Establishment and development of methods for genetic modifications in plants and their effect on the metabolism

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Abstract

The discovery of genome editing methods expanded the possibilities to genetically modify plants and allows the targeted manipulation of plant genomes. In recent years, the pharmaceutical demand of cannabinoids, the psychoactive compounds of the plant *Cannabis sativa* L., increased continuously, but cultivation of the plant is still restricted by law in many countries. On the one hand, the heterologous production of the cannabinoids in other plant species like tobacco to cover the high demand would be an alternative. Today, tobacco represents a well-established model plant for biotechnological processes and heterologous production of proteins, only the alkaloid (nicotine) content prevent many applications. On the other hand, the production of distinct cannabinoids in the plant would be a great option to treat several diseases precisely. For this purpose, genetic engineering of the plant is necessary, which is poorly understood in *Cannabis*.

In this regard, the objective of this study was to establish methods for stable and transient genetic modifications of *Cannabis* and to generate a tobacco host plant without interfering alkaloids for heterologous protein production.

In this thesis, a nicotine-free, non-transgenic tobacco plant was generated by knocking out a whole gene family responsible for the last step in nicotine biosynthesis, with the use of the CRISPR-Cas9 method. Furthermore, a transformation protocol of axillary buds of *Cannabis* plants with *Agrobacterium tumefaciens* and subsequent regeneration was established and led to transgenic plants, showing a chimeric pattern. Additionally, a transient approach for post transcriptional gene silencing was established for the first time to be able to study gene functions in *Cannabis.*

This study serves as a basis for upcoming research on transgenic *Cannabis* plants and shows the powerful opportunities in plant biotechnology using the CRISPR-Cas9 method.

Zusammenfassung

Die Entdeckung von Methoden zur Editierung von Genen geht mit einer Erweiterung der Möglichkeiten zur genetischen Veränderung von Pflanzen einher und macht eine gezielte Manipulation des Pflanzengenoms möglich. In den letzten Jahren stieg der pharmazeutische Bedarf an Cannabinoiden, den psychoaktiven Substanzen der Pflanze *Cannabis sativa* L., kontinuierlich an, jedoch ist der Anbau der Pflanze in vielen Ländern gesetzlich eingeschränkt. Zum einen wäre die heterologe Produktion der Cannabinoide in anderen Pflanzenarten, wie Tabak, zur Deckung des hohen Bedarfs eine Alternative. Gegenwärtig fungiert Tabak als Modellpflanze für biotechnologische Prozesse und die heterologe Produktion von Proteinen, wobei der hohe Anteil an Alkaloiden (Nikotin) für viele Anwendungen einen Nachteil darstellt. Zum anderen stellt die Produktion von bestimmten Cannabinoiden in der Pflanze eine gute Möglichkeit dar, verschiedene Krankheiten zielgerichteter zu behandeln. Hierzu müssen genetische Veränderungen an dem Genom der Pflanze vorgenommen werden. Die hierfür notwendigen Methoden stehen jedoch bisher nicht für *Cannabis* zur Verfügung.

Im Hinblick darauf war das Ziel dieser Arbeit die Etablierung von transienten und stabilen genetischen Veränderungen von *Cannabis* und die Erzeugung einer Tabakpflanze mit reduziertem Nikotin- bzw. Alkaloidgehalt als Ausgangspflanze für die heterologe Produktion von Proteinen.

In dieser Arbeit wurde eine nikotinfreie, nicht-transgene Tabakpflanze durch das Ausschalten einer Genfamilie, verantwortlich für den letzten Schritt der Nikotinbiosynthese, mit der CRISPR-Cas9 Methode erzeugt. Des Weiteren wurde ein Transformationsprotokoll für Achselknospen von *Cannabis* mittels *Agrobacterium tumefaciens* und anschließender Regenration entwickelt, welches zu transgenen, chimären Pflanzen führte. Zusätzlich wurde zum ersten Mal ein transienter Ansatz für poststranskriptionelles Gen-Silencing, zur Analyse von Genfunktionen in *Cannabis* etabliert.

Die vorliegende Studie kann als Grundlage für nachfolgende Arbeiten an transgenem *Cannabis* verwendet werden und zeigt die beeindruckenden Möglichkeiten der CRISPR-Cas9 Methode in der Pflanzenbiotechnologie auf.

Chapter 1 Introduction

Parts of Chapter 1.2 were published in:

Schachtsiek, J., Warzecha, H., Kayser, O. and Stehle, F**.** (2018) Current Perspectives on Biotechnological Cannabinoid Production in Plants. *Planta Medica*, 84, 214–220.

1.1. Genetic modifications in plants

Since more than 30 years plant genetic engineering represents a central technology that increased the basic knowledge of plant biology enormously. After the first transformations of plant cells with bacterial resistance genes (Fraley et al., 1983), several different techniques were developed, leading to a boost in plant research progress (Ferrer et al., 2016). In general, plant cells can either be transformed transiently or stable. In the latter possibility, the DNA is integrated in the genome, whereas in a transient approach integration is not required. The T-DNA is expressed in the nucleus without integration and is degraded after a few days (Hwang et al., 2017). For stable transformation, regeneration of transformed plant tissues is necessary to obtain stable expressing transgenic plants, which is time consuming. Nevertheless, the transgene is expressed in cells of the regenerated plant and in following generations. In transient transformation approaches, the regeneration of plants is not necessary, because the transgene is directly expressed in the transformed tissue, mostly leaves. This results in a high protein production within some days, because of a higher copy number of the transformed genes (Canto, 2016; Ferrer et al., 2016). Moreover, a selection marker is not required.

Plant transformation can be done either with the use of *Agrobacterium tumefaciens* or with particle bombardment of the plant cells. The biolistic bombardment method is done with a particle gun. Particles, like gold, are coated with the DNA and shot with high pressure into the plant cells (Finer et al., 1992). The soil pathogen *Agrobacterium tumefaciens* naturally infects wounded tissue of plants and induces grown galls via the transfer of ssDNA, so called T-DNA into the plant cells (Hwang et al., 2017). This mechanism is utilized to transfer DNA sequences of choice into plant cells using a binary vector system. The Ti-helper plasmids carry the *vir*-genes, whereas the binary vector carries the T-DNA. Infection of the host with *A. tumefaciens* can only occur if both plasmids are present in the strain (Hoekema et al., 1983). The *A. tumefaciens*-mediated gene transfer, represents the most frequently used method, because of some drawbacks of the biolistic method e.g. the damage of the plant tissue (Canto, 2016). This induces plant defense mechanisms, leading to changes in primary and secondary metabolism (Savatin et al., 2014).

In the last years several methods for genetic modifications in plants were developed (Figure 1-1), including methods for overexpression of genes, investigation of protein-protein interactions, as well as gene silencing methods (RNAi or VIGS) and gene editing like CRISPR-Cas9. In the following, they are explained in more detail.

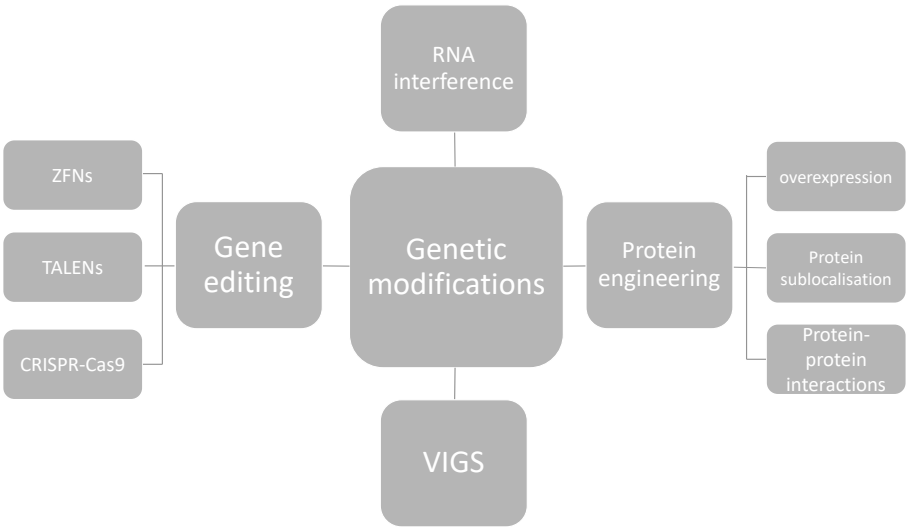

Figure 1-1 Overview of important methods for genetic modifications in plants: Plant genomes can be modified in different ways. With RNA interference (RNAi) and virus-induced gene silencing (VIGS) transcription of genes is silenced. With protein engineering it is possible to overexpress genes, to visualize protein localizations or to investigate protein-protein interactions. Gene editing opens the possibility to specifically integrate heterologous genes into the genome or knockout genes completely.

1.1.1. Protein engineering

Engineering of proteins describes a versatile tool to investigate localizations of proteins as well as protein-protein interactions. Furthermore, overexpression of proteins is possible by using a strong promotor. Protein engineering is often used for fundamental research studies and can be applied to target gene sequences with a reporter gene, e.g. a fluorescent protein or the beta-glucuronidase (GUS). The GUS activity can be visualized with 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) as a substrate directly in the plant tissue (Jefferson et al., 1987) and gives evidence for the localization of gene expression of the tagged protein in the plant. With fluorescence proteins tagged to a gene, it is possible to localize gene expression within a cell with fluorescence microscopy. To study protein-protein interactions, the bimolecular fluorescence complementation (BiFC) is used, which is based on the formation of a fluorescent complex out of two non-fluorescent parts of the yellow fluorescent protein (YFP). The coding sequences of the genes are fused to the splitted YFP fragments. Fluorescence is only visible if both proteins interact with each other (Schütze et al., 2009).

Overexpression of heterologous genes in plants is done transiently or stable. For stable expression, the gene is expressed under a strong promotor, like the cauliflower mosaic virus promotor (CaMV) 35 S or the UBQ1-promotor (Holtorf et al., 1995). It was reported that

translational enhancers as well as the right choice of terminators led to higher expression rates of heterologous proteins in plants (Limkul et al., 2015). With the overexpression of the NHX1 gene from *Arabidopsis thaliana* encoding for a vacuolar Na+/H+ antiport in tomato, the stable transgenic plants were more tolerant to salt stress than the wild type. The salt was just accumulated in the foliage, but not in the fruits (Zhang and Blumwald, 2001). Tobacco plants are designated as model plants for recombinant protein production because of their quick growth and high susceptibility for agroinfiltration. The plant can for example be used to overexpress human antibodies, or proteins like erythropoietin (EPO) or interleukin-10 (IL-10), used in the treatment of anaemia or as anti-inflammatory agents, respectively (Conley et al., 2011). It is also possible to produce recombinant proteins transiently with the use of virus-based vectors, e.g. the tobacco mosaic virus (TMV) or the potato virus X. The gene of interest is cloned into the vector with the virus, is delivered into plant cells via *Agrobacteria* and the virus infects the plant systemically (Marillonnet et al., 2004). This method was improved several times to enhance the production of recombinant proteins. With removal of the coat protein, the virus is not able to infect the plant systemically, but with a method called magnifection, protein expression was faster and synchronous in almost all areas of the plant. For magnifection, the whole plant is dipped into an *Agrobacterium*-solution and a weak vacuum is applied and slowly released to transform nearly every cell of the plant (Marillonnet et al., 2005). Based on these findings a company, Icon Genetics, based in Germany developed an advanced method trademarked as magnICON® technology, used in many business entities all over the world, especially for *Nicotiana benthamiana* (Klimyuk et al., 2012). Recently, it could be shown that a combination of geminiviral replication and two terminators the transient protein expression was enhanced in several plant species; making this application an alternative to the TMV-based MagnICON® technology (Yamamoto et al., 2018).

As already mentioned, the transformation of plant cells with *Agrobacteria* is the most used tool in plant molecular biology approaches. Either the infiltration of the bacteria transformed with the desired binary plasmid into the plant cells is done with vacuum or directly into the leaves of a plant with a needleless syringe, which is called agroinfiltration.

1.1.2. RNA interference (RNAi)

Except from overexpression, plant scientists are also interested in downregulation of gene expression, to investigate functions of genes. This is for example possible with the use of RNA interference (RNAi), first discovered in plants as a mechanism to silence invading viruses, preferably double stranded RNA (dsRNA) (Brodersen and Voinnet, 2006). Researchers

developed strategies to use this mechanism for post transcriptional gene silencing (PTGS) of a gene of interest. For the creation of a dsRNA, the gene fragment of interest is cloned both in sense and in antisense orientation into a vector under control of a strong promotor (Figure 1-2). Between the target sequences, an intron serves as linker sequence. The transcribed RNA forms a dsRNA linked with a single stranded hairpin loop, which triggers the RNA silencing process, because it is recognized as invading dsRNA by the plant (Chuang and Meyerowitz, 2000; Wesley et al., 2001).

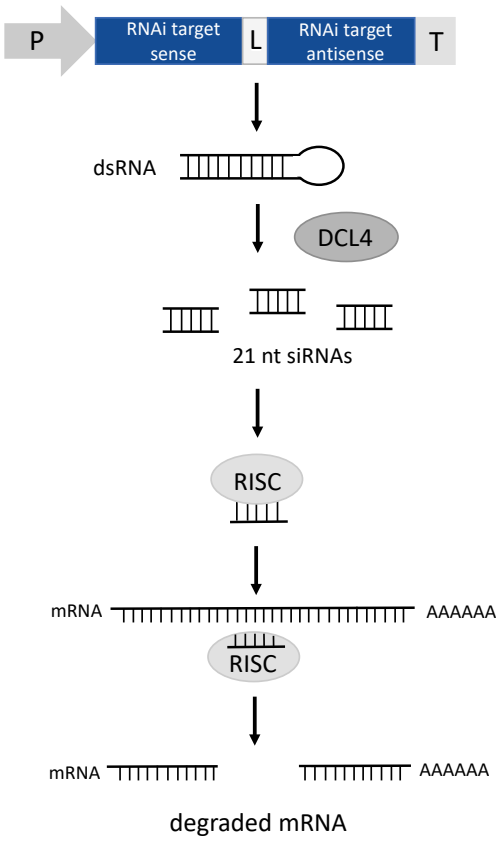

Figure 1-2 Mechanism of RNA interference in plants: modified from Waterhouse and Helliwell (2003)**.** Constructs with the sense and antisense RNAi target linked with a linker sequence are transcribed into RNA. A dsRNA with a linker-loop is built, which is recognized by the dicer-like enzyme 4 (DCL4). The enzyme cuts the dsRNA into small interfering RNAs (siRNA). The RNA-induced silencing complex (RISC) is guided by siRNAs to the complementary mRNA, which is degraded. Abbreviations: P - promotor, T - terminator, L - linker.

The recognition of the dsRNA is done by the dicer-like enzyme 4 (DCL4), a RNase type III enzyme, which cleaves the dsRNA into small interfering RNAs (siRNA) with a length of 21 nucleotides (Dunoyer et al., 2005). The siRNAs are recognized by the RNA-induced silencing complex (RISC). Subsequently, the double stranded siRNAs are melted into single-stranded once and the antisense strand is used by the RISC complex to screen for complementary sequences, which will be cleaved (Waterhouse and Helliwell, 2003). The RISC complex of

plants is not understand completely until now, but a major enzyme in the complex is the RNA-binding protein Argonaute 1 (Ago1), which selectively recruits the siRNAs in plants (Baumberger and Baulcombe, 2005; Qi et al., 2005).

Beside the elucidation of gene functions, RNAi is used for metabolic engineering of plants. A breakthrough in this field of research was the knockdown of the codeinone reductase (COR) family with one RNAi-construct in opium poppy, resulting in the accumulation of the precursor alkaloid reticuline, which is non-narcotic. The opium alkaloids morphine, codeine, oripavine and thebaine were just detectable in low amounts (Allen et al., 2004). With RNAi it was also possible to produce seedless tomato fruits by either silencing of the *AUCSIA* genes, expressed in ovaries of tomatoes, or the *Chalcone synthase (CHS)*, the first gene in flavonoid synthase pathway (Molesini et al., 2009; Schijlen et al., 2007). Furthermore, researchers were able to reduce the caffeine content of coffee plants up to 70 % by silencing the *theobromine synthase* gene with RNAi (Ogita et al., 2003). Thus, there is the opportunity to use this method to investigate gene functions in an easy and time saving manner, but also to engineer whole pathways. Nevertheless, a complete knockout of the gene expression is not possible.

1.1.3. Virus-induced gene silencing

Virus-induced gene silencing (VIGS) is a fast method to analyze gene functions in plants transiently by the use of the innate plant defense system against virus infections (Baulcombe, 1999; Ratcliff et al., 1999) and is based on RNA silencing (chapter 1.1.2). Most plant viruses replicate via dsRNA, which is recognized by post transcriptional gene silencing (PTGS). This leads to the cleavage of the dsRNA and the generation of siRNA molecules (Zamore, 2001), that are recognized by the RISC complex leading to degradation of the invading RNA and a slower virus replication (Ding and Voinnet, 2007; Waterhouse and Fusaro, 2006; Figure 1-2). This will lead to an amplification of the siRNA enabling a transport throughout the plant. Therefore silencing can be observed in distant cells and often in most of the parts of the plant (Kalantidis et al., 2008). VIGS exploits this RNA defense mechanism by mimicking a virus infection based on the delivery of artificial siRNA molecules to a plant cell. Thus, targeting of plant specific mRNA molecules is possible, resulting in a suppression of the gene expression and a reduction of the amount of the corresponding encoded protein.

After the discovery of this method in the 1990s, many different plant viruses were tested for VIGS (Lange et al., 2013). In the beginning the TMV or the potato virus X were used, but these viruses were not able to infect meristems, whereas the tobacco rattle virus (TRV) seems to infect all plant tissues (Waterhouse and Helliwell, 2003). Thus, the TRV is the most commonly

used virus for VIGS approaches. The virus genome consists of two RNAs, the RNA1 and RNA2, encoding for the RNA-dependent RNA polymerase (RdRp) and the movement protein (MP) and the coat protein (CP), respectively. cDNA clones of these RNAs can be cloned on separate binary vectors for agroinfiltration of plants to knockdown the gene of interest (Ratcliff et al., 2001). The schematic construction of the T-DNA is shown in Figure 1-3.

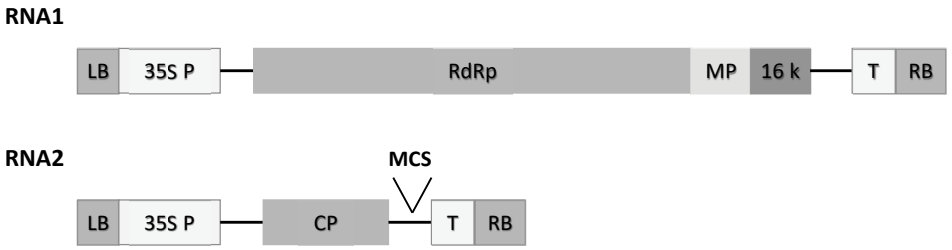

Figure 1-3 T-DNA constructs for TRV-based VIGS: modified from Ratcliff et al. (2001). cDNA copies of the RNAs 1 and 2 of TRV are cloned into binary vectors between the left border (LB) and the right border (RB) under control of the cauliflower 35 S promotor. RNA1 consists of the RNA-dependent RNA polymerase (RdRp), the movement protein (MP) and the 16k protein. RNA2 was modified and only consists of the coat protein (CP) and a multiple cloning site (MCS) for insertion of gene of interest sequences.

RNA1 consists of the RdRp, the movement protein and the 16k protein under control of the 35 S promotor. The RNA2 consists of the coat protein under control of the 35 S promotor. Downstream of the coat protein the non-essential genes encoding for proteins 29.4k and 32.8k were replaced by a multiple cloning site.

The establishment of the method in different plants was mainly done by targeting genes with a visible phenotype, if the gene is knocked down. The most frequently used gene for this purpose is the *phytoene* desaturase (*PDS*). The PDS encodes an enzyme catalyzing the first step in the lycopene biosynthetic pathway. The knockdown of the transcript will result in white leaves due to a photo bleaching effect (Kumagai et al., 1995). This is the result of a negative feedback regulation, inhibiting the chlorophyll biosynthesis (Qin et al., 2007). Another commonly used gene is the *magnesium chelatase subunit I* (*ChlI*), encoding for one of three subunits (ChlH, ChlD and ChlI) of the magnesium chelatase (MgCh), the primary enzyme for chlorophyll biosynthesis. ChlI is of significant importance, since its binding to ChlD activates ChlH, thereby regulating the chlorophyll synthetic pathway (Tanaka and Tanaka, 2007; Willows, 2003). A loss of function in ChlI results in a “yellow” phenotype since no chlorophyll biosynthesis is possible.

Efficient establishment and down regulation of the *PDS* gene, leading to a bleaching phenotype in new developed leaves, was done in several model plants like *N. benthamiana*, *A. thaliana* and tomato (Burch-Smith et al., 2006; Liu et al., 2002a, 2002b). The knockdown of *ChlI* or

ChlD as a marker for successful VIGS was used e.g. in cotton pea and sorghum (Luo et al., 2013; Singh et al., 2018; Tuttle et al., 2008).

As already mentioned, TRV represents the most frequently used virus for VIGS. However, the virus is not suitable for all plants to induce VIGS efficiently, especially shown for rosid species (Ma et al., 2008). One exception is *A.thaliana* (Lange et al., 2013). For other plant species, alternative virus systems like the geminivirus were used, which belongs to the group of ssDNA viruses. The begomoviruses, one genus of the geminiviruses, are mostly used in VIGS experiments and consist of two circular ssDNA molecules; DNA-A and DNA-B. The DNA-A encodes genes for virus replication and DNA-B encodes for a nuclear shuttle protein and the movement protein (Vanitharani et al., 2005). In contrast to RNA viruses, these viruses replicate bidirectional, with the use of the host RNA II polymerase (Hanley-Bowdoin et al., 1999). VIGS was successful in different plant species, e.g. *A. thaliana*, *N. benthamiana*, cassava and cotton (Fofana et al., 2004; Turnage et al., 2002; Tuttle et al., 2008). Like RNAi, gene silencing with VIGS, does not knockout genes completely, but is sufficient for analysing gene functions fast and easy, because gene silencing effects occur approximately three to four weeks after application of the virus.

1.1.4. Targeted genome editing

In the last decade, targeted genome editing methods have been emerged as an important tool for genetic modifications in many organisms and redefined the boundaries of research. The basis of genome editing is the usage of chimeric nucleases, composed of the fusion of a sequence-specific binding domain to the DNA and a non-specific module for DNA cleavage (Gaj et al., 2013). With the induction of the chimeric nuclease-based DNA double-strand break (DSB) at a precise position, DNA repair mechanisms like homology directed repair (HDR) or error prone non-homologous end joining (NHEJ) are activated (Gaj et al., 2013; Wyman and Kanaar, 2006), leading to gene replacements or knockout of genes, respectively. HDR is the minor pathway in plant somatic cells for DNA repair, whereas DSB are more frequently repaired by NHEJ (Schmidt et al., 2019). Before the discovery of the CRISPR-Cas9 mediated gene editing method (chapter 1.1.4.2), the Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) were commonly used for gene editing in several organisms.

1.1.4.1. ZNFs and TALENs

Zinc-finger nucleases in general consist of typically three zinc finger domains, each binding to three base pairs of the DNA and a DNA-cleavage domain derived from the *Fok*I restriction

enzyme at the C-terminus (Figure 1-4 A). Since *Fok*I is only active as a dimer, a second ZFN is necessary, binding the opposite DNA strand (Osakabe and Osakabe, 2015). For precise targeting of DNA, the binding sites to the DNA is changed. Furthermore, two three-finger molecules can be linked with a linker peptide, offering the possibility to target 18 base pairs specifically (Liu et al., 1997). Customized ZFN were used e.g. in *A. thaliana* or tobacco (Lloyd et al., 2005; Wright et al., 2005). In the latter, it was also possible to use HDR to replace genes (Wright et al., 2005). However, it is possible that zinc fingers interact with nucleotides close to the binding site, which causes unpredictable binding, leading to time consuming testing of each customized nuclease (Schmidt et al., 2019).

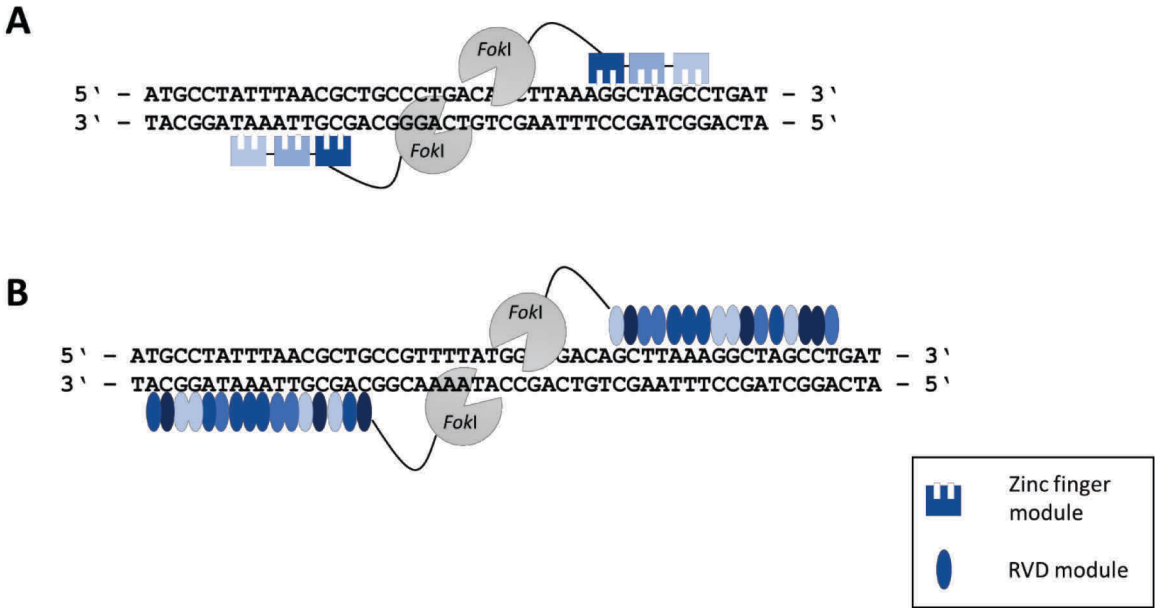

Figure 1-4 Gene editing methods using *Fok*I cleavage domain for DSB induction: modified from Osakabe and Osakabe (2015). **(A)** zinc-finger nucleases (ZFNs) consist of the *Fok*I cleavage domain and three zinc-finger domains, each binding three base pairs**. (B)** transcription activator-like effector nucleases (TALENs) are composed of the *Fok*I domain and repeat-variable di-residues (two amino acids) binding to distinct nucleotide bases.

Like ZNFs, the TALE nucleases consist of a *Fok*I cleavage domain, which needs dimerization to be active. Instead of zinc-finger domains for DNA recognition, so called transcription activator-like effectors (TALEs), are linked to the cleavage domain (Figure 1-4 B). TALE proteins are originally derived from *Xanthomonas* and can bind to specific DNA sequences (Weeks et al., 2016). Native TALE consists of 12-27 repeat units with a length of 34 amino acids (Boch and Bonas, 2010). The amino acid 12 and 13 of each repeat are called repeat-variable di-residues (RVDs), because they are highly variable. Pending on the combination of amino acids, one of the four nucleotide-bases is targeted, e.g. NI (asparagine-isoleucine) recognizes adenine, NG targets a thymine, cytosine is recognized by the amino acid

combination histidine and aspartic acid (HD) and NN targets adenine or guanine (Boch et al., 2009). These codes are then used to design TALENs for targeting consecutive DNA bases to induce a DSB at a desired position. TALEN-based gene editing was applied efficiently to various plant species, e.g. to produce disease-resistant rice (Li et al., 2012b) or for targeted mutagenesis of several genes in *Arabidopsis* (Christian et al., 2013). TALEN-mediated gene replacement was shown in tobacco (Zhang et al., 2013). For the customized assembly of TALE arrays, PCR-based approaches are nearly impossible, because of the similarity of the repeat sequences (Voytas, 2013). Therefore, tools based on Golden Gate assembly were developed for easier assembly of the TALE arrays. (Li et al., 2012a; Sanjana et al., 2012). Gene editing with TALENs is highly specific, but the time-consuming assembly represents a drawback.

With the first publications of efficient gene editing of plant genomes, especially of different crops, with the CRISPR-Cas9 method in 2013, this technique developed as a state-of-the-art technology, now used in many plant species (Puchta, 2017). Thus, this method is explained in more detail.

1.1.4.2. CRISPR-Cas9

In bacteria and archaea clustered, regularly interspaced, short palindromic repeats (CRISPR) in combination with CRISPR-associated (*cas*) genes provide immunity against plasmids and viruses by the uptake of so-called protospacers, represented by short DNA fragments of the foreign DNA (Barrangou et al., 2007; Gasiunas et al., 2012). For gene editing, the principle of the type II CRISPR-Cas system from *Streptococcus pyogenes* is used, in which the Cas9 protein is responsible for cleavage of DNA (Figure 1-5).

After the uptake of the protospacer sequences by the bacteria, they are integrated into the repetitive elements, the CRISPR loci. When new protospacers are added, the CRISPR repeat is duplicated, leading to a repeat-spacer-repeat structure (Wiedenheft et al., 2012). These arrays are transcribed into pre CRISPR RNAs (pre-crRNAs) and processed into small transcripts, the CRISPR RNAs (crRNAs). They consist of the above-mentioned conserved repeat sequence and the protospacer, complementary to the invading DNA.

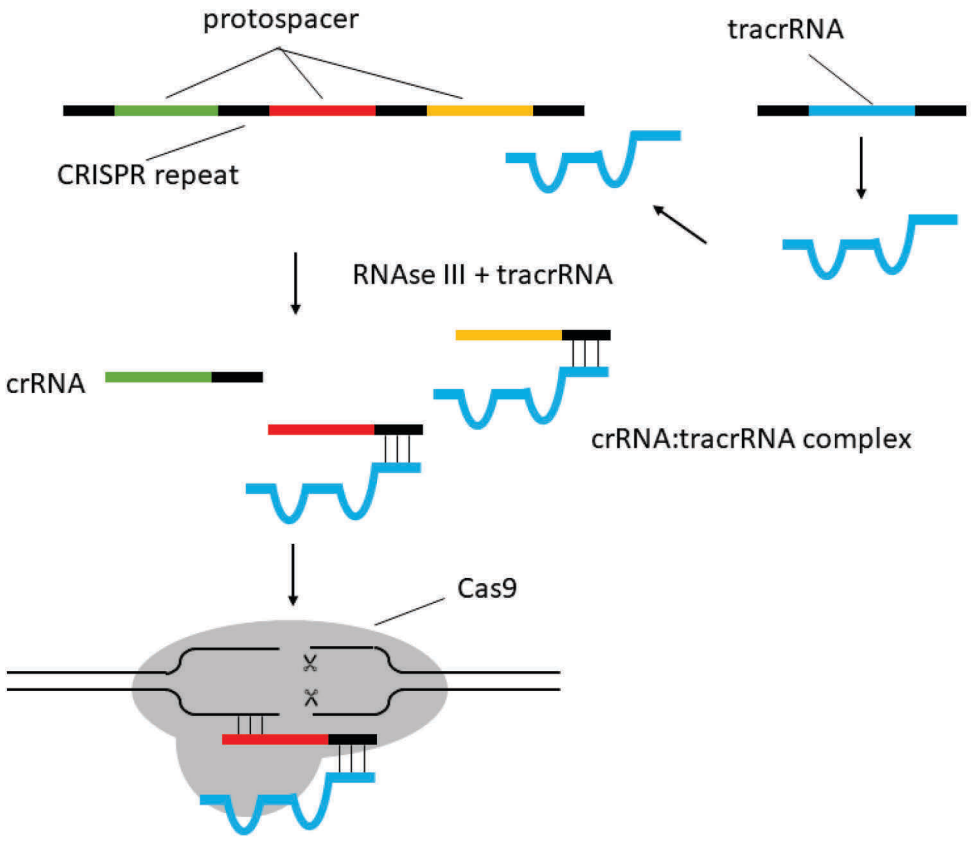

Figure 1-5 Naturally occurring type II CRISPR-Cas9 system in *S. pyogenes*: modified from Sander and Joung (2014). Protospacers were integrated separated by CRISPR repeats into the CRISPR locus, which is transcribed and processed to CRISPR RNAs (crRNAs) with the help of *trans*-activating crRNAs (tracrRNAs). Both RNAs form a complex, which guides the nuclease Cas9 to the invading DNA and introduces double strand breaks to eliminate the invading DNA.

In vicinity to the CRISPR loci, the *cas* genes, coding for endonucleases, as well as the *trans*-activating CRISPR RNA (tracrRNA) are transcribed. On the one hand the tracrRNA triggers the processing of the pre crRNA by an RNase III enzyme, on the other hand it can form a complex with the mature crRNA, because it consists of a stretch of 20-25 nucleotides complementary to the repeat sequence of the guiding crRNA (Figure 1-5; Deltcheva et al., 2011; Jinek et al., 2012). The complex of crRNA and tracrRNA is necessary for the cleavage of foreign targeted DNA by the Cas9 proteins (Jinek et al., 2012). Cas9 proteins recognize the cleavage site via the protospacer adjacent motif (PAM) at the 3' end of the crRNA and the 20-nucleotide sequence of the crRNA; complementary to the target DNA. The PAM is composed of three nucleotides with the structure NGG and Cas9 cleaves the DNA three base pairs upstream of the PAM to create a DSB (Gasiunas et al., 2012; Jinek et al., 2012).

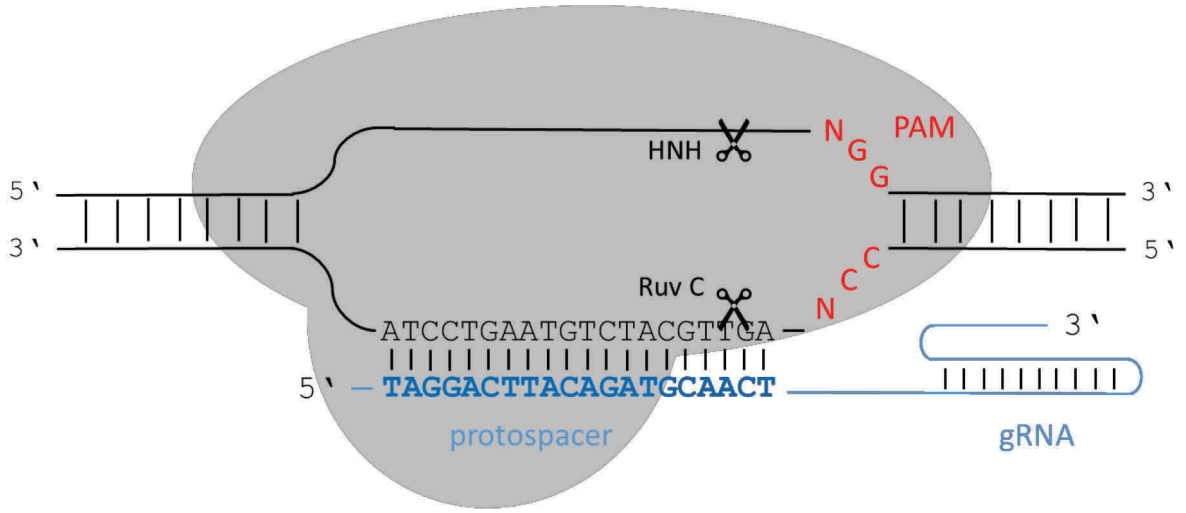

Figure 1-6 CRISPR-Cas9 principle with the use of a chimeric guide RNA: modified from Puchta (2017). A chimeric RNA composed of the tracrRNA and crRNA, linked with a loop, can bind the target DNA sequence and the recruited Cas9 endonuclease cleaves the DNA three base pairs upstream of the PAM sequence. Cas9 consists of two cleavage domains (HNH and RuvC) each responsible for cleavage of one strand.

Cas9 enzymes contain two nuclease domains: (i) the HNH domain, responsible for the cleavage DNA strand, complementary to the crRNA; (ii) the RuvC nuclease domain, which is required for cleavage of the non-complementary strand (Figure 1-6). Only with both nuclease domains, a DSB of the DNA is possible. Furthermore, a seed sequence of about 8-12 nucleotides at the 3' end of the protospacer is crucial for recognition by the crRNA and cleavage by Cas9 (Jinek et al., 2012; Sapranauskas et al., 2011).

Since the Cas9 protein is guided to the target DNA sequence through the crRNA:tracrRNA complex, researchers created a single crRNA:tracrRNA chimera with a linker loop (Jinek et al., 2012), with the intention to use this chimeric RNA in gene editing experiments to target a DNA sequence of choice. This RNA chimera is called guide RNA or single guide RNA (gRNA/sgRNA) and since its invention, it is used in almost all gene editing experiments and represents a breakthrough in molecular biology. It consists of the 20 base pair target DNA sequence without the PAM, which is complementary to the gene region, where the double strand break should occur and the structural component, which represents parts of the crRNA and the tracrRNA (Figure 1-6). When using this method, selection of the 20 base pair target sequence requires appropriate proof. A target sequence unique in the genome is recommended, to reduce the possibility of off target effects by a more precise Cas9 DNA-cleavage.

After the invention of the guide RNA the CRISPR-Cas9 system was used in many organisms, e.g. human cell lines, mice or yeast (Cong et al., 2013; DiCarlo et al., 2013; Mali et al., 2013). If Cas9 is mutated at the catalytic residue of RuvC (D10A), only one DNA strand is cleaved, resulting in DNA nicks (Gasiunas et al., 2012; Jinek et al., 2012). Researchers used this

knowledge to enhance the genome editing specificity, by creating double nicks with the use of two gRNAs target sites in a gene next to each other. Both, NHEJ and HDR were possible with this method (Ran et al., 2013).

During the same time, first success concerning CRISPR-Cas9 mediated gene editing could be shown in model plants like *N. benthamiana* and *A. thaliana* (Li et al., 2013; Nekrasov et al., 2013) as well as in different important crop plants, like wheat and rice (Shan et al., 2013; Wang et al., 2014). One of the biggest advantages of this method represents the marker-free mutation of the genome without any footprints left behind. For plants, it was shown, that due to mendelian segregation, integrated foreign DNA fragments are removed in sexually reproducing species, like *A. thaliana* (Fauser et al., 2014). In most studies, CRISPR-Cas9 is applied for knockouts of specific genes. DSB are generated at distinct positions and with NHEJ the DNA is repaired in an error-prone manner, leading to frame shifts in the reading frame by insertions or deletion of base pairs of variable lengths. One example represents the knockout of a master regulator gene in fruit ripening of tomato (Ito et al., 2015). Moreover, researchers were able to create resistance to powdery mildew in bread wheat by CRISPR-Cas9 mediated knockouts (Wang et al., 2014). Although HDR is not the preferred repair mechanism in plants, researchers were successful with the integration of complete genes with the use of the CRISPR-Cas9 method. Beside the *cas9* gene and a suitable gRNA, cells have to be transformed with a fragment composed of the insert of interest and homologous flanking regions. This was successfully done e.g. with the gene *acetolactate synthase 1* (*ALS1*). With the CRISPR-Cas9-mediated exchange of a mutated gene sequence with amino acid changes into the genomes of maize and rice, a resistance against the herbicide chlorsulfuron was achieved (Sun et al., 2016; Svitashev et al., 2015).

With the discovery of the CRISPR-Cas9 method, metabolic engineering of plants became more and more attractive, since a complete knockout of genes or the knock-in of heterologous genes or mutated genes is possible. Metabolic engineering in plants was already shown by the targeted mutagenesis in the starch branching enzyme (*SBEIIb*) leading to a high amylose rice (Sun et al., 2017). Furthermore, Alagoz et al. were able to use the CRISPR-Cas9 method in Opium Poppy for the manipulation of the benzylisoquinoline alkaloids, like morphine (Alagoz et al., 2016). However, the mentioned examples only targeted one gene. For the implementation of more than one gene or the mutation of several genes, multiplex genome editing approaches are necessary. This is realized by targeting several genes at the same time with different gRNAs expressed on one vector, as already demonstrated in tomato. They were able to target five genes of the GABA shunt metabolic pathway leading to an enhanced GABA content of 19-fold (Li et

al., 2018). These first studies show that metabolic engineering in plants with using the CRISPR-Cas9 method can be a future alternative in plant synthetic biology.

1.2. Model plants

"Plant synthetic biology is an emerging discipline (Staniek et al., 2013). To date, only a few examples are published, like the production of the cyanogenic glucoside dhurrin in *Arabidopsis* (Kristensen et al., 2005) or the provitamin A biosynthesis in potato (Diretto et al., 2007). Tobacco is the most commonly used model plant to produce proteins heterologously. Molecular genetics and the genetic transformation are well established even in chloroplasts (Jube and Borthakur, 2007)." (Schachtsiek et al., 2018). In contrast to this, molecular genetics are poorly understood in other plants, like *Cannabis sativa* L., which becomes more and more important regarding the pharmaceutical potential of the cannabinoids.

1.2.1. *Cannabis sativa* L.

"*Cannabis sativa* L. (*Cannabaceae*) belongs to the group of dioecious plants (male and female flowers on different plants) possessing an allogamous (cross fertilization) nature." (Schachtsiek et al., 2018). Since 6000 years the plant is grown all over the world, e.g. for its fibers for textiles or oil production from seeds (Chandra et al., 2017; Zuardi, 2006). Furthermore, the plant "contains a number of psychoactive chemical compounds, the cannabinoids, which possess a significant pharmaceutical potential. Recently, the usage of *Cannabis* for medicinal purposes was legalized in many countries. Thus, the study on the influence of different cannabinoids in combination with other *Cannabis*-derived compounds with respect to the treatment of various diseases becomes increasingly important." (Schachtsiek et al., 2018)

1.2.1.1. *Cannabis* as medicine

"In recent years the use of *Cannabis*, at least for medicinal purposes, was decriminalized in many US states (Fasinu et al., 2016) as well as in most states of the European Union (Bifulco and Pisanti, 2015)." (Schachtsiek et al., 2018). In July 2018, Canada legalized *Cannabis* for recreational purposes as a second country in the world after Uruguay in 2015 (Cox, 2018). "This resulted in an increased demand for well-characterized *Cannabis* varieties for the treatment of several diseases and disease symptoms like posttraumatic stress, vomiting during anti-neoplastic chemotherapy, tremor in multiple sclerosis, epilepsy, and others (Ablin et al., 2016). However, in the past *Cannabis* varieties were bred specifically to increase the amount of Δ^9-tetrahydrocannabinol (Δ^9-THC) with the focus on psychotropic effects. But in the light of the

medicinal use of *Cannabis* the focus has entirely changed since a strong psychedelic effect is a clear drawback of most of the currently available *Cannabis* plants. Specifically, cannabidiol (CBD)-rich so-called medicinal *Cannabis* plants became available and also a limited number of plant-derived pharmaceuticals (e.g. Sativex, Bediol, Bedrocan) are already approved. Even more pharmaceutical applications are under investigation (Carlini, 2004; Pertwee, 2006) but these new entities require more studies on the influence of the different cannabinoids in combination with the endogenous terpene and phenolic compound profile (Andre et al., 2016), and also the breeding of the corresponding plants having those properties needs to be intensified." (Schachtsiek et al., 2018).

