA), single-strand annealing (SSA) and break-induced replication (BIR) [44]. DSBR was initially described to explain crossover events during meiosis and gene conversion [45]. SDSA is used for mitotic DSB repair [46] and is the predominant repair mechanism for two ended DSBs via HR [47]. In plants evidence has been found for the occurrence of DSBR, SDSA and SSA [48].

HR invariably starts off with the 5' resection of the ends resulting in large 3' ssDNA stretches that can search for complementary sequences. Repair via SDSA starts with a 3' end invading a homologous double-strand forming a D-loop. This is followed by repair synthesis from the 3' end using the newly paired strand as a template. The "X" shaped structure formed at the border between the hetero- and homoduplex of the D-loop is called a Holiday junction. After elongation, in SDSA the invading strand is displaced from the D-loop structure and anneals back to the 3' homologous strand that was formed by resection of the other end of the DSB. Thus in SDSA the donor molecule remains unaltered and gene conversion without loss of sequence information is the final result of the reaction [49]. SDSA is preferably used for HR repair in mitotic cells in the S and G2 phase, when a sister chromatid is available as a template. DSBR is mainly used for the repair of DSB breaks in meiotic cells. In this case both DNA ends invade a homologous chromosome to copy genetic information and this results in a double Holiday junction that may be resolved into a crossover or a non-crossover product.

SSA is a repair mechanism that can be used when two homologous sequences are in close proximity and arranged in a tandem orientation. Repair via SSA starts with resection and the production of 3' single-stranded overhangs. This is followed by immediate pairing of the single stranded complementary sequences found near the break site and trimming of the any remaining 3' ends [50]. SSA is not conservative in contrast to SDSA and DSBR and leads to deletion of one repeat and the intervening sequence. Up to one out of three DSBs in an artificial genomic region with tandemly arranged duplications was repaired via SSA after DSB induction [51].

Site-specific nucleases

To induce targeted DSBs, four different classes of SSNs have been developed: homing endonucleases (HEs), zinc finger nucleases (ZFNs), TAL effector nucleases (TALENs) and the CRISPR/Cas RNA guided endonuclease encoded by the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system (Fig. 1) [52].

The first class of SSNs are the HEs which are a class of proteins typically encoded for by introns or inteins and are considered selfish genetic elements, much like transposons [53]. This class of proteins can be divided into eight different subclasses: LAGLIDADG, H-N-H, His-Cys, GIY-YI G, PD(D/E)xK, HJ resolvase-like, EDxHD and Vsr-like [54]. All of these different classes of HEs have large DNA sequence recognition sites varying in length

between 12 to 40 basepairs. Because the DNA binding domain and the cleavage domain of HEs overlaps [55] creating HEs with new DNA binding specificities is challenging. For HEs of the LAGLIDADG class progress has however been made in altering the DNA recognition and cleavage site [56,57]. HEs were the first to be used for targeted mutagenesis in *Arabidopsis* and maize and instrumental in showing that DSB induction enhances targeted mutagenesis and gene targeting in plants (see Table 1).

In ZFNs the DNA binding domain consisting of zinc fingers is fused to the non-specific nuclease domain of the FokI nuclease. The zinc-finger array responsible for DNA binding is created by combining multiple zinc-finger domains, of the C_2H_2 class of zinc-fingers, that were discovered as part of the transcription factor IIIA [58]. This class of zinc-fingers consists of two β-sheets and an α-helix, which fits directly into the major groove of double strand DNA. The side chains from the N-terminal part of this helix contact the edges of the basepairs. Changing the amino acids in the α-helix may alter the affinity for different DNA sequences. Each zinc-finger domain recognizes 3bp of DNA [59]. Zinc-fingers have been created for the recognition of most of the 64 possible triplets [60–62]. By fusing the FokI nuclease domain to the zinc-finger array a zinc-finger nuclease (ZFN) is created. Two ZFNs are usually constructed that bind the DNA at opposing sites of the target sequence, each making a nick in one of the DNA strands leading together to a staggered DSB. A problem that can arise is the formation of a homodimer consisting of two ZFNs for one of the half sites. As a consequence, a DSB may be induced in the genome at a position which was not the initial target, possibly leading to toxicity [63]. Miller et al. [64,65] have designed complementary FokI cleavage domain variants that together function as an obligate heterodimer. The widespread application of ZFNs is however limited because of the limited selectivity conferred by the zinc-finger modules, and the complex context-dependent interactions between neighboring zinc-finger modules [66,67]. Moreover designing ZFNs typically involves multiple rounds of testing without necessarily resulting in a nuclease that performs optimally [66,68–70]. ZFNs have been used for genome editing in *Arabidopsis*, tobacco, soybean and maize (see Table 1).