1.2.1.2. The cannabinoid biosynthesis

The cannabinoids belong to the chemical class of terpenophenolics and bind to CB_1 and CB_2 receptors in human brains (Gertsch et al., 2010; Marzo et al., 2004). In recent years, several plant-derived compounds were discovered that have the ability to bind to the mentioned receptors in humans, leading to the new term phytocannabinoids (Gertsch et al., 2010). One example represents the phytocannabinoid perrottetinenic acid, discovered in the New Zealand liverwort *Radula marginata*, which has a highly similar structure to the *Cannabis*-derived cannabinoid Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA) (Toyota et al., 2002).

The expression of several genes involved in the cannabinoid biosynthesis, including the tetrahydrocannabinolic acid synthase (THCAS), the gene responsible for the conversion of cannabigerolic acid (CBGA) to THCA, was detected in glandular trichomes of *C. sativa* L. leading to the suggestion that the cannabinoid synthesis takes place in the glandular trichomes (Marks et al., 2009). Glandular trichomes appear in two different shapes, mainly on female flowers and additionally on anthers of male plants, the bulbous and the capitate shape (Dayanandan and Kaufman, 1976).

"The cannabinoid biosynthetic pathway can be divided into the monoterpene (geranyldiphosphate; GPP), polyketide (olivetolic acid; OA), and an actual cannabinoid forming part (Figure 1-7)." (Schachtsiek et al., 2018).

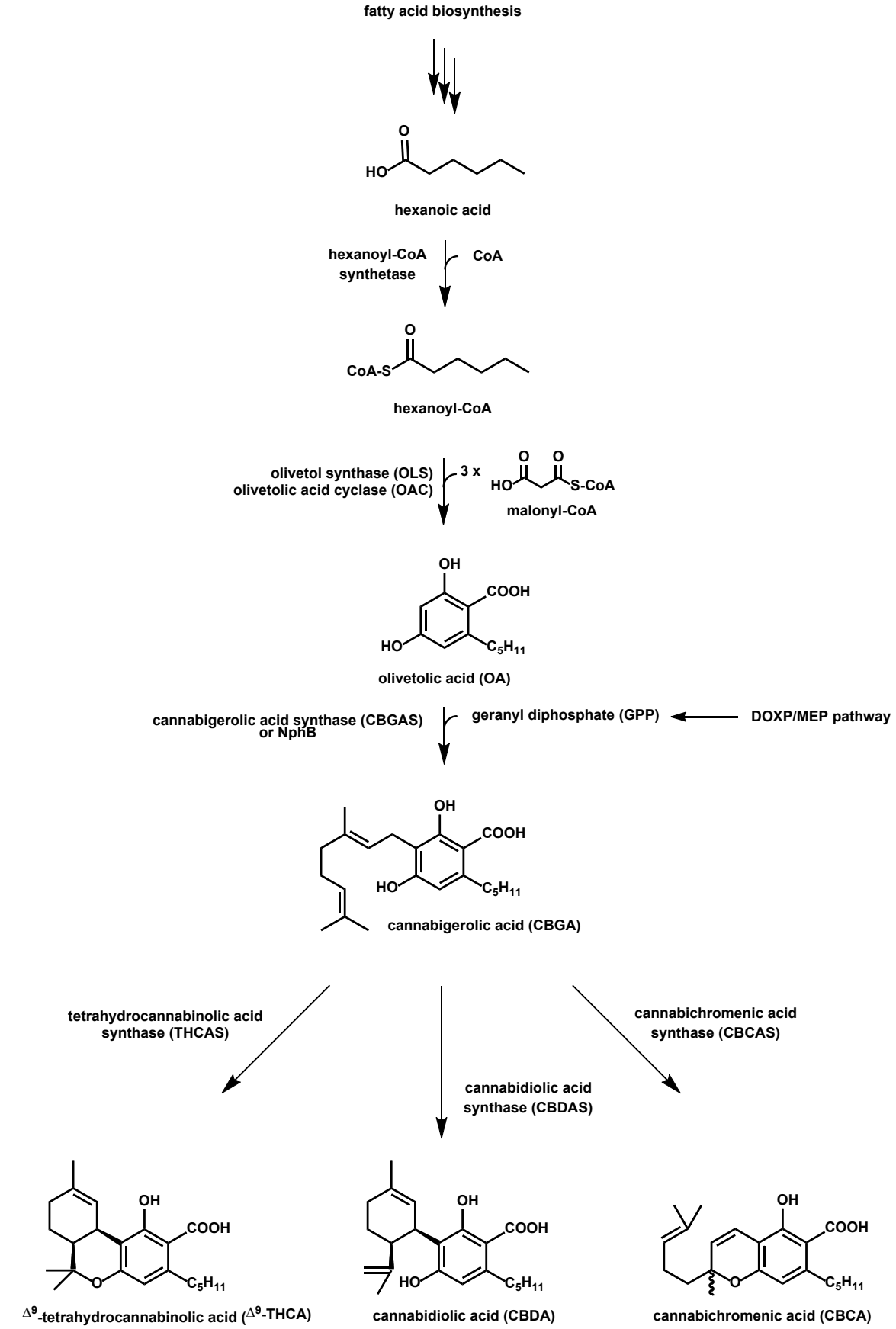

Figure 1-7 Biosynthesis of cannabinoids in *C. sativa L.*: "The central precursor of cannabinoid biosynthesis, CBGA, is converted by three enzymes CBDAS, CBCAS, and THCAS resulting in the main cannabinoids that accumulate in the head cells of glandular trichomes of *C. sativa L.*". modified from Schachtsiek et al. (2018).

As a precursor for the cannabinoid biosynthesis, hexanoyl-CoA was identified, which is synthetized by a hexanoyl-CoA synthetase, the acyl activating enzyme (AAE), in the trichomes of the plant from hexanoic acid (Stout et al., 2012). Hexanoyl-CoA serves as a precursor for olivetolic acid biosynthesis in the polyketide pathway. Together with three molecules of malonyl-CoA, the polyketide synthase OLS (olivetol synthase), catalyzes the reaction from hexanoyl-CoA into the intermediate substances (Taura et al., 2009), which are directly converted into olivetolic acid by the olivetolic acid cyclase (OAC; Gagne *et al.*, 2012).

"The isoprene biosynthesis, the building blocks of monoterpenes, is realized in plants either by the mevalonate (MEV) or the 1-deoxy-D-xylulose 5-phosphate (DXP) pathway, whereby the latter is mainly involved in secondary metabolism (Eisenreich et al., 2004)." (Schachtsiek et al., 2018). It was shown that the isoprene precursors isopentyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) predominantly derived from the DXP-pathway in the *C. sativa* L. cannabinoid biosynthesis (Fellermeier et al., 2001). The DXP-pathway starts with a condensation reaction of pyruvate and glyceraldehyde-3-phosphate followed by six additional enzymatic steps to form IPP and DMAPP (Lange, 2015; Tholl, 2015). The formation of GPP by a condensation reaction of IPP and DMAPP is catalyzed by a GPP synthase (Bouvier et al., 2000).

The first catalysis step in the cannabinoid forming part of the biosynthesis is the formation of cannabigerolic acid (CBGA) from olivetolic acid, which is C-prenylated by GPP. The reaction is catalysed by the aromatic prenyltransferase CBGA synthase (Degenhardt et al., 2017). The central precursor for the cannabinoid biosynthesis CBGA is then used as substrate for the oxidative cyclization towards the formation of THCA, cannabidolic acid (CBDA) and cannabichromenic acid (CBCA). These reactions are catalyzed by the THCA synthase, CBDA synthase and the CBCA synthase, respectively (Morimoto et al., 1998; Shoyama et al., 2012; Taura et al., 1996). THCA synthase is supposed to be synthesized and stored in the glandular trichomes (Sirikantaramas et al., 2005). Recently, the storage of THCA in glandular trichomes of drug-type plants was confirmed by coherent anti-Stokes Raman scattering (CARS) microscopy. Furthermore, CBDA was found in the secretory cavity of fiber-type trichomes (Ebersbach et al., 2018). Moreover, it was also shown that THCAS and CBDAS are able to convert CBGA into THCA, CBDA and CBCA dependent on the pH (Zirpel et al., 2018).

If the cannabinoids are exposed to light or heat (smoking, baking) they are non-enzymatically converted to their decarboxylated forms THC, CBD and CBC.

1.2.1.3. Biotechnological production chassis

"Since seeds are highly heterozygous, conventional breeding is costly and time consuming. *In vitro* plant propagation in combination of modern genome editing tools like CRISPR (clustered regularly interspaced short palindromic repeats) Cas9 (CRISPR-associated) opens the possibility to modify the metabolic pattern of *Cannabis*. One prerequisite is to establish a reliable transformation and regeneration protocol resulting in transgenic female *Cannabis* plants (Figure 1-8) that can be subsequently propagated via vegetative cuttings." (Schachtsiek et al., 2018). In general, transformation of plant tissues is mainly either done with i) the biolistic method (Finer et al., 1992), or ii) infiltration with *A. tumefaciens* (Gelvin, 2003). Transformed tissues are then incubated on plant tissue culture media supplemented with distinct plant growth regulators for callus induction and further development of shoots and roots, leading to a transgenic (T_0) plant (Figure 1-8).

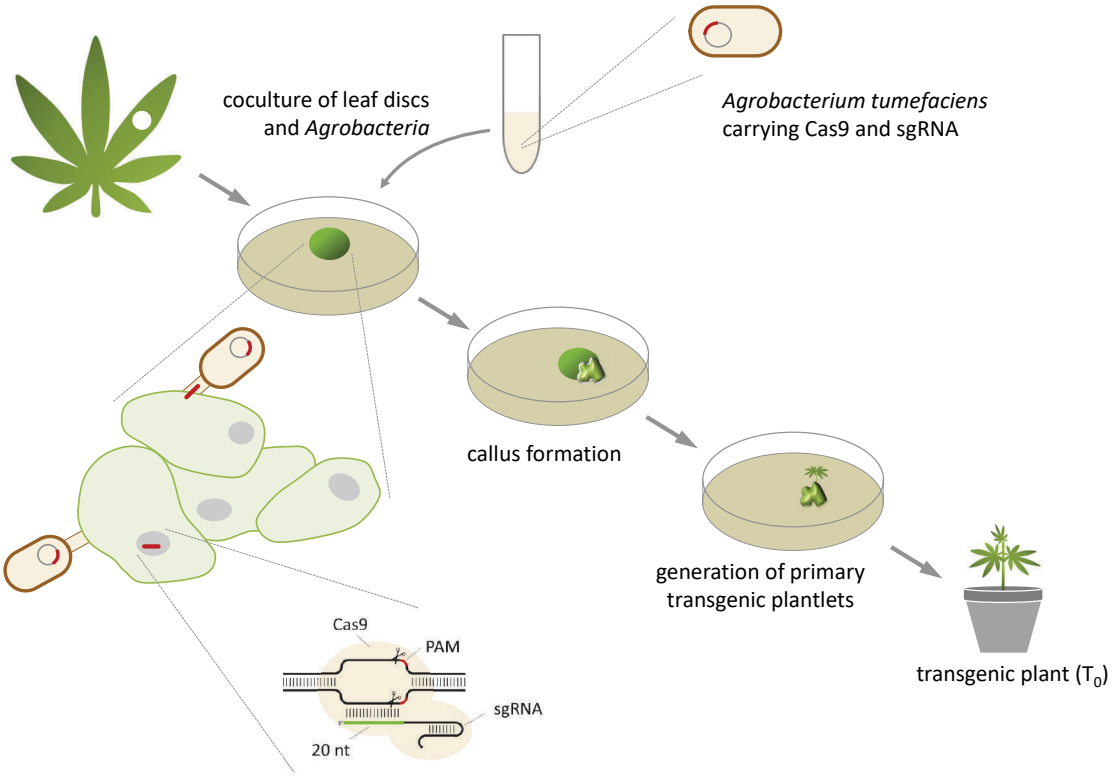

Figure 1-8 Generation of transgenic *Cannabis* plants: "The *Cannabis* leaves are transformed by *Agrobacterium* carrying Cas9 and sgRNA. Alternatively, leaves can be transformed before leaf discs are cut or callus cultures can be inoculated with *Agrobacterium*, respectively. Using *Agrobacteria*-delivering transient viral vectors non-transgenic plants will be obtained. CRISPR - clustered regularly interspaced short palindromic repeats; CAS9 - CRISPR-associated; PAM - protospacer adjacent motif; sgRNA – single guide RNA". modified from Schachtsiek et al. (2018).

"Alternatively, one can make use of a different host producing the desired cannabinoids heterologously. This can be achieved by either transferring the cannabinoid pathway into a different host plant (e.g. tobacco) or in microorganisms like yeast. For the latter possibility, it was recently shown that the tetrahydrocannabinolic acid (THCA) forming enzyme (Zirpel et al., 2015) in combination with a soluble prenyltransferase can be simultaneously produced in *Saccharomyces cerevisiae* and *Komagataella phaffii* in their active form (Zirpel et al., 2017). This marks an important step towards the total biosynthesis of cannabinoids in a microbial platform organism and was recently reviewed in detail elsewhere (Carvalho et al., 2017)." (Schachtsiek et al., 2018).

1.2.1.4. *Cannabis* cell cultures

"To reach high yields of secondary metabolites, plant cells are often cultivated as callus, hairy root, or cell suspension culture for biotechnological approaches. However, with the intention to produce high amounts of cannabinoids with the use of cell culture systems, difficulties were reported in the past. Cannabinoids were neither detectable in *C. sativa* cell suspension cultures nor in callus cultures so far (Flores-Sanchez et al., 2009; Pacifico et al., 2008). Additionally, it is documented that cannabigerolic acid (CBGA) and THCA were toxic to *C. sativa* cell suspension cultures at concentrations of 50 µM (Sirikantaramas et al., 2005). Furthermore, it could be shown that cannabichromenic acid (CBCA) and THCA caused cell death in *Cannabis* leaf cells and suspension cultures (Morimoto et al., 2007). The toxicity of the cannabinoids might be the reason why they are produced and stored in glandular trichomes in *C. sativa.*
Regarding an increased cannabinoid production, this would imply the generation of, for example, callus cultures with trichomes on their surface." (Schachtsiek et al., 2018) One can also think of the possibility to glycosylate the cannabinoids to reduce their toxicity. Glycosylated metabolites become more water soluble and a transport to the vacuole is enabled (Härtl et al., 2017). "Since now, nothing was reported toward this direction. Thus, cell culture systems do not seem to be the best strategy to produce high or selective yields of cannabinoids. It would be more efficient to use genetically engineered *Cannabis* plants for this purpose. Nevertheless, cell culture systems, especially callus cultures, represent the basis of plant regeneration, which would be necessary when following the mentioned strategy. Callus cultures are described as undifferentiated totipotent cells derived from wounded plant tissue. The use of growth regulators like auxins and cytokinins can lead to a differentiation of the cells and might induce the growth of shoots or roots." (Schachtsiek et al., 2018). For the development of shoots and roots, the formation of meristemoids on the callus cultures is necessary, which can

differentiate into shoot or root primordia. "Different studies on *Cannabis* cell culture systems have been already done (Table 1-1).

Table 1-1: Overview of the current research on *Cannabis* cell culture, regeneration and transformation. Modified from Schachtsiek et al. (2018).

cell culture system	explant tissue	plant regeneration	transformation	reference
callus	young leaves, petioles, internodes, axillary buds	yes	no	(Slusarkiewicz-Jarzina et al., 2005)
callus, suspension cultures	stem, leaves	no	yes	(Feeney and Punja, 2003)
direct organogenesis	nodal segments (axillary buds)	yes	no	(Lata et al., 2009)
callus	leaves	yes	no	(Lata et al., 2010)
direct organogenesis	axillary buds	yes	no	(Lata et al., 2016)
callus	shoot tips	yes	yes	(MacKinnon et al., 2001)
callus	hypocotyl	yes	yes	(Sirkowski, 2012)
callus	cotyledons	yes	no	(Chaohua et al., 2016)
hairy roots	hypocotyl	no	yes	(Wahby et al., 2013)
suspension cultures	leaves	no	no	(Flores-Sanchez et al., 2009)
callus	leaves	no	no	(Pacifico et al., 2008)
suspension cultures	stem, leaves	no	no	(Sirikantaramas et al., 2005)
callus	cotyledons	yes	no	(Movahedi et al., 2015)
callus	axillary buds	yes	no	(Lata et al., 2009)
callus	cotyledons, stem, root	yes	no	(Wielgus et al., 2008)

Successful callus induction for *C. sativa* could be shown by different working groups. By using different explants, callus induction was most efficient when using petioles as explants (Slusarkiewicz-Jarzina et al., 2005). Feeney and Punja (Feeney and Punja, 2003) used stem and

leaf segments to produce callus cultures with kinetin and 2,4-dichlorophenoxyacetic acid." (Schachtsiek et al., 2018). Initiation of callus cultures from cotyledons could be achieved with the plant growth regulators thidiazuron (TDZ) and indole-butyric acid (Movahedi et al., 2015).

1.2.1.5. Transgenic *Cannabis* plants

"With regard to use *Cannabis* plants for biotechnological processes to selectively produce cannabinoids or other useful products, a genetically engineered *Cannabis* plant would be desirable to reach these goals. In recent years, some transformation and plant regeneration studies were already done" (Schachtsiek et al., 2018), summarized in Table 1-1. "The latter could be successfully shown by using petioles as explants and the growth regulator dicamba to initiate callus growth and induce shoots. However, the efficiency of completely regenerated plants was very low (Slusarkiewicz-Jarzina et al., 2005). With the use of the cytokinin meta-topolin, the group of ElSohly (Lata et al., 2016) was successful. They were able to establish a one-step protocol for plant regeneration out of axillary bud explants with direct organogenesis. According to a patent application, regeneration of *Cannabis* plants was also possible by the use of hypocotyls as explant tissue. Regeneration was done in a combination of zeatin and 6-benzylaminopurine (Sirkowski, 2012). Moreover, plant regeneration was possible by using cotyledons as explants and a medium consisting of the synthetic cytokinin thidiazuron (TDZ) for shoot regeneration and indole-butyric acid for rooting of the plantlets (Chaohua et al., 2016)." (Schachtsiek et al., 2018). Likewise, TDZ was used alone or in combination with naphthaleneacetic acid (NAA) to regenerate *Cannabis* shoots from callus cultures, followed by rooting (Lata et al., 2009, 2010). Noticeable, in many protocols TDZ is used for regeneration of *Cannabis* plants. "Recently, scientist showed that using TDZ for plant regeneration causes high levels of DNA methylation in callus cultures. Additionally, methylation events were more polymorphic than in normal leaf tissue (Ghosh et al., 2017). This leads to the production of plant variants in plant tissue culture. There is evidence that DNA methylation is involved in cell differentiation and gene regulation, which can lead, for example, to chromosome breakage or the activation of transposable elements (Kaeppler et al., 2000; Park et al., 2009). This indicates that TDZ is not suitable as growth regulator for plant regeneration" (Schachtsiek et al., 2018), especially if engineered plants with e.g. a distinct cannabinoid profile should be used in medicinal purposes. "By now, transformation studies on *Cannabis* were exclusively done via *Agrobacteria* infiltration. Wahby et al. (2013) used the hypocotyl of seedlings as explants for transformation with *Agrobacterium tumefaciens* as well as *Agrobacterium rhizogenes*. Transformation of the generated hairy root cultures could be verified by a β-glucuronidase

reporter assay. Successful transformation of callus suspension cultures of hemp with *A. tumefaciens* could be shown as well. However, regeneration of plantlets from transformed calli was not yet achieved (Feeney and Punja, 2003). According to the mentioned patent application, transformation of *Cannabis* plants with *A. tumefaciens* was possible by the use of hypocotyls as explant tissue. Their application should be used to express fluorescent proteins or genetic markers in the plant (Sirkowski, 2012)." (Schachtsiek et al., 2018)

Regarding the lack of established genetic tools and a suitable protocol for the generation of transgenic *Cannabis* plants, it might make sense to transfer the cannabinoid pathway to other plant species for the production of cannabinoids. On the one hand, tools for genetic engineering need to be well established in the host plant and on the other hand, trichomes on the surface of the leaves are essential. Since tobacco plants fulfill these criteria, they represent a suitable host plant for cannabinoid production.

1.2.2. Tobacco

Plants of the genus *Nicotiana* referred to as tobacco belong to the family of *Solanaceae*. Cultivated tobacco (*Nicotiana tabacum* L.) is used to produce tobacco products. With approximately 90 %, nicotine represents the major alkaloid in the plant, whereas the other alkaloids (nornicotine, anabasine and anatabine) mainly represent the other 10 % of the alkaloids (Dewey and Xie, 2013; Saitoh et al., 1985). *N. tabacum* is naturally allotetraploid supposed to be derived from the hybridization of ancestors of *N. sylvestris* (maternal) and *N. tomentosiformis* (paternal), both diploid species (Kajikawa et al., 2017; Leitch et al., 2008; Sierro et al., 2013). Tobacco species are commonly used as model organisms in scientific studies, whereby *N. benthamiana* represents the most prominent one. It serves as a model organism in plant-pathogen interactions (Goodin et al., 2008) and many tools for functional genomics, like RNAi, VIGS or CRISPR-Cas9 are well established (Derevnina et al., 2019), which can be transferred to *N. tabacum* as well. Because of the advantage as a well understood model organism, it would be possible to transfer whole pathways from other organisms into tobacco plants, to achieve high production rates. One example would be the transfer of the cannabinoid pathway of *C. sativa* L. since the growing of a high number of *Cannabis* plants is still limited in most countries. Due to the increasing market on cannabinoids as pharmaceuticals, the heterologous production would be an alternative.

1.2.2.1. Nicotine-free tobacco as an advantage in biotechnological production chassis

"One major drawback of tobacco as pharmaceutical production chassis is the high nicotine and alkaloid content." (Schachtsiek et al., 2018). Thus, a tobacco plant without any alkaloids, especially nicotine, would be a preferable host plant for metabolic engineering studies.

Most genes involved in the nicotine biosynthesis are known and well characterized (Figure 1-9, Dewey and Xie, 2013). After nicotine is synthesized in the roots (Dawson and Solt, 1959) it is transported via the xylem to aerial parts of the plants, mainly to the leaves (Hashimoto and Yamada, 2003; Wink and Roberts, 1998). There, it is stored in the vacuoles (Saunders, 1979). Nicotine itself consists of a pyridine and a *N*-methylpyrrolidine ring, which derive from two independent primary metabolic pathways (Dewey and Xie, 2013).

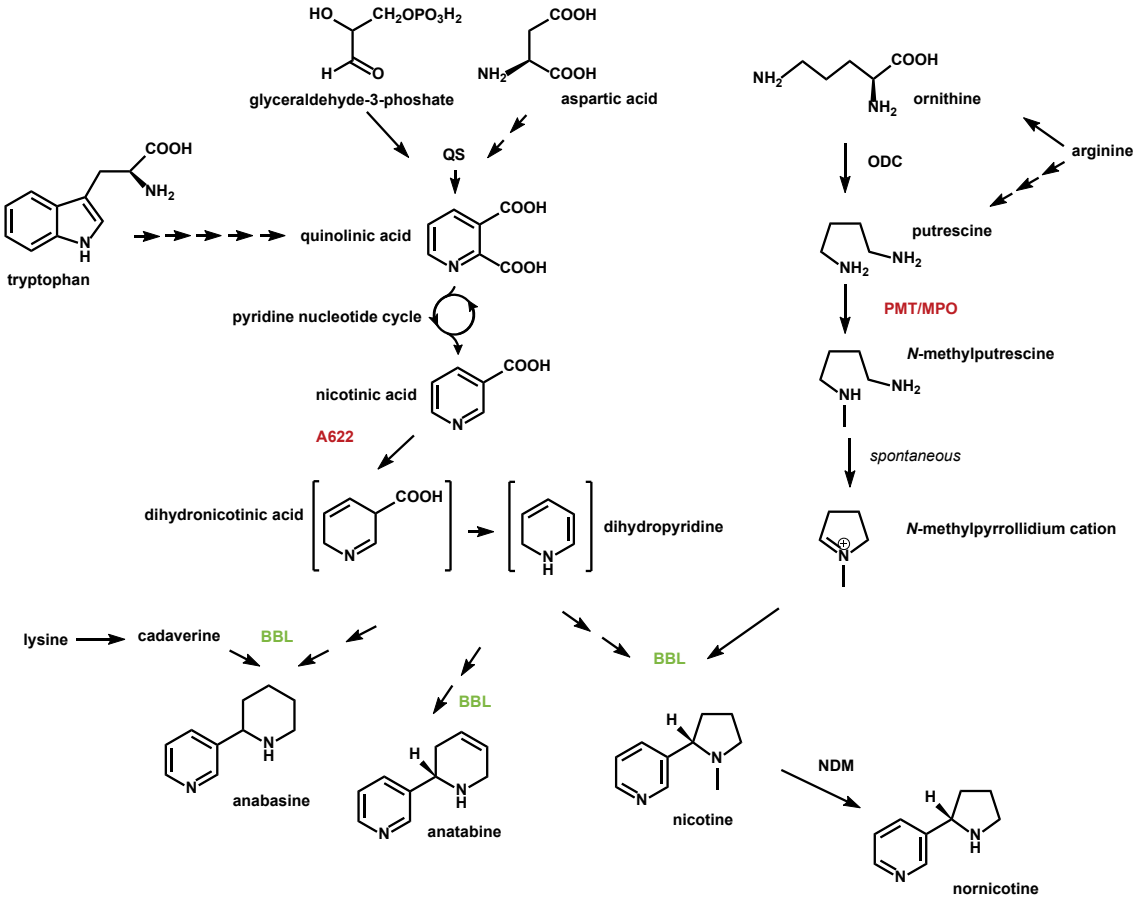

Figure 1-9 Alkaloid biosynthesis pathway in tobacco: modified from Dewey and Xie (2013). Alkaloid biosynthesis consists of two independent pathways, the pyrrolidine and a pyridine pathway. Different enzymes involved in the biosynthesis were already used as targets for studies on alkaloid reduction in the plant. Knockdown of genes in red led to side effects in the plant, downregulation of genes marked in green led to a successful reduction of the alkaloid content without side effects. Abbreviations: QS - qinolinate synthase, A622 - NADPH dependent oxido-reductase, ODC -ornithine decarboxylase, PMT - putrescine *N*-methyltransferase, BBL - berberine bridge enzyme-like, NDM - nicotine *N*-demethylase

1.2.2.1.1. The nicotine biosynthesis

The first step of the biosynthesis of the *N*-methylpyrrolidine ring is the formation of putrescine. This is achieved either through a decarboxylation reaction of ornithine catalyzed by the ornithine decarboxylase (ODC) directly or through an independent pathway over arginine, which is decarboxylated to agmatine via the arginine decarboxylase (ADC). Agmatine is then converted to putrescine over two additional enzymatic steps (Fuell et al., 2010). There is evidence that in tobacco the direct formation of putrescine through ODC is preferred, because the downregulation of ODC led to decreased nicotine levels (DeBoer et al., 2011), whereas the downregulation of ADS had just minor effects (Chintapakorn and Hamill, 2007).

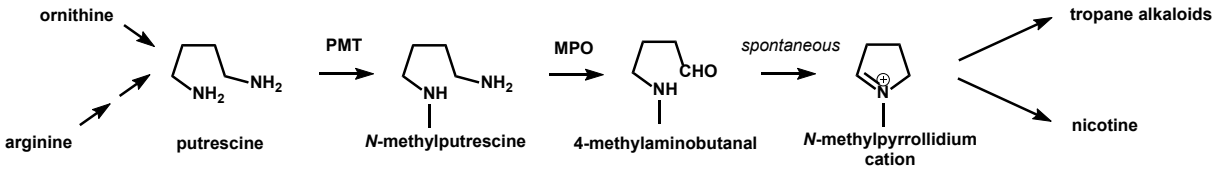

Figure 1-10 Formation of the pyrrolidine ring in the nicotine biosynthesis: modified from (Katoh et al., 2007). Putrescine is methylated by PMT to form *N*-methylputrescine. With a deamination reaction, *N*-methylputrescine is converted to 4-methyl-aminobutanal, which spontaneously non-enzymatically forms the *N*-methyl-pyrrolinium cation. This is the precursor for the formation of nicotine.

The following methylation reaction from putrescine to *N*-methylputrescine is catalyzed by the putrescine *N*-methyltransferase (PMT), a key regulatory enzyme in the biosynthesis of nicotine controlling the transition from primary to secondary metabolism (Dewey and Xie, 2013). In *N. tabacum* five isoforms of *PMT* are known which are expressed in the roots. Furthermore the expression level of the *PMTs* correlates with the nicotine content of different tobacco varieties (Riechers and Timko, 1999). *N*-methylputrescine is further deaminated by an *N*-methylputrescine oxidase (MPO) to form 4-methyl-aminobutanal. The spontaneous conversion of 4-methyl-aminobutanal to the *N*-methyl-pyrrolinium cation (Katoh et al., 2007; Mizusaki et al., 1972), generates the precursor for nicotine and tropane alkaloids, the pyrrolidine ring (Figure 1-10).

The biosynthesis of the pyridine ring starts with the oxidation of aspartic acid to α-iminosuccinat. Together with glyceraldehyde 3-phosphate quinolinic acid is formed in a condensation reaction by the quinolinate synthase (QS) (Figure 1-9; Katoh et al., 2006; Dewey and Xie, 2013).

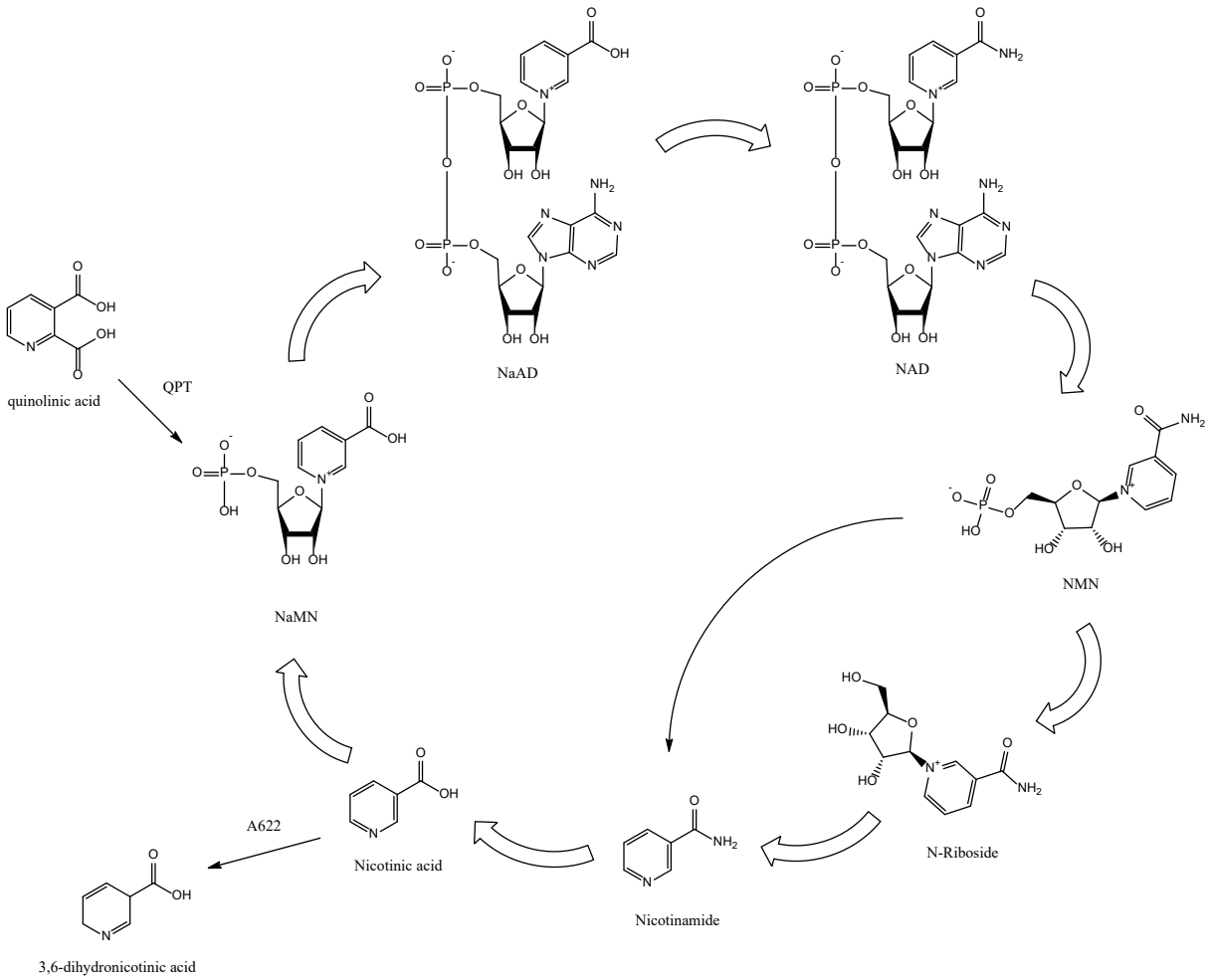

Figure 1-11 The pyridine nucleotide cycle in the nicotine biosynthesis: modified from Wagner et al., (1986) Nicotinic acid is formed via the pyridine nucleotide cycle. The substrate NaMN is catalyzed from quinolinic acid by the QPT. Abbreviations: QPT - quinolinate phosphoribosyltransferase, NaMN - nicotinic acid mononucleotide; NaAD - nicotinic acid adenine dinucleotide; NAD - Nicotinamide adenine dinucleotide; NMN - nicotinamide mononucleotide; A622 - NADPH dependent oxido-reductase

Afterwards, the quinolinate phosphoribosyltransferase (QPT) is responsible for the conversion of quinolinic acid to nicotinic acid mononucleitid (NaNM), which enters the pyridine nucleotide cycle to form nicotinic acid (Figure 1-11). NaMN is converted to nicotinic acid adenine dinucleotide (NaAD) by adenylylation with the enzyme nicotinate mononucleotide adenylyltransferase (NaMNAT) followed by the conversion to NAD by the NAD synthase. An NAD-pyrophasphatase converts NAD to nicotinamide mononucleotide (NMN). This can be converted either directly to nicotine amide or indirectly via nicotinamide-riboside (N-riboside). The nicotineamide deaminase catalyzes the formation of nicotinic acid from nicotinamid. (Noctor et al., 2006; Wagner et al., 1986)

The last steps in the nicotine biosynthesis remained unclear for a long period. In 2009, research of two groups gave evidence that A622, a phosphatidylinositol-4-phosphate (PIP)-family

member of NADPH-reductases, is able to use nicotinic acid as substrate for the formation of 3,6-dihydronicotinic acid, which represents the intermediate from the pyridine-pathway for alkaloid biosynthesis (DeBoer et al., 2009; Kajikawa et al., 2009). In 2011 the group of Kajikawa presented evidence that the final oxidation step to produce nicotine, as well as anatabine and anabasine is catalyzed by flavoproteins of the berberine bridge enzyme-like (BBL) family, which are localized in the vacuole (Kajikawa et al., 2011). In first studies four different *BBL* genes (*BBLa – BBLd.1*) could be found in the genome of *N. tabacum*. Expression of *BBLa, BBLb and BBLc* genes could be detected in tobacco roots (Kajikawa et al., 2011). In total, the *BBL* gene family consists of six genes, since two additional genes (*BBLd.2* and *BBLe*) were discovered recently. *BBLb*, *BBLd.1* and *BBLe* originate from *N. tomentosiformis* and *BBLa*, *BBLc* and *BBLd.2* derive from *N. sylvestris* (Kajikawa et al., 2017). The formation of nornicotine is catalyzed by the nicotine N-demethylase (NDM), which uses nicotine as substrate (Xu et al., 2006a).

1.2.2.1.2. Target genes for the nicotine-free tobacco plant

Since two pathways are involved in the biosynthesis of nicotine, several target genes can be considered for the modulation of the overall alkaloid yield in tobacco, especially the generation of a nicotine-free plant by knockout studies. In the last years, many studies already dealt with the knockdown of genes involved in the biosynthesis of nicotine, mainly to investigate their function. Nevertheless, the results of the studies give evidence towards the right target gene for a nicotine-free plant. An overview of the nicotine biosynthesis and the considered target genes are shown in Figure 1-9. Genes marked in red were designated as a non-suitable target gene.

The downregulation of the *ODC* gene, which is involved in the early part of the pyrrolidine ring formation, led indeed to a reduction of the nicotine level, but simultaneously the anatabine yield was increased (DeBoer et al., 2011). The next step in the synthesis of the pyrrolidine ring is catalyzed by the PMT, which represents an important regulatory enzyme (chapter 1.2.2.1.1). By using different RNA silencing methods e.g. RNAi or antisense a reduced nicotine level could be achieved along with the occurrence of increased anatabine levels (Chintapakorn and Hamill, 2003; Steppuhn et al., 2004; Wang et al., 2008, 2009). Beside the low nicotine content other studies obtained abnormalities of leaves and inflorescence when reducing the expression of *PMT* in *N. sylvestris* plants (Sato et al., 2001). Suppressed expression of the genes encoding for *N*-methylputrescine oxidase (MPO) in hairy root cultures of tobacco resulted in a decrease of nicotine but again in an increase of anatabine (Shoji and Hashimoto, 2008) similar to the *PMT* suppression experiments. The downregulation of the gene encoding for the enzyme A622,

was not successful in whole plants. A reduced alkaloid content was only achieved in hairy root cultures and BY-2 cells (Kajikawa et al., 2009). The *BBL*-genes are responsible for the last oxidation step in nicotine biosynthesis. The knockdown of the genes *BBLa* - *BBLc* by RNAi resulted in significant reductions of the alkaloid content of hairy root cultures and transgenic plants by 94 % and 95.5 %, respectively (Kajikawa et al., 2011). EMS-induced mutations in the genes *BBLa* - *c* led to a 13-fold reduction in total alkaloid levels in field-grown tobacco (Lewis et al., 2015). Except from a lower yield of cured leaves, no increase of other alkaloids or any phenotypic effects were detected. Nevertheless, the nicotine level was not reduced completely. This can be explained by the fact that the gene *BBLd.1*, as well as the newly identified genes *BBLd.2* and *BBLe* were not considered in the RNAi and EMS experiments (Kajikawa et al., 2017). Thus, the *BBL* genes represent promising targets for knockout studies concerning a low nicotine yield.

1.2.2.2. Heterologous production of cannabinoids in tobacco

As already mentioned, tobacco is used as a model plant since many years, mainly for the heterologous production of proteins, especially antibodies from *N. benthamiana* (Holtz et al., 2015). Tobacco plants possess a lot of glandular trichomes on their leaves, which are known for the biosynthesis and storage of terpenes, especially diterpenes (Huchelmann et al., 2017). The presence of glandular trichomes in combination with the well understood molecular genetics and heterologous protein production, tobacco plants represent a suitable host for the heterologous production of cannabinoids e.g. tetrahydrocannabinolic acid (THCA). For the production of cannabinoids, IPP and DMAPP are needed as precursors for GPP. Both metabolites are already produced naturally in tobacco plants in high amounts (Huchelmann et al., 2017). A brief description of the cannabinoid biosynthesis and the particular building blocks for the formation of e.g. THCA is given in chapter 1.2.1.2.

The importance of the glandular trichomes on the surface of tobacco leaves becomes clear, "since the cannabinoids CBGA and THCA co-cultivated with tobacco and *C. sativa* cells caused cell death of all cells within 24 h (Sirikantaramas et al., 2005), suggesting that both substances are highly toxic. Therefore, a secretion of the THCAS into the secretory cavity of trichomes is mandatory for biotechnological approaches to ensure a high THCA titer. This can be achieved by using trichome-specific promoters as described for the engineering of triterpenes in *Nicotiana tabacum* trichomes (Jiang et al., 2016) or the taxadiene synthase (Rontein et al., 2008)." (Schachtsiek et al., 2018)

"Finally, the formation of the different cannabinoids like THCA, cannabidiolic acid (CBDA) and cannabichromenic acid (CBCA) can be achieved by the addition of one respective enzyme (Figure 1-7). First, THCAS expression studies in tobacco hairy root cultures were already performed, demonstrating that active enzyme can be produced in tobacco (Sirikantaramas et al., 2004). Subsequently, the THCAS was fused to GFP and expressed in transgenic tobacco plants (Sirikantaramas et al., 2005), revealing that the fusion protein was localized in the head cells of tobacco trichomes." (Schachtsiek et al., 2018) Recently, THCAS targeted to the endoplasmic reticulum was successfully expressed transiently in *N. benthamiana* leaves, producing THCA and CBCA (Geissler et al., 2018). "Together, this makes tobacco a promising candidate to function as plant-based chassis organism for a heterologous cannabinoid production." (Schachtsiek et al., 2018).

1.3. Scope of the thesis

In the last years, metabolic engineering of plants, especially to produce pharmaceuticals, became more and more important. The discovery of the gene editing method CRISPR-Cas9 opened new possibilities for genetically engineering plants by introducing heterologous genes or to knockout genes completely. The heterologous production of proteins in plants, like antibodies, is mostly done in tobacco, a model plant in biotechnology. Since the plant has trichomes on the surface of the leaves, a heterologous production of cannabinoids from *C. sativa* L., which have a high pharmacological potential, would be possible to cover the future high demand, because the cultivation of the plant is still restricted in many countries.
Since medicinal use of *C. sativa* L. is legalized in many countries, it would be favorable to produce distinct cannabinoids in the plant, to be able to treat various diseases efficiently. For this purpose, it is necessary to understand the mechanism of cannabinoid biosynthesis completey, which is possible with the use of genetic engineering techniques. Until now, genetic modifications of *C. sativa* L. are poorly understood, due to the lack of reliable transformation and regeneration protocols.

Hence, the aims of this thesis are:

1) To create a tobacco plant suitable to produce pharmaceuticals without interference of alkaloids, by knocking out the nicotine biosynthesis with the CRISPR-Cas9 method. With the knockout a nicotine-free, non-transgenic plant can be obtained.

2) To develop and establish a transformation and regeneration protocol for *C. sativa* L., for the generation of stable transgenic plants. Beside the investigation of suitable regeneration conditions, different parameters for transformation with *A. tumefaciens* will be evaluated and optimized.

3) To develop a protocol for virus-induced gene silencing in *C. sativa* L for the analysis of gene functions in the plant by post translational gene silencing, as a transient and fast alternative to stable transgenic plants.

Chapter 2 Results and Discussion

The results from chapter 2.1 were published in:

Schachtsiek J. and Stehle F. Nicotine-free, nontransgenic tobacco (*Nicotiana tabacum* L.) edited by CRISPR-Cas9 (2019). *Plant Biotechnology Journal*, doi: 10.1111/pbi.13193

Schachtsiek J. and Stehle F. Dataset on nicotine-free, nontransgenic tobacco (*Nicotiana tabacum* L.) edited by CRISPR-Cas9 (2019). *Data in Brief,* 26, 104395, doi: 10.1016/j.dib.2019.104395

The chapter "Results and Discussion" is divided into three parts:

1) Nicotine-free, non-transgenic tobacco (*Nicotiana tabacum* L.) edited by CRISPR-Cas9 (chapter 2.1)
2) Development of a regeneration and transformation protocol for *Cannabis sativa* L. (chapter 2.2)
3) Virus-induced gene silencing in *Cannabis sativa* L. (chapter 2.3)

2.1. Nicotine-free, non-transgenic tobacco (*Nicotiana tabacum* L.) edited by CRISPR-Cas9

Since plants of the *Nicotiana* species represent a well-known model organism for heterologous production of proteins and metabolites, a plant without any alkaloids, especially nicotine, would be a preferable host plant for metabolic engineering studies. Moreover, nicotine represents a precursor of tobacco specific nitrosamines (TSNAs), which are known as carcinogens in tobacco products. The TSNAs *N*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are formed out of nornicotine and nicotine in air-cured tobacco through nitrosation reactions and have been found to be strong carcinogenic (Hecht, 1998). Thus, a reduction of these substances would be favorable as well. Until now, the reduction of the nicotine content or TSNAs were only achieved with knockdown studies, e.g. RNAi (Kajikawa et al., 2011; Lewis et al., 2008, 2010; Wang et al., 2009). In recent years, the CRISPR-Cas9 method became the most used technique to genetically modify numerous of organisms. With this method it is possible to knockout genes in plants completely, as already shown e.g. for *N. tabacum* (Gao et al., 2014). As already mentioned, the *BBL*-genes, responsible for the last oxidation step in nicotine formation, represent suitable targets for the generation of nicotine-free tobacco (chapter 1.2.2.1.2). To obtain a plant free of nicotine all six known *BBL*-genes must be knocked out, in the best case with the use of just one guide RNA targeting all *BBL*-genes at once.

2.1.1. Determination of the target sequence for the *BBL* gene knockout

Since the six *BBL* genes should be knocked out at once, it was necessary to search for a target sequence identical in the coding sequence of all known *BBL* genes. Therefore the six published coding sequences for *BBLa*, *BBLb*, *BBLc, BBLd.1*, *BBLd.2* and *BBLe* (Kajikawa et al., 2011, 2017) were aligned and suitable regions were evaluated according to the relevant criteria for a

suitable target sequence including a BLAST search of the target sequences against the genome. Figure 2-1 shows the chosen target sequence for the desired knockout. Except from the PAM sequence, the target sequence is identical in all six *BBL* genes. Mismatches according to other parts of the *N. tabacum* genome were excluded with a BLAST search.

Figure 2-1 Alignment of all *BBL* gene family members: "The six gene sequences of the *BBL* gene family were aligned and possible gene targets were evaluated resulting in a single target sequence identical in all six genes." Modified from Schachtsiek and Stehle (2019b)

2.1.2. Tobacco transformation and regeneration

The chosen 20 base pair target sequence for the knockout of the *BBL* genes was cloned into the pChimera vector (Fauser et al., 2014) between the Ubiquitin 6-26 promotor from *A.thaliana* and the chimeric sgRNA. This gene cassette was cloned into the final transformation vector pCas9-TPC (Fauser et al., 2014), which consists of an *Arabidopsis* codon-optimized version of Cas9, which is driven by the Ubiquitin 4-2 promotor from *Petroselinum crispum*. Additionally, the vector consists of a *bar* resistance cassette for selection of transformed plants. The *bar* genes encodes for a phosphinothricin acetyltransferase (PAT) originally from *Streptomyces hygroscopicus*, which acetylates phosphinothricin resulting in a detoxification of the herbicide (De Block et al., 1987).

After transformation, ten plants were regenerated and denoted as T_0 generation. Rooted plantlets were cultivated in a plant chamber for self-pollination to produce T_1 seeds. Obtained seeds from each T_0 plant were germinated on wet tissue paper to test if they are transgenic. This was done by the application of a 100 mg L^{-1} phosphinothricin (PPT)-solution three times every two days.

Figure 2-2 Selection for positive transformants in T_1-generation: "Seeds from T_0 – plants were germinated on tissue paper and sprayed with a 100 mg L^{-1} PPT solution three times every two days. Seedlings of the wild type (left) died, seedlings from a transformed and regenerated plant (right) survived." modified from Schachtsiek and Stehle (2019a)

Since the transformed T-DNA contains a *bar* resistance cassette, transgenic seedlings should survive the treatment with the PPT-solution. To be sure that the chosen PPT-concentration is sufficient, wild type seedlings were sprayed as well as a control. Grown T_1-seedlings survived incubation with a PPT-solution (Figure 2-2).

2.1.3. Analysis of the nicotine content of T_0 and T_1 plants

To evaluate which of the ten regenerated, transgenic plants seem to be the best candidate for a low nicotine level, at first, the nicotine content was determined from leaves of T_0 plants and wild type leaves as a reference. Extraction of alkaloids was done from dried, grounded leaves and the nicotine level was analyzed with GC-FID.

As shown in Figure 2-3 A the nicotine amount varied between the tested regenerated plants. Whereas the nicotine level of plant T_0 5 was not reduced, plants T_0 1 and T_0 4 revealed a low nicotine level of 0.9 and 0.7 mg g DW^{-1}, respectively, which represents a reduction of 95 % and 96 % compared to the wild type. Beside these two plants, the plant T_0 3 was chosen as well to investigate the development of the nicotine level in further generations. This plant had a medium nicotine level of 5.5 mg g DW^{-1}, which corresponds to a reduction of 65 %. The chosen plants were further analyzed in T_1 generation.

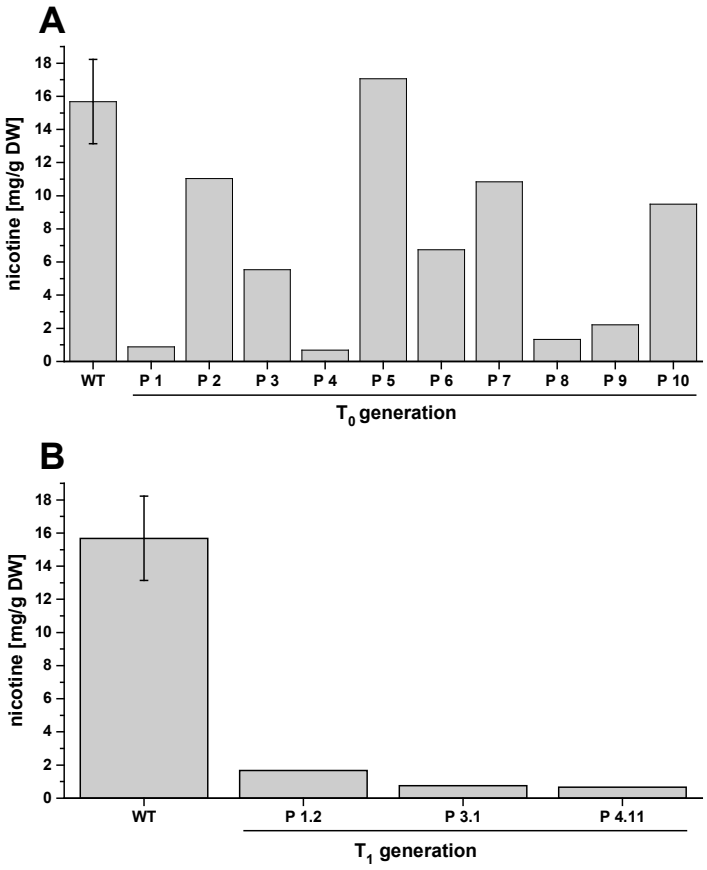

Figure 2-3 Amount of nicotine in wild type, T_0 and T_1 plants: "**(A)** The amount of nicotine was measured with GC-FID of 200 mg dried and grounded leaf material of plants extracted with MTBE. Amount of nicotine was calculated as mg per gram dry weight (DW). **(B)** Amount of nicotine in extracts of T_1 plants." modified from Schachtsiek and Stehle (2019a)

Figure 2-3 B shows the nicotine level of the plants with the lowest nicotine level. Whereas the nicotine yield in plant T_1 1.2 did not decrease further, a decrease in nicotine yield could be observed for plant T_1 3.1 from a 65 % reduction in T_0 generation to a 95 % reduction in T_1 generation. The nicotine level of plant T_1 4.11 was as low as in T_0 generation. Due to the high reduction of the nicotine level in the T_1 plant 3.1 in comparison to the T_0 plant, this plant was chosen to be analyzed more in detail.

2.1.4. Nicotine can still be detected if not all *BBL* genes are knocked out

The presence of nicotine in low amounts in the plant T_1 3.1 was confirmed by GC-MS measurements (Figure 2-4) by analyzing the extracted ion chromatogram (EIC) of nicotine (*m/z* value of 162.23).

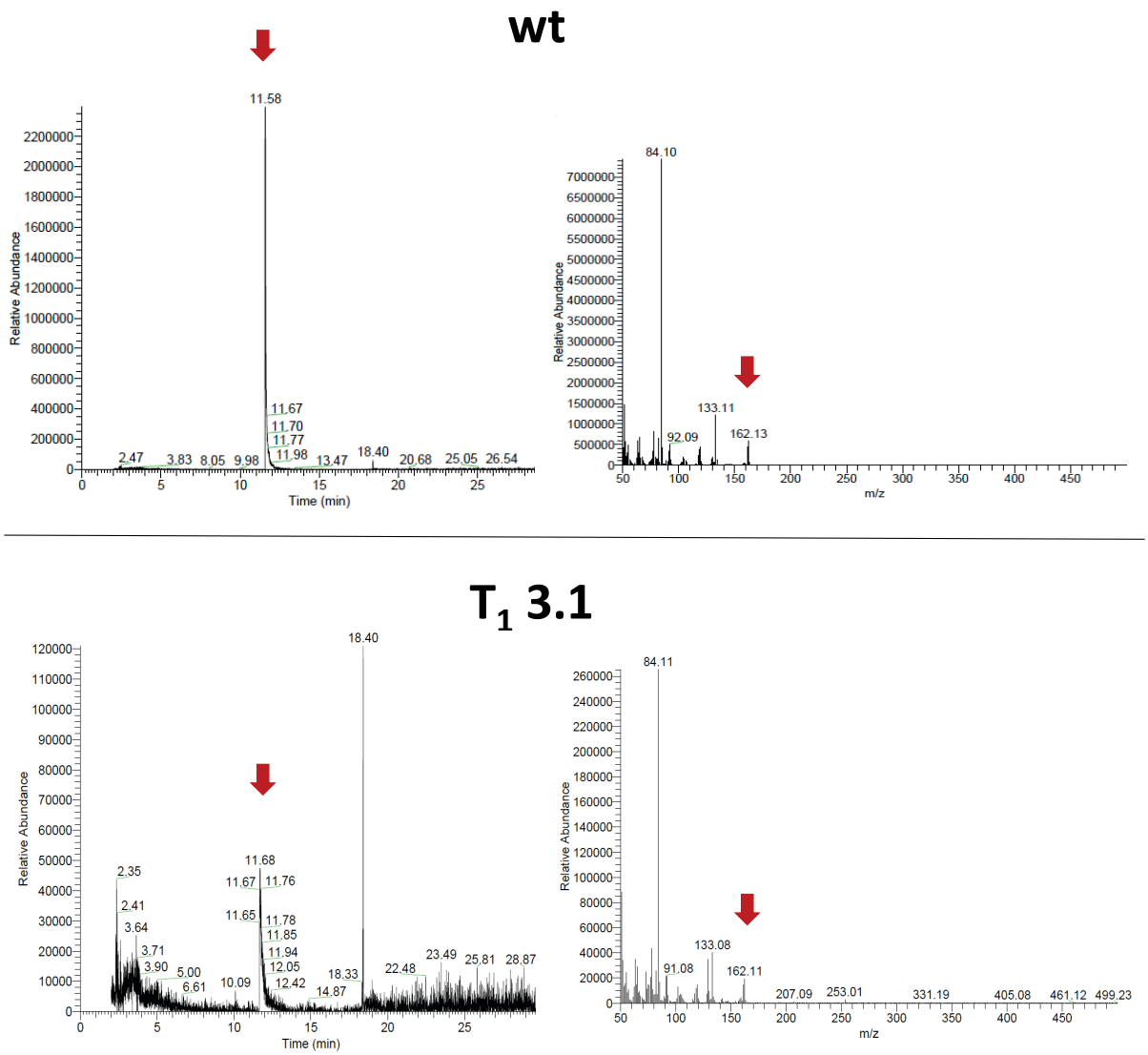

Figure 2-4 GC-MS analysis of plant extracts regarding the mass of nicotine: "Extracts of the wild type (wt) and plant T_1 3.1 were analyzed with GC-MS measurements by the analysis of peaks corresponding to the *m/z* of nicotine (162.23 g/mol). Extracted Ion chromatogram (EIC; left) of the mass of nicotine and the corresponding mass spectra are shown (right)." modified from Schachtsiek and Stehle (2019a)

According to these results, this plant was analyzed on genomic level, to check if all six genes of the *BBL* gene family were knocked out. Therefore, genomic DNA was isolated from leaves of the plant; gene fragments of the six *BBL* genes were amplified and cloned into the vector pDionysos (Stehle et al., 2008) to enable the sequencing of single amplicons of each gene at the target region. Because of the cloning of single amplicons into the vector, it can be seen if the wild type gene could still be amplified or if each *BBL* gene is knocked out.

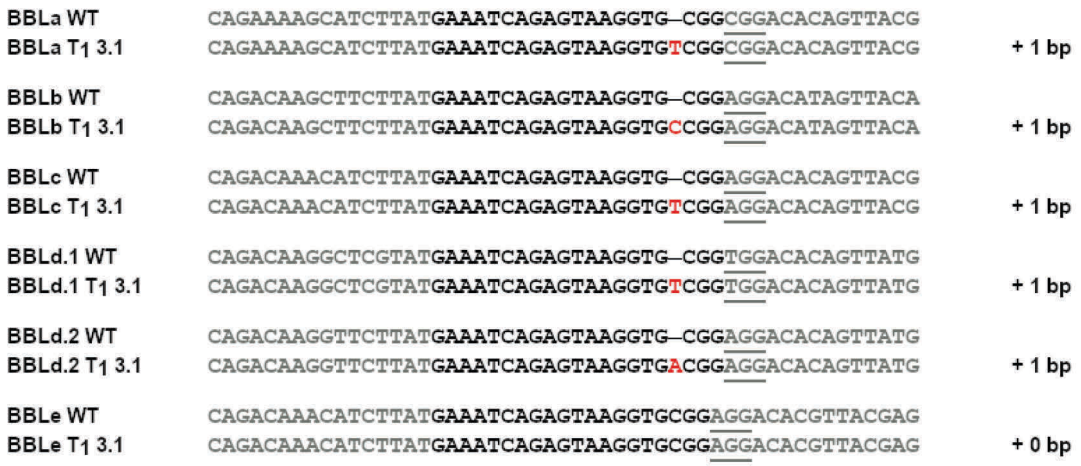

Figure 2-5 Analysis of plant T_1 3.1 on genomic level: "Fragments of all six *BBL* genes (*BBL*a – *BBL*e) of the wild type (WT) and plant T_1 3.1 were amplified and cloned into a vector by Gibson Assembly for sequencing." modified from Schachtsiek and Stehle (2019a)

The *BBL* genes *BBLa* to *BBLd.2* showed an insertion of one base pair three base pairs upstream of the PAM sequence, leading to a frame shift in the reading frame of each gene, resulting in a knockout (Figure 2-5). However, no mutation could be detected by sequencing the *BBLe* gene (Figure 2-5), which might be the reason for still detectable nicotine in this plant.

2.1.5. Nicotine-free tobacco plant

In order to obtain tobacco plants with a knockout in all six *BBL* genes the plant T_0 4 was chosen, since the nicotine content was already low in T_0 and T_1 plants (Figure 2-3). Therefore, the following generations up to the T_3 generation were analyzed according to their nicotine content. Although the nicotine content was not altered in the plant T_1 4.11 compared to the T_0 4 plant, a further reduction of the nicotine level was observed in T_2 plant 4.11.1 and plant T_3 4.11.1.2 (Figure 2-6).

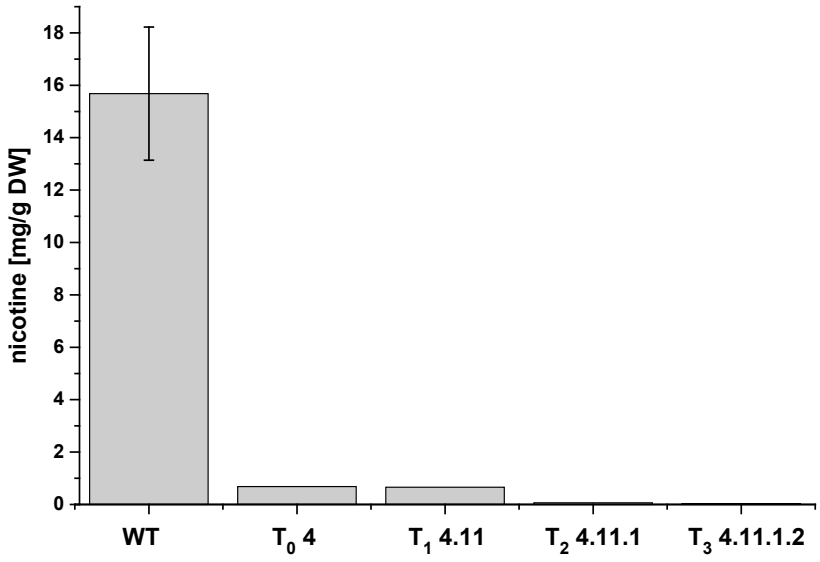

Figure 2-6 Comparison of nicotine content of plant 4 in all generations: "The amount of nicotine was quantified with GC-FID from 200 mg of dried and grounded leaf material of plants extracted with MTBE. Amount of nicotine was calculated as mg per gram dry weight (DW)." modified from Schachtsiek and Stehle (2019b)

The GC analysis of the nicotine content resulted in minimal peaks with the retention time of nicotine. Since the peaks were too small for an automated peak detection, a manual analysis of the peak area was performed. To ensure if the peak corresponds indeed to nicotine a GC-MS measurement of the T_3 plant 4.11.1.2 was performed and an *m/z* of 162.23 identical to nicotine could be detected but with a signal-to-noise ratio intensity of nearly 1:1 (Figure 2-7).

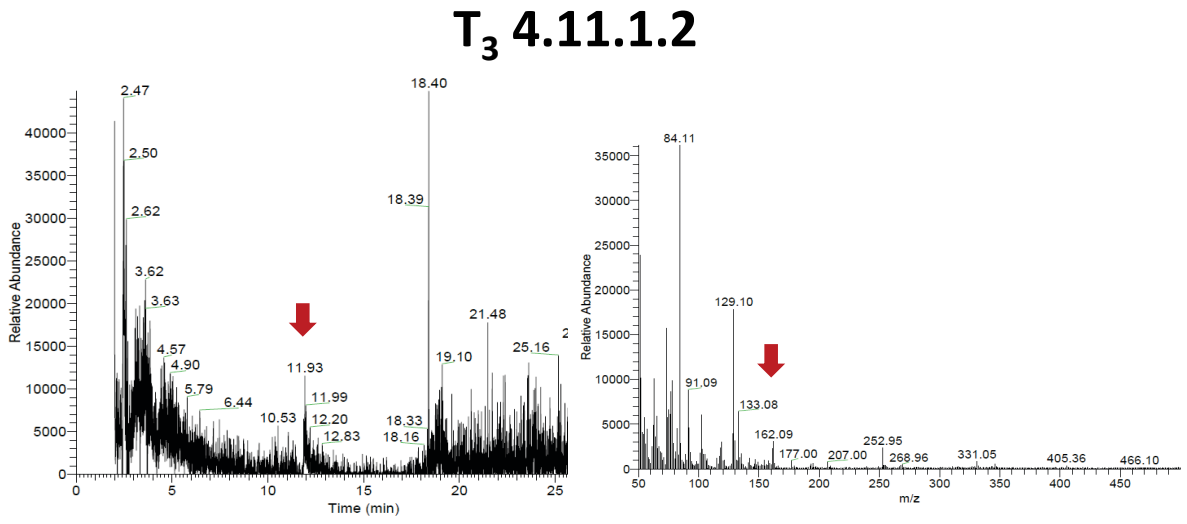

Figure 2-7 GC-MS analysis of plant extract of plant T_3 4.11.1.2 regarding the mass of nicotine: "Extract of plant T_3 4.11.1.2 was analyzed with GC-MS measurements by the analysis of peaks corresponding to the *m/z* of nicotine (162.23 g/mol). Extracted Ion chromatogram (EIC; left) of the mass of nicotine and the corresponding mass spectra is shown (right)." modified from Schachtsiek and Stehle (2019a)

Nevertheless, the manual fitted peak area of the GC-FID measurements was used to calculate the residual nicotine content. In comparison to the wild type, only 0.4 % of nicotine was detectable in the plant T_2 4.11.1 resulting in 0.06 mg g DW^{-1} of nicotine. The analysis of the T_3 plant 4.11.1.2 showed nearly similar results. The nicotine level in this plant was calculated as 0.04 mg g DW^{-1} which is equivalent to a reduction of 99.7 %. With these results, this plant was considered as nicotine-free.

In a last step, the plant was analyzed on genomic level by cloning the *BBL* fragments in the vector pDionysos (Stehle et al., 2008) to confirm the knockout of the *BBL* gene family. Different from the results of the genomic analysis of plant T_1 3.1 (section 2.1.4), it could be shown that all six *BBL* genes are knocked out in the nicotine-free plant (Figure 2-8 A and B). In all gene sequences a single nucleotide three base pairs upstream of the PAM sequence was inserted, which results in a frameshift, i.e. a knockout. However, for *BBLa* sequencing results showed either the insertion of the base guanine (G) or thymine (T), whereas sequencing results of the other *BBL* genes always showed the same base pair insertion (Figure 2-8 A). To confirm these results, fragments of the *BBL* genes were amplified with PCR from genomic DNA and send directly to sequencing without prior cloning into a vector. The previous results could be confirmed by analyzing the sequencing trace of the samples (Figure 2-8 B). Except from *BBLa*, the sequencing trace of the fragments showed a distinct signal peak for the appropriate base insertion. For *BBLa* the sequencing trace showed a double peak for thymine and guanine, which is consistent with the results from the cloning experiments, where both base pair insertions were found. The genomic sequences of the wild type genes were not detectable in any sample, which indicates that all copies of the genes were knocked out.

Due to mendelian segregation, it is possible to find plants, which do not carry the transformation cassette including the *bar* resistance, anymore. To prove if the nicotine-free plant is non-transgenic, leaf discs were cut out, surface sterilized and put on MS-medium with PPT for selection. As a control, a T_1 generation plant harboring the T-DNA cassette was used. After two weeks, leaf discs of the T_1 plant were still green and even started to grow bigger, whereas the leaf discs of the nicotine-free plant died (Figure S IV-1). This result was additionally confirmed with PCR by using primers, which bind inside the transformation cassette (Figure 2-8 C). As a positive control, genomic DNA from a T_0 plant was used. Neither for the wild type, nor for the tested nicotine-free plant a PCR product was obtained. Thus, the nicotine-free plant was declared as non-transgenic.

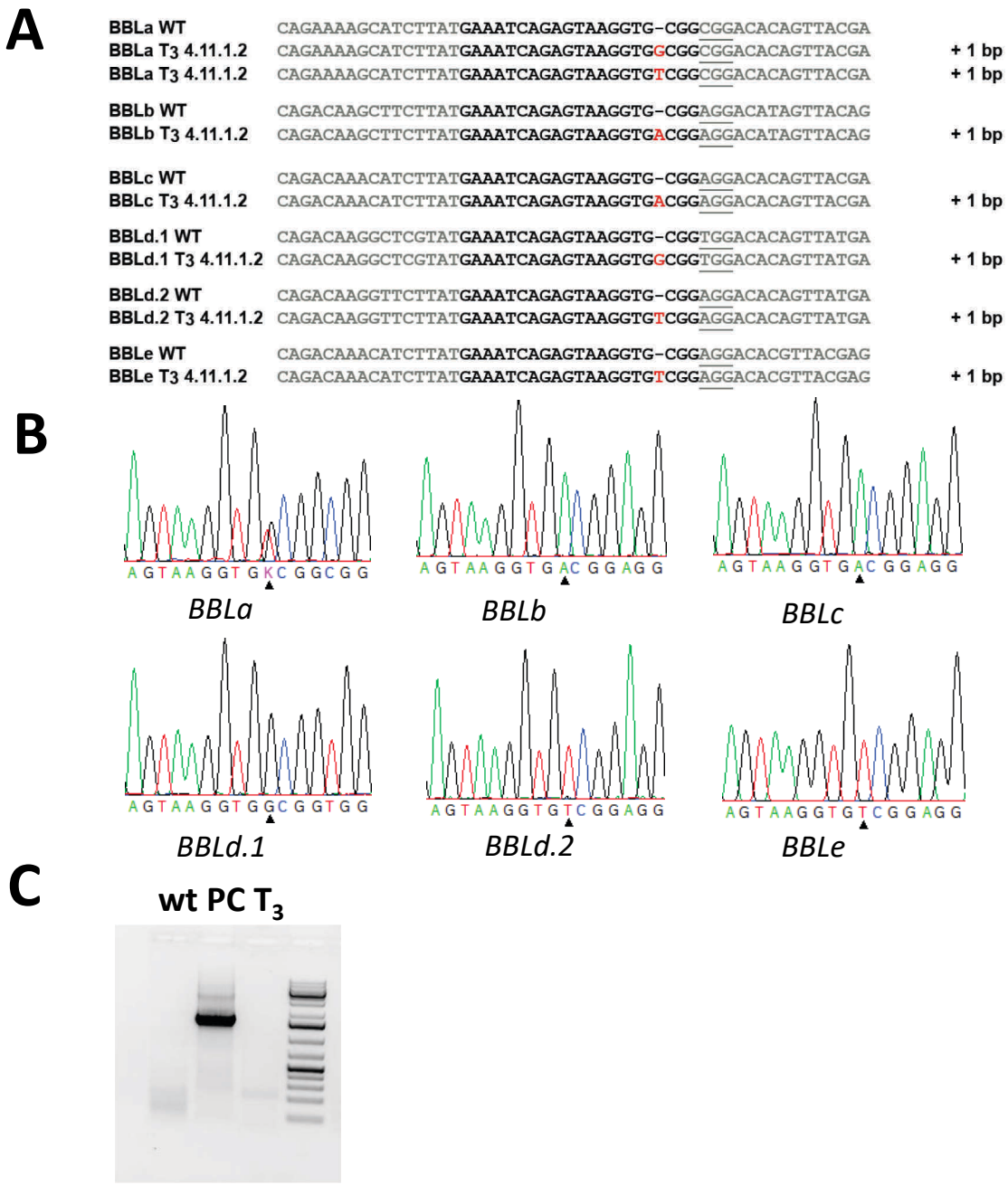

Figure 2-8 Genomic analysis of plant T_3 4.11.1.2 (A) "Genomic DNA was isolated from the plant T_3 4.11.1.2 and a wildtype plant for the amplification of fragments of the six *BBL* genes. The fragments were cloned into a vector for easier sequencing. One base pair insertion was observed in all six genes; for *BBLa* two different base pair insertions could be observed **(B)** sequencing of fragments of genomic DNA of plant T_3 4.11.1.2 without cloning into a vector to verify the results obtained from vector sequencing **(C)** amplification of the T-DNA cassette to test if plant T_3 4.11.1.2 (T_3) is still transgenic. As a positive control (PC) an T_0 plant was used, as a negative control the wild type (wt)." modified from Schachtsiek and Stehle (2019b)

2.1.6. Effect of the *BBL*-knockouts regarding phenotype, other alkaloids and primary metabolism

Since it could be proven that it was possible to create a nicotine-free plant by CRISPR-Cas9, which is non-transgenic as well, in a last step it was investigated if the knockout of the *BBL* gene family has any impact on the level of other alkaloids as well as the phenotype of the plant and the primary metabolism. The analysis of the GC-FID measurements showed, that in wild type extracts the alkaloids nornicotine and anabasine are present in a very low yield, indicated by no detectable peaks at the expected retention time. For anatabine a small peak was detectable. In comparison, the chromatogram of the nicotine-free plant showed no additional peaks related to anabasine or nornicotine as well as a reduced peak area of anatabine (Figure S IV-2). This led to the conclusion, that the knockout of the *BBL* genes had no negative impact on the alkaloid yield overall. Additionally, no changes in the phenotype were observed (Figure S IV-3). ^{1}H-NMR measurements were performed to evaluate changes in the primary metabolism. The wild type and the nicotine-free plant showed no substantial changes in the analyzed spectra (Figure S IV-4 and Figure S IV-5). Peaks between 3 and 5 ppm show a slightly lower peak area in the nicotine-free tobacco plant. Additionally, the comparison of the spectra verified the GC-MS data regarding the nicotine content (Figure S IV-4, Figure S IV-5 and Figure S IV-6).

2.1.7. Discussion

In recent years, the CRISPR-Cas9 method became the state-of-the-art technique for gene editing in several plant species. This approach demonstrates gene editing by using a single guide RNA target sequence to knockout a complete gene family of six genes, the *BBL* genes, responsible for the last oxidation step in the nicotine biosynthesis in *N. tabacum*, resulting in a nicotine-free tobacco plant. Since the genes from the *BBL* family of *N. tabacum* originated from *N. sylvestris* and *N. tomentosiformis,* in total 12 loci were knocked out simultaneously. Gene editing with CRISPR-Cas9 of polyploid plant species was already shown in polyploid oilseed rape and allohexaploid *Camelina sativa* (Braatz et al., 2017; Jiang et al., 2017) as well as different polyploid *Solanum tuberosum* species (Nadakuduti *et al.*, 2018). The overall results of the study show that the design of the gRNA using highly conserved regions within a gene family opens the possibility to knock out all members of the respective gene family. Certainly, to use this kind of approach a suitable gRNA target sequence is required that fulfills all known criteria (Liang et al. 2016), including stretches of homologous sequences within the targeted gene family. In case of the *BBL* gene family of *N. tabacum* just one sequence segment fulfilled all required criteria (Figure 2-1). The utilization of one or at least a few gRNAs opens the

possibility to knockout complete gene families in plants in a single step to elucidate the functions of the encoded proteins. A similar approach was done to generate low-gluten wheat by targeting the 33-mer peptide in the a-gliadin gene family responsible for coeliac, resulting in a reduced immunoreactivity by 85 % when 35 of 45 identified genes were knocked out (Sánchez-León et al., 2018). Using RNAi to knockdown gene expression often results in almost no clear effect, if gene families are targeted. Reasons are e.g. the overlapping and redundant activities of the respective proteins that diminish the reduction of transcript/protein levels. In contrast to classical mutant crossing approaches, to elucidate the function of gene families the use of a CRISPR-Cas9 approach demonstrates an easy and time saving alternative.

Nevertheless, the use of the CRISPR-Cas9 tool does not relieve from the necessity to screen several generated plant mutants to ensure that all loci are knocked-out. The knockout of the *BBL* gene family revealed a huge variation in the nicotine levels among the analyzed T_0 plants (Figure 2-3). Although the T-DNA integrates randomly, in theory it would be possible to obtain non-transgenic, nicotine free plants in T_1 generation. But since the nicotine content in T_0 plants was never under 4 % with regard to the wild type it was decided to select for transgenic T_1 plants, to prolong the time of *BBL*-gene editing. In the following T_1 and T_2 generations, a further decrease of the nicotine levels was observed. This indicates that not all six genes were knocked out immediately after integration of the T-DNA cassette. The knockout of all 12 loci was even not possible in T_1 generation at least for the analyzed plants. One should keep this in mind designing CRISPR-Cas9 mediated gene editing in tobacco. The knockout of single loci seems to be very efficient, but the knockout of larger gene families might take one or two generations.

To check if indeed all 12 loci were knocked out by the used method, a PCR based method was applied. The combination of cloning of the amplicons in a vector followed by Sanger sequencing and the direct sequencing of the PCR product provides a clear picture without the necessity to perform whole genome sequencing. This is only possible since the results show, that in all cases the same number of bases was inserted surprisingly three base pairs upstream of the PAM sequence. In contrast, in *Arabidopsis* and rice insertions or deletions of several base pairs were observed after non-homologous end joining (NHEJ) events (Jiang et al., 2013). Thus, variations in this position can easily be seen by looking at the trace of the Sanger sequencing results of the PCR products (Figure 2-8 B). This result was additionally verified by the cloned amplicons (Figure 2-8 A). This clearly shows, that the PCR-based method is suitable to detect any non-modified gene loci in tobacco.

The loss of the T-DNA cassette by self-crossing generates non-transgenic plants. Although they are designated as GMO plants in Europe by law (European Court of Justice, verdict C528/16),

they can serve as source plants to introgress this trait into commonly used smoking tobacco varieties to reduce the nicotine content outside of Europe. Many approaches addressing the reduction of the alkaloid yield in tobacco species by knockdown of genes involved in the alkaloid biosynthesis have been done. In several approaches a reduction in the nicotine level could be achieved, but as a side effect the level of other alkaloids, especially anatabine, were increased (Shoji and Hashimoto, 2008; Wang et al., 2009). In this approach, no increase of any alkaloid was detectable, which is in coincidence with studies regarding the knockdown of the *BBL* gene family (Kajikawa et al., 2011; Lewis et al., 2015). ^{1}H-NMR measurements indicate that the knockouts do not lead to severe changes in the primary metabolism of the plant, which is important due to the possible usage as host plant for synthetic biology studies or heterologous expression of partial or complete pathways.

2.2. Development of a regeneration and transformation protocol for *Cannabis sativa* L.

The plant *Cannabis sativa* is known for its psychoactive compounds, the cannabinoids, with high pharmaceutical potentials. For investigation of the effect of distinct or several combinations of cannabinoids it would be favorable to be able to genetically modify the plant. However, genetic modification of *C. sativa* L. is poorly understood because of the non-availability of protocols, which combine plant transformation and regeneration. Only a few protocols for either transformation or regeneration with low efficiencies are available (chapter 1.2.1.5). The goal of this part was to develop protocols for successful plant transformation and regeneration, which can be used for further studies.

2.2.1. Regeneration of *Cannabis* plants out of callus cultures

The basis of the development of a suitable plant regeneration protocol is the initiation of embryogenic callus cultures with the potential to develop shoots. Several parameters need to be tested, including combinations of different plant growth regulators (PGRs), the carbon source as well as additional substances like amino acids, which are known from other plant species for successful plant regeneration. Beside the regularly used Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) for plant tissue cultures, a modified version without ammonium nitrate (referred to as MS modified) was tested. However, some plant species require a lower concentration or the absence of macro nutrients like ammonium nitrate for effective plant regeneration (Decruse and Seeni, 2002; Ishioka and Tanimoto, 1990). For this reason, the modified version of the MS medium was considered for regeneration experiments. Furthermore, callus initiation was done with different plant tissues, like leaves, petioles and shoot tips.

2.2.1.1. Evaluation of different carbon sources for callus initiation

It is known that the carbon source, which is used in the plant cell culture media, has a great influence on the success of plant regeneration as well as the efficiency. Furthermore, they are necessary for developmental processes, like embryogenesis, shoot proliferation and root induction (Yaseen et al., 2013). However, many studies regarding callus induction or plant regeneration only use sucrose as carbon source without considering other sources. In a first step it should be evaluated which carbon source might be the most adequate for callus induction and regeneration of a plant. Beside sucrose, the sugars maltose and glucose were chosen. First

experiments showed that it is possible to induce callus cultures from petioles and leaves with the combination of benzylaminopurine (BA) and naphthaleneacetic acid (NAA). This combination of plant growth regulators was used to initiate callus cultures on MS medium with either maltose or sucrose as carbon source for a comparison regarding the growth and vitality of the callus cultures.

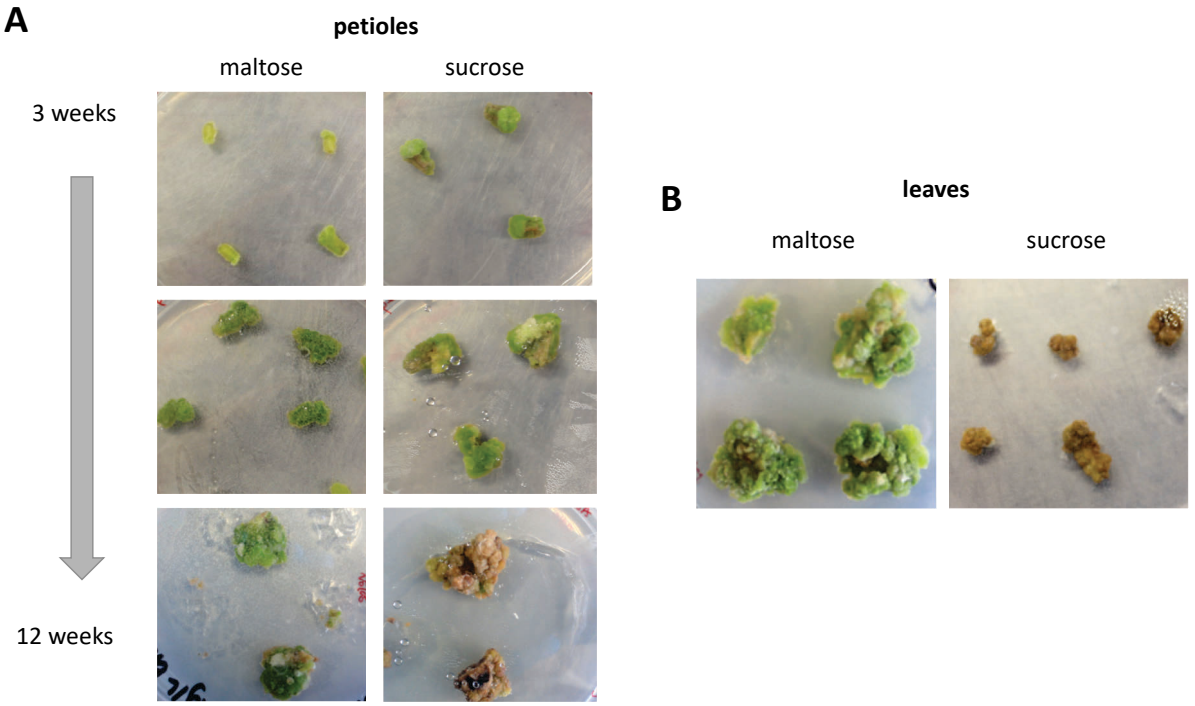

Figure 2-9 Comparison of the growth of *C. sativa* callus cultures in MS medium with maltose or sucrose as carbon sources: modified from [a]. petioles (A) and leaves (B) of *C. sativa* were surface sterilized and transferred to callus induction medium (1 mg L^{-1} BA and 0.2 mg L^{-1} NAA) either supplemented with maltose or sucrose as carbon source. The development of the callus cultures was analyzed over time.

Growth of callus cultures was studied over a period of twelve weeks. During this time callus cultures were sub-cultivated every three to four weeks to fresh medium. In the beginning of callus initiation from petioles, callus growth was faster in comparison to petioles incubated on medium with maltose. The same was noticed as well after six weeks of culture. Surprisingly, after twelve weeks of culture callus cultures grown on medium with maltose were still green and healthy whereas callus cultures on medium with sucrose started to die, indicated by the brown color (Figure 2-9 A). The same was observed for callus cultures initiated from leaves. After ten weeks of cultivation callus cultures grown on medium with maltose were still green and healthy whereas those, grown on medium with sucrose were small and almost no green parts were left (Figure 2-9 B).

In the beginning of callus initiation sucrose might be the better alternative because of the faster growth of the callus cultures, but if callus cultures are maintained for a longer time, maltose

would be a better choice. Thus, a combination of maltose and sucrose was tested and compared to the previous results (Figure 2-10).

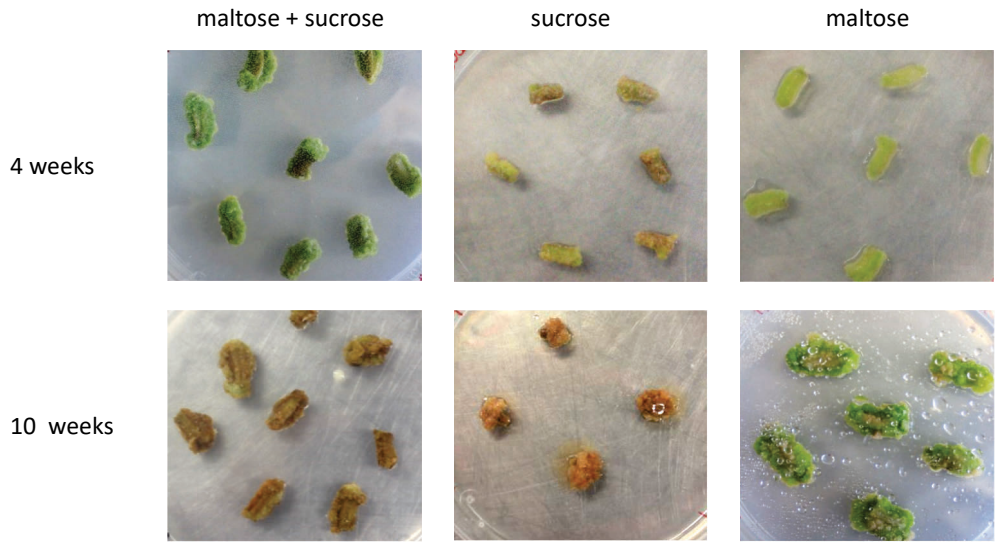

Figure 2-10 Comparison of the growth of *C. sativa* callus cultures in MS medium with different carbon sources: modified from [a]. Petioles of *C. sativa* were surface sterilized and transferred to callus induction medium (2 mg L^{-1} BA and 0.4 mg L^{-1} NAA) either supplemented with maltose or sucrose or a mixture of both as carbon source. The development of the callus cultures was analyzed over time.