The third class of artificial restriction enzymes called TAL effector nucleases (TALENs) has been developed as the successor of ZFNs. Transcription activator-like effectors (TALEs) are produced by plant pathogenic bacteria in the genus *Xanthomonas* and are transferred via type III secretion systems to the host, where they function as important virulence factors that act as transcriptional regulators [71–73]. The DNA binding domain of these TALEs contains multiple 30-35 amino acid long repeats that each recognizes a single base pair of DNA. Two hyper variable amino acids found at positions 12 and 13 of these repeats, known as the repeat-variable di-residues (RVDs), determine the base specificity [74,75]. Crystallization of the TALE DNA-binding domain revealed how the TALE wraps around the sense strand of the DNA as a right-handed super helix with each repeat forming a left-handed helix-loop-helix structure [76]. The structure also revealed that the 13th amino acid of the RVD determines the base specificity by interacting with the major groove, and that the 12th amino acid of the RVD stabilizes the loop in the helix-loop-helix structure [76,77]. By fusing the DNA binding domain of TALEs to the nuclease domain of FokI, TALENs are constructed that just like ZFNs can be designed to bind at opposing sites of the target sequences and create a DSB upon dimerization of the nuclease domain [78]. TALENs have been used to modify the genome of the following plants: *Arabidopsis,* barley, *Brachypodium*, cucumber, maize, rice, soybean, tobacco, tomato and wheat (see Table 1).

The newest addition to the family of SSNs is the CRISPR\Cas system derived from a prokaryotic adaptive immune system that cleaves DNA in a sequence dependent manner. In prokaryotes this RNA based defense system provides protection from foreign invading nucleic acids, such as viruses and plasmids [79–81]. The CRISPR/Cas system is made up of a Cas gene operon and CRISPR arrays. Immunity is acquired by integrating short fragments of invading DNA known as spacers between two flanking identical repeats at the proximal region of CRISPR loci. Transcripts of these CRISPR arrays, including the spacers, are processed into small interfering CRISPR RNAs (crRNAs) approximately 40 nucleotides in length [82]. Together with transactivating RNAs (tracrRNAs) these crRNAs form a complex which enables the Cas enzyme to cleave homologous double-stranded DNA sequences, known as protospacers, in previously encountered invading DNA [83,84].

There are three distinct classes of CRISPR/Cas systems from which a type II class system from *Streptococcus pyogenes* has mostly been used for genome editing, because only one protein (Cas9) is sufficient for nuclease activity in combination with the two RNA molecules [85]. A requirement for cleavage is the presence of a conserved protospacer adjacent motif (PAM) downstream of the target DNA, with either a 5'-NGG-3' or 5'-NAG-3' motif [85–87]. The quick development of the CRISPR/Cas system into a tool for genome editing started with the discovery that a 20 nucleotide change in the crRNA was sufficient to reprogram the DNA target specificity [85], and the subsequent development of a single chimeric guide RNA that combines the target specificity of the crRNA with the structural properties of the tracrRNA. The first reports of the CRISPR/Cas system being used for genome editing in different eukaryotes followed shortly thereafter [88–92]. The Cas9 protein has two active sites in a RuvC and a HNH domain, respectively, each of which induces a single-strand break which combined create a DSB. To transform Cas9 into a nickase that induces a single strand break point mutations have been introduced into the RuvC (D10A) and HNH (H840A) domains [85,86,93]. Using a nickase instead of a nuclease strongly decreases the frequency of off target mutations, while still increasing the frequency of HDR [94,95]. The CRISPR/Cas system has been used successfully for genome editing in the following plants: *Arabidopsis*, *Camelina*, common wine grape, tobacco, maize, petunia, orchids, potato, red sage, rice, sweet orange, tomato, sorghum, watermelon wheat and (see Table 2).

Using site-specific nucleases for genome editing in plants

The outcome of repair of a DSB is dependent on which repair pathway is used. In plants and many other higher eukaryotes repair via NHEJ is the predominant pathway used for the repair of DSBs. Repair via HR, but also via NHEJ often results in perfect repair of the lesion, but therefore also restores the target site for the SSNs allowing for the induction of a new DSB. This process can therefore cycle between DSB induction and repair until imperfect repair via NHEJ results in a small deletion or insertion that destroys the recognition site of the site specific nuclease.

When SSNs are used to introduce DSBs into a gene, mutations can be induced that affect gene function. All four classes of SSNs have been used to this end in both model plants as well as in a wide range of crop species (for an overview of see Table 1). High mutation frequencies have been found for instance when poplar leaf discs were transformed with a constitutive CRISPR/Cas construct resulting in 89% of leaf discs transformed showing an albino shoot indicative of mutations created in the phytoene desaturase gene [96]. Maize plants regenerated from calli that constitutively expressed the CRISPR/Cas system were found to have mutations frequencies up to 70-100% [97].

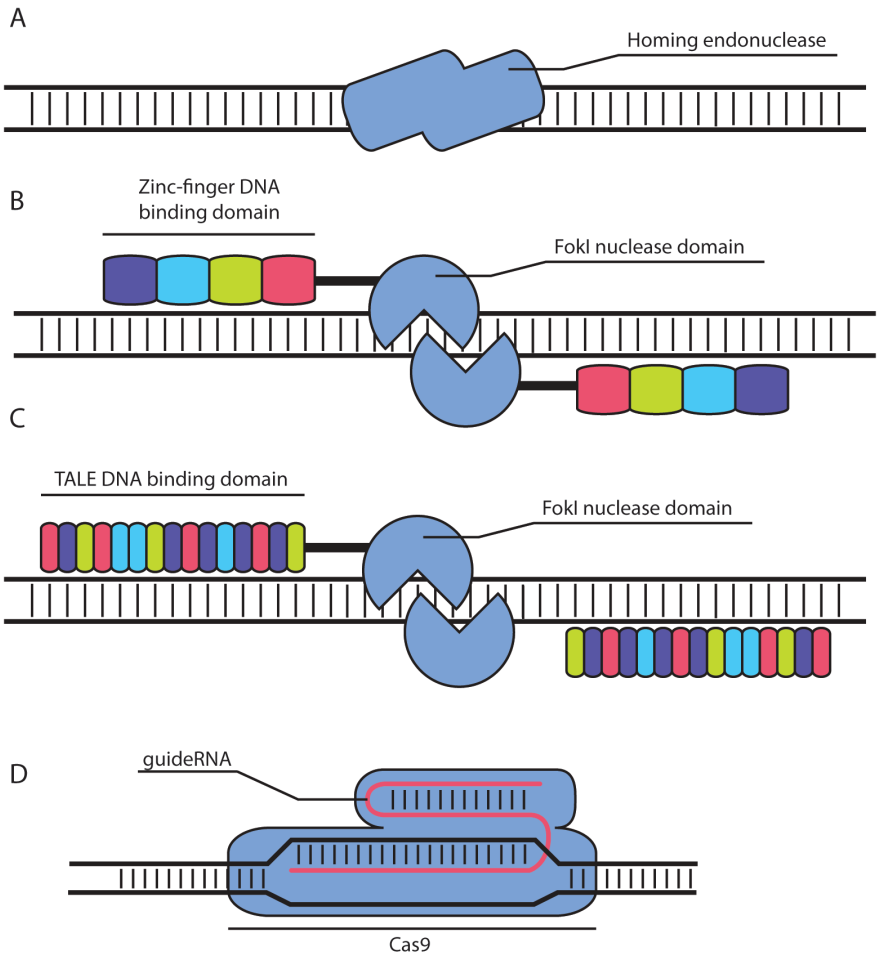

Figure 1. Site-specific nucleases used for genome editing. (**A**). Homing endonuclease with overlapping DNA binding and cleavage domain. (**B**) Zinc-finger nuclease (ZFN) composed of multiple zinc-fingers that form the DNA binding domain fused to the FokI nuclease domain. (**C**) TALEN composed of the DNA binding domain of transcription activator-like effectors (TALEs) fused to the FokI nuclease domain. **(D**) CRISPR/Cas composed of the Cas9 protein and the sgRNA which determines the base specificity with a 20bp sequence that is complementary to the target sequence.

Mutations created in the T0 generation however did not show expected inheritance patterns and are not always inherited by the T1 progeny [98,99]. In *Arabidopsis* chimerism was found to occur in T1 plants obtained after floral dip transformation [100,101]. More complex genetic modifications can be achieved when SSNs are used to induce two DSBs simultaneously, including specific deletions [19,69], inversions [102], duplications [102] and translocations [103]. In plants ZFNs, homing endonucleases and CRISPR/Cas have been used to create targeted chromosomal deletions [20,104–106].

When a DSB is induced and simultaneously an artificial repair template lacking the nuclease restriction site is present, this may be used for HDR, and the specific mutations present in the template introduced into the genome. In plants this has been accomplished in the following species: *Arabidopsis*, tobacco, tomato, soybean, rice, potato, wheat and maize. Similarly by using a repair template containing novel genes these can be inserted in this way at the DSB site (gene targeting). In plants the introduction of DSBs near the site of the desired recombination has been shown to greatly increase the frequency of HDR. The introduction of a SSB by engineered nickases also increases the frequency of HDR, although the increase is less pronounced as with the introduction of a DSB [107,108]. Repair via HDR has been used in plants for targeted modifications of genes in model species as well in several crop species using ZFNs [109–112], TALENs [113,114] and the CRISPR/Cas system [115–120].