After four weeks of culture, the callus cultures derived from petioles and incubated on medium with maltose as well as maltose and sucrose in a combination looked green and healthy whereas callus cultures grown on both sugars were bigger and had a darker green color. However, callus growth was less if sucrose was used as a carbon source and the explants started to die. After cultivation of ten weeks on medium with either both sugars or solely sucrose, explants and callus cultures were almost dead. In contrast to this, callus cultures incubated on maltose were still green and growth was not inhibited, leading to the assumption that maltose might be the best carbon source for callus growth.

Since incubation of calli on medium with maltose grow slowly, glucose should be tested as an alternative carbon source. Growth of callus cultures was analyzed after eight weeks. As shown in Figure 2-11 A calli derived from leaves grown on medium with glucose had a pale green color compared to calli grown on medium with maltose. Additionally, no clear differences according to the growth of the calli was visible. However, when using different plant growth regulators (PGRs) like kinetin and 2,4-D, in combination with glucose as carbon source, growth of callus cultures is strongly reduced and nearly inhibited (Figure 2-11 B).

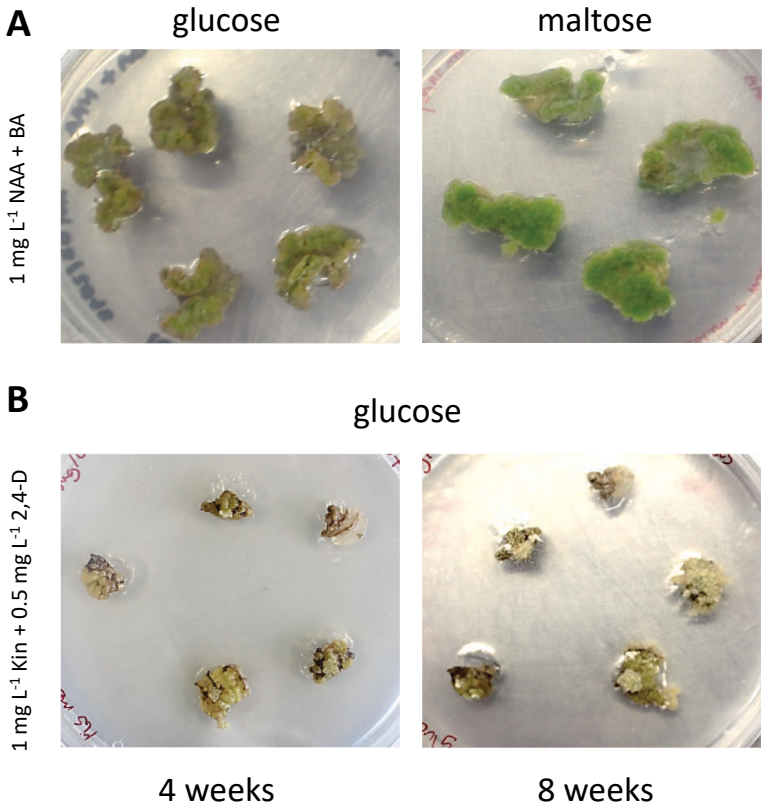

Figure 2-11 Comparison of the growth of *C. sativa* callus cultures in MS medium with glucose or maltose as carbon sources: (A) modified from [a]. Leaves of *C. sativa* were surface sterilized and transferred to callus induction medium (1 mg L^{-1} BA and 1 mg L^{-1} NAA) either supplemented with glucose or maltose as carbon source. Pictures were taken eight weeks after callus induction. **(B)** Callus cultures from leaves, four or eight weeks on callus induction medium (1 mg L^{-1} Kinetin and 0.5 mg L^{-1} 2,4-D) supplemented with glucose.

With consideration of all results regarding the carbon source, it can be concluded that maltose seems to be the best carbon source for green and healthy callus cultures for long time experiments, whereas calli grown on medium with sucrose can be used if fast callus growth is needed. Furthermore, the results indicate that the use of glucose as carbon source is not suitable for all callus induction conditions and is pending on the used plant growth regulators.

2.2.1.2. Callus initiation for plant regeneration

Callus growth is initiated by the application of PGRs to wounded plant tissue. In general, two main classes of PGRs are described: Cytokinins promote plant cell division and shoot elongations, whereas auxins are more involved in root initiation. Typically used cytokinins for callus induction are kinetin and BA alone or in combination with typically used auxins like 2,4-D or NAA. In some studies, also gibberellic acid (GA_3) or the synthetic hormone thidiazuron (TDZ) with a cytokinin-like effect are used to initiate callus and promote regeneration.

In the past some protocols for plant regeneration for *C. sativa* L. have already been published (Lata et al., 2010; Slusarkiewicz-Jarzina et al., 2005). In first experiments, these protocols were tested for successful callus initiation and regeneration of plantlets. Leaves and petioles were surface sterilized and transferred to the published media. However, none of the published conditions led to regeneration of shoots. Only callus cultures were initiated, as shown in Figure 2-12.

Figure 2-12 Callus cultures obtained from already published protocols for *C. sativa* regeneration: (A) Callus cultures after five weeks of cultures in MS medium with sucrose and 3 mg L^{-1} dicamba derived from petioles. **(B)** Six-week old callus cultures initiated from shoot tips incubated on MS medium with 3 mg L^{-1} dicamba. **(C)** Callus cultures on MS medium with maltose twelve weeks after callus initiation supplemented with 1 µM TDZ and 0.5 µM NAA, initiated from leaves

Petioles and shoot tips were used as explants incubated on MS medium with 3 mg L^{-1} of the synthetic auxin dicamba as described by Slusarkiewicz-Jarzina et al. (2005). After five to six weeks of culture, callus tissue developed at the wounding sites of the petioles (Figure 2-12 A) and green and healthy callus cultures were grown from shoot tips (Figure 2-12 B). Further incubations did not lead to the formation of shoots. If conditions published by Lata et al. (2010) were used, callus growth was initiated, but shooting could not be observed (Figure 2-12 C).

Since it was not possible to regenerate shoots with already published protocols, screening of new PGR combinations was necessary. For this purpose, the commonnly used PGRs kinetin, BA, NAA and 2,4-D were used. Experiments were done with leaves and petioles as explants and as carbon sources maltose as well as sucrose were used. Although maltose was designated as a better carbon source for callus development (section 2.2.1.1), sucrose was still applied in sceening experiments, because it could be shown that shoot initiation or the development of an embryogenic callus can be dependent on the carbon source (Yaseen et al., 2013).
At first BA and 2,4-D were used as PGRs alone between concenrations of 0.5 mg L^{-1} and 5 mg L^{-1} to initiate callus cultures. However, it was not possible to generate healthy callus

cultures. Surprisingly, after six weeks on MS medium with sucrose and 1 mg L^{-1} 2,4-D, formation of roots instead of shoots on the callus could be observed (Figure 2-13). This indicates, that it was possible to initiate meristem-like structures, the meristemoids, which are necessary for the development of plant organs, but roots were developed instead of the desired shoots.

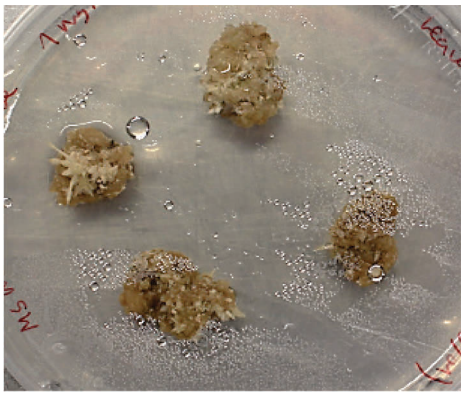

Figure 2-13 Formation of roots on callus cultures incubated on 2,4-D: Callus cultures derived from leaves of *C. sativa* L. show root formation after six weeks of culture in MS medium with 1 mg L^{-1} 2,4-D and sucrose.

Due to these results, callus induction was done with combinations of PGRs, using a cytokinin in combination with an auxin in different concentrations with the goal to find suitable conditions for shoot formation. At first, callus cultures were initiated with BA and NAA. This combination of PGRs was already used for the evaluation of the best carbon source and healthy and green callus cultures were obtained, with this combination. Table 2-1 shows the tested concentrations of BA and NAA and summarizes the results regarding callus formation with petioles and leaves as explants and their appearance in MS medium with maltose after ten weeks of culture. In general, callus induction was successful for all tested combinations, either resulting in medium quality or high quality calli. Best callus formation was obtained with concentrations of 1 or 2 mg L^{-1} BA and 0.2 mg L^{-1} NAA or 1 mg L^{-1} BA and NAA whereas the combination of 3 mg L^{-1} BA and 1 mg L^{-1} NAA led to just low amounts of callus tissue. Notably, callus cultures induced on MS medium with sucrose as carbon source did not survive that long or were of poor quality.

Table 2-1 Overview of the tested combinations and concentrations of BA and NAA and their impact on callus formation and appearance in MS medium with maltose: modified from [a]. Leaves and petioles were surface sterilized, wounded and transferred to callus induction medium with maltose. Quality of the callus were evaluated after ten weeks of culture. + low amount of callus/ slightly brown; ++ medium amount of callus/slightly brown; +++ big callus formation/healthy callus; / not tested

NAA / BA	0.1	0.2	0.4	1	2	3
1	++	+++	++	+++	/	++
2	++	+++	++	/	++	/
3	/	/	/	+/++	/	/
4	++	++	/	/	/	/
5	++	/	/	/	/	/

As a second combination of PGRs for callus induction and possible formation of shoots kinetin and 2,4-D were chosen. An overview of the tested concentrations and their impact on the callus formation is given in Table 2-2. All tested combinations led to formation of callus cultures, whereas most of them are of poor quality after ten weeks in culture. Healthy and green callus cultures were obtained with concentrations of 1 mg L^{-1} kinetin and 0.5 mg L^{-1} 2,4-D, as well as 1 mg L^{-1} kinetin and 2,4-D or 2 mg L^{-1} kinetin and 1 mg L^{-1} 2,4-D. High concentrations of either kinetin or 2,4-D led to poor callus growth.

Table 2-2 Overview of the tested combinations and concentrations of Kinetin and 2,4-D and their impact on callus formation and appearance in MS medium with maltose: modified from [a]. Leaves and petioles were surface sterilized, wounded and transferred to callus induction medium with maltose. Quality of the callus were evaluated after ten weeks of culture. + low amount of callus/slightly brown; ++ medium amount of callus/slightly brown; +++ big callus formation/healthy callus; / not tested

2,4-D / Kinetin	0.5	1	2	3	4	5
1	+++	++/+++	/	/	+	+
2	++	++/+++	/	++	+	+
4	+	+	+	+	++	+

As already observed for the combination of the PGRs BA and NAA, calli cultivated on MS medium supplemented with sucrose and the different combinations of kinetin and 2,4-D were

only poorly developed and died after almost approximately six weeks, indicated by a yellow/brown colour.

Regarding the overall goal to regenerate plants out of the callus cultures, calli derived from both tested PGR combinations with the bests results were compared according to their appearance. Whereas callus cultures incubated on MS medium with 1 mg L^{-1} BA and NAA looked normal (Figure 2-14 A), callus cultures induced on medium with 1 mg L^{-1} kinetin and 0.5 mg L^{-1} 2,4-D had a different appearance (Figure 2-14 B). Calli developed emerging structures, which were analysed in more detail with a binocular. Different types of structures were found. On the one hand, some part of the callus cultures looked like bubbles of different shapes, on the other hand some parts developed structures, which look somehow like the beginning of the development of plant organs e.g. roots or shoots, indicating a previous induction of a meristemoid callus.

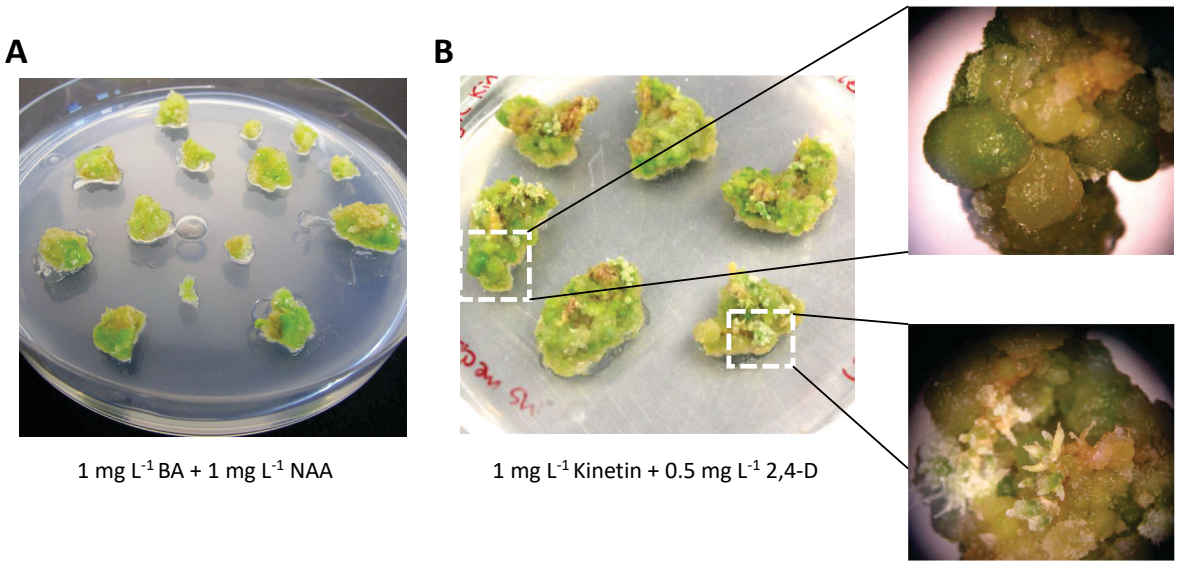

Figure 2-14 Comparison of best grown callus cultures regarding their appearance: in parts modified from [a]. Callus cultures were induced from leaves either by (A) NAA and BA or (B) kinetin and 2,4-D. Appearance was compared by analyzing newly built structures with a binocular.

However, no further development of the newly built structures was observed. Nevertheless, these findings demonstrate that it is indeed possible to initiate meristems and meristem-like structures on cannabis callus cultures. It also demonstrated that callus cultures induced with kinetin and 2,4-D can develop new, meristem-like structures on the surface, probably capable of differentiating into roots or preferably shoots.

The most promising combinations of the PGRs BA and NAA as well as kinetin and 2,4-D were additionally tested for callus and shoot induction with the MS modified medium, lacking ammonium nitrate, in maltose and sucrose. In contrast to the results from the full MS medium,

green and healthy callus cultures were obtained in MS modified medium supplemented with sucrose and 2 mg L^{-1} BA + 0.4 mg L^{-1} NAA and 1 mg L^{-1} BA + 0.1 mg L^{-1} NAA (Figure 2-15) after ten weeks of culture. In contrast to this, callus cultures incubated on MS modified medium with maltose as a carbon source supplemented with 2 mg L^{-1} kinetin and 0.5 mg L^{-1} 2,4-D developed very slowly in the same timeframe (Figure 2-15).

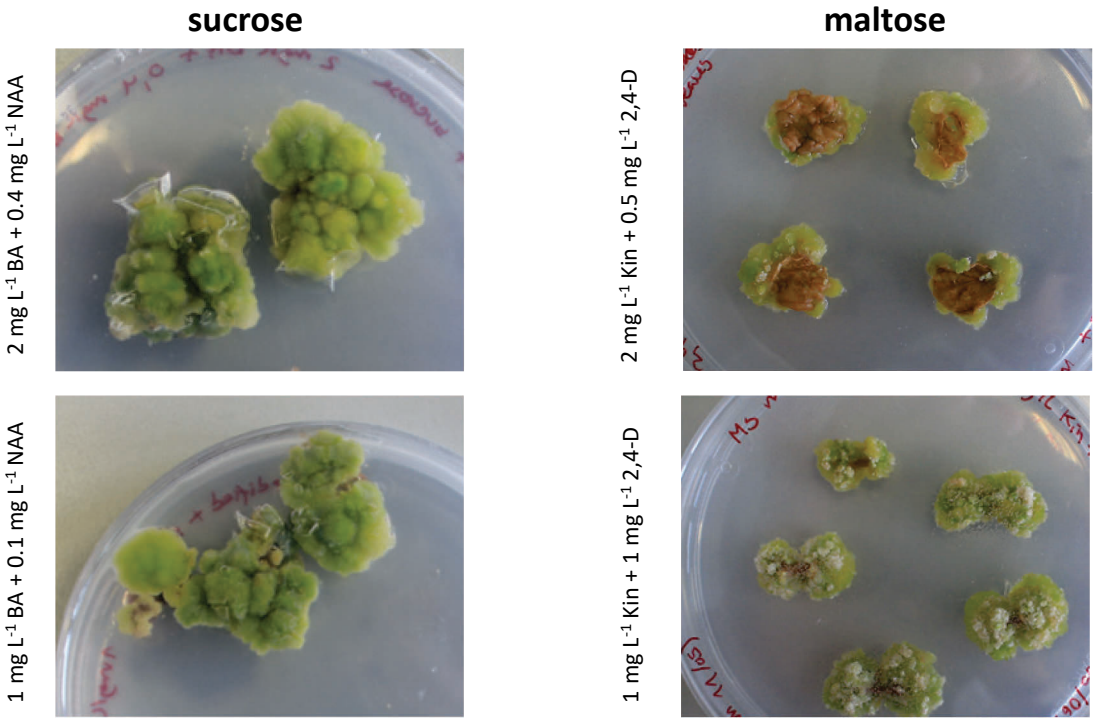

Figure 2-15 Callus cultures induced in MS modified Medium with sucrose or maltose as carbon source: modified from [a]. Leaves and petioles were surface sterilized and transferred to MS modified medium with either sucrose or maltose and different combinations of PGRs. Pictures were taken ten weeks after callus induction.

Interestingly, calli induced on MS modified medium with maltose and 1 mg L^{-1} kinetin and 2,4-D grew faster and developed emerging structures on the surface as already observed on callus cultures incubated on MS medium with maltose and 1 mg L^{-1} kinetin and 0.5 mg L^{-1} 2,4-D (Figure 2-14). Callus cultures with the previously mentioned combination of PGRs, showed emerging structures on the surface of the callus cultures in MS modified medium as well. The newly built structures were analyzed using a binocular (Figure 2-16). On the surface of callus cultures of both conditions, meristem-like structures were detected, which can develop into shoot or root primordia. Additionally, beginnings of roots were visible on calli grown on MS modified medium with 1 mg L^{-1} kinetin and 0.5 mg L^{-1} 2,4-D. A further development of the meristems into shoots was not obsesrved.

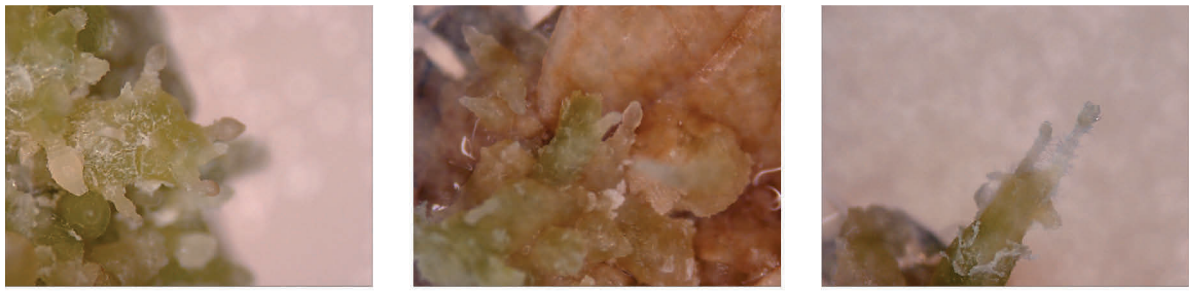

Figure 2-16 Meristem and root-like structures observed on callus cultures in MS modified medium: modified from [a]. Callus cultures induced and incubated on MS modified medium with maltose and the PGRs kinetin and 2,4-D developed new structures on the surface and were analyzed with a binocular.

2.2.1.3. Additional substances for shooting

Since no formation of shoots were observed in the conducted experiments with PGRs, the addition of substances known for the promotion of shoots in plant tissue culture were tested. Shooting is often promoted using silver nitrate, proline and glutamine as well as copper sulfate. The mentioned substances were tested on callus cultures initiated either on MS medium with BA and NAA or kinetin and 2,4-D. Beside leaves and petioles, shoot tips were used as explants, to test a different type of tissue for shoot initiation as well. The used substances and their concentrations are listed in Table 2-3.

Table 2-3 Concentrations of different substances for shoot initiation in callus cultures: Callus cultures were induced on MS medium with sucrose or maltose and either BA + NAA or kinetin + 2,4-D. Callus cultures were transferred to the same media with addition of the listed substances.

concentration [mg L^{-1}]			
$AgNO_3$	Proline	Glutamine	$CuSO_4$
2.5	50	50	2.5
5	100	100	5
	250	250	
	500	500	

With all tested combinations, no shoot formation was initiated, but some other observations were made, illustrated in Figure 2-17. If callus cultures were incubated on medium with 1 mg L^{-1} BA and NAA with addition of 250 mg L^{-1} proline, a friable callus was developed and some darker green spots were observed on the callus cultures. If callus cultures were transferred to media with the addition of either 2.5 or 5 mg L^{-1} silver nitrate root formation was initiated. Pending on the medium and silver nitrate concentration the root formation was more or less

distinct. The application of 2.5 mg L^{-1} silver nitrate lead to thin roots similar to hairy roots, whereas roots in medium with 5 mg L^{-1} silver nitrate appeared thicker. Callus cultures incubated on medium with 1 mg L^{-1} BA and 0.1 mg L^{-1} NAA with the addition of 5 mg L^{-1} silver nitrate even developed long and thick roots after four weeks of culture (Figure 2-17).

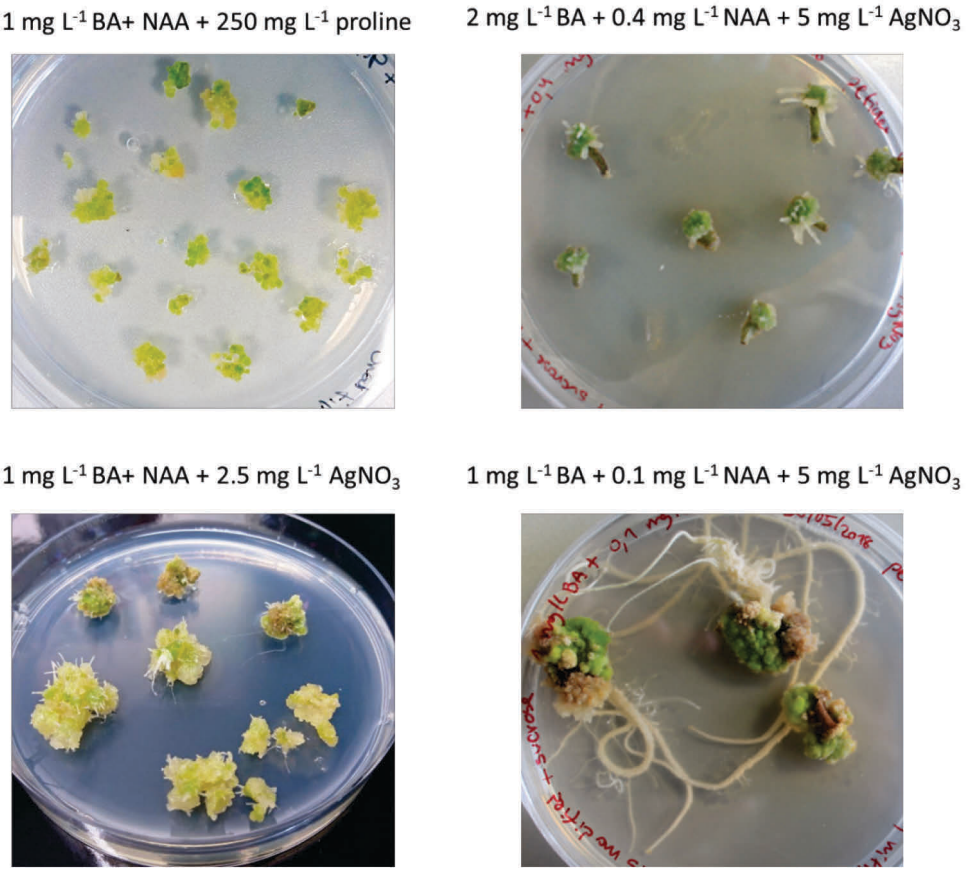

Figure 2-17 Callus cultures incubated on shoot induction media: in parts modified from [a]. Callus cultures were initiated from shoot tips or petioles and transferred to shoot induction media supplemented with proline or silver nitrate ($AgNO_3$).

Thus, it was not possible to initiate shoots from callus cultures within these experiments. Nevertheless, conditions for the growth of healthy callus cultures were found for further experiments. Additionally, it was possible to initiate meristems or meristem-like structures, which are necessary for further development of shoots, roots and other plant organs. This represents a promising approach and the basis for further studies, regarding the formation of shoots instead of roots from the meristem-like structures.

2.2.2. Direct organogenesis of *Cannabis* plants

Although several conditions for callus induction and shoot regeneration out of callus cultures were tested, so far it was not possible to regenerate *Cannabis* plants with this method. Therefore, it was necessary to think about other strategies to obtain regenerated plants for later planned transformation experiments. As already mentioned in chapter 1.2.1.4 the group of ElSohly published a one-step protocol for direct organogenesis of *C. sativa* plants (Lata et al., 2016). They used MS medium with solely meta-topolin (mT), an aromatic cytokinin as plant growth regulator. According to their protocol, the newly built shoots do not need to be transferred to rooting medium. Roots should be built directly from shoots in the same medium. For own experiments a modified version of the published protocol was used. In first trials, axillary buds from four to six-week-old *C. sativa* L. plants were harvested and surface sterilized. After wounding of the explants, they were placed on regeneration medium. Different to the published medium, no activated charcoal was used and the light intensity was set to normal *Cannabis* growing conditions (130 µmol m^{-2} s^{-1}) instead of 52 µmol m^{-2} s^{-1}.

Table 2-4 Tested conditions for shooting out of axillary buds with direct organogenesis: Explants were surface sterilized and transferred to MS medium with 3 % sucrose on agar plates with the listed conditions. Abbreviations: PGR (plant growth regulators), meta-topolin (mT), BA (benzylaminopurine), NAA (naphthaleneacetic acid)

Basic shooting condition	Additional PGR
MS medium + 0.5 µM mT	-
MS medium + 1.0 µM mT	-
MS medium + 1.5 µM mT	-
MS medium + 2 µM mT	0.5 mg L^{-1} BA; 1 mg L^{-1} BA
MS medium + 2 µM mT	1 mg L^{-1} NAA
MS medium + 2 µM mT	0.5 mg L^{-1} BA + 0.5 mg L^{-1} NAA
MS medium + 3 µM mT	-
MS medium + 3 µM mT	0.5 mg L^{-1} BA; 1 mg L^{-1} BA
MS medium + 3 µM mT	0.5 mg L^{-1} BA + 0.5 mg L^{-1} NAA
MS medium + 3 µM mT	1 mg L^{-1} BA + 0.5 mg L^{-1} NAA
MS medium + 4 µM mT	-
MS medium + 5 µM mT	-

Beside the published concentration of 2 µM meta-topolin other concentrations and different combinations with other phytohormones were tested for shoot induction (Table 2-4). Best

results for shooting out of axillary buds were obtained with 2 µM mT with the addition of 1 mg L^{-1} BA after one to two weeks of incubation on the media (Figure 2-18 A).

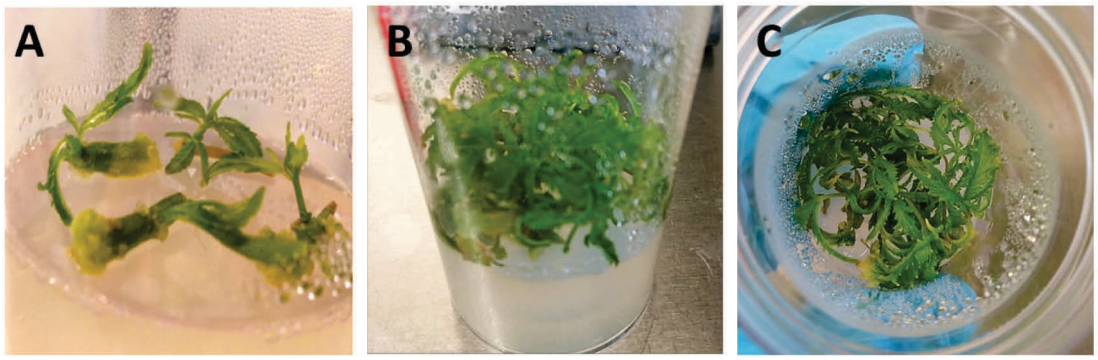

Figure 2-18 Direct organogenesis of shoots out of axillary buds: Axillary buds from *C. sativa* L. plants were cut off from four to six-week-old plants. After sterilization, they were wounded and incubated on MS medium with 2 µM meta-topolin and 1 mg L^{-1} BA. Shoots appeared after one to two weeks **(A).** After four to six weeks, shoots were multiplied and elongated **(B** and **C**) and big enough for rooting.

Shoots were grown out of the wounded shoot meristem at the axillary buds. When the shoots were further incubated for two to four weeks on the same medium shoots elongated and multiplied (Figure 2-18 B and C). With this size, the shoots were big enough for rooting.

Table 2-5 List of tested conditions for rooting of *Cannabis* shoots obtained from direct organogenesis: After four to six weeks, regenerated shoots were transferred to different rooting media. MS media always contained 3 % sucrose. Abbreviations: NAA (naphthaleneacetic acid), IAA (indole-3-acetic acid), IBA (indole-3-butryic acid)

Rooting condition	**Hormone concentration [mg L^{-1}]**
MS medium + NAA	0,2, 0.5, 1
MS medium + IAA	0.5, 1
MS medium + IBA	0.5, 1
½ MS medium + NAA	0.5, 1
½ MS medium + IAA	0.5, 1
½ MS medium + IBA	0,2, 0.5, 1

Auxins, like NAA, IAA and IBA are known to promote root initiation in tissue cultures (Gaspar et al., 1996). For rooting of the obtained shoots several concentrations of the known plant hormones for rooting were tested in either MS medium or ½ MS medium, which is often used to initiate rooting in plant tissue cultures (Table 2-5). Before the shoots were transferred to rooting media, a vertical cut was made in the stem, as it is done for cuttings of *C. sativa* L. as an additional trigger to initiate rooting.

Successful rooting was achieved in ½ MS medium with the addition of 0.5 mg L^{-1} IBA after approximately four to six weeks of incubation under normal growth conditions (Figure 2-19 A). As a second approach, elongated shoots were transferred to a cutting propagator normally used for vegetative propagation of *Cannabis* cuttings. Like the tissue culture approach, a little vertical cut was made to promote rooting. In the cutting propagator, the shoots were sprayed continuously with nutrition solution (chapter 4.2.2.3) under normal long day-conditions. After approximately ten days of incubation, first roots were visible. Further incubation in the cutting propagator led to the elongation and branching of the roots (Figure 2-19 B).

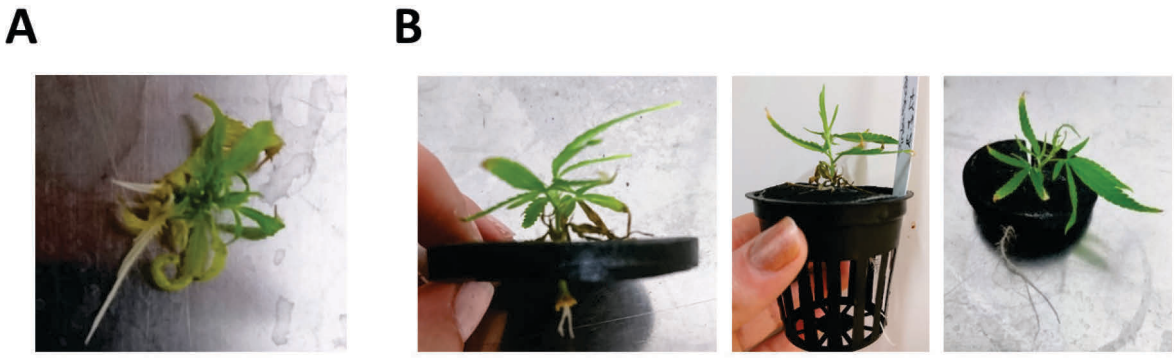

Figure 2-19 Rooting of micro propagated shoots: (A) Regenerated shoots from tissue culture were transferred to ½ MS medium with 3 % sucrose an 0.5 mg L^{-1} IBA. Shoots appeared four to six weeks after the transfer. **(B)** Regenerated shoots were transferred to a cutting propagator for rooting. Roots appear around ten days after transfer.

To be sure that the rooted plantlets can grow to a fully expanded plant, plantlets from both rooting approaches were transferred either from the tissue culture medium or from the cutting propagator to small pods for growing on hydroculture. As shown in Figure 2-20 rooted plantlets looked green and healthy. However, the plantlet from tissue culture (Figure 2-20 A; right) did not grow as fast as the plantlet from the cutting propagator (Figure 2-20 A; left). After transfer to bigger pots both plants developed almost equal, looked healthy and did not show any abnormalities (Figure 2-20 B).

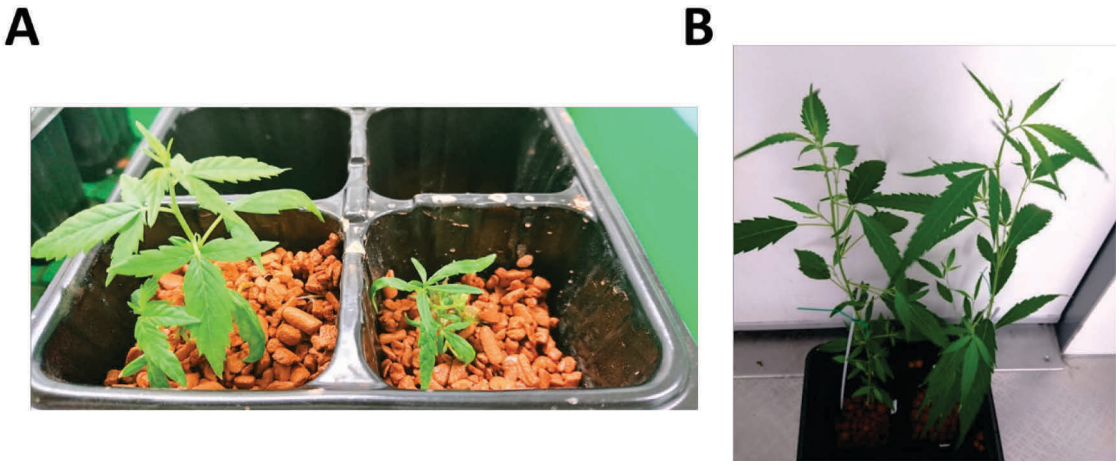

Figure 2-20 Further growing of the *C. sativa* plantlets obtained from direct organogenesis: (A) Plantlets were further grown on hydroculture **(B)** Expanded plants in medium size pots. Left plant: cutting propagator, right plant: tissue culture with 0.5 mg L^{-1} IBA

Thus, regeneration of *Cannabis* plants out of axillary buds with direct organogenesis was successful. Based on the established protocol, experimental studies concerning a transformation protocol with use of axillary buds as explants were conducted.

2.2.3. Development of an *Agrobacterium*-mediated transformation protocol

There are mainly two common approaches for the transformation of plant cells or plant tissues. One approach represents the biolistic transformation with a particle gun (Finer et al., 1992). Particles, like gold, are coated with the desired DNA and "shot" with high pressure into the plant cells. The other approach is the delivery of DNA into plants cells with the use of *A. tumefaciens*, leading to an integration of a T-DNA cassette randomly in the plant genome.
For the transformation of *Cannabis*, the latter method was chosen. On the one hand, some studies already showed successful transformation of *Cannabis* hypocotyls or callus cultures with *A. tumefaciens* (Feeney and Punja, 2003; Wahby et al., 2013) and on the other hand, a particle gun system would be much more expensive than the transformation with *A. tumefaciens*.

2.2.3.1. Susceptibility of *Cannabis* plants for transformation with *A. tumefaciens*

As already mentioned, it was already possible to transform *Cannabis* tissues with *A. tumefaciens* (Feeney and Punja, 2003; Wahby et al., 2013). However, in our department different *A. tumefaciens* strains were available. Thus, it was necessary to test whether it was possible to transform *Cannabis* plants with the available strains. For this purpose, one of the available *A. tumefaciens* strain (GV3101) was transformed with a well characterized binary

plasmid for GFP expression (pBIN-ER-GFP-Kan), targeted to the endoplasmic reticulum (ER) (Nelson et al., 2007). The plasmid consists of a T-DNA cassette with ER-*gfp5* under the control of a double 35S promotor and a *bar* resistance cassette.

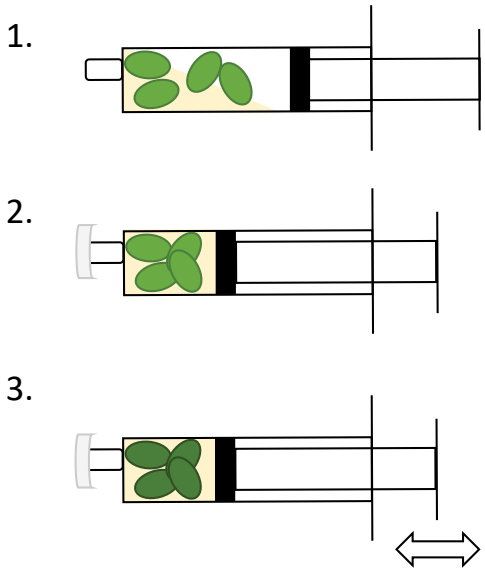

Figure 2-21 Syringe-based vacuum infiltration method of *A. tumefaciens*: Leaf discs were cut out from surface sterilized leaves and transferred to a syringe. Infiltration solution with *A. tumefaciens* was added (1.) and after removing the air, the syringe was sealed with parafilm (2.). Vacuum was generated by pulling the plunger and released rapidly and the leaf discs are soaked with the medium (3.).

Due to the structure of a normal *Cannabis* leaf with many leaf veins in small distances, it is not easy to infiltrate the leaves with a small syringe, as it is done for other plant species. As an alternative, an approach based on vacuum infiltration was tested according to an existing protocol for the infiltration of leaf discs (Matsuo et al., 2016). Leaf discs from surface sterilized leaves were cut out with a cork borer and transferred to a syringe, which was filled with the *Agrobacterium* solution. After removing the air completely, the syringe was sealed and vacuum was generated by pulling the plunger. Infiltration was done by releasing the plunger immediately. This was repeated until the whole leaf discs were soaked with the infiltration medium completely which can be seen by a darker green color (Figure 2-21).

For infiltration, the optical density of the bacteria was set to 0.1. After three days of incubation under normal growing conditions, expression of *gfp* was tested by fluorescence microscopy. Pictures were taken from the abaxial site of the leaves. As a control, leaf discs were transformed with the *A. tumefaciens* strain GV3101 without the plasmid.

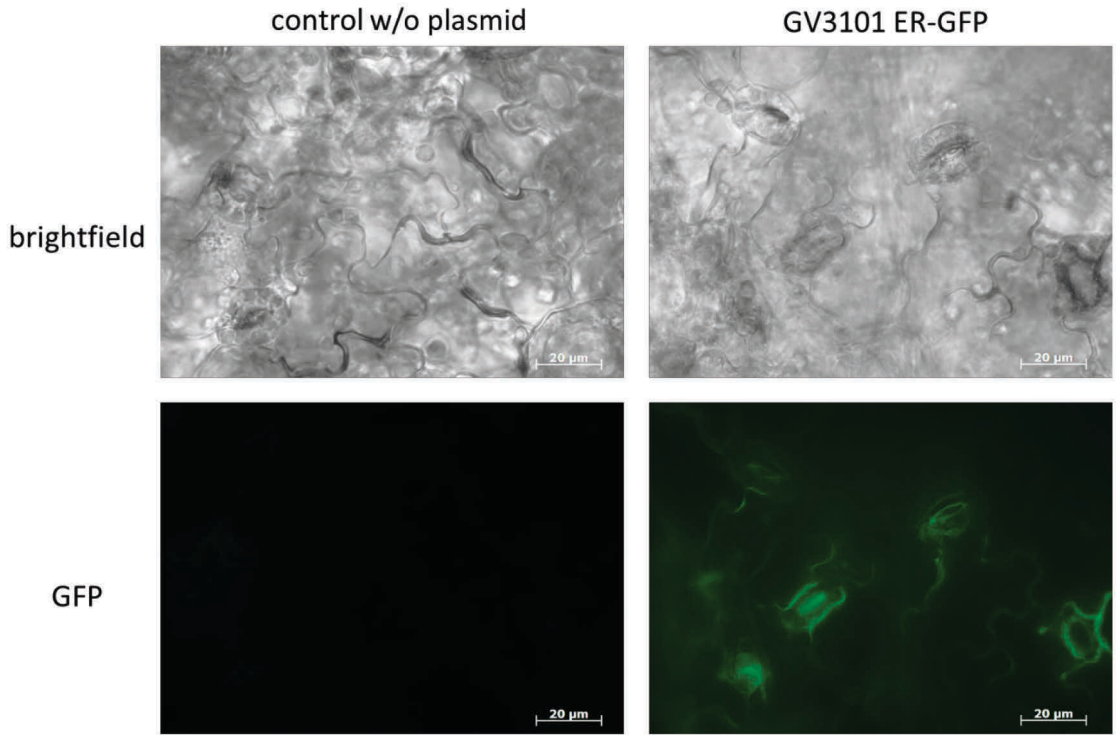

Figure 2-22 Transient transformation of *C. sativa* L. leaves with a vector expressing *gfp* targeted to the ER: Leaf discs of *C. sativa* L. were vacuum infiltrated with *A. tumefaciens* GV3101 carrying the plasmid pBIN-ER-GFP (Nelson et al., 2007) or without the plasmid as a control. Pictures were taken in the bright field and with a GFP-filter (GFP).

As shown in Figure 2-22, no green fluorescence signal for the control leaves, only infiltrated with GV3101 without a plasmid was detected as a background signal. In contrast to this fluorescence was detectable in leaf discs, which were infiltrated with *Agrobacteria* carrying the GFP-plasmid, especially in stomata and on the edges of the epidermal leaf cells. There results confirm the findings of the previous studies, that it is generally possible to transform *C. sativa* L. cells with *A. tumefaciens*. Furthermore, it was proven that the available strain GV3101 could be used for transformation experiments.