Delivery of site-specific nucleases

Although the choice for the delivery system used will often be based on efficiency and feasibility considerations, the resulting expression levels and concentration of the nuclease will also impact the outcome. In plants several methods of delivery have been used for either constitutive or transient expression of SSNs. To obtain plants that constitutively express SSNs varying plant tissues have been transformed using biolistic or *Agrobacterium* based methods (Table 1 and Table 2). These methods, although relatively easy, have some drawbacks. Integration of transgenes may disrupt endogenous genes at the site of transgene integration. The presence of transgenes increases the administrative burden in regulatory processes needed for marketing of genetically modified crops. Furthermore, depending on the position in the genome gene expression levels might be influenced and constitutive expression of the SSN may lead to off target effects. Therefore methods are being developed for the transient expression of SSNs, for instance by controlling them by inducible or cell specific promoters. Alternatively methods for the direct delivery of nuclease mRNA have also been developed [90,121,122], as well for the direct delivery of SSN proteins in human cells and drosophila [88,123,124] and tobacco protoplasts for which direct introduction of I-SceI and TALEN protein was reported [125]. In addition, preassembled CRISPR/Cas complexes of purified Cas9 protein and sgRNA have been transfected into protoplasts of *Arabidopsis*, tobacco, rice and lettuce [126]. Direct protein delivery does however require the isolation and purification of large quantities of SSNs and the isolation of protoplasts. Therefore, a system that eliminates the isolation and purification steps but would still directly deliver the SSN as a protein would have value in both academic and commercial settings. The *Agrobacterium* system may fulfill this promise.

Table 1. Overview of use of homing endonucleases, zing finger nucleaes and TALENs in plants

Site specific nuclease	Transformation method	Mutation type	Reference
Homing endonucleases			
A. thaliana	*Agrobacterium* (Floral dip)	TM / Gene excision	[185]
Z. mays	*Agrobacterium* (Embryo)	TM	[186]
Z. mays	*Agrobacterium* (Embryo)	TM	[187]
Zinc finger nucleases			
A. thaliana	*Agrobacterium* (Floral dip)	TM	[188]
A. thaliana	*Agrobacterium* (Floral dip)	TM	[189]
A. thaliana	*Agrobacterium* (Floral dip)	TM	[190]
A. thaliana	*Agrobacterium* (Floral dip)	TM / GT	[109]
A. thaliana	*Agrobacterium* (Floral dip)	TM / GT	[110]
G. max	*Agrobacterium* (Hairy roots)	TM	[191]
N. tabacum	Protoplast electroporation	TM / GT	[111]
Z. *mays*	*Agrobacterium* (Embryo)	TM / Gene addition	[112]
TALENs			
A. thaliana	*Agrobacterium* (Floral dip)	TM	[192]
A. thaliana	*Agrobacterium* (Floral dip)	TM	[193]
A. thaliana	PEG-protoplast transfection	TM	[194]
B. distachyon	PEG-protoplast transfection	TM	[195]
G. max	*Agrobacterium* (Hairy roots)	TM	[196]
G. max	*Agrobacterium* (Hairy roots)	TM	[197]
H. vulgare	*Agrobacterium* (Embryonic pollen), Particle bombardment	GT	[198]
H. vulgare	*Agrobacterium* (Embryonic pollen)	TM	[199]
H. vulgare	*Agrobacterium* (Embryonic pollen)	TM	[200]
H. vulgare	Particle bombardment	GT	[198]
N. benthamiana	mRNA transfection	TM	[201]
N. benthamiana	PEG-protoplast transfection	GT	[202]
N. tabacum	PEG-protoplast transfection	TM / GT	[113]
O. sativa	*Agrobacterium* (Callus)	TM	[203]
O. sativa	*Agrobacterium* (Callus)	TM	[204]
O. sativa	Particle bombardment	GT	[205]
O. sativa	PEG-protoplast transfection	TM	[120]
O. sativa	PEG-protoplast transfection	TM	[206]
S. lycopersicum	*Agrobacterium* (Seedlings)	TM / GT	[114]
S. tuberosum	PEG-protoplast transfection	TM	[207]
T. aestivum	PEG-protoplast transfection	TM	[208]
Z. mays	*Agrobacterium* (Embryo)	TM	[209]

TM = targeted mutagenesis, GT = gene-targeting, CD = Chromosomal deletions

Table 2. Overview of use CRISPR/Cas in plants

CRISP/Cas9	Transformation method	Mutation type	Reference
A. thaliana	*Agrobacterium* (Floral dip)	TM	[210]
A. thaliana	*Agrobacterium* (Floral dip)	TM	[211]
A. thaliana	*Agrobacterium* (Floral dip)	TM	[212]
A. thaliana	*Agrobacterium* (Floral dip)	TM	[213]
A. thaliana	*Agrobacterium* (Floral dip)	TM	[214]
A. thaliana	*Agrobacterium* (Floral dip)	TM	[215]
A. thaliana	*Agrobacterium* (Floral dip)	TM	[216]
A. thaliana	*Agrobacterium* (Floral dip)	TM	[101]
A. thaliana	*Agrobacterium* (Floral dip)	TM	[217]
A. thaliana	*Agrobacterium* (Floral dip)	TM	[218]
A. thaliana	*Agrobacterium* (Floral dip)	TM	[219]
A. thaliana	*Agrobacterium* (Floral dip)	TM / GT	[116]
A. thaliana	*Agrobacterium* (Floral dip)	TM / GT	[220]
A. thaliana	*Agrobacterium (Floral dip)*	TM (SSA)	[106]
A. thaliana	*Agrobacterium* (Floral dip)	TM (SSA)	[221]
A. thaliana	*Agrobacterium* (Leaf infiltration)	CD	[222]
A. thaliana	*Agrobacterium* (Leaf infiltration)	TM	[223]
A. thaliana	PEG-protoplast transfection	TM	[126]
A. thaliana	PEG-protoplast transfection	TM	[224]
A. thaliana	*PEG-protoplast transfection / Agrobacterium* (Floral dip)	TM / GT	[115]
Camelina sativa	*Agrobacterium* (Floral dip)	TM	[225]
C. lanatus	PEG-protoplast transfection	TM	[226]
C. sinensis	*Agrobacterium* (Leaf infiltration)	TM	[227]
C. sinensis	*Agrobacterium* (Cotelydons)	TM	[228]
C. sativus	*Agrobacterium* (Cotelydons)	TM	[229]
Chrysanthemum morifolium	*Agrobacterium* (Callus)	TM	[230]
Dendrobium officinale	*Agrobacterium* (protocorns)	TM	[231]
G. max	*Agrobacterium* (Hairy roots)	TM	[232]
G. max	*Agrobacterium* (Hairy roots)	TM	[233]
G. max	*Agrobacterium* (Hairy roots)	TM	[196]
G. max	*Agrobacterium* (Hairy roots)	TM	[234]
G. max	PEG-protoplast transfection	TM	[235]
G. max	Particle bombardement	TM / GT	[236]
G. max	*Agrobacterium* (Hairy roots)	TM	[237]
Gossypium hirsutum L.	*Agrobacterium* (Hypocotyl/cotyledonary petiole)	TM	[238]
Gossypium hirsutum L.	*Agrobacterium* (Hypocotyl/cotyledonary petiole)	TM	[239]
Gossypium hirsutum L.	*Agrobacterium* (Hypocotyl/cotyledonary petiole)	TM	[240]
Hordeum vulgare	*Agrobacterium* (Embryo)	CD	[241]
L. sativa	PEG-protoplast transfection	TM	[126]
M. truncatula	*Agrobacterium* (Hairy roots)	TM	[242]
M. truncatula	*Agrobacterium* (Hairy roots)	TM	[237]
Marchantia polyorpha	*Agrobaceterium* (spores)	TM	[243]
N. attenuata	PEG-protoplast transfection	TM	[235]
N. attenuata	PEG-protoplast transfection	TM	[126]
N. benthamiana	*Agrobacterium* (Cas9) / guideRNA (CaLCuV virus)	TM	[244]
N. benthamiana	*Agrobacterium* (Cas9) / guideRNA (TVR)	TM	[245]
N. benthamiana	*Agrobacterium* (Cas9) / guideRNA (TYLCV virus)	TM	[246]
N. benthamiana	*Agrobacterium* (Leaf infiltration)	TM	[247]
N. benthamiana	*Agrobacterium* (Leaf infiltration)	TM	[223]
N. benthamiana	*Agrobacterium* (Leaf infiltration)	TM / CD	[104]
N. benthamiana	*Agrobacterium* (Leaf infiltration)	TM	[248]