Notably, the detected fluorescence was not as strong as expected, with the regard to the used double 35S promotor, which represents a very strong promotor. With respect to the development of a transformation protocol it would be much easier to be able to detect green fluorescence e.g. with a portable UV lamp to screen fast and efficiently for positive transformants without using a fluorescence microscope. For this purpose, the expression of GFP should be targeted to a different cell organelle.

2.2.3.2. Construction of a binary plasmid for GFP expression targeting the vacuole

As already mentioned above, it would be favorable to obtain better green fluorescence signals from transformed *Cannabis* cells. As the ER represents a small part in plant cells, the idea was to enlarge the area of green fluorescence. This could be achieved by the exchange of the signal peptide for the ER by a signal peptide targeting the GFP to the vacuole, which represents a large organelle in plant cells. For targeting of GFP into the vacuole, the signal peptide of phaseolin, one of the major proteins of legumes, which accumulates in the storage vacuoles, was used. Studies showed that the four c-terminal amino acid residues (alanine-phenylalanine-valine-tyrosine; AFVY) are essential for sorting of proteins into the vacuolar lumen of the plant cell (Frigerio et al., 1998). If these four residues were missing fluorescence was only detected in the apoplast when RFP with the signal peptide was transformed (Hunter et al., 2007).

Due to these findings, a GFP expression vector was created with the n-terminal vacuolar signal peptide of phaseolin and the four residues AFVY at the c-terminus of GFP. The plasmid pFGC-Pt-GFP-Basta (Nelson et al., 2007) served as the backbone of the new plasmid. Beside the *bar* resistance cassette, the plasmid consisted of GFP with a signal peptide for the plastids which was cut out with the restriction enzymes *Nco*I and *Xba*I. The GFP with the vacuolar signal peptide of phaseolin (24 amino acids) and the c-terminal sequence were amplified by PCR using overhang primers with the pFGC-Pt-GFP-Basta plasmid as a template. The GFP fragment (Figure 2-23 A) was cloned into the cut vector backbone via Gibson Assembly. The final vector is shown in Figure 2-23 B.

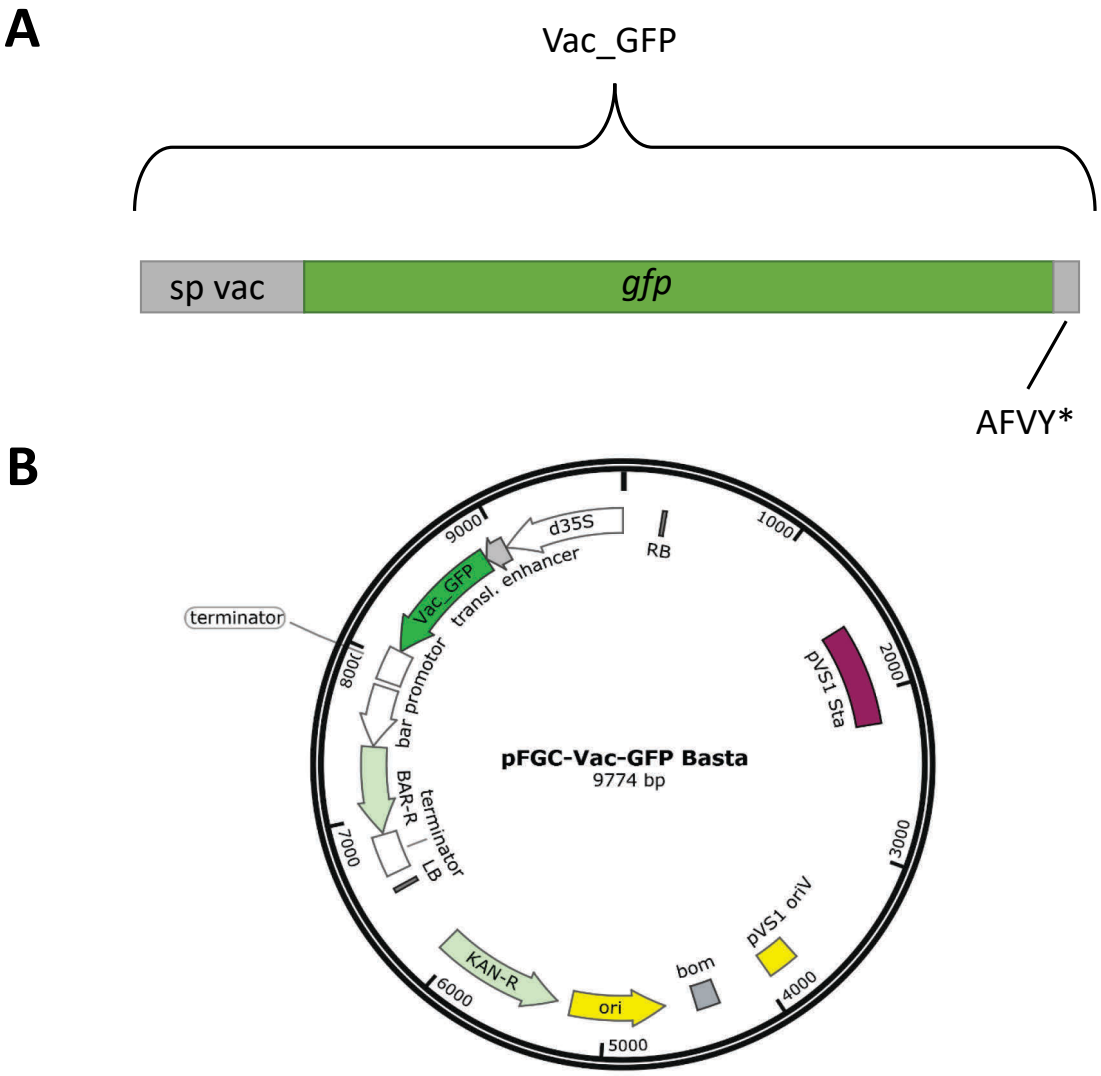

Figure 2-23 Fragment and vector used for the transformation experiments of *C. sativa* L. expressing *gfp* targeted to the vacuole: The signal peptide for targeting the plastids of the vector pFGC-Pt-GFP Basta was exchanged by a signal peptide targeting the vacuole **(A)**, resulting in the vector pFGC-Vac-GFP Basta **(B).** The star (*) represents the stop codon.

The constructed vector was transformed into the *A. tumefaciens* strains GV3101 and LBA4404 followed by the transformation of *C. sativa* L. leaf discs with the vacuum-based syringe method (Figure 2-21). Fluorescence microscopy was done after three days of incubation of the leaf discs under normal growth conditions. As shown in Figure 2-24 fluorescence was detected for both strains. However, if leaf discs were infiltrated with strain GV3101, GFP expression was only detected at the edges of the epidermal cells like it was shown with the ER-targeted GFP. In contrast to this, cells infiltrated with strain LBA4404 showed expression of GFP over the whole cells, like expected. Although the leaf discs were infiltrated nearly completely, fluorescence was not detected in every cell.

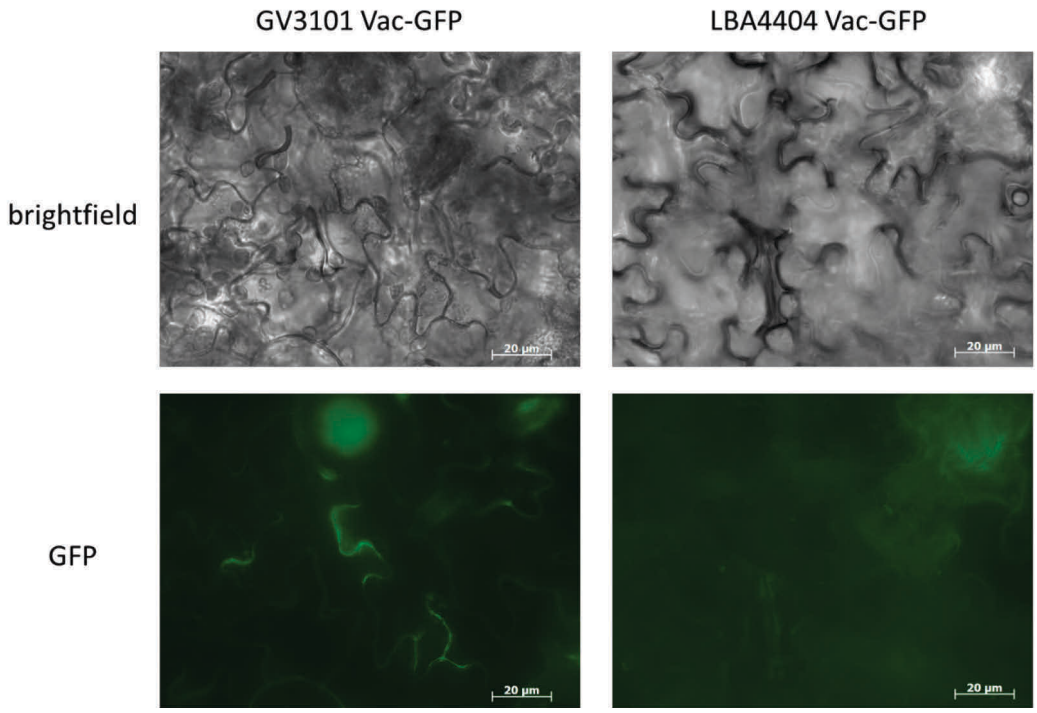

Figure 2-24 Transient transformation of *C. sativa* L. leaves with a vector expressing *gfp* targeted to the vacuole: Leaf discs of *C. sativa* were vacuum infiltrated with *A. tumefaciens* GV3101 and LBA4404 carrying the plasmid pFGC-Vac-GFP Basta. Pictures were taken in the bright field (BF) and with a GFP-filter (GFP).

Nevertheless, it was shown that transient transformation of leaf discs of *C. sativa* L. with a plasmid for targeting GFP-expression to the vacuole was successful for both used strains. According to these results, it was decided to use the generated plasmid with the vacuolar target signal for upcoming transformation experiments.

2.2.3.3. Development of a transformation protocol of *C. sativa* L. axillary buds

As already mentioned in chapter 1.2.1.5, except from two patent applications, no combined transformation and regeneration protocol for *C. sativa* L. was published until now. Based on the results of the vacuum-based transformation method for transient expression of GFP in *C. sativa* L. (section 2.2.3.1) and the developed direct organogenesis protocol (section 2.2.2) a protocol for stable transformation with the use of axillary buds as explants should be developed. In this protocol both methods should be combined and the following steps should be included:

1) Cultivation of *A. tumefaciens*
2) Infiltration of axillary buds
3) Co-cultivation of axillary buds with *A. tumefaciens*
4) Incubation on selection medium and direct organogenesis

In each of the listed steps, testing of different parameters and conditions were necessary. This included the optimal optical density for transformation, as well as an appropriate infiltration buffer and the used transformation method. Furthermore, the time for co-cultivation had to be optimized.

During the optimization process two different infiltration buffers were tested. One was already used for the transformation of *N. tabacum* (section 2.1; Sparkes *et al.*, 2006) and the other was used for the transformation of *A. thaliana* (Burch-Smith et al., 2006). In the following, they are denoted as infiltration buffer 1 and 2, respectively. Composition of the buffers are listed in section 4.2.2.8. Transformation of axillary buds was either done with the vacuum-based syringe method (Figure 2-21) or with the use of a desiccator to generate vacuum for infiltration. An overview of all tested parameters in each step are listed in Table 2-6.

Table 2-6 Overview of the different steps for the transformation protocol of *C. sativa* L. axillary buds and the parameters for optimization

Step in the protocol	Optimization parameters
Cultivation of *A. tumefaciens*	Optical density (OD_{600nm}) – 0.5, 1, 1.5, 2, 3
Infiltration of axillary buds	Infiltration buffer – Infiltration buffer 1 (Sparkes et al., 2006) – Infiltration buffer 2 (Burch-Smith et al., 2006) Infiltration method – syringe based (Matsuo et al., 2016) – desiccator
Co-cultivation of axillary buds and bacteria	Co-cultivation time – 1 day to 5 days Concentration of acetosyringone – 200, 300 µM Incubation in antibiotic solution after co-cultivation – Selection of antibiotic – Incubation time and concentration
Selection of transformants	Concentration of PPT – 3 and 6 mg L^{-1}

2.2.3.3.1. Preliminary experiments

Before first transformation experiments were done, a suitable concentration of PPT for the eventual selection of transgenic shoots was investigated. Axillary buds were incubated on MS medium supplemented with two different concentrations of PPT (3 mg L^{-1} and 6 mg L^{-1}). After one week of infiltration, explants incubated on both conditions were already dead (Figure S

V-1) suggesting that the lower concentration of 3 mg L^{-1} would be sufficient for selection. Therefore, 3 mg L^{-1} PPT was used for selection of transformants.

Beside the syringe-based infiltration method, which was already proven as a suitable transformation method for *C. sativa* L. leaves (section 2.2.3.1), a second infiltration method should be tested. It was decided to use a desiccator to create vacuum with a vacuum pump. With a slow release of the vacuum, it was expected that the explants will be soaked with the infiltration medium. To test some conditions for infiltration, small plants were used, because it is easier to see successful infiltration on leaves then on axillary buds. The plants were dipped upside down in a beaker filled with normal water (Figure 2-25) and transferred to a desiccator. Vacuum was created for one, two, three or five minutes, held for ten minutes and released slowly afterwards, resulting in infiltration of the leaves after three and five minutes of creating vacuum with the pump. Figure 2-25 shows successfully infiltrated leaves after creation of three minutes vacuum, indicated by a darker green color of the leaves. Since vacuum-treatment results in stress for the plant or explants it was decided to create the vacuum for three minutes, held for 10 minutes, before slow release of the vacuum for further transformation experiments with axillary buds

Figure 2-25 Vacuum infiltration of *C. sativa* L. leaves with a desiccator: To test the best infiltration conditions with a desiccator, small plants were placed upside down in a beaker filled with water (left). Vacuum was created for different timeframes to find the best conditions. Vacuum was held for ten minutes and released afterwards. Infiltration of the leaves is indicated by a darker green colour (right).

Furthermore, it was necessary to find suitable conditions for elimination of the *Agrobacteria* after co-cultivation with antibiotics. For this purpose, first transformation experiments were done with an optical density (OD_{600nm}) of 0.5, 0.7 or 1 in infiltration buffer 1 with the syringe method and co-cultivated for one to four days. Afterwards the explants were washed with water and transferred to an antibiotic solution.

At first a combination of timentin (150 mg L^{-1}) and carbenicillin (300 mg L^{-1}) was tested. Explants were incubated with continuous shaking on a rocker for four hours and transferred to shooting medium, supplemented with the same concentrations of antibiotics, afterwards. After incubation of one to two weeks, growth of the bacteria was observed. Therefore, the incubation time with the antibiotic solution was increased to six or seven hours. On the one hand, bacterial growth was inhibited, but on the other hand, explants died very fast and no shooting was observed. As an alternative to timentin and carbenicillin, the antibiotic cefotaxime was tested with a concentration of 500 mg L^{-1} and incubation times of three to four hours. After transfer to shooting medium supplemented with cefotaxime, no growth of bacteria was visible within two weeks. Thus, it was decided to use 500 mg L^{-1} cefotaxime to eliminate the bacteria with an incubation time of three to four hours.

First transformation experiments of axillary buds were done either with the syringe-based method or with the desiccator. Optical densities between 0.3 and 1 were used and different co-cultivation times between one and five days were tested. Although bacterial growth on transformed explants was eliminated with the optimized washing conditions, plant regeneration was not successful at all. Only a few shoots were growing from axillary buds and the rest of the explants died (Figure S V-2). After cutting off the shoots from the explants and the transfer to new selection medium, they died or did not grow further. It was suggested, that the harsh treatment of vacuum infiltration and washing with antibiotics in combination with selection pressure resulted in too much stress for the explants and growing of shoots was not possible anymore or very slow. Because the main goal of this study was the development of a transformation protocol, it was decided to exclude the selection with PPT for further optimizations of the protocol. As shown in chapter 2.2.3.2, the fluorescence in transformed leaves with the *Agrobacterium* strains GV3101 and LBA4404 was not present in every cell and was not very strong. According to these results, the upcoming optimizations should be done with the *A. tumefaciens* strain AGL1, which is described as hypervirulent (Lazo et al., 1991), for enhancing the chance of successful transformation.

2.2.3.3.2. Optimization of transformation conditions

In a new approach, conditions for transformation of axillary buds should be optimized regarding the cell density for transformation, the infiltration buffer as well as the infiltration method and the co-cultivation time.

For first optimizations of the transformation conditions, the cells were diluted to optical densities (OD_{600nm}) of 0.5, 1, 1.5 and 2 in infiltration buffer 1, respectively. Axillary Buds were

harvested, surface sterilized and infiltrated with the syringe-based method. After infiltration, the explants were transferred to agar plates for co-cultivation with the *Agrobacteria.* The co-cultivation medium consisted of MS medium with 3 % sucrose, 2 µM mT and 1 mg L^{-1} BA as well as 200 µM acetosyringone. Co-cultivation was done for one to five days in the dark. Taken together, in this first approach 25 different conditions were tested. As expected, the growth of *Agrobacteria* increased with the time of co-cultivation.

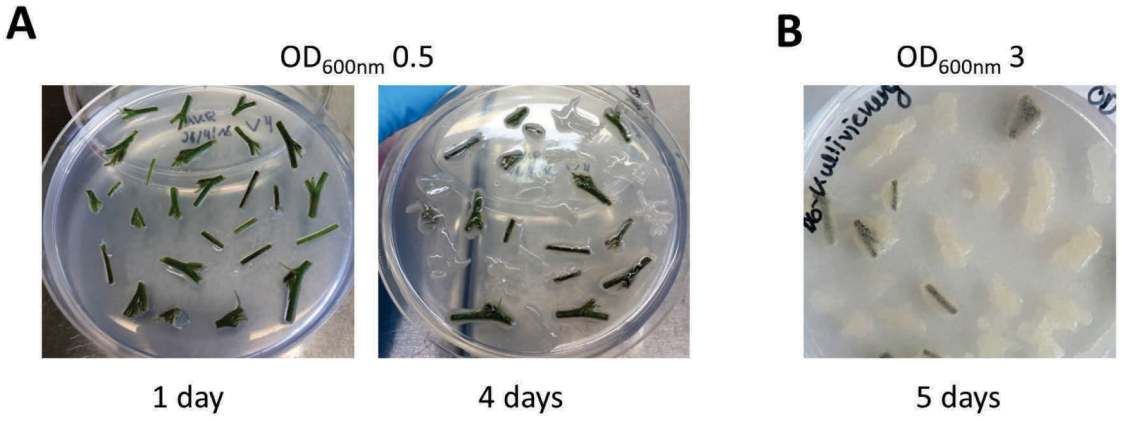

Figure 2-26 Transformed axillary buds after different co-cultivation times: (A) in parts modified from [b]. Axillary buds after co-cultivation for one or four days transformed with Agrobacteria with an $OD_{600\ nm}$ of 0.5. **(B)** axillary buds after co-cultivation for five days transformed with Agrobacteria with an $OD_{600\ nm}$ of 3.

Figure 2-26 A shows exemplarily explants transformed with an OD of 0.5 after one day of co-cultivation compared to four days of co-cultivation. After one day, nearly no growth of bacteria was visible and the explants were still healthy and green. In contrast to this, explants were overgrown with bacteria after four days of co-cultivation and the color changed to dark green. Figure 2-26 B shows explants after transformation with OD 3 after five days of co-cultivation. Explants were completely overgrown and did not look healthy anymore. After co-cultivation, explants were transferred to shooting medium with cefotaxime for regeneration of shoots.

No regeneration of shoots was obtained from explants co-cultivated for four or five days for all tested conditions. Except from axillary buds transformed with OD 3, highest regeneration efficiencies of 30 % to 40 % could be achieved after one day of co-cultivation. If transformed axillary buds were co-cultivated for two days, the highest transformation efficiency was obtained with an OD of 0.5 of about 25 %. Except from a shooting efficiency of 30 %, if explants were transformed with OD 1, co-cultivation for three days resulted in shooting efficiencies between 5 % and 17 %. Shooting efficiencies are summed up in Figure 2-27. No shooting was observed for explants transformed with OD 2 and 3 and a co-cultivation time for three days.

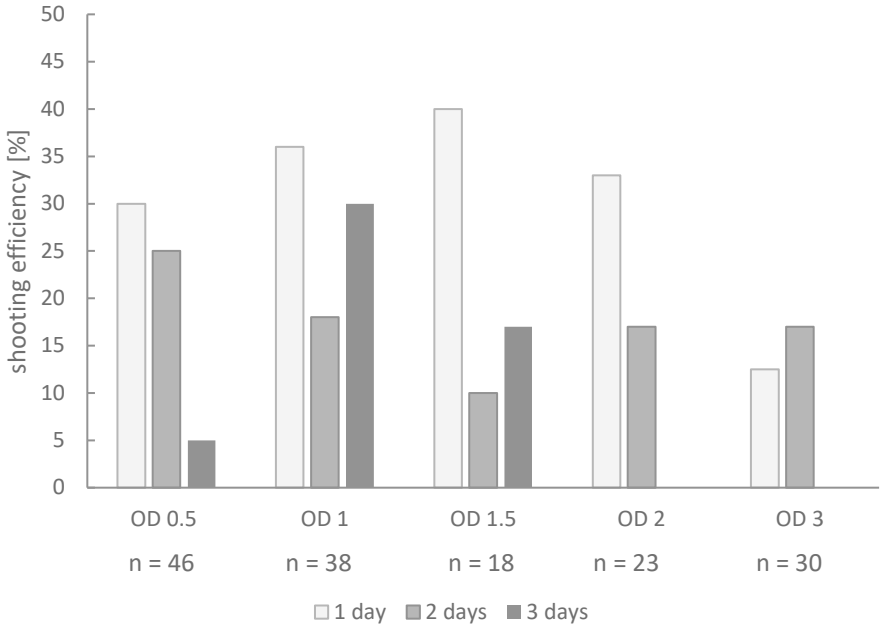

Figure 2-27 Shooting efficiencies for the different optical densities and co-cultivation times (syringe-based method): Axillary buds of *C. sativa* L. were surface sterilized and transformed with different concentrations of *Agrobacteria* (0.5- 3). Co-cultivation was done in the dark for one to five days. Only results from co-cultivation of one day to three days are shown.

Regenerated shoots were transferred to bigger cultivation jars for further growth. During the incubation time, reinfection of some shoots with *Agrobacteria* was observed, as shown exemplarily in Figure S V-3. These shoots were washed again in a cefotaxime solution and transferred afterwards to new medium. To avoid reinfection again, the shooting medium was supplemented with cefotaxime in a concentration of 100 mg L^{-1}. Since the additional washing step means again stress to the newly developed shoots, it was decided to keep cefotaxime as a permanent feature in the shooting medium after transfer to bigger jars in a lower concentration of 100 mg L^{-1}.

As a next step, the second chosen infiltration method with a desiccator should be tested in combination with the other selected infiltration buffer 2. Like the syringe-based approach, optical densities (OD_{600nm}) from 0.5 to 3were tested with co-cultivation times from one day to five days, resulting in 25 different conditions. Axillary buds were harvested, surface sterilized and transferred to falcon tubes filled with the *Agrobacterium* solution. Treatment with vacuum was done as described above (chapter 2.2.3.3.1) and explants were transferred to co-cultivation medium. After co-cultivation, the explants were washed with the antibiotic solution and transferred to shooting medium.

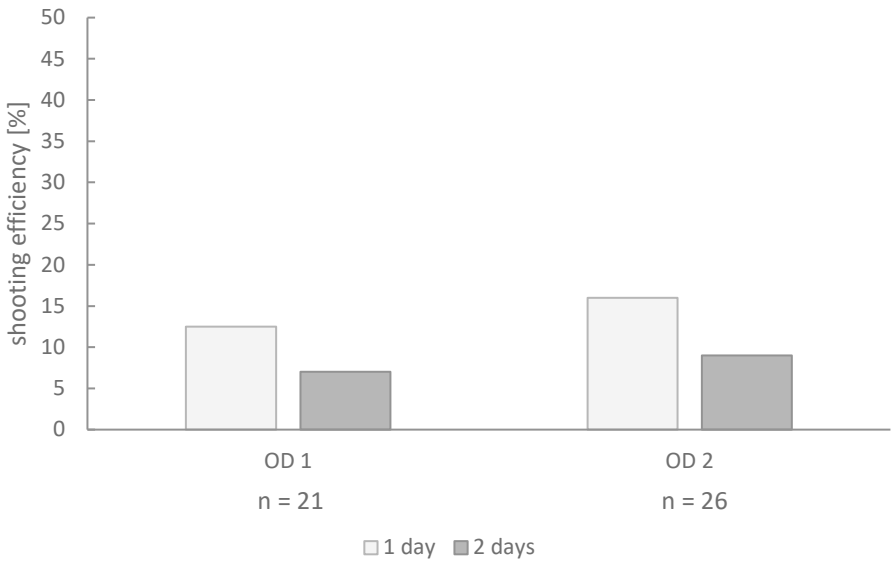

Figure 2-28 Shooting efficiencies of the vacuum-based infiltration method with a desiccator: Axillary buds of *C. sativa* L. were surface sterilized and transformed with different concentrations of *Agrobacteria* (0.5- 3). Co-cultivation was done in the dark for one to five days. Only results of conditions with successful shooting are shown.

As expected, no shoots were grown from explants co-cultivated for four or five days. However, results for shooting efficiencies differ from the results obtained with the syringe. Beside from explants transformed with OD 1.0 and 2.0 and co-cultivated for one or two days, no shooting was observed at all. Additionally, the shooting efficiencies were not as high compared to the syringe-based method. Shoots could only be regenerated with efficiencies between 7 % and 16 % (Figure 2-28).

Regarding the low shooting efficiencies for only a few of the tested conditions, the syringe-based method represents the better alternative for infiltration of axillary buds than the usage of a desiccator for infiltration.

2.2.3.3.3. Detection of GFP fluorescence in regenerated shoots

Although, results indicate that the syringe-based infiltration method was more efficient than using a desiccator for the infiltration of axillary buds, it should be evaluated, if obtained shoots from both approaches are transgenic or not. The shoots were tested for GFP expression, indicated by green-fluorescence. A small leaf was harvested from a regenerated shoot and analyzed with a fluorescence microscope. A regenerated shoot, which was not transformed with *Agrobacteria*, was used as a control.

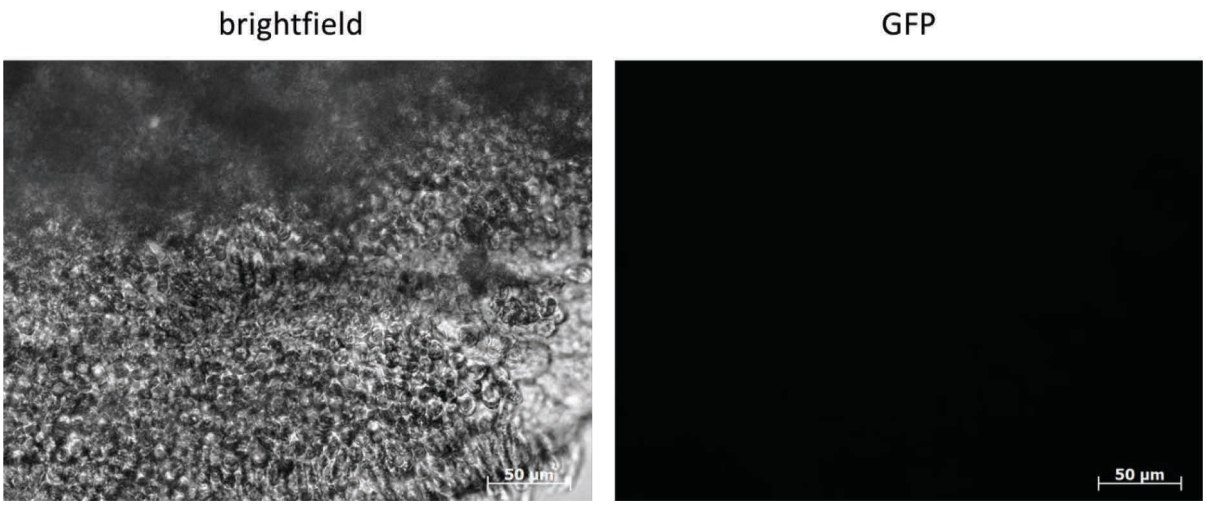

Figure 2-29 Microscopic picture of a regenerated wild type *C. sativa* L. leaf: shoots were regenerated from *C. sativa* L. axillary buds and a leaf was harvested for microscopic analysis. The brightfield and GFP channel are shown separately.

As shown in Figure 2-29 no fluorescence was detectable in regenerated wild type plants. Therefore, it should be possible to clearly distinguish between non-transformed and transformed plants.

Leaves infiltrated either with the syringe-based method or with the desiccator-based method were compared. As an example, microscopic pictures for explants infiltrated with OD_{600nm} of 1.0 and a co-cultivation time are described (Figure 2-30). For both tested leaves from regenerated shoots, green fluorescence was observed (Figure 2-30). However, in all analyzed samples only spots of green fluorescence were detected, especially in areas around the stomata of the leaves. Although the GFP expression was not detectable in all leaf cells, the results clearly show a successful transformation of axillary buds with both used methods resulting in chimeric plants.

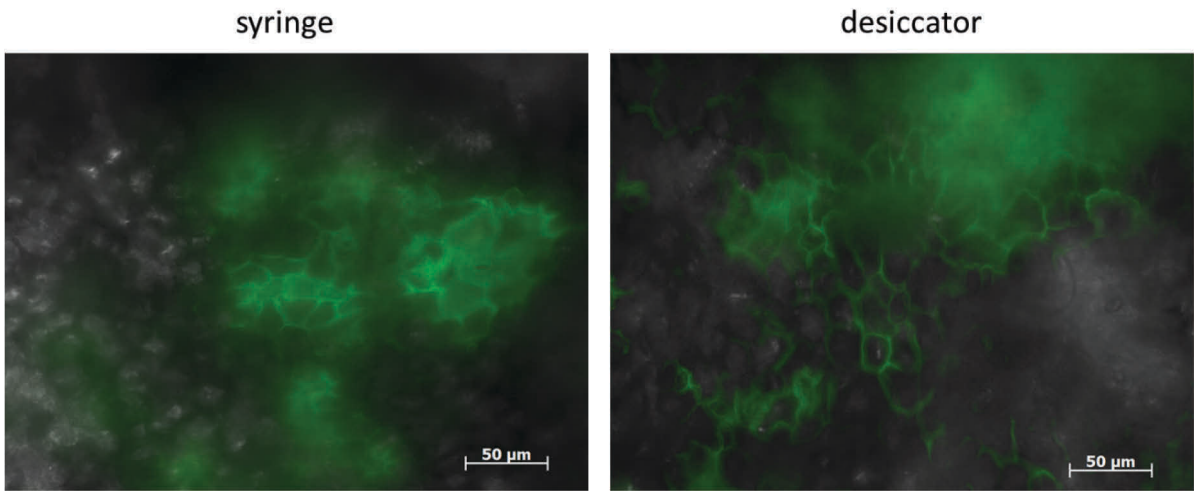

Figure 2-30 Fluorescence microscopy pictures of transformed and regenerated *C. sativa* L. plants: modified from [b]. Axillary buds of *C. sativa L.* were transformed the plasmid pFGC-Vac-GFP Basta either with the syringe-based method or with vacuum generated in a desiccator. Fluorescence pictures were taken from leaves of regenerated shoots. Pictures show the overlay of the brightfield and the GFP channel.

2.2.3.3.4. The effect of acetosyringone on the transformation efficiency

First results showed indeed that the used transformation methods of axillary buds in general can be used to generate transgenic *Cannabis* plants. Nevertheless, it was only possible to create chimeric plants. Thus, it was necessary to optimize the protocol regarding a higher number of transformed cells in the regenerated shoots. For this purpose, the acetosyringone concentration in the co-cultivation medium was increased to 300 µM instead of 200 µM. Moreover, acetosyringone (20 µM) and MES (10 mM), as described by Burch-Smith et al. (Burch-Smith et al., 2006) were added to the medium used for cultivation of *Agrobacteria*. Acetosyringone is known for activation of the *vir* genes, which are necessary for the activation of the T-DNA transfer (Stachel et al., 1985), leading to the hypothesis that a higher acetosyringone concentration leads to an increase of the transformation efficiency resulting in a higher number of transformed cells in the regenerated shoots.

Regarding the previous results from the optimization experiments, the syringe-based method in combination with infiltration buffer 1 was used, because of the better shooting efficiencies. Optical densities between OD_{600nm} 0.5 and 2 were used and co-cultivation was done for one or two days in co-cultivation medium with 300 µM acetosyringone in the dark. After around four weeks, first shoots were analyzed with a fluorescence microscope. Figure 2-31 exemplarily shows the microscopic pictures from a leaf harvested from a shoot transformed with OD_{600nm} of 0.5 and a co-cultivation time of one day. In comparison to the axillary buds co-cultivated on medium with 200 µM acetosyringone, GFP expression was detected in a broad area of the

leaves from regenerated plants, indicating a higher transformation efficiency when using higher acetosyringone concentrations. These results indicate as well, that low concentrations of *Agrobacteria*, e.g. OD_{600nm} 0.5 or 1, are sufficient for successful transformation of axillary buds.

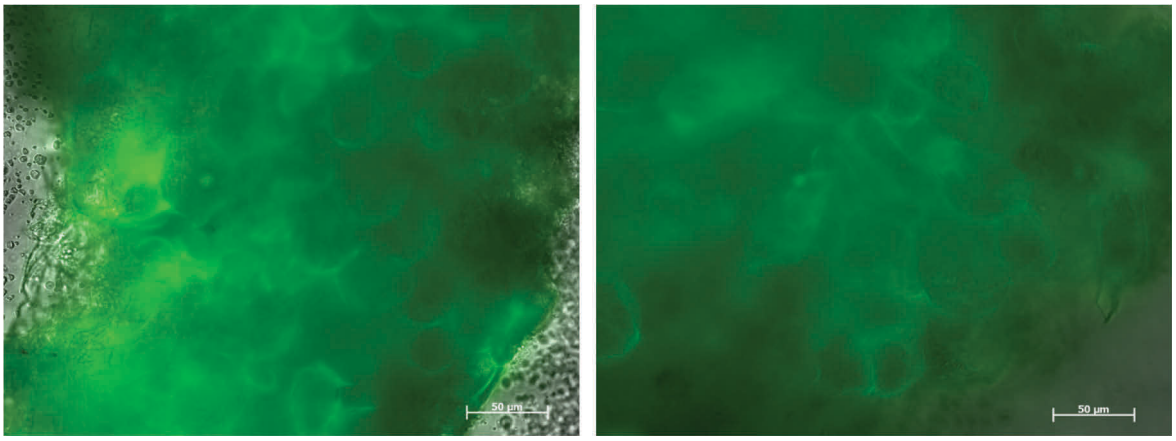

Figure 2-31 Fluorescence microscopy pictures of transformed and regenerated *C. sativa* L. plants co-cultivated with 300 µM acetosyringone: in parts modified from [b]. Axillary buds of *C. sativa L.* were transformed the plasmid pFGC-Vac-GFP Basta with the syringe-based method and co-cultivated for one day in medium with 300 µM acetosyringone. Fluorescence pictures were taken from leaves of regenerated shoots. Pictures show the overlay of the brightfield and the GFP channel.

Thus, according to the presented results, transformation of *Cannabis* axillary buds should be done with a low optical density of *Agrobacteria* and a short co-cultivation time of one or two days, to obtain a high number of shoots. Furthermore, co-cultivation on MS medium with 300 µM acetosyringone led to better transformation results and it is necessary to incubate the transformed explants in an antibiotic solution to get rid of the remaining *Agrobacteria*.

2.2.4. Discussion

Regeneration of *C. sativa* L. plants from callus cultures was not successful although many conditions were tested to initiate callus growth as well as shoot formation. Beside the evaluation of the best carbon source, the best combination of PGRs was evaluated. Furthermore, it was tested, if shoots can be initiated with additives, like amino acids or silver nitrate (chapter 2.2.1). The use of different carbon sources in the callus induction medium showed differences regarding the growth rate, the vitality as well as the lifetime of the callus cultures. Callus cultures induced on MS medium with sucrose as carbon source grew faster, then those on maltose in the first weeks, whereas callus cultures on MS medium with maltose had a longer lifetime (chapter 2.2.1.1). This might be due to the fast metabolization of sucrose leading to hypoxia in the cells (De Paiva Neto and Otoni, 2003). This again has an impact on the pH of the plant tissue culture medium, which is an important parameter for callus growth and

regeneration of plants (Leifert et al., 1992). In contrast to this, maltose has a 20-fold lower metabolization rate then sucrose resulting in a slower absorption and metabolism (Blanc et al., 2002) and in a slower callus growth as observed in the performed experiments. Experiments showed that the secretion rate of phenolic compounds, which are toxic for the cells, was higher in callus cultures grown on sucrose, fructose or glucose than in callus cultures grown on maltose (Kumar et al., 2015).

Some published protocols for *Cannabis* plant regeneration are available (Table 1-1), but unfortunately none of the tested protocols worked in our laboratory. Beside the fact that the efficiency of the published protocols is not high, it seems that regeneration of *Cannabis* plants is highly dependent on the used variety. While hemp varieties like Fedora 17 of Finola are not suitable for plant regeneration because of very slow callus growth, varieties of the drug-type plants seem to be better candidates. Since there are many varieties available through intensive breeding, it will be complicated and time consuming to find suitable conditions for regeneration from callus cultures of more than just one variety.

Some of the tested conditions led to the differentiation of the callus cultures cells e.g. to undefined structures maybe developing into roots or shoots (Figure 2-14 B) and to already differentiated roots (Figure 2-17). These findings indicate that the induced callus cultures somehow could differentiate into different cell types, but the optimal conditions to promote shoot initiation are not available. The most promising result were obtained in MS modified medium supplemented with 1 mg L^{-1} kinetin and 0.5 mg L^{-1} 2,4-D and maltose as a carbon source. With these conditions it was possible to induce meristem-like structures (Figure 2-16), indicating an induction of somatic embryogenesis. Unfortunately, no further development was observed. To obtain a fully regenerated shoot, a new screening for conditions might be necessary, which support and promote somatic embryogenesis leading to shoot formation. Although experiments presented in this study were not successful, somatic embryogenesis and the resulting shoot formation were already successfully done with leaves and cotyledons as explants with the use of TDZ (Table 1-1; Chaohua et al., 2016; Lata et al., 2010). Regarding the goal to create transgenic *Cannabis* plants for further studies e.g. on gene regulation, the use of this substance is not favorable because it causes high polymorphic DNA methylation (Ghosh et al., 2017; Schachtsiek et al., 2018).

Different from the regeneration of *Cannabis* plants out of callus cultures, the alternative approach for plant regeneration with direct organogenesis from axillary buds was successful, leading to fully regenerated plants (Figure 2-20) using an already existing protocol (Lata et al.,

2016) with some modifications (chapter 2.2.2). However, rooting of the obtained shoots was only possible in ½ MS medium and a small vertical cut in the stem of the shoot like it is done for cuttings, indicating that this cut enhances root formation. However, first roots were observed four to six weeks after the transfer to rooting medium. These findings are in coincidence with published protocols for *Cannabis* regeneration using the identical rooting conditions with the same timeframe of appearance of first roots (Chaohua et al., 2016; Lata et al., 2010).

The developed direct organogenesis protocol was used as a basis for the development of a transformation protocol of axillary buds, leading to transgenic *Cannabis* plants. With the use of GFP, as a marker gene, it was shown that *A. tumefaciens*-mediated transformation of *Cannabis* is generally possible, either using an ER-targeting signal peptide or a signal peptide for targeting the vacuole (Figure 2-22; Figure 2-24). The latter enhances the fluorescence within the cell, since the vacuole represents a big compartment in the plant cell. However, in most cases, fluorescence was still only detectable at the edges of the cells. This indicates, that the chosen vacuolar targeting signal of phaseoline (Frigerio et al., 1998), one of the most abundant enzymes of legumes, might not work properly in *C. sativa L.*. Still, it was possible to use the constructed vector for transformation experiments of axillary buds. After the development of a transformation protocol, including several optimization steps, GFP expression indicated by fluorescence can be used as an indicator for successful transformation of *C. sativa* L.. However, fluorescence was only achieved utilizing the hypervirulent *A. tumefaciens* strain AGL1. Commonly used strains GV3101 and LBA4404 were not suitable for transformation, indicating that a hypervirulent strains is necessary for high transformation rates. Changes in transformations efficiencies while using different *A. tumefaciens* strains is already known from other plant species like tomato, corn or rice or sorghum (Cao et al., 2014; Chetty et al., 2013; Cho et al., 2014; Do et al., 2016). Best results during the optimization process was archived with the use of optical densities for transformation of 0.5 or 1.0 with just one day of co-cultivation (chapter 2.2.3.3.3). Nevertheless, fluorescence was not detected in every cell of the regenerated plants (Figure 2-30), which might be due to the fact that not every cell of the shoot meristem, which is multicellular, of the axillary bud was transformed by *Agrobacteria.* Indeed, the increase of the acetosyringone concentration led to better transformation results (Figure 2-31), but the shoots still consist of non-transformed cells. The *Agrobacterium*-mediated transformation of explants from citrus plants, flax and *Lesquerella fendleri* resulted in chimeric regenerated plants as well (Chen, 2011; Domínguez et al., 2004; Dong and McHughen, 1993). It is suggested, that the high frequency of chimeric plants after transformation is caused by

efficient protection from the selection agent of the non-transformed cell by the surrounding transformed cells (Chen, 2011; Domínguez et al., 2004; Dong and McHughen, 1993). In our experiments, the application of a selection agent in the medium after transformation resulted in no shoot regeneration, leading to the decision to regenerate shoots without the selection agent for development of the protocol. The combination of selection pressure, vacuum infiltration as well as antibiotic treatment of the axillary buds might cause too much stress to the explants, resulting in no shoot formation. Nevertheless, one should keep in mind that in the future selection pressure is needed for the reduction of chimeric regenerated plants.

2.3. Virus-induced gene silencing in *Cannabis sativa* L.

The increased demand in *Cannabis* as a medicinal plant in recent years led to a tremendous interest in understanding the biosynthetic routes of *Cannabis* metabolites. Since there is no established protocol to generate stable gene knockouts in *Cannabis* until now, the use of a virus-induced gene silencing (VIGS) method, resulting in a gene knockdown, to study gene functions is desirable. To get access to a fast and easy method to study the function of genes in *C. sativa* L., VIGS should be established. For this purpose, two visible markers should be used as target genes: i) the endogenous *phytoene desaturase* (*PDS*) gene and ii) the *magnesium chelatase subunit I* (*ChlI*).