N. benthamiana	*Agrobacterium* (Leaf infiltration)	CD	[222]
N. benthamiana	PEG-protoplast transfection	TM	[115]
N. benthamiana	PEG-protoplast transfection	TM	[224]
N. benthamiana	PEG-protoplast transfection	TM	[249]
N. tabacum	*Agrobacterium* (Leaf infiltration)	TM	[250]
N. tabacum	*Agrobacterium* (Leaf disc)	TM	[251]
N. tabacum	PEG-protoplast transfection	TM	[252]
N. tabacum	PEG-protoplast transfection	TM	[253]
N. tabacum	PEG-protoplast transfection	TM / CD	[105]
N. tabacum	PEG-protoplast transfection / *Agrobacterium*(Leaf disc)	TM	[254]
O. sativa	*Agrobacterium* (Callus)	TM	[255]
O. sativa	*Agrobacterium* (Callus)	TM	[250]
O. sativa	*Agrobacterium* (Callus)	TM	[210]
O. sativa	*Agrobacterium* (Callus)	TM	[256]
O. sativa	*Agrobacterium* (Callus)	TM	[257]
O. sativa	*Agrobacterium* (Callus)	TM	[211]
O. sativa	*Agrobacterium* (Callus)	TM	[258]
O. sativa	*Agrobacterium* (Callus)	TM	[259]
O. sativa	*Agrobacterium* (Callus)	TM	[260]
O. sativa	*Agrobacterium* (Callus)	TM	[261]
O. sativa	*Agrobacterium* (Callus)	TM	[262]
O. sativa	*Agrobacterium* (Callus)	TM	[263]
O. sativa	*Agrobacterium* (Callus)	TM	[264]
O. sativa	*Agrobacterium* (Callus)	TM	[265]
O. sativa	*Agrobacterium* (Callus)	TM	[266]
O. sativa	*Agrobacterium* (Callus)	TM	[267]
O. sativa	*Agrobacterium* (Callus)	TM	[98]
O. sativa	*Agrobacterium* (Callus)	TM	[268]
O. sativa	*Agrobacterium* (Callus)	TM	[269]
O. sativa	*Agrobacterium* (Callus)	TM	[270]
O. sativa	*Agrobacterium* (Callus)	TM	[271]
O. sativa	*Agrobacterium* (Callus)	GT	[272]
O. sativa	*Agrobacterium* (Particle bombardment)	TM / GT	[273]
O. sativa	PEG-protoplast transfection	TM	[126]
O. sativa	PEG-protoplast transfection	TM	[274]
O. sativa	PEG-protoplast transfection	GT / GI	[275]
O. sativa	PEG-protoplast transfection / *Agrobacterium*	TM / CD	[20]
O. sativa	PEG-protoplast transfection / *Agrobacterium*	TM / CD	[104]
O. sativa	PEG-protoplast transfection / *Particle bombardement*	TM	[120]
Petunia hybrida	PEG-protoplast transfection ribonucleoproteins	TM	[276]
Petunia hybrida	*Agrobacterium* (Leaves)	TM / CD	[106]
Physcomitrella patens	PEG-protoplast transfection	TM	[277]
Physcomitrella patens	PEG-protoplast transfection	TM	[278]
P. tomentosa	*Agrobacterium* (Leaf disc)	TM	[96]
P. tomentosa	*Agrobacterium* (Leaf disc)	TM	[279]
P. tomentosa	*Agrobacterium* (Leaf disc)	TM	[280]
S. bicolor	*Agrobacterium* (Leaf infiltration)	TM	[223]
Salvia miltiorrhiza	*Agrobacterium* (Hairy roots)	TM	[281]
Scopelophila cataractae	PEG-protoplast transfection	TM	[278]
S. lycopersicum	*Agrobacterium* (Cotyledon segments)	CD	[282]
S. lycopersicum	*Agrobacterium* (Cotyledon segments)	TM	[283]
S. lycopersicum	*Agrobacterium* (Cotyledon segments)	TM	[114]
S. lycopersicum	*Agrobacterium* (Cotyledon segments)	TM	[284]
S. lycopersicum	*Agrobacterium* (Cotyledon segments)	TM	[285]
S. lycopersicum	*Agrobacterium* (Cotyledon segments)	TM	[286]
S. lycopersicum	*A. rhizogenes*	TM	[287]
S. tuberosum	*Agrobacterium* (Callus)	TM / GT	[119]

S. tuberosum	*Agrobacterium* (Stem segments)	TM	[288]
S. tuberosum	PEG-protoplast transfection	TM	[289]
T. aestivum	Particle bombardement	TM	[290]
T. aestivum	PEG-protoplast transfection	TM	[291]
T. aestivum	PEG-protoplast transfection	TM / GT	[120]
T. aestivum	PEG-protoplast transfection	TM	[208]
T. aestivum	PEG-protoplast transfection / Particle bombardement	TM	[292]
T. aestivum	*Agrobacterium* (Leaf infiltration)	TM	[293]
Vitis vinifera	*Agrobacterium* (Embryo)	TM	[294]
Z. mays	*Agrobacterium* (Embryo)	TM	[99]
Z. Mays	*Agrobacterium* (Embryo) / Particle bombardement	TM / GT / GI	[295]
Z. mays	*PEG-protoplast transfection* / Agrobacterium(Embryo)	TM	[296]
Z. mays	*PEG-protoplast transfection* / Agrobacterium(Embryo)	TM	[217]
Z. mays	PEG-protoplast transfection	TM	[297]
Z. mays	PEG-protoplast transfection	TM	[223]
Z. mays	PEG-protoplast transfection ribonucleoproteins	TM	[298]

TM = targeted mutagenesis, GT = gene-targeting, CD = Chromosomal deletions

Part 2

Agrobacterium

Agrobacterium tumefaciens is known as the causative agent of crown gall disease in plants since the beginning of the 20th century. Crown galls arise at the site of infection and are the result of uncontrolled cell division. They can be excised and propagated in vitro without exogenous plant hormones [127]. Crown galls produce opines, amino-acid derivatives, which can be used by *Agrobacterium* as a nitrogen and carbon source and therefore the galls form a favorable niche for the bacterium [128]. The genetic basis for the uncontrolled growth of crown galls is the transfer of a copy of the T-region of the a large Tumor inducing (Ti) plasmid from the bacterium into plant cells. Processing of this T-region in the bacterium results in production of single stranded copies known as T-strands (transfer strands) that are transferred through a virB/virD4 type IV secretion system (T4SS) directly into the host cells. Upon entry into the host cell the T-strand is directed towards the nucleus and eventually may be integrated into the host genome [129]. The genes on this transferred DNA (T-DNA) encode for several proteins involved in the production and activation of plant hormones and the production of opines [130,131].