2.3.1. Identification of candidate genes and siRNA prediction

Phytoene desaturase (*PDS*) and *magnesium chelatase subunit I* (*ChlI*) genes are suitable visible marker genes for the functional analysis using the virus induced gene silencing (VIGS) approach. For this purpose, *PDS* and *ChlI* genes were identified from *C. sativa* L.. Therefore, *AtPDS* (NM_001340908.1) and *AtChlI* (NM_117962.3) from *Arabidopsis thaliana* were used as reference sequences to find the homologous sequence in the *Cannabis* draft genome as well as transcriptome resources. First a command line standalone BLAST+ for linux (version 2.6.0+) was used for the homology search. The "BLASTDB" algorithm was utilized to create a database for the *Cannabis* draft genome and transcriptomic data. Then "blastn" was used to find the respective homologous sequences (Figure 2-32).

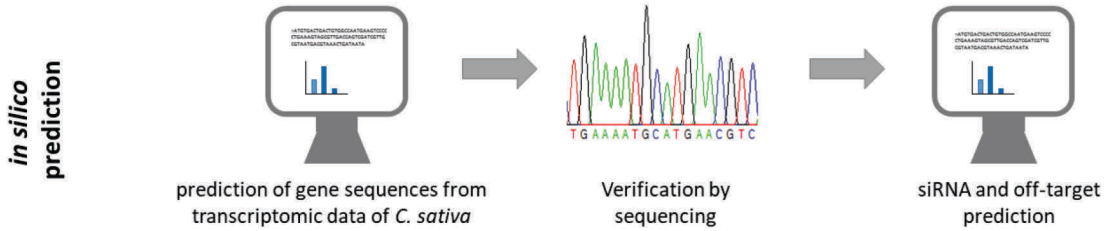

Figure 2-32 *In silico* prediction of target genes for VIGS of *C. sativa* L.: based on the *A. thaliana* gene sequences of *PDS* and *ChlI*, gene sequences from C: sativa l. were predicted and cloned into a vector for verification of the sequences by sequencing. The verified sequences were used for siRNA and off-target prediction, to find suitable target fragments for the silencing experiments.

The resulting sequences that had at least 60 % identity and a stringent e-value of 1e-05 were retained. To further verify these candidate sequences, BLAST+ was used to compare these sequences against NCBI non-redundant protein (nr), nucleotide (nt) and Swiss-Prot (UniProtKB) databases using the blastx algorithm. Only the sequences which showed

significant similarity (> 60 %) with *ChlI* and *PDS* from other plant species were retained and considered as the homologous sequence in *C. sativa* L.

The identified *Cs-ChlI* and *Cs-PDS* homologous coding sequences each have more than 80 % sequence similarity both at nucleotide and protein level with the corresponding *Arabidopsis* sequence. To validate the computational predicted sequences *(Cs-PDS and Cs-ChlI)*, primers were designed to amplify the coding sequences from cDNA using PCR. The amplified fragments were cloned into the vector pDionysos (Stehle et al., 2008) by Gibson assembly and sent for sequencing. The sequencing results showed 99.9 % similarity with the predicted sequences. Both candidate sequences can be found in Table S VI-2.

As a next step it was evaluated, which fragments of the predicted gene sequences are suitable targets for the gene silencing experiment. It is important to select fragments with minimal or no off-target effects, i.e. siRNAs should only bind to the desired target gene and not to others. Furthermore, the optimum length of the target fragment for efficient VIGS with minimal off-target effects is 200 – 400 nucleotides (Senthil-Kumar and Mysore, 2014). The gene sequences for *Cs-PDS* and *Cs-ChlI* were used to predict siRNAs analyzed with the publicly available bioinformatics tool pssRNAit (http://plantgrn.noble.org/pssRNAit/). In total 16 siRNAs and 259 off-targets for *ChlI* and 21 siRNAs and 347 off-targets for *PDS* were predicted. Subsequently, all predicted siRNAs as well as the off-targets were scanned against the previously established *Cannabis* database (described above). All siRNAs that have potential binding sites other than Cs-*PDS* and Cs-*ChlI* candidate genes, so called off-target sites, were filtered out. Finally, two *Cs-ChlI* and four *Cs-PDS* siRNAs were used to design the silencing fragments. The siRNAs binding site containing gene fragments were selected that have the length of 150-400 bp and GC content ranging from 30-70 % to be tested in the post translational gene silencing (PTGS) experiment (Table S VI-3). The designed silencing fragments for PDS had a length of 392 bp for PDS-1 (1236-1628), 375 bp for PDS-2 (492-867), 335 bp for PDS-3 (173-508) and 368 bp for PDS-4 (760-1128). Similarly, designed silencing fragments for each *ChlI* having the length of 300 bp at position 108-407 for ChlI-1 and 427-726 for ChlI-2.

2.3.2. The *Cotton leaf crumple virus* is suitable to induce PTGS in *Cannabis*

As already mentioned, it could be shown that the TRV, which is often used in VIGS experiments, is not that efficient in rosid species, except from the model plant *A. thaliana* (Lange et al., 2013). Since *C. sativa* L. also belongs to the rosids, an alternative virus system had to be found. The *Cotton leaf crumple virus* (*CLCrV*) belongs to the geminiviruses and therefore consists of a bipartite genome of ssDNA. It could be efficiently used for VIGS in

cotton plants, using *PDS* and *ChlI* as targets to visualize gene silencing (Tuttle et al., 2008). Beside vectors for biolistic transformation, the group designed vectors for an agroinfiltration-based method and made the vectors available for scientific use by depositing the vectors at Addgene, a plasmid repository (Tuttle et al., 2012). Thus, this virus-system was chosen to test VIGS in *C. sativa* L..

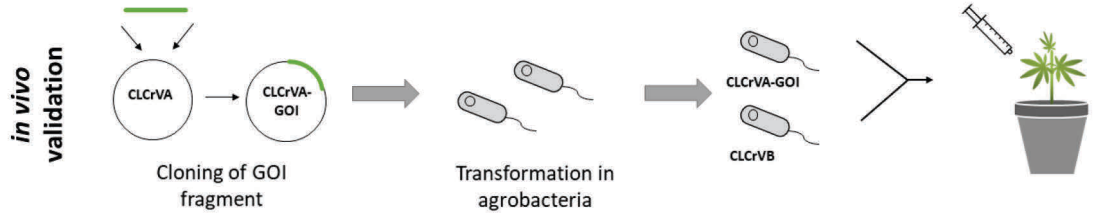

Figure 2-33 Workflow of a VIGS experiment: The predicted target fragments of the gene of interest (GOI) were cloned into the pCottonA vector, subsequently transformed into *A. tumefaciens*. Together with *Agrobacteria* transformed with the vector consisting of DNA-B of the virus (pCottonB), plants were transformed with a needless syringe.

The chosen gene silencing fragments were cloned into pJRT.Agro.CLCrVA.008 (referred to as pCottonA) behind the coat protein promotor to ensure transcription of the corresponding fragments as described by Tuttle et al. (2008). For the development of the method, at first only the *PDS* gene was targeted. The four resulting plasmids for silencing of the *PDS* gene (pCotton-PDS1 to pCotton-PDS4) were transformed in *A. tumefaciens* strains LBA4404, GV3101 and AGL1 to evaluate which *Agrobacterium* strain is the most effective one. In combination with the plasmid carrying the DNA-B component of the virus pJRT.Agro.CLCrVB1.3 (referred to as pCottonB) the *Agrobacteria* were infiltrated in seedlings of *C. sativa* L. at a ratio of 1:1 with different optical densities varying from OD_{600nm} of 0.5 to 5 (Figure 2-33) into the cotyledons and first leaves of the seedlings. However, no phenotype was visible by infiltrating the seedlings with either the *Agrobacterium* strains LBA4404 or GV3101 with different optical densities. In contrast to this, after infiltration of the plants with the *Agrobacterium* strain AGL1, representing a hypervirulent strain (Lazo et al., 1991), with an optical density of 4.0, a phenotype was visible on the leaves after approximately 3-4 weeks after infiltration (Figure 2-34) for the fragments *PDS-1* and *PDS-2*.

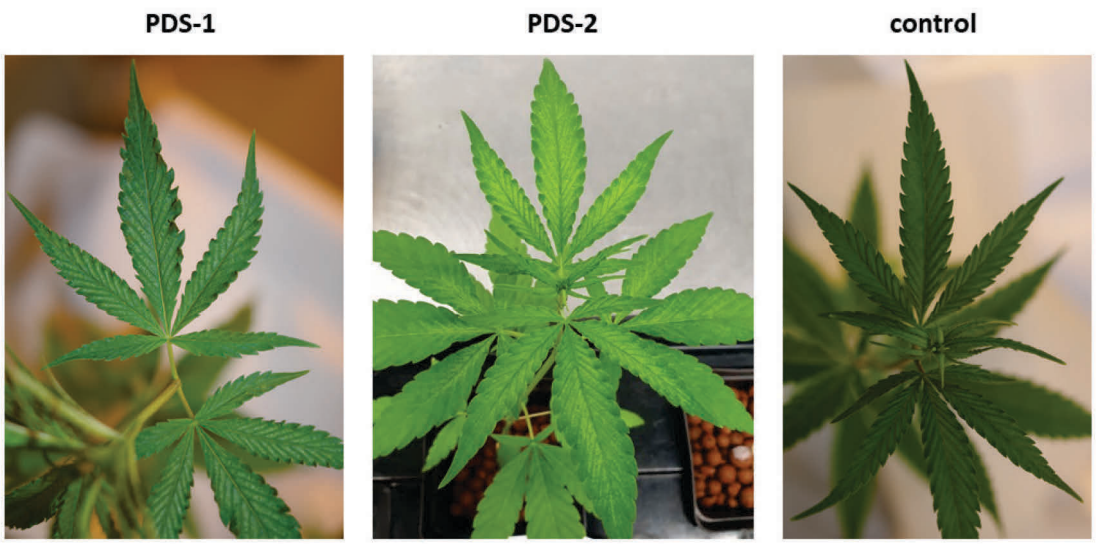

Figure 2-34 Phenotype of *C. sativa* L. plants infiltrated with pCotton-PDS constructs: Seedlings were co-infiltrated with AGL1 pCotton-PDS1, PDS2 and pCottonA as control and AGL1 pCottonB with an optical density of 4.0 and a ratio of 1:1. After three to four weeks, phenotypic effects were visible, except from the control plants.

The infiltrated plants developed leaves spotted with white dots all over the leaves, which can be described as a weak phenotype. No phenotype was observed when plants were infiltrated with fragments *PDS-3* and *PDS-4*. Control plants infiltrated with the empty vector as a control (pCottonA) showed no altered phenotype (Figure 2-34).

To verify VIGS, same experiments were performed for silencing of the *ChlI* gene. Since, infiltration with the *A. tumefaciens* strains GV3101 and LBA4404 was not successful before, plants were only infiltrated with the *A. tumefaciens* strain AGL1 transformed with the two designed plasmids containing the *ChlI-1* and *ChlI-2* fragments (referred to as pCotton-ChlI1 and pCotton-ChlI2) with an optical density (OD_{600nm}) of 4.0 at the 4 leaf-state. Cotyledons and first real leaves were infiltrated at the same time.

Figure 2-35 Phenotype of *C. sativa* L. plants infiltrated with pCotton-ChlI2: Seedlings were co-infiltrated with AGL1 pCotton-ChlI2 and AGL1 pCottonB with an optical density of 4.0 and a ratio of 1:1. After three to four weeks, first phenotypic effects were visible (left). 1-2 weeks later, bleaching of the leaves continued (right).

A weak phenotype was visible approximately four weeks after infiltration on the leaves of plants infiltrated with both constructs, i.e. pCotton-ChlI1 and pCotton-ChlI2. Exemplarily, Figure 2-35 shows the phenotype of a plant infiltrated with pCotton-ChlI2 in an early stage (left) and two weeks later (right). In the beginning, only small areas of the leaves showed the desired phenotype. Later, newly developed leaves where spotted with yellow dots all over the surface of the leaves, but still the phenotype was weak. A more closer view on the white and yellow spots is shown in Figure 2-36 A.

2.3.3. Verification of the knockdown with qPCR

For verification of the visible phenotypes, which indicate a successful down regulation of the genes, quantification of the transcript levels was done. Total RNA was isolated from leaves, which showed the desired phenotype and from leaves of the empty vector infiltrated plants as a control. After cDNA synthesis, qPCR experiments were performed. Until now, only a few protocols for quantitative real-time PCR for *C. sativa* L. where established. The most crucial factor for the analysis of the transcript level represents the selection of reference genes. In this approach, a combination of two reference genes was used to minimize errors in normalization and to make the results more reliable. All already published studies concerning qPCR in *C. sativa* L. only referred to one reference gene. Furthermore, the experiments were not done

exclusively with leaf samples, but with other tissues from the plant (Docimo et al., 2013; Mangeot-Peter et al., 2016).

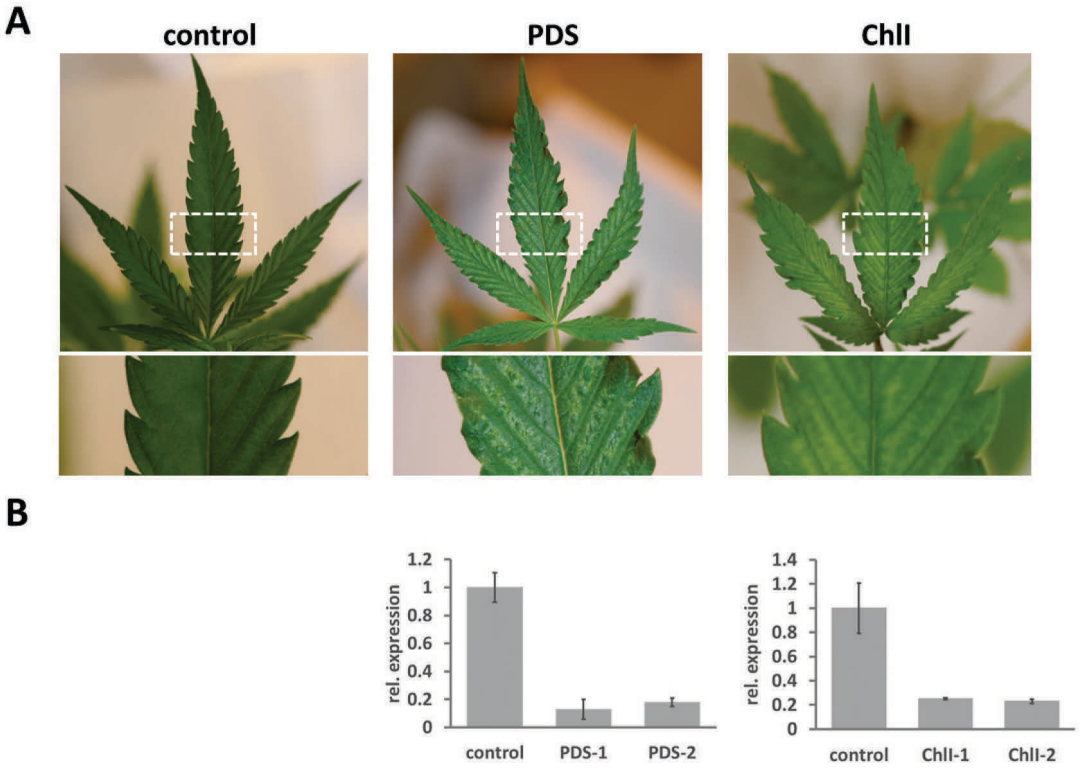

Figure 2-36 *CLCrV*-based VIGS in *C. sativa* L. (A) plants were infected with *CLCrV*-vectors, carrying fragments of *PDS* and *ChlI* or the empty vector as control. Phenotypes could be identified 3-4 weeks after infiltration**, (B)** quantification of the expression level of *PDS* and *ChlI* in plants showing a phenotype were analyzed by qPCR and normalized relative to the expression levels of *eIFa* and *ubq5***.**

Thus, a new qPCR protocol with the evaluation of the best combination of reference genes had to be established first. For this purpose, nine reference genes were selected that could be previously identified in the established cannabis database and tested for stable expression in both samples. Beside commonly used reference genes like *actin2*, *tubulin1, 18S rRNA*, *glyceraldehyde 3-phsophate dehydrogenase* (*GAPDH)*, *elongation factor 1α* (*EF1α)*, *eukaryotic initiation factor a* (*eIFa)*, *ubiquitin 5* and 10 (*ubq5*, *ubq10)*, the gene coding for yellow leaf specific protein 8 (YLS8) was evaluated. This gene was found to be suitable in qPCR experiments in hop (*Humulus lupulus* L.) (Štajner et al., 2013), a plant from the Cannabaceae family. To find the best reference genes, experiments were done with three biological replicates and three technical replicates for each gene. C_t-values of all genes for both samples were analyzed with the NormFinder tool to find the most suitable reference gene combination (Andersen et al., 2004). Lowest stability value, i.e. most stable expression, was

obtained for the combination of *ubi5* and *eIFa* (Table S VI-6). According to this, *ubq5* and *eIFa* were used in all qPCR experiments as reference genes.

To confirm the successful down regulation of *ChlI* and *PDS*, leaves with a visible phenotype were chosen as samples for quantification of the expression level. Leaves of an empty vector control plant served as control. As shown in Figure 2-36 B, the relative expression level of the *PDS* gene was reduced by 87 % and 82 % when plants were infiltrated with *Agrobacteria* transformed with pCotton-PDS1 and pCotton-PDS2, respectively. The analysis of the expression level of *ChlI* also showed a reduction of 75 % when fragment *ChlI-1* was transformed and a reduction of 77 % for fragment *ChlI-2*. These results demonstrate the possibility to knockdown genes with the VIGS-method in *C. sativa* L. plants.

2.3.4. Discussion

Up to date no reliable cannabis regeneration and transformation protocol to enable stable transformants is available (Schachtsiek et al., 2018). Nevertheless, to be able to analyze the function of individual genes a virus-based transient silencing method was evaluated. It could be shown for the first time that VIGS, using the *Cotton leaf crumple virus,* can be applied in *C. sativa* L. plants to enable knockdowns. For transformation of the plasmids the *Agroinfiltration* method was used. Indeed, only *Agrobacterium* strain AGL1-transformed *Cannabis* plants showed a desired phenotype. Transformation with the commonly used strains LBA4404 and GV3101 did not show any effects. This might indicate that the virulence power of these both strains is not sufficient to transform *C. sativa* L. to obtain visible phenotypes. The strain AGL1 is designated as hypervirulent (Lazo et al., 1991), which, together with a high applied bacteria concentration (OD_{600nm} 4.0), leads to a successful transformation in *C. sativa* L.. However, the predicted siRNAs/silencing fragments did not result in a desired phenotype in all cases or similar gene knockdown efficiencies. One reason might be that the used siRNA prediction tool (pssRNAit) is useful for RNAi silencing approaches but not to identify the longer needed silencing fragments for VIGS (Fernandez-Pozo et al., 2015). Since no VIGS-construct prediction tool for *Cannabis* is available, it is necessary to test several siRNA/silencing fragments regarding their silencing efficiency.

The observed weak phenotype showed only white and yellow spotted leaves instead of a complete bleaching of the green color (Figure 2-34**,** Figure 2-35 and Figure 2-36). This is in accordance to other reports utilizing the *Cotton leaf crumple virus*-system for gene knockdowns (Tuttle et al., 2008, 2012). In contrast, using the *tobacco rattle virus* (TRV) mostly a complete

bleaching is observed, as shown e.g. in *Nicotiana benthamiana* or *A. thaliana* (Burch-Smith et al., 2006; Liu et al., 2002a). But due to a very low efficiency of VIGS in most of the rosid species the application of TRV in *Cannabis* is not possible (Lange et al., 2013; Ma et al., 2008). For the analysis of the expression level of the targeted genes, it was necessary to establish a quantitative real-time PCR protocol for the best combination of reference genes. After the analysis of a set of potential reference genes with the NormFinder tool (Andersen et al., 2004), the genes *ubq5* and *eIFa* were selected as best combination of reference genes. The sequences of the identified reference genes are now available to design future qPCR experiments of different cannabis varieties or different tissues, respectively.

According to the weak phenotype, it is assumed that the down regulation of the target genes is not as high as shown in studies utilizing the TRV. Interestingly, our results with a down regulation from 75 % to 87 %, show similar effects of down regulation in comparison to other studies. VIGS down regulated genes in *N. benthamiana* and *A. thaliana* resulted in a reduction of 78 % to 95 % (Burch-Smith et al., 2006; Liu et al., 2002a). In tomato down regulation of 78 % of the *PDS* gene was achieved (Liu et al., 2002b) and for barley the expression level of genes was reduced to 75 % to 90 % by using the *Foxtail Mosaic Virus* (Liu et al., 2016).

This shows, that despite the weak phenotype, the developed VIGS-method in *C. sativa* L. can be used to perform reverse genetic approaches to identify unknown gene functions. This will help to elucidate unknown biosynthetic routes and will contribute to a deeper knowledge of medical *Cannabis*. Moreover, the established method is also suitable for the evaluation of endogenous miRNAs and their corresponding targets (Tiwari et al., 2014; Yan et al., 2014). This opens the possibility to elucidate gene functions and to decipher the regulatory network of altering transcription levels for a better understanding of the regulation of biosynthetic routes and plant development.

Chapter 3 Conclusion and Outlook

3.1. Nicotine-free, non-transgenic tobacco (*Nicotiana tabacum* L.) edited by CRISPR-Cas9

Tobacco represents a model plant, widely used in science for synthetic biology or the study of plant pathogen interactions. This leads to the advantage of the availability of many genetic tools to genetically modify the plant, like CRISPR-Cas9. The high alkaloid content, especially the nicotine content might be a drawback for heterologous protein expression in tobacco. With the use of the CRISPR-Cas9 method, it was possible to knockout the whole *BBL*-gene family of six genes with a single gRNA target sequence. This gene family is responsible for the last oxidation step in the formation of nicotine and the complete knockout led to a reduction of the nicotine content of 99.7 % in the T_3 generation, which did not carry the T-DNA cassette anymore due to Mendelian segregation. Thus, the plant was designated as nicotine-free and non-transgenic and no changes in the phenotype occurred.
This approach can also be transferred to *N. benthamiana* varieties that are commonly used as heterologous production platforms, to reduce the overall alkaloid content. Together with the fact that the nicotine-free plant does not show any phenotype this opens the possibility to use these plants as model plants for metabolic engineering studies without any interference of alkaloids. So far, *N. benthamiana* is already used for the large scale production of antibodies (Holtz et al., 2015) and a nicotine-free plant carries the potential to expand the production spectrum to the biotechnological production of plant made pharmaceuticals beyond antibodies, like the production of cannabinoids.

So far it was clearly shown, that all six genes in the plant were knocked out by the analysis of amplicons specific for each gene by PCR and sequencing. Although no phenotypic effects could be identified, it is still possible that off-target mutations are present in the plant. To be sure, that this is not the case, whole genome sequencing would be necessary. This also reveals, if beside off target mutations, fragments of the foreign T-DNA served as filler DNA in the DNA repair processes after the double strand breaks (Gorbunova and Levy, 1997). There are already methods available how to scope with these problems. One can use a method, established for bread wheat, using transient expression of CRISPR-Cas9 DNA or RNA, designated as transgene-free as well (Zhang et al., 2016). In another approach ribonuleoprotein complexes of Cas9 and *in vitro* transcribed gRNA were transformed to plant cells, leading to no detectable off-target mutations (Liang et al., 2017; Woo et al., 2015). Since the European Court of Justice 2018 declared plants, created with the CRISPR-Cas9 method, still as genetically modified organisms (GMOs), introgression of this approach into other tobacco varieties would only be

possible outside of Europe, although, with the mentioned methods above, no foreign DNA is transferred to the cells.

3.2. Development of a regeneration and transformation protocol for *Cannabis sativa* L.

Due to the pharmaceutical potential of the cannabinoids, the interest in the plant *C. sativa* L. is increasing continuously. To get more insight into the metabolism as well as the effect of distinct cannabinoids, genetic modifications would be favorable. In order to modify the genome, a transformation and regeneration protocol is necessary. Regeneration by the standard approach through callus cultures was not successful so far, but promising results could be obtained when callus cultures were induced on MS modified medium with maltose and the PGRs kinetin and 2,4-D. Meristem-like structures developed from callus cells indicating a starting somatic embryogenesis. It needs to be evaluated how somatic embryogenesis can be promoted in cannabis callus cultures to obtain shoots out of callus cultures by testing again different combinations or concentrations of PGRs or other additive substances as well as different pH ranges. It should also be considered to test sorbitol or mannitol as osmotic regulators, known to increase callus induction, shoot proliferation or somatic embryogenesis (Jeannin et al., 1995; Kadota et al., 2001; Sairam et al., 2003).

Based on the developed direct organogenesis protocol from axillary buds of *Cannabis sativa* L. an *Agrobacterium*-mediated transformation protocol with the use of vacuum infiltration was developed. After several optimization steps it was possible to regenerate shoots expressing *gfp*, indicated by green fluorescence. Although the regenerated shoots consist of non-transformed and transformed cells and are therefore chimeric, to our knowledge this is the first protocol combining cannabis transformation and regeneration. Still, optimizations are necessary; especially to avoid non-transformed cells in regenerated shoots. This can be achieved by using selection pressure again, but this is accompanied with new strategies for application of selection pressure e.g. in lower concentrations or at a later stage of the regeneration process. Another approach would be the reduction of chimeric tissue by several cycles of direct organogenesis of the transformed shoot axillary buds (Roux et al., 2001), which would be time consuming and selection pressure would be recommended as well.

Regarding the goal to genetically modify *C. sativa* L., a transformation and regeneration protocol from callus cultures would be desired, because the regenerated shoot is mostly derived

from a single cell, avoiding the development of chimeric plants. Using the developed transformation and regeneration method for e.g. CRISPR-Cas9 mediated knockouts or other modifications, no satisfying conclusion regarding gene functions would be possible. Nevertheless, the method can be used to test e.g. if gene editing e.g. CRISPR-Cas9 would be possible by analysis of the transformed shoots by PCR and sequencing.

3.3. Virus-induced gene silencing in *Cannabis sativa* L.

Genetic modifications in *C. sativa* L. to investigate gene functions are poorly understood, due to the absence of reliable regeneration and transformation protocols. Virus-induced gene silencing (VIGS), based on PTGS, represents a fast and easy alternative to elucidate gene functions in plants transiently. For this purpose, VIGS was established in *C. sativa* L. using an agroinfiltration approach. With the *A. tumefaciens* strain AGL1 and binary vectors consisting of the *Cotton leaf crumple virus* (*CLCrV*), gene silencing of *PDS* and *ChlI*, both involved in chlorophyll biosynthesis, was possible for the first time. Beside the reduction of the transcription level between 75 % and 87 %, the desired phenotype on the leaves, i.e. bleaching of the leaves and yellow leaves, could be observed. However, only white and yellow spots were visible, leading to a weak phenotype. Nevertheless, the VIGS method represents a suitable alternative to study gene functions in *C. sativa* L., but optimizations are still necessary to enhance gene silencing effects and to use this method as reliable alternative for genetic modifications until stable transgenic *Cannabis* plants, without the chimeric pattern (chapter 2.2.3.3) are available.

It was shown, that removal of the apical shoot meristem directly after infiltration of the *Agrobacteria* led to higher silencing efficiencies, due to a more efficient spread of the virus, in tobacco and flax (Chantreau et al., 2015; Wijdeveld et al., 1992). The use of this technique might also be efficient in *Cannabis*. Results indicate that infection efficiencies of *CLCrV* were higher in low temperatures (Tuttle et al., 2008). Although plants were already cultivated at 22 °C during the day and 18 °C in the night, cultivation in even lower temperatures might also increase virus infection. Moreover, testing of other virus systems for gene silencing should be considered for better silencing results, either other geminiviruses like the *cabbage leaf curl virus* (*CbLCV*) or the *Apple latent spherical virus* (*ALSV*) a cheravirus. After optimization of the gene silencing in *Cannabis*, it would also be possible to use the system to investigate the role of distinct miRNAs, which have regulatory functions on the transcript level. This can be

done by either mimicking the target of the miRNA to silence the miRNA activity (Yan et al., 2014) or by overexpressing the miRNA (Tiwari et al., 2014).

Overall, the study shows, that it is indeed possible to modify the transcription level of *C. sativa* L., which moves the *Cannabis* research into the molecular age for the understanding of gene functions in the plant.

Chapter 4 Material and Methods

Parts of chapter 4.1 were published in:

Schachtsiek J. and Stehle F. Dataset on nicotine-free, nontransgenic tobacco (*Nicotiana tabacum* L.) edited by CRISPR-Cas9 (2019). *Data in Brief,* 26, 104395, doi: 10.1016/j.dib.2019.104395

4.1. Material

The used chemicals were mainly purchased from VWR (Germany), Sigma-Aldrich (Germany), Carl Roth (Germany), Duchefa (The Netherlands) and Applichem (Germany).

The isolation of plasmids from *E. coli* DH5α was done with the Nucleospin® Plasmid (No Lid) Kit (Macherey-Nagel, Germany). Plasmid isolation from *A. tumefaciens* was done according to an extended protocol of the same Kit provided by the manufacturer. Isolation of genomic DNA was done with the Nucleospin® Plant II Kit (Macherey-Nagel, Germany) and total RNA was isolated using the Nucleospin® RNA plus Kit (Macherey-Nagel, Germany). Purification of gel pieces and PCR products was done with the NucleoSpin® Gel and PCR Clean-Up Kit (Macherey-Nagel, Germany).

Restriction enzymes were purchased from New England Biolabs® (NEB; Germany).

4.2. Methods

The upcoming methods chapter will be divided into three parts according to the structure in the results and discussion part as follows:

1) Nicotine-free, non-transgenic tobacco (*Nicotiana tabacum* L.) edited by CRISPR-Cas9 (4.2.1)
2) Development of a regeneration and transformation protocol for *Cannabis sativa* L. (4.2.2)
3) Virus-induced gene silencing in *Cannabis sativa* L. (4.2.3)

4.2.1. Nicotine-free, non-transgenic tobacco (*Nicotiana tabacum* L.) edited by CRISPR-Cas9

4.2.1.1. Strains used in this study

As a host organism for all cloning experiments, the *Escherichia coli* strain *DH5α* was used and cultivated in LB medium (5 g L^{-1} yeast extract, 10 g L^{-1} peptone, 10 g L^{-1} sodium chloride; pH 7.0 and 20 g L^{-1} agar for solid medium) and the appropriate antibiotics at 37 °C and 200 rpm. The *Agrobacterium tumefaciens* strain GV3101::pMP90 (referred to GV3101) was used for transformation experiments of *N. tabacum* leaves. Cultivation was done in YEB medium (1 g L^{-1} yeast extract, 5 g L^{-1} beef extract, 5 g L^{-1} sucrose, 5 g L^{-1} peptone, 0.5 g L^{-1} $MgSO_4 \cdot 7\ H_2O$;

pH 7.0 and 15 g L^{-1} agar for solid medium) at 28 °C in addition of 50 µg mL^{-1} gentamycin, 25 µg mL^{-1} rifampicin and appropriate antibiotic, if the strain was transformed with a plasmid. Strains used in this study are listed in Table 4-1.

Table 4-1 Strains used forCRISPR-Cas9 experiments

Strain	Plasmid	Reference
A. tumefaciens GV3101	Ti helper plasmid pMP190	(Koncz and Schell, 1986)
A. tumefaciens GV3101	Ti helper plasmid pMP190; pCas9- BBL	This study

4.2.1.2. Plasmid construction and transformation

All plasmids constructed in this study were created with restriction/ligation or the Gibson Assembly method (Gibson et al., 2009).

"For the delivery of the CRISPR cassette to *N. tabacum* plants with *A. tumefaciens* a binary vector system was used (Fauser et al., 2014). The chosen sgRNA target sequence (GAAATCAGAGTAAGGTGCGG) for the *BBL* genes was cloned into the vector pChimera according to the author's instructions and the resulting vector was named pChimera-BBL. The gRNA chimera was subsequently cloned into the vector pCas9-TPC according to the author's instructions resulting in the vector pCas9-BBL which was used for transformation experiments after verification by sequencing (Seqlab, Germany)." (Schachtsiek and Stehle, 2019a)

The transformation of *A. tumefaciens* with the plasmid pCas9-BBL was done according to an existing protocol (Höfgen and Willmitzer, 1988).

"For the verification of the targeted mutagenesis on genomic level, gene sequences of the six *BBL* genes that include the target site were amplified with specific primers for each gene from genomic DNA of wildtype and transgenic plants. For sequencing, the gene fragments were either cloned into the vector pDionysos (Stehle et al., 2008), cutted with *Xba*I, by using the Gibson Assembly method or the PCR product was directly sequenced." (Schachtsiek and Stehle, 2019a) Used primers can be found in the supplementary material (Table S IV-1). For the amplification of the gene fragments the Q5® High-Fidelity 2x Master Mix (NEB, Germany) was used and assembly of the fragments was done with the 2x Gibson Assembly Master Mix (NEB, Germany) according to manufacturer's instructions.

All constructs were transformed into *E. coli* DH5α with a standard transformation method including heat-shock (Inoue et al., 1990).

Used plasmids of the study can be found in Table 4-2.

Table 4-2 Plasmids used for CRISPR-Cas9 experiments and verification of knockout

Plasmid	Resistance	Reference
pChimera	Ampicillin	(Fauser et al., 2014)
pCas9-TPC	Spectinomycin	(Fauser et al., 2014)
pChimera-BBL	Ampicillin	This study
pCas9-BBL	Spectinomycin	This study
pDionysos	Ampicillin	(Stehle et al., 2008)
pDionysos-BBLa	Ampicillin	This study
pDionysos-BBLb	Ampicillin	This study
pDionysos-BBLc	Ampicillin	This study
pDionysos-BBLd1	Ampicillin	This study
pDionysos-BBLd2	Ampicillin	This study
pDionysos-BBLe	Ampicillin	This study

4.2.1.3. Cultivation of *N. tabacum* plants

"N. tabacum L. plants of the variety "Virginia Smoking Tobacco" (Strictly Medicinal Seeds LLC, United States) were cultivated on hydro culture at 25 °C under long day conditions (18 h light/6 h dark) with a light intensity of 110 µM m^{-2} s^{-1}. For germination, seeds were surface sterilized with a sodium hypochlorite solution (1 % active chlorine) and a few drops of Tween 20 for 10 minutes and washed three times with water before they were plated out on Murashige and Skoog Medium (4.4 g L^{-1} Murashige and Skoog Medium with Gamborg's Vitamins, 30 g L^{-1} sucrose, pH 5.8)." (Schachtsiek and Stehle, 2019a) Transgenic plants from the T_0 to T_3 generation were cultivated equally.

4.2.1.4. Plant transformation and regeneration

"Transformation of *N. tabacum* leaves and the followed plant regeneration were done according to an existing protocol with minor changes (Sparkes et al., 2006). Plants were infiltrated with the *A. tumefaciens* strain GV3101 transformed with the plasmid pCas9-BBL with an $OD_{600\ nm}$ of 0.1 and incubated for 3 days at long day conditions. For plant regeneration infiltrated leaves were surface sterilized, cut into pieces and incubated on shooting medium (2.15 g L^{-1} Murashige and Skoog Medium with Gamborg's Vitamins, 30 g L^{-1} sucrose, 0.1 mg L^{-1} indole-3-butyric acid, 0.8 mg L^{-1} benzylaminopurine, 250 mg L^{-1} carbenicillin , 6 mg L^{-1} DL-phosphinothricin (PPT),

8 g L^{-1} agar, pH 5.2) under long day conditions. Developed shoots were transferred to rooting medium (2.15 g L^{-1} Murashige and Skoog Medium with Gamborg's Vitamins, 30 g L^{-1} Sucrose, 0.5 mg L^{-1} indole-3-butyric acid, 250 mg L^{-1} carbenicillin, 6 mg L^{-1} DL-phosphinothricin (PPT), 8 g L^{-1} agar, pH 5.2) for approximately 10 days." (Schachtsiek and Stehle, 2019a) After rooting, the plantlets were transferred to small pots and cultivated as described in section 4.2.1.3.

4.2.1.5. Test for transgenic plants

"Seeds from regenerated plants were collected and seeded out for growing the T_1 generation. To test if these plants were transgenic, 7 days-old plantlets were sprayed with a solution of 100 mg L^{-1} PPT three times every two days. Survived plants were considered as transgenic and grown for further experiments.

Plants in T_2 generation can be tested for the loss of the CRISPR-Cas9 cassette. For this purpose leaves of T_2 generation plants were surface sterilized for ten minutes with sodium hypochlorite (0.5 % active chlorine) and a few drops of Tween 20 followed by three washing steps with water. Leaf discs were cut out with a cork borer and incubated on selection medium (4.4 g L^{-1} Murashige and Skoog Medium with Gamborg's Vitamins, 30 g L^{-1} sucrose, 6 mg L^{-1} PPT; pH 5.8) under standard growing conditions. Additionally, validation of the loss of the CRISPR cassette was done with PCR. Genomic DNA of the plants was isolated and amplification of the CRISPR cassette was done by the use of primers SS43 and SS61." (Schachtsiek and Stehle, 2019a)

4.2.1.6. Plant extracts

"For the extraction of alkaloids a modified version of the extraction protocol from Lewis et al. (2015) was used. For the extraction 50 mg or 100 mg of ground leaves with 1 mL of a 2 N NaOH to moisten the sample in a glass vessel with a screw-cap. After 15 minutes of incubation time 5 mL of methyl tert-butyl ether (MTBE) containing 0.4 mg ml^{-1} quinoline used as an internal standard were added to the sample. Samples were incubated for 2.5 h with shaking at 200 rpm. For layer separation the glass vessels were stored without shaking overnight. The MTBE layer was used for gas chromatographic analysis." (Schachtsiek and Stehle, 2019a)

4.2.1.7. Gas chromatographic analysis

"Measurements of plant extracts were done with an Agilent Technologies 7890A GC system equipped with a flame ionization detector (FID) set to 300 °C and a VF-5ms column (CP8944; 30 m x 0.25 mm, ID 0.25 µm). H_2 flow was set to 30 mL min^{-1}, Air Flow to 400 ml min^{-1} and

N_2 flow to 30 mL min^{-1}. Injector temperature was set to 250 °C and 1 µL of the sample was injected in splitless mode. Initial oven temperature was set to 110 °C, held for 1 minute and increased afterwards to 200 °C with a rate of 10°C/min followed by an increase to 300 °C in steps of 25 °C/min. The temperature of 300 °C was held for 10 minutes.

For GC-MS measurements a Thermo Scientific Trace GC Ultra system with an ISQ mass spectrometer and a TG-SQC column (Thermo Scientific; 15 m x 0,25 mm, ID 0,25 µm) was used. Injector temperature was set to 90 °C and 1 µL of the sample was injected in splitless mode. Initial oven temperature was set to 60 °C for 1 min, followed by an increase of the temperature to 200 °C with a rate of 10 °C/min. Afterwards the temperature was increased to 300 °C with a rate of 25 °C/min which was held for 10 minutes. Helium was used as a carrier gas with a flow of 0.7 ml min^{-1}." (Schachtsiek and Stehle, 2019a)

4.2.1.8. ^{1}H-NMR analysis

"For NMR analysis, 20 mg of freeze-dried leaf material of the wild type and the nicotine-free plant or 10 mg of nicotine standard were mixed with 1 mL methanol-D4 and vortexed for one minute. After ultrasonication for 15 minutes the samples were centrifuged at 13,000 g for 5 minutes. Around 600 µL of the supernatant was filled into a 3 mm NMR-tube. ^{1}H-NMR measurements were done at 25 °C and 600 MHz with the Bruker AV 600 Avance III HD (Cryoprobe) spectrometer. The data were analyzed using TopSpin 4.0." (Schachtsiek and Stehle, 2019a)

4.2.2. Development of a regeneration and transformation protocol for *Cannabis sativa* L.

4.2.2.1. Strains used in this study

The *E. coli* strain DH5α was used as a host organism for all cloning experiments. Cultivation was done in LB medium (5 g L^{-1} yeast extract, 10 g L^{-1} peptone, 10 g L^{-1} sodium chloride; pH 7.0 and 20 g L^{-1} agar for solid medium) at 37 °C and 200 rpm and appropriate antibiotics.

The *A. tumefaciens* strains GV3101, LBA4404 and AGL1 were cultivated in YEB medium (1 g L^{-1} yeast extract, 5 g L^{-1} beef extract, 5 g L^{-1} sucrose, 5 g L^{-1} peptone, 0.5 g L^{-1} $MgSO_4 \cdot 7 H_2O$; pH 7.0 and 15 g L^{-1} agar for solid medium) and used for transformation experiments. All cultivations were done at 200 rpm with temperatures of 28 °C for strains GV3101 and LBA4404 and 26 °C for strain AGL1. During cultivation the medium was supplemented with 25 µg mL^{-1} rifampicin and 50 µg mL^{-1} gentamycin for strain GV3101; 25 µg mL^{-1} rifampicin and 50 µg

mL^{-1} streptomycin for strain LBA4404 and 100 µg mL^{-1} carbenicillin for strain AGL1. If transformed with a plasmid the appropriate antibiotic was added additionally.

The used strains in this study are listed in Table 4-3.

Table 4-3 Strains used for transformation experiments in *C. sativa L.*

Strain	Plasmid	Reference
A. tumefaciens GV3101	Ti helper plasmid pMP190	(Koncz and Schell, 1986)
A. tumefaciens GV3101	Ti helper plasmid pMP190; pBIN-ER-GFP-Kan	This study
A. tumefaciens GV3101	Ti helper plasmid pMP190; pFGC-Vac-GFP Basta	This study
A. tumefaciens LBA4404	Ti helper plasmid pAL4404	(Hoekema et al., 1983)
A. tumefaciens LBA4404	Ti helper plasmid pAL4404; pFGC-Vac-GFP Basta	This study
A. tumefaciens AGL1	Ti helper plasmid pTiBo542ΔT	(Lazo et al., 1991)
A. tumefaciens AGL1	Ti helper plasmid pTiBo542ΔT; pFGC-Vac-GFP Basta	This study

4.2.2.2. Plasmid construction and transformation

The used and generated plasmid of this study are listed in Table 4-4. As a basis for the construction of a binary vector for *gfp* expression targeted to the vacuole, the vector pBIN-Pt-GFP-Basta (Nelson et al., 2007) was used. The vacuolar signal peptide (n-terminal) as well as the c-terminal peptide (AFVY) were added to the GFP sequence with a PCR-reaction using the Q5® High-Fidelity 2x Master Mix (NEB, Germany). The obtained fragment was cloned into the vector pBIN-Pt-GFP-Basta backbone, previously cutted with *Nco*I and *Xba*I, via Gibson assembly (Gibson et al., 2009) using the 2x Gibson Assembly Master Mix (NEB, Germany). Transformation of the assembled plasmid in *E. coli* DH5α was done according to a standard protocol with heat shock (Inoue et al., 1990). Correct assembly was confirmed by colony PCR with the Red-Taq DNA Polymerase 1.1x MasterMix (VWR, Germany) and sequencing (Seqlab, Germany). All used primers are listed in supplementary material Table S V-1.