Induction of the virulence genes

The transformation of plant cells starts with a large cascade of events, the first step of which is the detection of phenolic compounds released by wounded plant cells by the bacterium through the VirA/VirG two-component sensor/regulatory system. VirA is autophosphorylated upon interaction with these phenolic compounds [132–135]. Phosphorylation of VirG by VirA leads to the binding of VirG to the *vir*-boxes of *vir* genes, stimulating their expression [136]. In addition to the VirA/VirG system the chromosomally encoded periplasmic protein ChvE, acts synergistically with the VirA/VirG system to induce *vir* gene expression upon detection of specific sugars in the plant sap [137]. Activation of the *vir* system only occurs in medium with a low pH like plant sap and at moderate temperatures (below 30° degrees). Eight operons designated *VirA*, *B*, *C*, *D*, *E*, *F*, *G* and *H* are largely conserved on the virulence regions of different types of Ti-plasmid. [138–140].

T-DNA processing

The T-region on the Ti-plasmid is flanked by two imperfect direct repeats (borders) that are the only determinants that define the T-DNA, of which only the right border sequence is essential for transfer [141]. Both of these borders are recognized by the virulence proteins VirD1 and the relaxasome VirD2. A single stranded break is introduced by VirD2 which stays covalently attached to the 5'-end of the nick [142,143]. The accessory Vir protein VirD1 enhances the binding and nicking on supercoiled DNA, while VirC1 and VirC2 further increase the efficiency by attaching to the overdrive sequence found close to the right border [144,145]. Probably the T-strand is released by displacement synthesis with VirD2 still covalently attached to the 5' end. This nucleo-protein complex is recognized by the T4SS and transported into the host cell [146].

The type IV secretion system of Agrobacterium

The T4SS of *Agrobacterium* through which the T-strand is transported is a large multi-protein complex that spans the inner membrane, the periplasm and the outer membrane. The genes encoding the T4SS are located on the Ti-plasmid and are encoded by a large *virB* operon, consisting of 11 open reading frames, and the *virD4* gene. Studying the T4SS has led to a division of its components into energy providing subunits (VirB4, VirB11, VirD4), inner membrane channel components located predominantly in the inner membrane (VirB3, VirB6, VirB8), periplasmic and membrane outer components (VirB7, VirB9), connecting component (VirB10), pilus components (VirB2, VirB5) and the VirB1 transglycosylase (extensively reviewed [147]). The T4SS present in *Agrobacterium* also translocates separately the T-strand four other so called effector proteins (VirD5, VirE2, VirE3 and VirF) into the host cell [148–150]. Their translocation via the T4SS is dependent on a C-terminal translocation signal present in all translocated proteins that has a net positive charge with a consensus motif of R-X(7)-R-X-R-X-R-X-X(n)>[149]. Combined with VirD2, VirE2 aids in nuclear targeting of the T-strand [151,152]. The translocated effector protein VirE3 is highly conserved and has two potential NLS by which it binds to importin-α and is transported into the nucleus [153]. Upon mutation of VirE3 hardly any attenuation of virulence is observed, but combined with the inactivation of VirF the role of VirE3 in virulence becomes apparent [154]. Recently, it was shown that VirE3 is a transcription factor which upregulates the VIP1-binding F-box gene thereby removing the requirement for VirF [155]. A recent study focusing on the virulence protein VirD5 suggests that it binds to centromers/kinetochores and there may induce cycle arrest, chromosome missegregation and aneuploidy [156]. The VirF protein is an F-box protein [157] which together with homologues of the yeast protein Skp1 and Cullin forms a Skp-Cullin-F-box protein (SCF) complex [157]. This complex has been reported to target the host protein VIP1 and associated VirE2 for degradation by the 26S proteasome [158], which may be required for the uncoating of the T-DNA to enable integration into the host cell's genome. VirF also targets degradation of the transcription factors VBF3 and VBF4 [159]. Plant species that can be transformed by an *Agrobacterium* strain lacking virF have been shown to induce upon infection the expression of a plant F-box protein, VBF, that can functionally replace VirF [160].