Transformation of the binary plasmids into the different *A. tumefaciens* strains was done according to a standard protocol (Höfgen and Willmitzer, 1988).

Table 4-4 Plasmids used for transformation of *C. sativa L.*

Plasmid	Resistance	Reference
pBIN-ER-GFP-Kan	Kanamycin	(Nelson et al., 2007)
pBIN-Pt-GFP-Basta	Kanamycin	(Nelson et al., 2007)
pBin-Vac-GFP Basta	Kanamycin	This study

4.2.2.3. Cultivation of *C. sativa* L. plants

Cannabis sativa L. plants of the varieties Finola and Euphoria were grown from either seeds or cuttings of mother plants. Seeds of were germinated on wet tissue paper for 2-3 days and grown further on a semi-hydroponic system under long day conditions (16 h light/8 h dark) in the vegetative phase at 25 °C. As a nutrient solution, Flora Series (FloraGro, FloraMicro, FloraBloom, General Hydroponics, USA) was applied according to manufacturer's instructions. *Cannabis* plants of the variety Euphoria were propagated through cuttings.
Plants were grown with the permission of No. 458 64 16 issued by the Federal Institute for Drugs and Medical Devices (BfArM), Germany.

4.2.2.4. Surface sterilization of *Cannabis* explants for tissue culture

As explants for tissue culture leaves, petioles, axillary buds as well as shoot tips were used. The explants were harvested and surface sterilized with a sodium hypochlorite solution (0.5 % active chlorine) with addition of a few drops of Tween 20 for 10 minutes under occasional shaking followed by three washing steps with sterile water.

4.2.2.5. Initiation of *Cannabis sativa* L. callus cultures for plant regeneration

All callus cultures were initiated on either Murashige and Skoog (MS) Medium with Gamborg's Vitamins or modified MS medium without ammonium nitrate, referred to as MS modified medium. As carbon sources, 3 % sucrose, maltose or glucose were used. The medium was supplemented with 0.8 % agar and adjusted to a pH of 5.8. Different plant growth regulators (PGRs) in different concentrations were added to initiate callus as well as shoot regeneration. As auxins, 2,4-Dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA) and Dicamba were used. Kinetin (Kin) and Benzylaminopurine (BA) were used as cytokinins. Beside these growth regulators the cytokinin-like compound thidiazuron (TDZ) was tested for plant regeneration. Beside the mentioned PGRs, other substances, like proline, glutamine, silver nitrate, active charcoal, copper sulfate and casein were tested according to their effect on plant regeneration.

Incubation of surface sterilized explants (leaves, petioles, shoot tips) on various callus induction media was done under long day conditions with 25 °C and 130 µmol·s^{-1}·m^{-2} or in the dark.

4.2.2.6. Direct organogenesis of *C. sativa* L. axillary buds and micropropagation

Shooting

For direct organogenesis of axillary buds an existing protocol from the group of ElSohly was used (Lata et al., 2016) with minor modifications. Axillary buds were cut from the *C. sativa* L. variety Euphoria and surface sterilized according to chapter 4.2.2.4. Sterilized explants were cut again at the edges to wound the plant and transferred to shoot induction medium (MS-Medium with Gamborg's vitamins, 3 % sucrose, 2 µM meta-topoline, 1 mg L^{-1} benzylaminopurine, 0.8 % agar; pH 5.8). Tissue culture plates were incubated at long day conditions and 25 °C. Appearing shoots were cut off from axillary buds and transferred to bigger jars with the same medium for further growth of the shoots.

Rooting

For rooting, the elongated shoots were transferred to rooting medium (MS medium or ½ MS-medium with 3 % sucrose, different concentrations of indole-3-butryic acid, indole-3-acetic acid or 1-naphthaleneacetic acid, 0.8 % agar; pH 5.8) and incubated further on the same conditions.

Alternatively, the elongated shoots can be transferred to a cutting propagator. For initiation of rooting, a vertical cut in the stem was made.

4.2.2.7. *A. tumefaciens*-mediated transformation of *C. sativa* L. leaf discs

For transformation of leaf discs 30 mL of YEB Medium supplemented with 10 mM MES and 20 µM acetosyringone were inoculated with an *A. tumefaciens* culture transformed with the plasmid pFGC-Vac-GFP Basta in a 300 mL shaking flask with baffles. The culture was incubated for three days at 26 °C and 200 rpm with appropriate antibiotics in the medium.

Cultures were harvested by centrifugation at 3,000 x g for 20 minutes and the pellet was washed in infiltration buffer (50 mM MES, 2 mM Na_3PO_4 · 12 H_2O, 100 µM acetosyringone, 5 g L^{-1} glucose). After centrifugation for 10 minutes at 3,000 x g the washing step was repeated. The washed pellet was resuspended in approximately 20 mL infiltration buffer and the $OD_{600\ nm}$ was measured and the culture was diluted to an $OD_{600\ nm}$ of 0.5. Infiltration of surface sterilized leaf discs was done according to an existing protocol (Matsuo et al. 2016). After infiltration, the leaf

discs were washed for two hours in a 500 mg L^{-1} cefotaxime-solution and transferred to MS medium. The leaf discs were incubated in the dark for two days at 25 °C.

4.2.2.8. *A. tumefaciens*-mediated transformation of *C. sativa* L. axillary buds

A. tumefaciens cells were cultivated and harvested as described in chapter 4.2.2.7. Furthermore, experiments were done with two different infiltration buffer systems published by Burch-Smith *et al*. (10 mM MES, 10 mM $MgCl_2$, 200 µM acetosyringone; Burch-Smith *et al.*, 2006) or Sparkes *et al.* (50 mM MES, 2 mM $Na_3PO_4 \cdot 12\ H_2O$, 100 µM acetosyringone, 5 g L^{-1} glucose; Sparkes *et al.*, 2006). The $OD_{600\ nm}$ was measured and the cells were diluted to optical densities varying from 0.5 – 3. Surface sterilized axillary buds were wounded with a sterile needle. Vacuum-based transformation of axillary buds was either done with the method of Matsuo et al. (Matsuo et al., 2016) with a syringe or with a desiccator. For the syringe method, the vacuum was generated and released 1-2 times. While using the desiccator a vacuum was generated for three minutes, followed by an incubation time of 10 minutes and a slow release of the vacuum. Transformed explants were incubated for three to four hours in the dark in the *Agrobacterium*-solution and placed afterwards on agar plates with a co-cultivation medium (MS-Medium, 3 % sucrose, 2 µM meta-topoline, 1 mg L^{-1} BA) with varying acetosyringone concentrations from 200-300 µM. The explants were co-cultivated at 26 °C in the dark for 1-5 days. After co-cultivation, the explants were washed three times in sterile water and incubated further in a cefotaxime-solution (500 mg L^{-1}) for 3-4 hours. After the washing step, the explants were transferred to shoot-induction medium (chapter 4.2.2.6) with the addition of 500 mg L^{-1} cefotaxime to avoid bacterial growth and incubated under long day conditions at 25 °C until shoots appear.

4.2.2.9. Fluorescence microscopy

The fluorescence microscopy of the leaves *C. sativa* L. was done with the Axio Observer.D1 from Zeiss (Oberkochen, Germany). Microscopic pictures were taken with the AxioCamMR3 equipped with the Plan-Apochromat 100x/1.40 Oil M27 objective. For detection of GFP the reflectorblock 38 HE Green Fluorescent Prot was used. All pictures were taken from the ventral site of the leaf and were analyzed with the AxioVision software.

4.2.3. Virus-induced gene silencing in *Cannabis sativa* L.

4.2.3.1. Strains used in this study

Molecular cloning was done with the use of *E. coli* DH5α as a host strain cultivated in LB medium (5 g L^{-1} yeast extract, 10 g L^{-1} peptone, 10 g L^{-1} sodium chloride; pH 7.0 and 20 g L^{-1} agar for solid medium) with appropriate antibiotics at 37 °C and 200 rpm.

For transient transformation experiments, *A. tumefaciens* strains LBA4404, GV3101 and AGL1 were used. Cultivation of the strains was done in YEB medium (1 g L^{-1} yeast extract, 5 g L^{-1} beef extract, 5 g L^{-1} sucrose, 5 g L^{-1} peptone, 0.5 g L^{-1} $MgSO_4 \cdot 7 H_2O$; pH 7.0 and 15 g L^{-1} agar for solid medium). The strains LBA4404 and GV3101 were cultivated at 28 °C and 200 rpm with the addition of rifampicin (25 µg mL^{-1}) as well as streptomycin (50 µg mL^{-1}) for LBA4404 and gentamycin (50 µg mL^{-1}) for GV3101. The strain AGL1 was cultivated at 26 °C and 200 rpm with the addition of carbenicillin (100 µg mL^{-1}). If cultivation of strains was done with a transformed plasmid, the appropriate antibiotic was added as well.

An overview of the used strains in this study is given in Table 4-5.

Table 4-5 Strains used in this study for the transient transformation of *Cannabis* plants with CLCrV

Strain	Plasmid	Reference
A. tumefaciens GV3101	Ti helper plasmid pMP190	(Koncz and Schell, 1986)
A. tumefaciens GV3101	Ti helper plasmid pMP190; pJRT.Agro.CLCrVB1.3	This study
A. tumefaciens GV3101	Ti helper plasmid pMP190; pJRT.Agro.CLCrVA.008	This study
A. tumefaciens GV3101	Ti helper plasmid pMP190; pCotton-PDS1	This study
A. tumefaciens GV3101	Ti helper plasmid pMP190; pCotton-PDS2	This study
A. tumefaciens GV3101	Ti helper plasmid pMP190; pCotton-PDS3	This study
A. tumefaciens GV3101	Ti helper plasmid pMP190; pCotton-PDS4	This study
A. tumefaciens LBA4404	Ti helper plasmid pAL4404	(Hoekema et al., 1983)
A. tumefaciens LBA4404	Ti helper plasmid pAL4404; pJRT.Agro.CLCrVB1.3	This study
A. tumefaciens LBA4404	Ti helper plasmid pAL4404; pJRT.Agro.CLCrVA.008	This study
A. tumefaciens LBA4404	Ti helper plasmid pAL4404; pCotton-PDS1	This study
A. tumefaciens LBA4404	Ti helper plasmid pAL4404; pCotton-PDS2	This study
A. tumefaciens LBA4404	Ti helper plasmid pAL4404;	This study

	pCotton-PDS3	
A. tumefaciens LBA4404	Ti helper plasmid pAL4404; pCotton-PDS4	This study
A. tumefaciens AGL1	Ti helper plasmid pTiBo542ΔT; pJRT.Agro.CLCrVB1.3	This study
A. tumefaciens AGL1	Ti helper plasmid pTiBo542ΔT; pJRT.Agro.CLCrVA.008	This study
A. tumefaciens AGL1	Ti helper plasmid pTiBo542ΔT; pCotton-PDS1	This study
A. tumefaciens AGL1	Ti helper plasmid pTiBo542ΔT; pCotton-PDS2	This study
A. tumefaciens AGL1	Ti helper plasmid pTiBo542ΔT; pCotton-PDS3	This study
A. tumefaciens AGL1	Ti helper plasmid pTiBo542ΔT; pCotton-PDS4	This study
A. tumefaciens AGL1	Ti helper plasmid pTiBo542ΔT; pCotton-ChlI1	This study
A. tumefaciens AGL1	Ti helper plasmid pTiBo542ΔT; pCotton-ChlI2	This study

4.2.3.2. Plasmid construction and transformation

Molecular cloning in this study was done with Gibson assembly (Gibson et al., 2009) with the use of the 2x Gibson Assembly Master Mix (NEB, Germany) according to manufacturer's instructions. Used primers of this study can be found in Table S VI-1 and generated and used plasmids in Table 4-6.

Verification of predicted DNA sequences of PDS and ChlI

Predicted gene sequences of *PDS* and *ChlI* (see section 4.2.3.3) were amplified with the use of the Q5® High-Fidelity 2x Master Mix (NEB, Germany) from cDNA of *C. sativa* L. var. Finola. Amplified sequences were cloned into the vector pDionysos (Stehle et al., 2008) with the Gibson assembly method, which was cut with *Xba*I for linearization. The assembled vectors were transformed into *E. coli* DH5α using a standard protocol (Inoue et al., 1990). Correct assembly was verified by PCR with use of the Red-Taq DNA Polymerase 1.1x MasterMix (VWR, Germany) and sequencing.

Vector construction for agroinfiltration

The plasmid system for VIGS consists of two plasmids containing the geminivirus *Cotton leaf crumple virus* (*CLCrV*). The DNA-A of the virus is located on the plasmid pJRT.Agro.CLCrVA.008 (Addgene plasmid # 31809) and the virus DNA-B on the plasmid

pJRT.Agro.CLCrVB1.3 (Addgene plasmid # 37974). Both plasmids were a gift from Niki Robertson (Tuttle et al., 2012).

Table 4-6 Plasmids used and generated in this study for transient transformation of *C. sativa* L. with *CLCrV*

Plasmid	Resistance	Reference
pDionysos	Ampicillin	(Stehle et al., 2008)
pDionysos-PDS	Ampicillin	This study
pDionysos-ChlI	Ampicillin	This study
pJRT.Agro.CLCrVA.008	Kanamycin	(Tuttle et al., 2012)
pJRT.Agro.CLCrVB1.3	Kanamycin	(Tuttle et al., 2012)
pCotton-PDS1	Kanamycin	This study
pCotton-PDS2	Kanamycin	This study
pCotton-PDS3	Kanamycin	This study
pCotton-PDS4	Kanamycin	This study
pCotton-ChlI1	Kanamycin	This study
pCotton-ChlI2	Kanamycin	This study

For construction of the VIGS-vectors the plasmid pJRT.Agro.CLCrVA.008 was digested with *SpeI* and 300-350 bp fragments of *PDS* and *ChlI* were amplified from cDNA of *C. sativa* L. var. Finola with the Q5® High-Fidelity 2x Master Mix (NEB, Germany), cloned into the cut vector by the Gibson Assembly method to produce the plasmids pCotton-PDS1 to 4 and pCotton-ChlI1 and 2. Cloning was verified by sequencing after transformation of the assembled plasmids into *E. coli* DH5α according to a standard heat shock protocol (Inoue et al., 1990). Transformation of the constructed plasmids to the different Agrobacterium strains was done according to an existing protocol (Höfgen and Willmitzer, 1988).

4.2.3.3. Identification of candidate genes and the siRNAs

In order to identify the cannabis *PDS* and *ChlI* orthologs the draft genome of *C. sativa* L. latest version GCA_000230575.2 was downloaded from NCBI (van Bakel et al., 2011). Standalone blast+ for unix operating system was downloaded, from National Centre for Biotechnology Information (NCBI) https://www.ncbi.nlm.nih.gov/books/NBK52640/, installed and configured. BLASTDB was used to create the database from the draft genome and the in-house transcriptomic resources (not published). To search for the homologous sequences in *C. sativa* L. blastn was used against the database created (detailed methodology of standalone blast

described in (Hussain et al., 2018)). Finally, the identified candidate genes from *C. sativa* L. were used to predict the siRNAs from publicly available bioinformatics tool http://plantgrn.noble.org/pssRNAit/, keeping the criteria as mentioned in (Xu et al., 2006b). Based on the identified siRNAs six silencing fragments were designed for cloning and infiltration experiments (Table S VI-3). The silencing fragments were selected in a region of the gene containing the siRNA target site with a length between 150 and 400 base pairs and a GC content in the range from 30-70 %. Additionally, fragments within the range of 5' untranslated regions (UTRs), 3' UTRs and first 100 base pairs from the start codon were not considered.

4.2.3.4. Identification of *Cannabis* reference genes

In order to identify the reference gene for the qPCR experiment, *actin* (NM_001338359.1), *tubulin* (NM_106228), *GAPDH* (NM_101214.4), *EF1a* (NM_125432.4), *ubq10* (NM_178968.5), *eIFa* (NM_104305.4), *18S rRNA* (X16077.1), *ubq5* (NM_116090.3), and *yls8* (AB047811.1) coding sequences from *Arabidopsis thaliana* were downloaded from NCBI and "blastn" 2.6.0+ was used to find the homologous sequences in *C. sativa* L.. The sequences having the query coverage more than 80 %, e-value of 1e10-3 and the percentage of the homology greater than 70 % were further used to perform "blastx" against the NCBI non-redundant (nr) database. Candidate sequences that showed significant homology to their respective genes from other plants were considered as homologous sequences. The sequences can be found in Table S VI-4.

4.2.3.5. Evaluation of reference genes for qPCR

To test, which combination of reference genes is suitable for qPCR experiments, expression of the identified reference genes was tested according to stable expression with cDNA from wild type leaves and leaves from infiltrated plants in three biological replicates. For evaluation of the best combination of reference genes the tool NormFinder was used (Andersen et al., 2004). The primers used in this study can be found in Table S VI-5.

4.2.3.6. Cultivation of Cannabis sativa L.

C. sativa L. var. Finola plants were germinated on wet tissue paper for 2-3 days and cultivated afterwards on hydroculture at 25 °C on long day conditions (18 h light/6 h dark, light intensity 130 µM m^{-2} s^{-1}) using the Flora Series (FloraGro, FloraMicro, FloraBloom, General Hydroponics, USA) as a nutrient solution according to manufacturer's instructions. After

infiltration of the plants with *Agrobacterium tumefaciens* the temperature was switched to 22 °C during the day and 18 °C during the night period.
Plants were grown with the permission of No. 458 64 16 issued by the Federal Institute for Drugs and Medical Devices (BfArM), Germany.

4.2.3.7. Agroinfiltration of *C. sativa* L. plants

For agroinfiltration the transformed *A. tumefaciens* cells were cultivated in a 5 mL-preculture in YEB medium overnight at 26 °C or 28 °C and 200 rpm with 50 mg L^{-1} kanamycin and 100 mg L^{-1} carbenicillin. The cultivation of the main cultures was done according to an existing protocol (Burch-Smith et al., 2006) with minor changes. Cells from the preculture were inoculated in 30 mL YEB medium with with 50 mg L^{-1} kanamycin and 100 mg L^{-1} carbenicillin as well as 10 mM MES and 20 µM acetosyringone. The cells were grown overnight at 26 °C or 28 °C and 200 rpm. For the infiltration the cells were harvested by centrifugation and washed twice in infiltration buffer (10 mM MES, 10 mM $MgCl_2$, 200 µM acetosyringone). The cells were incubated at room temperature for 4 hours in the dark, after the OD_{600nm} was adjusted to 4. After incubation, cells containing DNA-B (pJRT.Agro.CLCrVB1.3) were mixed in a ratio of 1:1 with cells containing DNA-A with the desired gene fragments of *PDS* or *ChlI.* The mixture was infiltrated into *C. sativa* L. var. Finola seedlings at the 4-leaf stage in the cotyledons and primary leaves with a needleless syringe. After infiltration the plants were cultivated at 22 °C in the light and 18 °C in the dark.

4.2.3.8. cDNA synthesis and quantitative real-time PCR (qPCR)

Plant leaves were harvested and immediately frozen in liquid nitrogen. Total RNA was isolated from ground plant material with an additional DNase digestion step. 800 ng of RNA was used for cDNA synthesis with the LunaScript® RT Supermix Kit (NEB). For qPCR experiments, the cDNA was diluted to a concentration of 5 ng μL^{-1}.
qPCR experiments were performed in 20 µL reactions with the Luna® Universal qPCR Master Mix (NEB) according to manufacturer's instructions. Amplification was performed with the following parameters: 95 °C denaturation for 60 seconds; 40 cycles of denaturation at 95 °C for 15 seconds and extension at 60 °C for 30 seconds. Melting curves were measured afterwards. With the software StepOne V2.2.2 (Applied Biosystems) C_t values were calculated. For quantification, samples were normalized to *ubq5* and *eIFa* and relative fold change was calculated by the use of the $\Delta\Delta C_t$-method (Pfaffl et al., 2004).

Erklärung zur Reproduktion vorab veröffentlichter Inhalte

Teile dieser Arbeit sind bereits von der Autorin veröffentlicht worden oder stammen aus studentischen Arbeiten:

Kapitel 1	1.2	in Teilen modifiziert aus	[A]
Kapitel 2	2.1	in Teilen modifiziert aus	[B], [C]
	2.2.1	in Teilen modifiziert aus	[a]
	2.2.3.3	in Teilen modifiziert aus	[b]

Publikationen

[A] **Schachtsiek, J.,** Warzecha, H., Kayser, O. and Stehle, F**.** (2018) Current Perspectives on Biotechnological Cannabinoid Production in Plants. *Planta Medica*, 84, 214–220.

[B] **Schachtsiek J.** and Stehle F. Nicotine-free, nontransgenic tobacco (*Nicotiana tabacum* L.) edited by CRISPR-Cas9 (2019). *Plant Biotechnology Journal*, doi: 10.1111/pbi.13193

[C] **Schachtsiek J.** and Stehle F. Dataset on nicotine-free, nontransgenic tobacco (*Nicotiana tabacum* L.) edited by CRISPR-Cas9 (2019). *Data in Brief,* 26, 104395, doi: 10.1016/j.dib.2019.104395

Studentische Arbeiten

[a] de Ribeiro e Barreto Ferreira, I. (2018) "Studies on the development of callus cultures of *Cannabis sativa* L. regarding plant regeneration", Masterarbeit, TU Dortmund, Dortmund

[b] Rekend, A (2018) „Ausarbeitung eines Transformationsprotokolls für *Cannabis sativa* L. mittels *Agrobakterium tumefaciens"*, Bachelorarbeit, TU Dortmund, Dortmund

Industrieprojekte

Teilergebnisse aus Kapitel 3.2 bilden die Grundlage für ein industriegefördertes Projekt mit TRAIT Biosciences Inc. (Toronto, Kanada) zur Erstellung von transgenen Cannabispflanzen vom 01.01.2019 bis zum 30.04.2019. Ein Folgeprojekt über zwei Jahre ist ab Juni 2019 geplant.

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Supplementary Material

I. List of Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BA	6-benzylaminopurine
BBL	berberine bridge enzyme-like
BLAST	basic local alignment search tool
bp	base pairs
CaMV	cauliflower mosaic virus
Cas9	crispr associated 9
CBC	cannabichromene
CBCA	cannabichromenic acid
CBCAS	cannabichromenic acid synthase
CBD	cannabidiol
CBDA	cannabidiolic acid
CBDAS	cannabidiolic acid synthase
CBGA	cannabigerolic acid
CBGAS	cannabigerolic acid synthase
ChlI	magnesium chelatase subunit I
CLCrV	cotton leaf crumple virus
CRISPR	clustered, regularly interspaced, short palindromic repeats
DMAPP	dimethylallyl diphosphate
DSB	double strand break
FID	flame ionization detector
fwd	forward
GABA	gamma-Aminobutyric acid
GC	gas chromatography
GC-MS	gas chromatography mass spectrometry
GFP	green fluorescent protein
GMO	genetically modified organism
GPP	geranyl diphosphate
gRNA	guide RNA
HDR	homologous directed repair
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid

IPP	isopentenyl diphosphate
m/z	mass-to-charge ratio
miRNA	micro RNA
MS	Murashige and Skoog
NAA	1-naphthaleneacetic acid
NHEJ	non-homologous end joining
NMR	nuclear magnetic resonance
OA	olivetolic acid
OD	optical density
PAM	protospacer adjacent motif
PCR	polymerase chain reaction
PDS	phytoene desaturase
PGR	plant growth regulator
PPT	phosphinothricin
PTGS	post transcriptional gene silencing
qPCR	quantitative real-time PCR
rev	reverse
RISC	RNA-induced silencing complex
RNAi	RNA interference
rpm	rounds per minute
siRNA	small interfering RNA
TALEN	transcription activator-like effector nuclease
TDZ	thidiazuron
THC	tetrahydrocannabinol
THCA	tetrahydrocannabinolic acid
THCAS	tetrahydrocannabinolic acid synthase
TRV	tobacco rattle virus
UBQ	ubiquitin
VIGS	virus-induced gene silencing
YEB	yeast Extract Broth
YPF	yellow fluorescent protein
ZNF	zinc-finger nuclease

II. List of Tables

III. List of Figures

IV. Nicotine-free, non-transgenic tobacco (*Nicotiana tabacum* L.) edited by CRISPR-Cas9

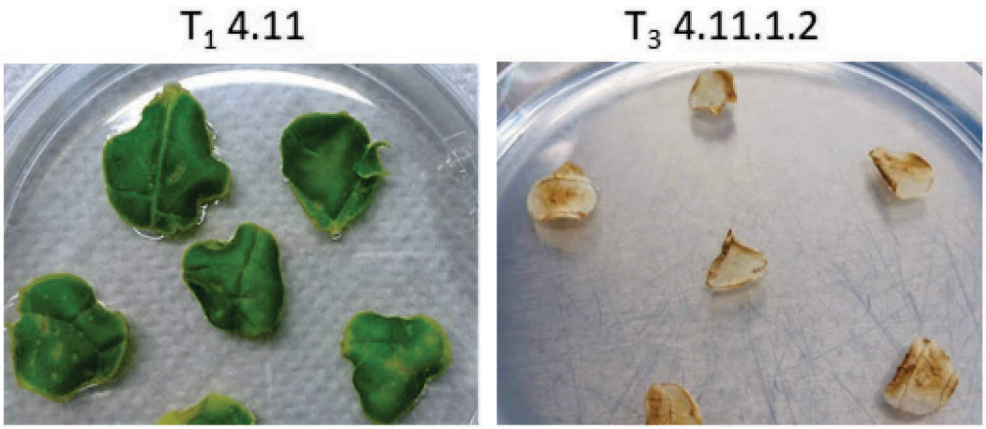

Figure S IV-1 Test for non-transgenity of the nicotine-free plant: "Leaf discs of the plants T_1 4.11 and T_3 4.11.1.2 were incubated in MS-medium with 6 mg L^{-1} PPT to test for the presence of the transformation cassette including the selection marker." Modified from Schachtsiek and Stehle (2019a)

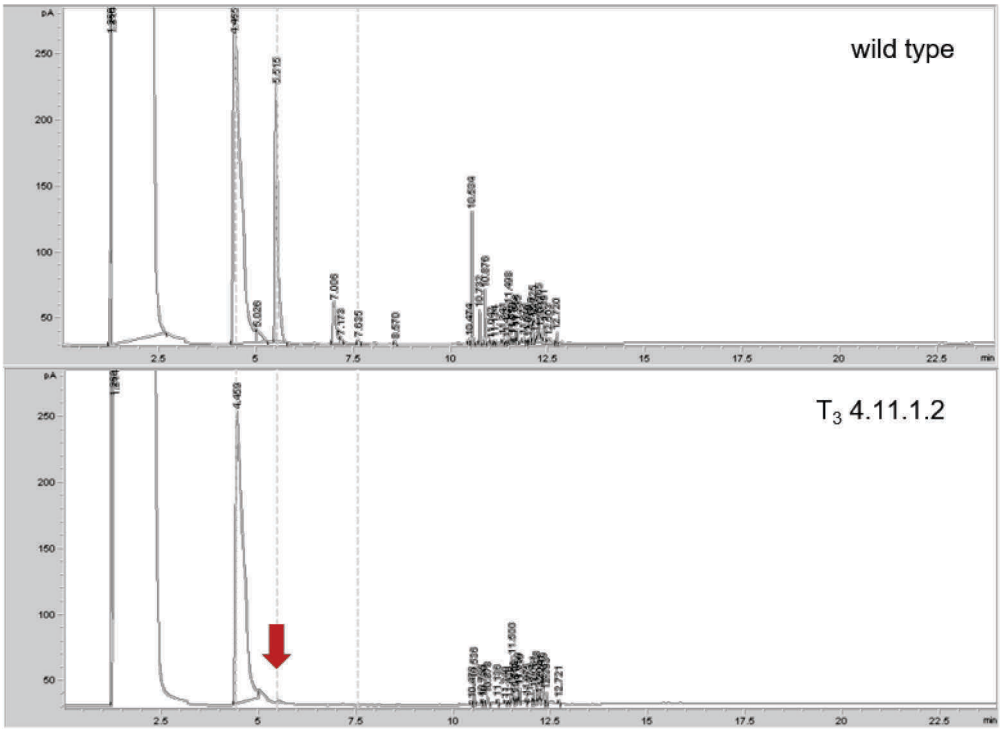

Figure S IV-2 GC chromatogram of crude extracts of *Nicotiana tabacum* leaves: "comparison of crude extracts of tobacco leaves of the wild type and the nicotine-free plant (T_3 4.11.1.2). The red arrow marks the retention time of nicotine. (1.8 min - injection peak; 4.4 min - internal standard (quinoline); 5.5 min – nicotine; 7.6 min – anatabine)". modified from Schachtsiek and Stehle (2019a)

Figure S IV-3 Comparison of the phenotype of the wild type and the nicotine-free plant (T_3 4.11.1.2) modified from Schachtsiek and Stehle (2019b)

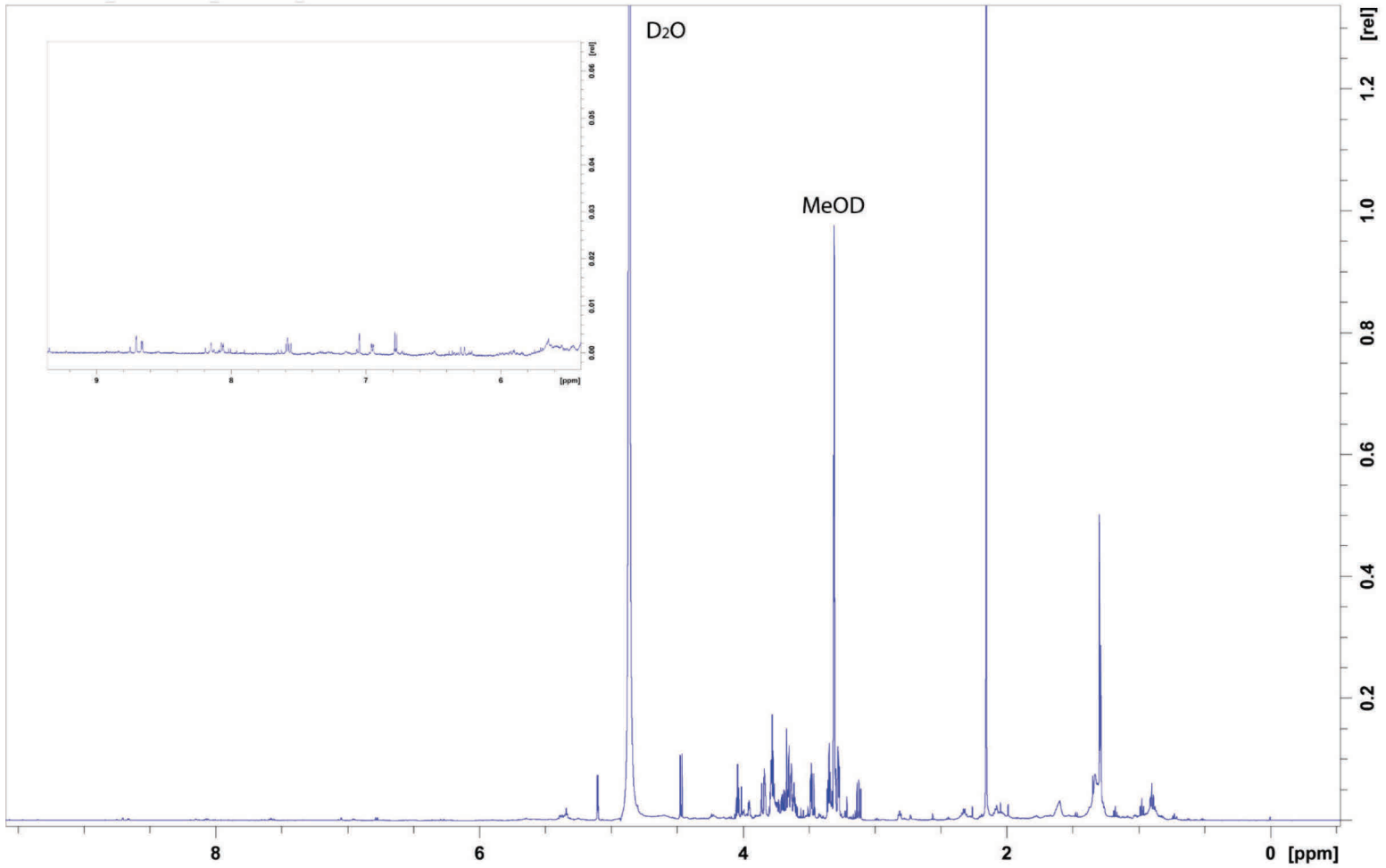

Figure S IV-4 ^{1}H-NMR spectrum of wild type leaf-extract in MeOD: "20 mg leaf material of wild type leaves were freeze-dried and extracted in MeOD. The region of aromatics (6 ppm-9 ppm) is highlighted in the zoom-in figure." modified from Schachtsiek and Stehle (2019a)

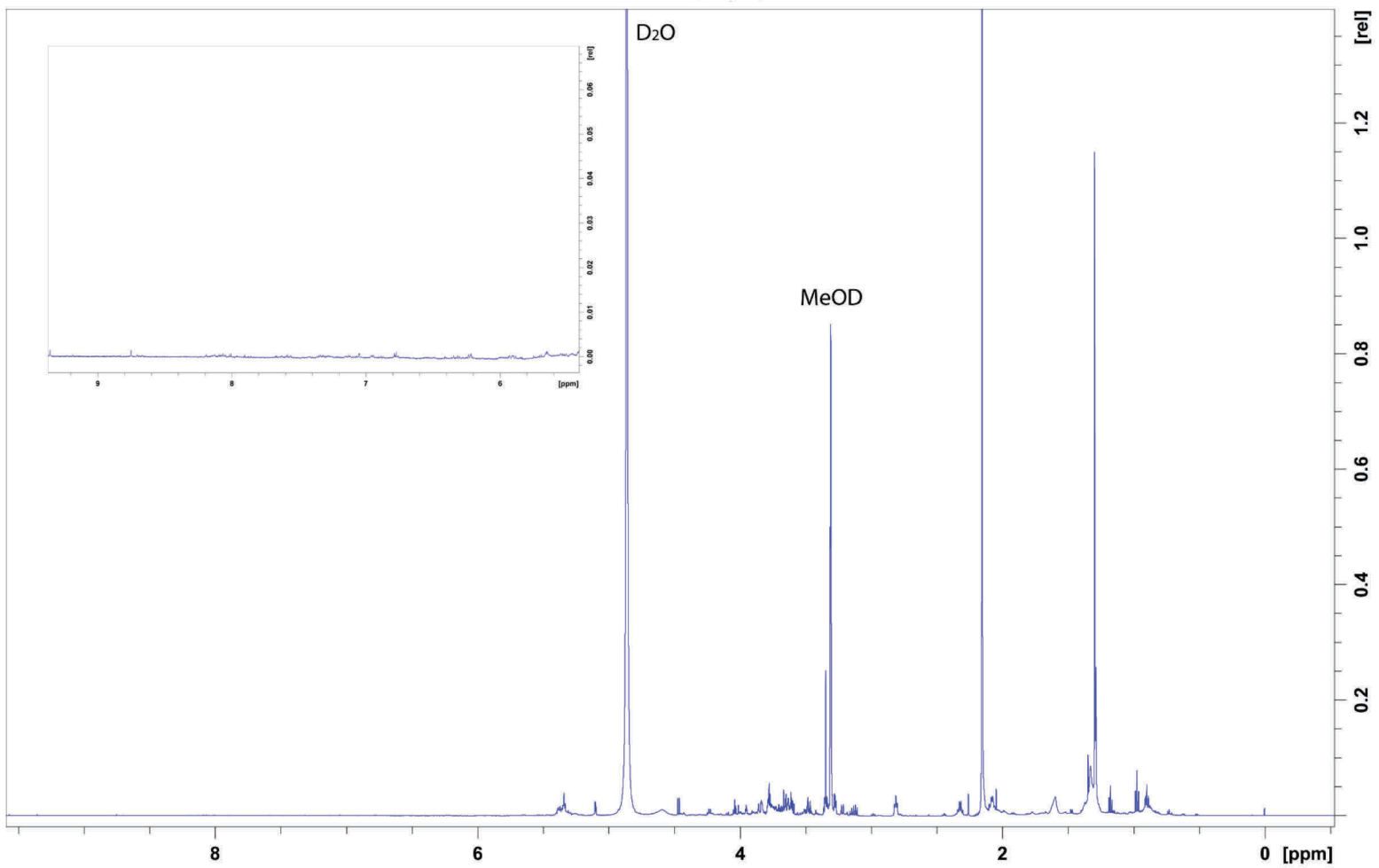

Figure S IV-5 ^{1}H-NMR spectrum of nicotine-free plant leaf-extract in MeOD: "20 mg leaf material of nicotine-free plant leaves were freeze-dried and extracted in MeOD. The region of aromatics (6 ppm-9 ppm) is highlighted in the zoom-in figure. modified from Schachtsiek and Stehle (2019a)

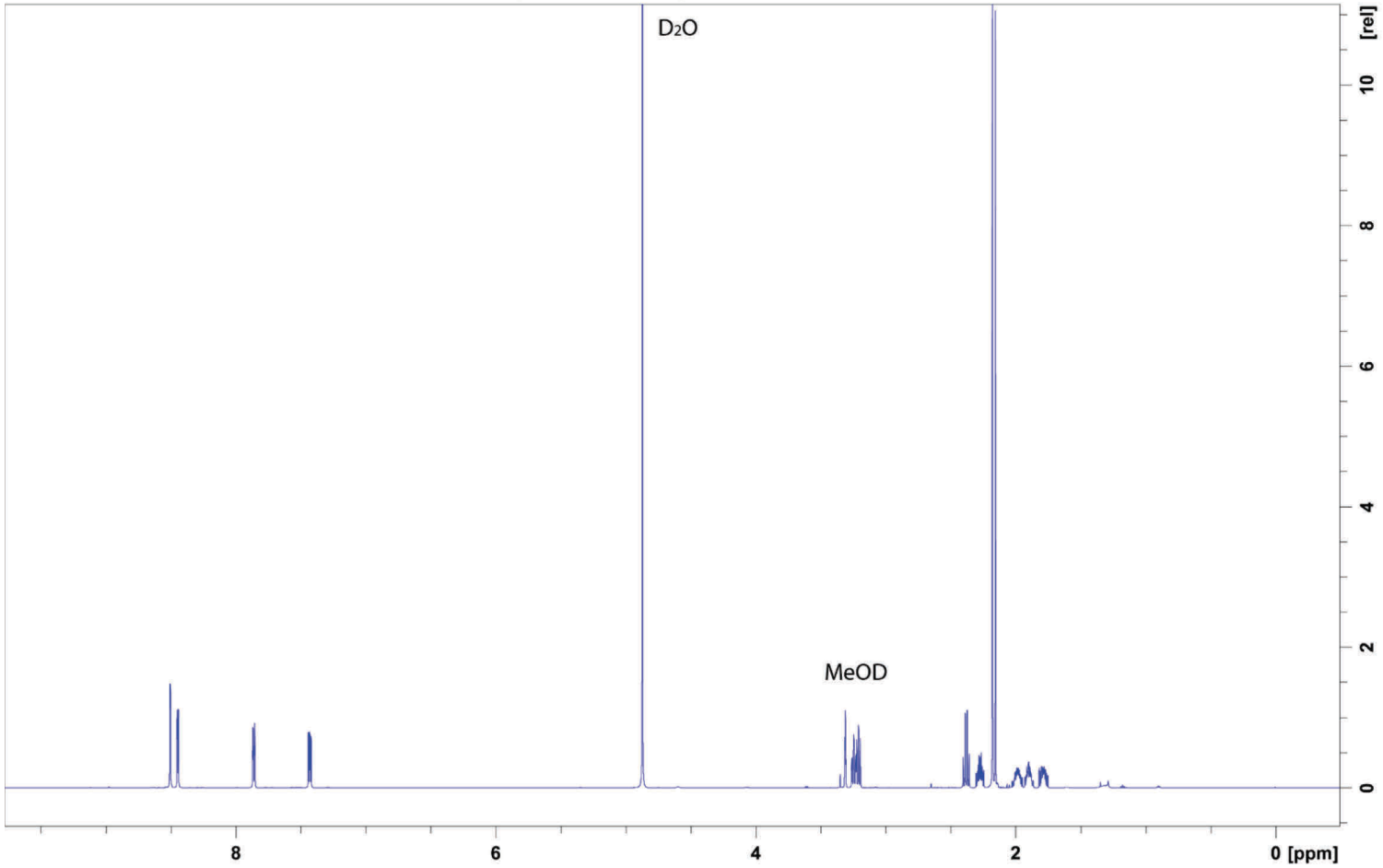

Figure S IV-6 ^{1}H-NMR spectrum of nicotine in MeOD. modified from Schachtsiek and Stehle (2019a)

Table S IV-1 Primer used in this study (modified from Schachtsiek and Stehle (2019a)

Primer Name	Sequence (5'→ 3')	purpose
BBL_gRNA_fwd	ATTGGAAATCAGAGTAAGGTGCGG	sgRNA target
BBL_gRNA_rev	AAACCCGCACCTTACTCTGATTTC	
SS129	CACAGGAAACAGCTATGAC	Colony PCR pChimera-BBL
SS42	TCCCAGGATTAGAATGATTAGG	Colony PCR; Sequencing pChimera-BBL
SS43	CGACTAAGGGTTTCTTATATGC	Colony PCR;
SS61	GAGCTCCAGGCCTCCCAGCTTTCG	Sequencing pCas9-BBL
BBLa_Gib_fwd	TCACACTGGCGGCCGCTCGAGCATGCATACTGC TACTGGAGCTGTTAC	Gibson Assembly pDionysos
BBLa_Gib_rev	ATAACTAATTACATGATGCGGCCCTTGCAGGTC TCAGCAGTACTC	
BBLb_Gib_fwd	TCACACTGGCGGCCGCTCGAGCATGCATCTCTG CTACTGCAACTAGTGGA	Gibson Assembly pDionysos
BBLb_Gib_rev	ATAACTAATTACATGATGCGGCCCTATTTCCTCC TCCGCCACCTC	
BBLc_Gib_fwd	TCACACTGGCGGCCGCTCGAGCATGCATTGGAG CAGGAGAAGGAGT	Gibson Assembly pDionysos
BBLc_Gib_rev	ATAACTAATTACATGATGCGGCCCTGGGCAACG TATTGTTTGGA	
BBLd1_Gib_fwd	TCACACTGGCGGCCGCTCGAGCATGCATTTCGG TCTCTGCAACAAC	Gibson Assembly pDionysos
BBLd1_Gib_rev	ATAACTAATTACATGATGCGGCCCTGAAACTGG TCACGGTCTT	
BBLd2_Gib_fwd	TCACACTGGCGGCCGCTCGAGCATGCATCTCTT CAGCGTTTGCTCATA	Gibson Assembly pDionysos,
BBLd2_Gib_rev	ATAACTAATTACATGATGCGGCCCTCAAATCTA CCGAAACATCATCT	
BBLe_Gib_fwd	TCACACTGGCGGCCGCTCGAGCATGCATGGAGC AGGAGGAGTTACAAATC	

BBLe_Gib_rev	ATAACTAATTACATGATGCGGCCCTTGGCGTCA TCATTCTTAGCG	Gibson Assembly pDionysos
pDio_seq_fwd	CGGTTTGTATTACTTCTTATTC	Colony PCR
pDio_seq_rev	GATGTGGGGGGAGGGCGTGAATGTA	Colony PCR; sequencing
PCR_BBLa_fwd	ACTGCTACTGGAGCTGTTAC	amplification BBLa
BBLa_rev	TGCAGGTCTCAGCAGTACTC	
BBLa_seq_fwd	GCACCTTTCATGCCGAAACC	sequencing *BBLa*
PCR_BBLb_fwd	TGGAGCAGGAGGTGGAGTTG	amplification *BBLb*
BBLb_rev	ATTTCCTCCTCCGCCACCTC	
BBLb_seq_fwd	GCATCTTACATGCCGAAACC	sequencing *BBLb*
PCR_BBLc_fwd	TGGAGCAGGAGAAGGAGTTG	amplification *BBLc*
BBLc_rev	GGGCAACGTATTGTTTGGAG	
BBLc_seq_fwd	GCATCTAACATGCCGAAACC	sequencing *BBLc*
PCR_BBLd1_fwd	TTCGGTCTCTGCAACAACTC	amplification *BBLd1*
BBLd1_rev	GAAACTGGTCACGGTCTTGG	
BBLd1_seq	ATTCGCAGCGTGCTCTAAAC	sequencing *BBLd1*
BBLd2_seq	CCGTCTCTGCTACAAATCTC	sequencing *BBLd2*

V. Development of a regeneration and transformation protocol for *Cannabis sativa* L.

Table S V-1 Primers used for cloning of pBIN-Vac-GFP Basta

Primer name	Sequence (5‘ → 3‘)	purpose
GFP_vac_fwd	ATGATGAGAGCAAGGGTTCCACTCCTGTTGCTGGGA ATTCTTTTCCTGGCATCACTTTCTGCCTCATTTGCTA TGGGTAAAGGAGAACTTTTCAC	Generation of Vac-GFP
GFP_vac_rev	TCAGTACACAAAGGCGATAGATCTGTATAGTTCATC CATGCCATGTGTA	
Vac_GFP_(o) Vec_fwd	ATTTTCACCATTTACGAACGATAGCATGATGAGAGC AAGGGTTCC	Amplification of Vac-GFP
Vac_GFP_(o) Vec_rev	GAGAGACTGGTGATTTTTGCGGACTTCAGTACACAA AGGCGATAGA	
Seq_GFP_Vac _fwd	CCCTTATCTGGGAACTACTC	Sequencing of Vac-GFP; colony PCR
PCR_GFP_Va c_rev	GGACCTCGAGAATTCTCAAC	Colony PCR

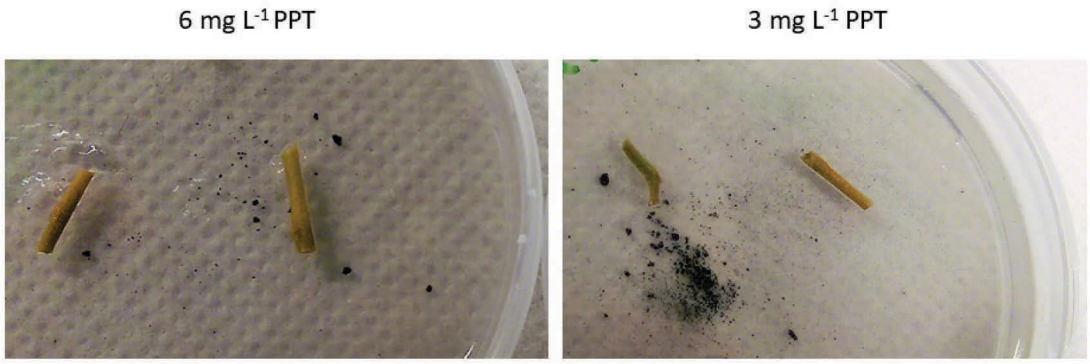

Figure S V-1 Testing of concentrations of PPT for testing of transgenic plants. Axillary buds were surface sterilized and transferred to shooting medium with either 3 mg L^{-1} or 6 mg L^{-1} PPT and incubated under normal growth conditions. Pictures were taken after 1 week of incubation.

Figure S V-2: Axillary buds transformed with A. tumefaciens and incubated on selection medium: Axillary buds were transformed with the strain LBA4404 and transferred to selection medium. Most of the explants died and grown shoots died shortly after transfer to new selection medium

Figure S V-3 Reinfection of transformed *C. sativa* shoots with *A. tumefaciens*: Regenerated shoots from transformed axillary buds were transferred to shooting medium. After approximately a week of incubation reinfection with *Agrobacteria* occurred.

VI. Virus-induced gene silencing in *Cannabis sativa* L.

Table S VI-1 Primers used in the study for construction of the vectors

Primer Name	Sequence 5' to 3'	Purpose
pDio_PDS_fwd	TCACACTGGCGGCCGCTCGAGCATGCATATGTCTCAGTGGGGTTCTGTT	Validation of *PDS*
pDio_PDS_rev	ATAACTAATTACATGATGCGGCCCTTCAACGACTTCCAGCCTCGG	
pDio_ChlI_fwd	TCACACTGGCGGCCGCTCGAGCATGCATATGGCATCCGTCCTTGGAA	Validation of *ChlI*
pDio_ChlI_rev	ATAACTAATTACATGATGCGGCCCTTCAGTTGAAAACCTCATAAAATTTCTC	
Gib-PDS1_fw	TGGCCAGTTAACGCTAGCGAATTCAATTGAAGAACACATATGATCAC	Gibson pCotton-PDS1
Gib-PDS1_rv	TAGAGCAAAATGGCATGCCTGCAGAACCTTCCATTGAAGCCAAA	
Gib-PDS2_fw	TGGCCAGTTAACGCTAGCGAATTCATCCCAACATACAGAACTTG	Gibson pCotton-PDS2
Gib-PDS2_rv	TAGAGCAAAATGGCATGCCTGCAGATGAAAGTTCATCTGGGTTAAT	
Gib-PDS3_fw	TGGCCAGTTAACGCTAGCGAATTCAAGAAGAAGGGTGTTTTGCCTTT	Gibson pCotton-PDS3
Gib-PDS3_rv	TAGAGCAAAATGGCATGCCTGCAGAAGTTCTGTATGTTGGGATAAGC	
Gib-PDS4_fw	TGGCCAGTTAACGCTAGCGAATTCAACCGTCAAAGATTGGATG	Gibson pCotton-PDS4
Gib-PDS4_rv	TAGAGCAAAATGGCATGCCTGCAGAGATATCAACTGGTGTTGCAAA	
Gib-ChlI1_fw	TGGCCAGTTAACGCTAGCGAATTCAGGTGACCCTTACAACTCAGA	Gibson pCotton-ChlI1
Gib-ChlI1_rv	TAGAGCAAAATGGCATGCCTGCAGAAGCAGCAGAATCCAATAAAACA	
Gib-ChlI2_fw	TGGCCAGTTAACGCTAGCGAATTCAGGGCAATGTGAATCGGTTGT	

Gib-ChlI2_rv	TAGAGCAAAATGGCATGCCTGCAGAGGAAGTAAATCAA CCAAAGACCTTACA	Gibson pCotton-ChlI2
SS129	CACAGGAAACAGCTATGAC	colony PCR
PCR_VIGS_rv	ACGTCCAGATCCGATTCAAC	colony PCR

Table S VI-2 Coding sequences of *PDS* and *ChlI* of C. sativa L. (Cs) var. Finola

Name	Sequence
CsPDS	ATGTCTCAGTGGGGTTCTGTTTCAGCTACTAACTTCAGCTCCCACCAGA GTCGTATCTTTGAATTTCCAAACGTAGGAACAGTACCCAGATGTTGTTT TTCATTGGGTTCCAAAAAAATGGCGTCTTTGGCTTTTGGGTCTAATCAA TTTATGCCTCCTGGTACAAGACTGAAGAAGAAGAAGGGTGTTTTGCCTT TAAAGGTGGTTTGTGTGGATTATCCAAGACCAGAGCTTGATAGTACTGT TAACTTTTTGGAAGCTGCGGCTTTGTCTGCTTCTTTTCGTGGCTCTCCT CGTCCAGCTAAGCCTTTGAAAGTTGTAATTGCTGGTGCAGGTTTGGCTG GTTTATCTACTGCAAAATACTTGGCAGATGCAGGTCATAAACCGATATT ACTCGAAGCAAGAGATGTTTTAGGTGGAAAGGTGGCAGCATGGAAAGAT GATGACGGTGACTGGTATGAGACCGGTCTACATATATTCTTTGGAGCTT ATCCCAACATACAGAACTTGTTCGGAGAGCTAGGTATTGATGATCGGTT GCAATGGAAAGAACATTCAATGATCTTTGCAATGCCCAGCAAGCCAGGA GAATTCAGCCGATTTGATTTCACCGATGCTCTGCCAGCTCCCTTAAATG GAATATGGGCCATTTTACGGAACAATGAGATGCTGACCTGGGCAGAAAA AGTCAAATTTGCAATTGGGCTTCTGCCTGCAATGGTTGGCGGACAGCCT TATGTCGAAGCTCAAGATGGTTTCACCGTCAAAGATTGGATGAGAAAGC AGGGCATACCTGATCGTGTAACGGATGAGGTCTTCATTGCCATGTCCAA GGCCTTAAACTTCATTAACCCAGATGAACTTTCAATGCAATGTATATTG ATTGCTTTAAACCGATTTCTTCAGGAGAAGCATGGTTCCAAGATGGCTT TCCTAGATGGAAATCCCCCAGAGAGACTCTGTATGCCCATAGTCGATCA TATCCAGTCATTGGGCGGTGAAGTCCGACTTAATTCCCGTATACAAAAA ATCGACCTGAATGATGATGGAACTGTAAAGAGATTTTTACTAACTAATG GTAGTGAAATCGAAGGGGATGCATATGTGTTTGCAACACCAGTTGATAT CCTCAAGCTTCTATTGCCCGAAAACTGGAAAGAGATTCCGTATTTCAAG AAATTGGATAAATTAGTTGGCGTCCCCGTTATTAATGTCCACATATGGT TCGACCGAAAATTGAAGAACACATATGATCACCTTCTTCTCAGCAGAAG TCCTCTCTTGAGTGTCTACGCCGATATGTCGGTAACTTGTAAGGAATAT TACAATCCAAACCAGTCTATGCTAGAGTTGGTTTTCGCACCAGCAGAAG AATGGATTTCGCGTAGTGACTCAGATATTATCGATGCTACGATGAAGGA GCTTTCTAAGCTCTTTCCTGATGAAATAGCAGCTGATCAGAGCAAAGCG AAAATCTTAAAGTACCATGTTGTAAAAACACCGAGGTCTGTCTACAAAA CTGTTCCTGATTGCGAACCTTGTCGCCCCAGACAAAGATCTCCTCTTGA AGGTTTCTATTTAGCAGGTGACTACACAAAACAAAAATATTTGGCTTCA ATGGAAGGTGCGGTTTTGTCAGGAAAGTTTTGTGCACAAGCAATTGTAC AGGATTATGAGTTGCTTGCTGCTCGGGGTCAGAGAATTTTGGCCGAGGC TGGAAGTCGTTGA
CsChlI	ATGGCATCCGTCCTTGGAACTTCATCGTCAGCAATCTTGGCTTCTCGCTTCCTG TCTTCTCCCTCCTCGAAGACTTCCAGTCCCTCTTTTTCTTTCAACCCAGGGCAG GGCAATGTGAATCGGTTGTATGGAGGAACCGGGATTCAGGTAAAAAAGGGGAGG TCTCAGTTCCACGTGGCAGTTACCAATGTTGCTACTGAAATCAACTCTGTGGAA

CAGGACGGGAAGCTTAAAGCTAAGAATAGTCAGAGGCCGGTTTACCCGTTTGCA
GCCATAGTAGGACAAGATGAGATGAAACTTTGTCTTCTCCTAAATGTGATTGAC
CCCAAGATTGGGGGTGTCATGATTATGGGTGATAGAGGAACTGGAAAATCCACC
ACTGTAAGGTCTTTGGTTGATTTACTTCCCGAAATTAAGGTTGTTTCTGGTGAC
CCTTACAACTCAGATCCAGAAGATCCAGAGTCTATGGGCGTGGAAGTCAGAGAG
AGTGTCTTGAAAGGGGACCAGCTTTCTGTTGTCTTGACTAAAATCAACATGGTT
GATTTACCTTTGGGTGCTACGGAAGATAGGGTATGCGGGACAATTGACATTGAG
AAAGCTCTGACTGAGGGTGTCAAAGCCTTTGAGCCTGGCCTTCTTGCAAAGGCC
AACAGAGGAATTCTATATGTAGATGAAGTAAATCTTTTGGATGATCACTTGGTT
GATGTTTTATTGGATTCTGCTGCTTCTGGTTGGAACACTGTGGAGAGAGAAGGT
ATTTCAATTTCACATCCTGCTCGGTTTATTTTGATTGGCTCGGGAAATCCAGAA
GAAGGGGAGCTCAGGCCACAACTTTTGGACCGTTTTGGAATGCATGCACAAGTG
GGTACCGTAAGGGATGCAGAGCTTAGAGTGAAGATTGTTGAGGAGAGATCTCGG
TTTGATAAAAATCCCAACGAATTCCGCGAATCATATGACGCCGAGCAAGAAAAA
CTGCAACAGCAAATTACTGCAGCTAGAAGTACTCTATCTTCTGTTCAGATCGAC
CATGAACTCAAAGTGAAAATTTCAAAGGTTTGTTCAGAGCTGAATGTTGATGGA
TTGAGAGGGGACATTGTAACAAACAGGGCTGCAAAAGCTTTGGCTGCTTTGAAG
GGAAGAGATAAAGTATCAACAGAGGATATTGCTACTGTCATCCCTAACTGCTTA
AGACATCGTCTTCGGAAGGATCCCTTGGAGTCAATTGATTCAGGTCTACTTGTC
ATTGAGAAATTTTATGAGGTTTTCAACTGA

Table S VI-3 Predicted siRNA sequences and silencing fragments

Name	siRNA	Sequence fragment
PDS-1_siRNA	CAUAUGAUCACCUUCUUCU	ATTGAAGAACACATATGATCACCTTCTT CTCAGCAGAAGTCCTCTCTTGAGTGTCT ACGCCGATATGTCGGTAACTTGTAAGGA ATATTACAATCCAAACCAGTCTATGCTA GAGTTGGTTTTCGCACCAGCAGAAGAAT GGATTTCGCGTAGTGACTCAGATATTAT CGATGCTACGATGAAGGAGCTTTCTAAG CTCTTTCCTGATGAAATAGCAGCTGATC AGAGCAAAGCGAAAATCTTAAAGTACCA TGTTGTAAAAACACCGAGGTCTGTCTAC AAAACTGTTCCTGATTGCGAACCTTGTC GCCCCAGACAAAGATCTCCTCTTGAAGG TTTCTATTTAGCAGGTGACTACACAAAA CAAAAATATTTGGCTTCAATGGAAGGT
PDS-2_siRNA	CCAACAUACAGAACUUGUU	TCCCAACATACAGAACTTGTTCGGAGAG CTAGGTATTGATGATCGGTTGCAATGGA AAGAACATTCAATGATCTTTGCAATGCC CAGCAAGCCAGGAGAATTCAGCCGATTT GATTTCACCGATGCTCTGCCAGCTCCCT TAAATGGAATATGGGCCATTTTACGGAA CAATGAGATGCTGACCTGGGCAGAAAAA GTCAAATTTGCAATTGGGCTTCTGCCTG CAATGGTTGGCGGACAGCCTTATGTCGA AGCTCAAGATGGTTTCACCGTCAAAGAT TGGATGAGAAAGCAGGGCATACCTGATC GTGTAACGGATGAGGTCTTCATTGCCAT GTCCAAGGCCTTAAACTTCATTAACCCA GATGAACTTTCA

PDS-3_siRNA	GCUUAUCCCAACAUACAGA	AGAAGAAGGGTGTTTTGCCTTTAAAGGT GGTTTGTGTGGATTATCCAAGACCAGAG CTTGATAGTACTGTTAACTTTTTGGAAG CTGCGGCTTTGTCTGCTTCTTTTCGTGG CTCTCCTCGTCCAGCTAAGCCTTTGAAA GTTGTAATTGCTGGTGCAGGTTTGGCTG GTTTATCTACTGCAAAATACTTGGCAGA TGCAGGTCATAAACCGATATTACTCGAA GCAAGAGATGTTTTAGGTGGAAAGGTGG CAGCATGGAAAGATGATGACGGTGACTG GTATGAGACCGGTCTACATATATTCTTT GGAGCTTATCCCAACATACAGAACT
PDS-4_siRNA	GUUUGCAACACCAGUUGAU	ACCGTCAAAGATTGGATGAGAAAGCAGG GCATACCTGATCGTGTAACGGATGAGGT CTTCATTGCCATGTCCAAGGCCTTAAAC TTCATTAACCCAGATGAACTTTCAATGC AATGTATATTGATTGCTTTAAACCGATT TCTTCAGGAGAAGCATGGTTCCAAGATG GCTTTCCTAGATGGAAATCCCCCAGAGA GACTCTGTATGCCCATAGTCGATCATAT CCAGTCATTGGGCGGTGAAGTCCGACTT AATTCCCGTATACAAAAAATCGACCTGA ATGATGATGGAACTGTAAAGAGATTTTT ACTAACTAATGGTAGTGAAATCGAAGGG GATGCATATGTGTTTGCAACACCAGTTG ATATC
ChlI-1_siRNA	AUCCCAUACGCCCUGUUAA CU	GGTGACCCTTACAACTCAGATCCAGAAG ATCCAGAGTCTATGGGCGTGGAAGTCAG AGAGAGTGTCTTGAAAGGGGACCAGCTT TCTGTTGTCTTGACTAAAATCAACATGG TTGATTTACCTTTGGGTGCTACGGAAGA TAGGGTATGCGGGACAATTGACATTGAG AAAGCTCTGACTGAGGGTGTCAAAGCCT TTGAGCCTGGCCTTCTTGCAAAGGCCAA CAGAGGAATTCTATATGTAGATGAAGTA AATCTTTTGGATGATCACTTGGTTGATG TTTTATTGGATTCTGCTGCT
ChlI-2_siRNA	GUCUCCGGCCAAAUGGGCA AA	GGGCAATGTGAATCGGTTGTATGGAGGA ACCGGGATTCAGGTAAAAAAGGGGAGGT CTCAGTTCCACGTGGCAGTTACCAATGT TGCTACTGAAATCAACTCTGTGGAACAG GACGGGAAGCTTAAAGCTAAGAATAGTC AGAGGCCGGTTTACCCGTTTGCAGCCAT AGTAGGACAAGATGAGATGAAACTTTGT CTTCTCCTAAATGTGATTGACCCCAAGA TTGGGGGTGTCATGATTATGGGTGATAG AGGAACTGGAAAATCCACCACTGTAAGG TCTTTGGTTGATTTACTTCC

Table S VI-4 Predicted sequences of reference genes

Name	Sequence
Cs_actin	ATGGCCGACGCAGAGGACATTCAGCCCCTCGTTTGTGACAATGGTACTGGAA TGGTGAAGGCTGGGTTTGCTGGCGATGATGCGCCCAGGGCAGTCTTTCCCAG TATTGTTGGTCGTCCTAGACACACTGGTGTTATGGTTGGAATGGGACAGAAG GACGCATACGTAGGTGATGAAGCCCAGTCTAAAAGAGGTATCCTTACTTTGA AATATCCCATCGAGCATGGTATTGTTAGCAACTGGGATGATATGGAGAAAAT TTGGCATCATACTTTCTACAATGAACTTCGTGTTGCCCCAGAAGAGCACCCA GTGCTTCTCACTGAGGCTCCTTTGAATCCTAAGGCTAACAGAGAAAAGATGA CCCAGATCATGTTTGAGACATTCAATGTTCCAGCTATGTATGTGGCCATCCA GGCAGTTTTGTCCCTTTACGCTAGTGGACGTACAACTGGTATCGTTCTGGAT TCTGGTGATGGTGTGAGTCACACTGTGCCAATCTACGAAGGTTATGCCCTTC CCCATGCCATTCTTCGTCTTGACCTTGCTGGGCGTGATCTCACTGATTCTTT GATGAAGATTCTCACTGAGAGAGGCTACATGTTCACCACCACTGCCGAACGG GAAATTGTCCGTGACATGAAGGAGAAGCTTGCTTATGTTGCCTTGGACTATG AGCAGGAACTCGAGACTGCCAAGAGCAGCTCCTCAGTTGAGAAGAACTACGA GTTGCCCGATGGCCAGATTATCACCATTGGAGCTGAGAGATTCCGATGCCCA GAAGTCCTCTTCCAACCATCCCTCATTGGAATGGAAGCTGCAGGAATTCACG AGACCACCTACAATTCCATCATGAAGTGTGATGTGGATATCAGAAAGGATCT CTACGGTAACATTGTTCTCAGTGGTGGTTCTACTATGTTCCCTGGTATTGCA GACAGGATGAGCAAGGAGATCACAGCTCTTGCACCAAGCAGCATGAAGATTA AGGTTGTGGCTCCACCGGAGAGAAAATACAGTGTCTGGATCGGAGGGTCCAT TCTTGCATCCCTCAGCACCTTCCAGCAGATGTGGATTTCCAAGGGCGAATAC GATGAGTCAGGTCCATCCATTGTCCACAGGAAGTGCTTCTAA
Cs_beta tubulin (partial)	ATGAGGGAAATCTTGCATATTCAAGGAGGTCAATGTGGTAACCAAATCGGTT CCAAGTTCTGGGAAGTAATCTGTAACGAGCATGGTGTAGACCCTACCGGGAG GTACCAGAGCGATGGTGCTGCTGATCTTCAGTTGGAGAGGATTAATGTCTAC TACAATGAGGCTTCTGGAGGAAGGTACGTTCCTCGGGCTGTTCTTATGGATC TCGAACCTGGAACTATGGATAGCATCAGATCGGGTCCTTATGGACAGATCTT TCGCCCTGATAACTTCGTTTTCGGCCAGTCCGGTGCTGGAAACAATTGGGCC AAAGGTCACTACACCGAAGGCGCTGAGTTGATCGATTCGGTACTTGATGTTG TTCGTAAAGAGGCTGAAAACTGTGACTGTCTTCAAGGTTTTCAGGTATGTCA CTCACTTGGAGGAGGGACTGGTTCTGGTATGGGAACACTTCTCATATCAAAG ATCAGAGAGGAATATCCAGACAGAATGATGCTCACATTCTCAGTTTTCCCTT CTCCAAAGGTCTCTGACACAGTTGTGGAACCATACAATGCCACCCTCTCGGT GCATCAACTGGTTGAAAACGCTGATGAGTGCATGGTTCTTGATAATGAAGCA CTTTATGATATTTGCTTCAGGACTCTAAAACTCAGCACACCAAGTTTTGGCG ACTTGAACCATTTGATATCTGCAACTATGAGTGGTGTAACTTGCTGCCTGAG GTTCCCTGGGCAACTCAACTCGGACCTTCGTAAGCTGGCTGTTAATTTGATT CCATTCCCGCGACTTCACTTCTTCATGGTGGGGTTTGCACCTCTGACTTCTC GTGGATCCCAACAGTACATCTCCCTCACTGTGCCAGAGCTTACTCAGCAAAT GTGGGATGCCAAGAACATGATGTGCGCAGCTGACCCTCGCCATGGCCGATAC CTGACTGCCTCGGCTATGTTCAGGGGTAAGATGAGTACTAAAGAGGTGGATG AACAGATGATCAATGTGCAAAATAAGAACTCATCTTACTTTGTTGAGTGGAT CCCAAACAACGTGAAATCAAGTGTTTGTGATATTCCACCATTGGGGCTTAAA ATGGCGTCTACCTTTGTTGGTAACTCAACGTCGATCCAGGAGATGTTCAGGA GGGTGAGCGAGCAGTTCACAGCTATGTTCCGTCGCAAGGCCTTTTTGCATTG GTACACAGGAGAAGGAATGGACGAGATGGAGTTCACAGAGGCAGAGAGTAAC ATGAATGATTTGGTCGCTGAGTATCAACAGTACCAGGACGCCACTGCTGATG AGGAAGGTGAATATGAAGAAGAAGAAGAA
Cs_18S_rRNA (partial)	TACCTGGTTGATCCTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCAT GCATGTGTAAGTATGAACTAATTCAGACTGTGAAACTGCGAATGGCTCATTA AATCAGTTATAGTTTGTTTGATGGTATCTGCTACTCGGATAACCGTAGTAAT TCTAGAGCTAATACGTGCAACAAACCCCGACTTCTGGAAGGGATGCATTTAT TAGATAAAAGGTCGACGCGGGCTCTGCCCGTTGCTCTGATGATTCATGATAA

	CTCGACGGATCGCACGGCCTTCGTGCCGGCGACGCATCATTCAAATTTCTGC CCTATCAACTTTCGATGGTAGGATAGTGGCCTACTATGGTGGTGACGGGTGA CGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACA TCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGACACGGGGAGGTA GTGACAATAAATAACAATACCGGGCTCTACGAGTCTGGTAATTGGAATGAGT ACAATCTAAATCCCTTAACGAGGATCCATTGGAGGGCAAGTCTGGTGCCAGC AGCCGCGGTAATTCCAGCTCCAATAGCGTATATTTAAGTTGTTGCAGTTAAA AAGCTCGTAGTTGGACCTTGGGTTGGGTCGATCGGTCCGCCTCCGGTGTGCA CCGGTCGGCTCGTCCCTTCTACCGGCGATGCGCTCCTGGCCTTAATTGGCCG GGTCGTGCCTCCGGTGCTGTTACTTTGAAGAAATTAGAGTGCTCAAAGCAAG CCTACGCTCTGTATACATTAGCATGGGATAACATCATAGGATTTCGGTCCTA TTCTGTTGGCCTTCGGGATCGGAGTAATGATTAACAGGGACAGTCGGGGGCA TTCGTATTTCATAGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACAA CTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTGGG GGCTCGAAGACGATCAGATACCGTCCTAGTCTCAACCATAAACGATGCCGAC CAGGGATTGGCGGATGTTGCTTTTAGGACTCCGCCAGCACCTTATGAGAAAT CAAAGTTTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGG AATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACCCA ACACGGGGAAACTTACCAGGTCCAGACATAGTAAGGATTGACAGATTGAGAG CTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAG CGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAG CTATGCGGAGG
Cs_GAPDH (partial)	GACAAGAAGATCAAGATCGGAATCAACGGTTTCGGAAGAATTGGACGTTTGG TTGCTAGAGTTGCTCTCCAGAGGGACGATGTTGAGCTTGTCGCTGTTAACGA TCCATTTATCACCACTGATTACATGACATACATGTTTAAGTACGATTCCGTT CATGGACCATGGAAGCATCATGAGCTTAAGGTCAAGGACTCTAAGACCCTTC TCTTCGGTGAGAAGCCCGTCACTGTTTTCGGTGTCAGGAACCCAGAAGAAAT CCCATGGGGCGAGACCGGTGCCGACTTTGTTGTTGAATCAACTGGAGTTTTC ACTGACAAGGACAAAGCTGCTGCTCACTTGAAGGGTGGTGCTAAGAAGGTTA TCATCTCTGCCCCAAGTAAAGATGCACCCATGTTCGTTGTTGGTGTCAATGA GAACGAATACAAGCCAGAGTACGATATTGTTTCCAATGCTAGTTGCACTACC AATTGCCTTGCCCCATTGGCCAAGGTTATCAACGACAGGTTTGGAATTGTTG AGGGTTTGATGACCACCGTCCACTCCATCACTGCTACCCAGAAAACTGTTGA TGGACCATCAAGCAAGGACTGGAGAGGCGGAAGAGCCGCTTCCTTCAACATC ATTCCCAGCAGCACTGGAGCTGCTAAGGCTGTTGGAAAGGTGCTCCCAGCTC TTAATGGTAAATTGACCGGTATGTCTTTCCGTGTTCCTACCGTCGATGTCTC AGTTGTTGACCTCACCGTCAGGCTTGCAAAGGCTGCAACCTACGACGACATC AAAAATGCCATCAAGGAGGAGTCTGAGGGCAAATTGAAGGGTATCTTGGGAT ACACCGAAGATGATGTTGTCTCTACCGACTTCATTGGTGACAGCAGGTCAAG CATCTTCGATGCCAAGGCCGGAATTGCTTTGAACGAGAACTTTGTGAAGCTT GTGTCGTGGTACGACAACGAATGGGGTTACAGTTCCCGTGTTGTTGACTTGA TTGTCCACAT
Cs_Ef1a	CACATCAACATCGTGGTCATCGGCCATGTCGACTCCGGCAAGTCGACCACCA CTGGTCACCTTATCTATAAGCTTGGAGGAATTGACAAGCGTGTGATTGAGAG GTTTGAGAAGGAAGCTGCTGAGATGAACAAGAGGTCATTCAAGTATGCTTGG GTTCTTGACAAGCTTAAGGCTGAGCGTGAGCGTGGTATCACCATTGACATTG CCCTGTGGAAGTTTGAGACCACCAAGTACTACTGCACAGTCATTGATGCTCC TGGCCATCGTGACTTTATCAAGAACATGATTACTGGTACTTCACAGGCTGAT TGTGCTGTTCTCATCATTGATTCCACCACTGGTGGTTTTGAAGCTGGTATCT CTAAGGATGGACAGACCCGTGAGCATGCTCTTCTTGCTTTCACCCTTGGTGT CAGGCAGATGATCTGTTGTTGTAACAAGATGGATGCCACCACACCCAAATAC TCCAAGGCCAGGTATGAGGAAATCGTGAAGGAAGTCTCTTCCTACTTGAAAA AGGTTGGGTACAACCCTGACAAAATCCCATTCGTTCCAATCTCTGGTTTCGA AGGAGACAACATGATTGAGAGGTCTACCAACCTTGACTGGTACAAGGGTCCA ACTCTTCTTGAAGCTCTTGACAATCTCTCTGAGCCCAAGAGACCCTCAGACA AGCCACTCCGTCTTCCACTTCAGGATGTCTACAAGATTGGTGGT

	ATTGGAACTGTGCCAGTGGGTCGTGTTGAGACTGGTGTCATCAAGCCTGGTA TGGTTGTCACCTTTGCTCCCACCGGTCTGACCACTGAGGTCAAGTCAGTTGA GATGCACCACGAGGCTCTTCTCGAGGCTCTTCCCGGTGACAATGTTGGGTTC AATGTTAAGAATGTGGCAGTTAAGGATCTCAAGCGTGGTTACGTTGCATCCA ACTCCAAGGATGATCCTGCCAAGGAGGCAGCCAACTTCACTGCTCAGGTCAT TATCATGAACCACCCCGGTCAAATTGGCAACGGCTATGCCCCAGTTCTCGAC TGCCACACCTCCCACATTGCTGTCAAGTTTTCTGAAATCCTAACCAAGATTG ACCGTAGGTCTGGTAAGGAGCTCGAGAAGGAACCCAAGTTCTTGAAGAATGG TGATGCAGGTATGGTTAAGATGGTTCCAACCAAGCCCATGGTGGTTGAAACC TTCGCTGAGTACCCACCACTCGGACGTTTTGCTGTCCGTGACATGCGTCAAA CCGTTGCTGTTGGTGTCATCAAGAGTGTGGAGAAGAAGGACCCATCAGGAGC TAAGGTGACCAAGTCTGCTGCCAAGAAGAAGTGA
Cs_eIFa (partial)	ATGGCAGGTTTAGCACCAGAAGGCTCTCAATTTGATGCTCGTCAGTATGATT CCAAGATGAGTGAATTACTGGAAAGTGATGGAAAAGATTTCTTTACATCATA TGATGAGGTTCATGAAAGTTTTGATGATATGGGTTTGCAAGAAAATCTTCTA AGAGGCATTTATGCATATGGTTTTGAGAAGCCATCTGCAATTCAGCAGAGAG GGATTGTCCCCTTTTGTAAAGGACTTGATGTGATTCAACAAGCACAGTCTGG AACTGGAAAGACTGCTACTTTCTGCTCTGGAATTCTGCAGCAGCTTGACTAT GGCTTACTTGATTGCCAGGCTTTGGTTCTTGCACCCACTCGAGAACTTGCCC AACAGATCGAGAAGGTTATGAGGGCTCTTGGTGATTATCTCGGTGTGAAGGT TCATGCATGTGTTGGTGGTACCAGTGTCCGTGAAGATCAACGTATTCTTTCC AGTGGAGTGCATGTTGTTGTTGGTACCCCTGGTCGTGTGTTTGACATGCTTC GGAGACAATCCCTGCGTCCTGATAACATCAAAATGTTTGTTTTGGATGAAGC TGATGAAATGCTTTCTAGGGGTTTCAAGGATCAGATTTATGACATTTTTCAA TTGCTACCGCCAAAGATTCAGGTCGGTGTGTTCTCTGCTACAATGCCACCTG AGGCTCTTGAGATCACCAGGAAGTTCATGAACAAACCTGTGAGAATCTTGGT GAAACGTGATGAGCTCACCCTCGAGGGTATCAAACAATTTCACGTCAACGTG GAGAAAGAGGAGTGGAAGCTCGAGACACTTTGTGATCTTTACGAAACATTGG CAATCACCCAGAGTGTTATCTTTGTTAACACAAGGCGAAAGGTCGATTGGCT CACAGACAAGATGCGCAGCAGAGATCACACAGTTTCCGCTACCCACGGAGAC ATGGACCAGAACACTAGGGACATCATCATGAGGGAATTCCGATCAGGTTCCT CTAGAGTCCTCATCACCACCGATCTCTTAGCTCGTGGTATTGATGTGCAACA GGTTTCTCTTGTTATAAACTTTGATCTTCCAACACAGCCCGAAAACTACCTC CATCGTATCGGTCGTAGTGGACGATTCGGGAGAAAGGGTGTTGCCATTAACT TTGTGACCAAAGATGATGAAAGAATGCTGTTCGATATTCAGAAGTTTTACAA TGTGGTTGTCGAAGAGCTGCCTGCAAATGTTGCTGA
Cs_UBQ5 (partial)	ATGCAGATCTTCGTGAAAACCCTAACGGGAAGACTATAACCCTAGAGGTTGA GTCTTCCGATACCATCGACAATGTCAAGGCGAAGATCCAGGACAAGGAGGGC ATCCCACCGGACCAGCAGCGTCTCATCTTCGCCGGGAAGCAGCTAGAGGACG GCCGAACCCTAGCCGATTACAACATCCAGAAGGAGTCGACTCTTCACTTGGT GCTCAGACTTAGGGGAGGAGCCAAAAAGAGGAAGAAGAAGACCTACACCAAG CCAAAGAAGATCAAACATAAGAAGAAGAAGGTGAAGCTCGCTCTTCTCCAGT TCTATAAGGTTGATGACTCCGGTAAGGTGCAGCGGCTGCGGAAGGAGTGTCC CAACGCTGAGTGCGGCGCCGGAACTTTCATGGCTAACCACTTCGACCGACAT TACTGCGGCAAGTGTGGTTTGACCTATGTGTATCAGAA
Cs_UBQ10 (partial)	ATCCAAGACAAGGAGGGTATCCCACCAGACCAGCAGAGACTTATCTTTGCCG GTAAGCAACTCGAGGATGGGAGGACTCTTGCTGATTACAATATTCAAAAGGA GTCTACTCTCCACTTGGTTCTCCGTCTTCGTGGTGGTATGCAGATTTTTGTA AAGACCCTTACAGGAAAGACAATAACCTTGGAGGTTGAGAGCTCCGACACTA TTGACAATGTCAAAGCAAAGATCCAAGACAAGGAAGGTATCCCACCAGATCA GCAGAGACTTATCTTTGCCGGTAAGCAACTCGAGGATGGAAGGACACTTGCT GACTACAACATTCAGAAGGAGTCCACACTTCATCTTGTGCTTCGTCTTCGTG GCGGTATGCAAATATTTGTGAAGACCCTTACAGGAAAGACCATCACCCTTGA GGTAGAAAGCTCGGATACAATTGACAATGTAAAGGCGAAAATTCAAGACAAG GAAGGAATCCCACCAGACCAGCAAAGACTTATCTTTGCCGGTAAGCAATTGG AAGATGGCAGGACTTTAGCT

	GATTACAACATCCAAAAGGAGTCGACCCTTCATTTGGTGCTGCGTCTGAGGG GTGGCATGCAGATCTTTGTGAAGACCTTAACTGGAAAGACCATCACTTTGGA GGTAGAAAGCTCGGATACAATAGACAATGTAAAGGCGAAAATTCAGGACAAA GAGGGCATCCCACCAGACCAGCAAAGACTTATCTTCGCCGGTAAGCAACTCG AGGATGGAAGGACTCTTGCAGATTACAACATCCAGAAGGAGTCTACTCTTCA CCTCGTGTTGCGTCTGAGGGGTGGTATGCAGATCTTTGTTAAGACCTTGACC GGAAAGACCATAACTTTGGAGGTGGAAAGTTCGGATACCATAGACAATGTAA AGGCGAAAATTCAGGACAAAGAGGGTATCCCACCAGACCAGCAGAGGTTGAT TTTTGCTGGTAAACAGTTGGAAGATGGTAGGACTTTGGCTGATTACAACATT CAGAAAGGAGTCTACTCTTCACCTTGTTCTCCGTCTTCGTGGCG
Cs_YLS8	ATGTCGTACTTGCTTCCACATTTGCACTCTGGATGGGCCGTAGATCAGGCCA TCCTCGCCGAGGAAGAGCGTCTCGTCATCATCCGATTTGGCCACGACTGGGA TGAGACCTGTATGCAGATGGATGAAGTTTTGTCATCAGTTGCTGAGACAATC AAAAACTTTGCAGTGATATACCTTGTCGACATCACTGAGGTTCCAGATTTCA ACACAATGTACGAGTTGTATGACCCATCTACGGTCATGTTTTTCTTCAGGAA CAAGCACATTATGATCGATCTCGGAACTGGAAACAATAATAAGATCAACTGG GCCCTCAAGGATAAGCAAGAGTTCATTGACATTGTTGAGACTGTGTACCGTG GAGCAAGGAAGGGACGGGGTCTTGTGATTGCTCCTAAGGATTACTCTACCAA ATATCGCTACTAA

Table S VI-5 Primer used for qPCR experiments

Primer name	**Sequence 5' to 3'**
ACT2_fwd	CTGCCGAACGGGAAATTGTC
ACT2_rev	AACTGAGGAGCTGCTCTTGG
TUB1_fwd	TTCCATTCCCGCGACTTCAC
TUB1_rev	GCGCACATCATGTTCTTGGC
18S_fwd	ACCATAAACGATGCCGACCAG
18S_fwd	TTCAGCCTTGCGACCATACTC
GAPDH_fwd	TGTCTTTCCGTGTTCCTACC
GAPDH_rev	TCAATTTGCCCTCAGACTCC
EF1a_fwd	AGCGTGGTATCACCATTGAC
EF1a_rev	AGCACAATCAGCCTGTGAAG
eIF4a_fwd	CAATCCCTGCGTCCTGATAAC
eIF4a_fwd	GACCTGAATCTTTGGCGGTAG
UBQ5_fwd	AAGCTCGCTCTTCTCCAGTTC
UBQ5_rev	CACACTTGCCGCAGTAATGTC
UBQ10_fwd	GGAAGGTATCCCACCAGATCAG
UBQ10_rev	CCACGAAGACGAAGCACAAG
YLS8_fwd	CTCGTCATCATCCGATTTGG
YLS8_rev	CCGTAGATGGGTCATACAACTC
PDS_fwd	CCAAGACCAGAGCTTGATAG
PDS_rev	ACCTGCACCAGCAATTAC

ChlI_fwd	CTTGGCTTCTCGCTTCCTGTC
ChlI_rev	TACCTGAATCCCGGTTCCTCC

Table S VI-6 Results from the NormFinder analysis of reference genes suitable for qPCR

Gene name	**Stability value**
ACT	0.256
EF1a	0.739
eIFa	0.169
GAPDH	0.359
TUB	1.077
UBQ5	0.233
UBQ10	0.517
YLS8	0.368
18 S	0.230

Best gene	eIFa
Stability value	0.169
Best combination of two genes	eIFa and UBQ5
Stability value for best combination of two genes	0.144

Curriculum vitae

Personal Information

Name	Julia Schachtsiek
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Academic education

06/2015 – present	**PhD student** TU Dortmund University, Dortmund, Germany Department of Biochemical and Chemical Engineering, Chair of Technical Biochemistry
06/2015 – present	**Research associate** TU Dortmund University, Dortmund, Germany Department of Biochemical and Chemical Engineering, Chair of Technical Biochemistry
10/2012 – 03/2015	**Master of Science (M.Sc.)** Degree in Molecular Cell Biology Bielefeld University, Bielefeld, Germany
10/2009 – 09/2012	**Bachelor of Science (B.Sc.)** Degree in Biology Bielefeld University, Bielefeld, Germany

School education

08/2000 – 06/2009	Immanuel-Kant-Gymnasium, Bad Oeynhausen, Germany Degree: Abitur

Working Experience

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Publications

Schachtsiek J, Warzecha H, Kayer O and Stehle F (2018) Current Perspectives on Biotechnological Cannabinoid Production in Plants. *Planta Medica*; 84, 214-220

Schachtsiek J. and Stehle F. Nicotine-free, nontransgenic tobacco (*Nicotiana tabacum* L.) edited by CRISPR-Cas9 (2019). *Plant Biotechnology Journal*, doi: 10.1111/pbi.13193

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