T-DNA transfer and integration

T-DNA transfer across the different T4SS components occurs in a tightly controlled manner in which the T-strand makes sequential contact with VirD4, VirB11, VirB6, VirB8, VirB9 and VirB2 [161]. Although the other components of the T4SS have not been found to interact with the T-strand they are essential [161–163]. The translocated VirE2 protein is thought to be responsible for protecting the T-strand against degradation by host cell proteases/nucleases after the T-strand has been transported to the hosts cytoplasm [164]. In the nucleus the T-strand is converted into a double strand form, which may be expressed for some time and then degraded. Integration into the genome leads to stable transformation and the continued expression that is necessary for tumorigenesis. The integration of the T-DNA occurs at a random position into the genome [165], which can result in mutation and variable expression due to position effects. The molecular mechanism of T-DNA integration was first studied in yeast where integration occurs efficiently via HR when the T-DNA contains yeast homologous sequences [166]. If no homology is present on the T-DNA integration in both

yeast and fungi occurs by NHEJ and is dependent on the C-NHEJ proteins Ku70, Ku80 and DNA ligase 4 [167,168]. Studies on the influence of these proteins on T-DNA integration in plants by different research groups mostly showed that their absence gave no or limited effects [30,169–171]. Disruption of multiple DNA repair pathways simultaneously also did not eliminate transformation [172–174]. Recently, it has been shown that the random integration of the T-strand occurs through a mechanism involving Pol θ [175].

Biotechnological applications of Agrobacterium

After the discovery of the genetic mechanism behind the transformation of plants by *Agrobacterium,* researchers focused on developing *Agrobacterium* into a tool for the introduction of foreign genes into plants. The genes that are naturally located between the borders are not involved in T-DNA transfer and these sequences can be replaced with other sequences of interest. The introduction of these genes of interest into the Ti-plasmid is however difficult due to its large size, low copy number and its inability to replicate in *E. coli*. The *vir* region and the T-DNA can however be separated on different plasmids without impacting *Agrobacterium*'s ability to form tumors [176]. The plasmid harboring the T-DNA, called the binary vector, contains at least one origin of replication that is functional in both *Agrobacterium* and *E. coli* and selectable markers for maintenance in *Agrobacterium* and *E. coli*, hereby allowing the easy cloning of genes on the T-region [176]. The plasmid with the intact *vir* region but lacking the T-region is known as the helper plasmid. The ability to introduce foreign genes into plant cells has made *Agrobacterium* an invaluable tool for plants scientists.

Using *Agrobacterium* plants have been created with enhanced tolerance to abiotic and biotic stress and pest resistance. Furthermore, *Agrobacterium* has been applied to better understand plant biology at a cellular and molecular level for example tagging various proteins with a fluorescent protein to visualize cell compartments [177]. In addition T-DNA insertion libraries have been made in which the T-DNA functions as an insertional mutagen and, by extension, a gene tag [178,179]. More recently SSNs have been introduced into plants, using *Agrobacterium*, for targeted gene disruption and gene targeting in plants (see Table 1). In recent years, *Agrobacterium* has also been used to modify plants for the production of useful proteins, such as edible vaccines and recombinant antibodies [177].

Although *Agrobacterium* only forms tumors on dicotyledonous plants, under laboratory conditions it is also capable of transforming monocotyledonous plants [180], yeast [166] and a wide range of fungi [181]. Because of its ease of use, low cost and precision it has become a preferred vector not only for the genetic modification of plants, but also of yeasts and fungi. As mentioned above *Agrobacterium* not only uses its T4SS for T-DNA transfer but also for the translocation of several effector proteins. It has been found that the translocation signal of these effector proteins can be attached to heterologous proteins such as the Cre recombinase and I-SceI to effectuate their transfer to plant cells [148,149,166,182–184].

Outline of this thesis

In **Chapter 2** we describe the application of CRISPR/Cas in *Agrobacterium*, to cure *Agrobacterium* from the promiscuous plasmid RP4 and from vectors with the replication unit of the octopine Ti plasmid. Furthermore we show that the Cas9 protein fused to a translocation signal recognized by the T4SS does not negate its ability to induce DSBs.

In **Chapter 3** yeast was used as a model organism to show that the Cas9 protein can be delivered through the T4SS of the plant pathogen *Agrobacterium*. The transfer of Cas9 was effectuated by fusion of a T4SS translocation peptide to the Cas9 protein.

In **Chapter 4** a method for targeted mutagenesis in *Nicotiana benthamiana* was developed, that is based on the translocation of the Cas9 protein through the T4SS of *Agrobacterium*. We show that concurrent transfer of Cas9 protein and a T-DNA encoding the sgRNA results in targeted mutations in the infiltrated leaves of *N. benthamiana*.

In **Chapter 5** we describe two novel approaches for the transient expression of the *Agrobacterium* derived isopentenyl transferase that can be used for the selection of transformed plants. The first consisted of the delivery of a T-DNA encoding the ipt gene into Pol-θ-deficient *Arabidopsis* mutants in which only transient expression of the T-DNA occurs but no integration. The second approach involved the direct delivery of the IPT protein through the *Agrobacterium* T4SS into *Arabidopsis.*

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