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MASTER OF SCIENCE THESIS

THE GENE KNOCK-OUT IN *N. benthamiana* L. AND *P. somniferum* L. BY USING RNA-GUIDED CRISPR/CAS9 SYSTEM

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ÖZET

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RNA-KILAVUZLU CRISPR/CAS9 SİSTEMİ KULLANILARAK *N. benthamiana* L. VE *P. somniferum* L.' DA GEN NAKAVT EDİLMESİ

Yağız ALAGÖZ

Çankırı Karatekin Üniversitesi
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Bitki genom modifikasyonları antik çağlardaki çifçilerin zamanından başlayarak günümüzün bilim insanları tarafından da klasik ıslah ve GM teknikleri uygulanarak insanlık tarafından yüzyıllardan beri yapılmaktadır. Şimdilerde, transkripsiyonel seviyedeki manipulasyonlar için sentetik yeni bir non-GM teknoloji geliştirildi. CRISPR sistemi, RNA kılavuzlu transiyent bir genom düzenleme aracıdır. Hedef spesifik olarak dizayn edilmiş bir kimerik sgRNA ve Cas9 endonükleazından oluşmakta olup, DNA da çift zincir kırıkları (DSB) oluşturarak genomda istenilen gen yada genlerde çerçeve kayması mutasyonu ile gen nakavtı yapar. CRISPR sistemi, DNA da kırık oluşumuna sebep olduktan sonra, bitkinin kendi tamir mekanizmaları olan HR ve NHEJ mekanizmalarını devreye sokar. Tip II CRISPR/SpCas9, yüksek oranda spesifik ve güvenilir bir sistem olup, bitki çalışmalarına adapte edilmiş diğer genom düzenleme teknolojileri arasındaki en uç teknolojidir. Bu çalışmada, en popüler tıbbi aromatik bitkilerden biri olan haşhaş (*Papaver somniferum* L.), ve iyi bilinen bir model organizma olan *Nicotina benthamiana* L.'nın genomları viral ve sentetik tabanlı olarak transkribe olan sgRNA ler kullanılarak CRISPR/Cas9 sisteminin uygulanabilirliği açısından incelenmiştir. *Pap4OMT2* ve *NbPDS* genlerisini hedefleyen sgRNA ler spesifik olarak dizayn edilmiş ve SpCas9 endonükleaz ile beraber istenilen gen segmentleri hedeflenmiş olup oluşan InDel ler gösterilmiştir. Böylece, CRISPR/Cas9 sisteminin model olmayan Papaver türlerinde uygulanabilirliği literatürde ilk kez gösterilmiştir.

2015, 83 sayfa

Anahtar kelimeler: Cas9, CRISPR, DSB, GM, HR, InDel, *NbPDS*, NHEJ, *Nicotina benthamiana* L., *Pap4OMT2*, *Papaver somniferum* L., sgRNA

ABSTRACT

MSc. Thesis

THE GENE KNOCK-OUT IN *N. benthamiana* L. AND *P. somniferum* L.
BY USING RNA-GUIDED CRISPR/CAS9 SYSTEM

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Genome modifications in plants have been doing for many centuries by mankind since from the times of ancient farmers to the scientists for now by applying selective breeding and GM techniques. Recently, a new synthetic non-GM technology has been developed for transcriptional-level manipulations. CRISPR system is an RNA-guided transient genome editing tool, that is generally composed of a target-specifically designed chimeric sgRNA and a Cas9 endonuclease, which forms DSBs in DNA and results a frame-shift mutation to knock-out any desired gene or genes in genome. CRISPR system employs genome repair mechanims of HR or NHEJ after introducing a cut into DNA and mediates genome editing by using organisms own repair mechanisms. Type II CRISPR/SpCas9 is a highly-specific and robust system which is a state of art among the other genome-editing technologies that is adapted to plant sicence. In this study, Opium poppy (*Papaver somniferum* L.), one of the most popular medicinal aromatic plants, and a well-known model organism *Nicotina benthamiana* L. genomes were investigated on the applicabilitiy of CRISPR/Cas9 system by using synthetic and viral based transcribed sgRNAs. sgRNAs targeting *Pap4OMT2* and *NbPDS* genes were specifically designed and used to target those specific gene segments with SpCas9 endonuclease and formed InDels were demonstrated. Thus, the applicability of CRISPR/Cas9 system in non-model Papaver species was shown for the first time in literature.

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Keywords: Cas9, CRISPR, DSB, GM, HR, InDels*NbPDS*, NHEJ, *Nicotina benthamiana* L., *Pap4OMT2*, *Papaver somniferum* L., sgRNA

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Kind regards.

Yağız ALAGÖZ
Cankiri, Aug 2015

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SYMBOLS INDEX

%	Percent
º	Celcius Degree
α	Alpha
µL	Microliter
mL	Milliliter
L	Liter
µM	Micromole
mM	Millimole
M	Mole
~	Approximation

ABBREVIATIONS

Arg	Arginine-rich region
Cas9	CRISPR-associated protein 9
CRISPR	Clusterly Regulated Interspaced Short Palindromic Repeats
crRNA	CRISPR RNA
CTD	C-terminal domain
DNA	Deoxyribonucleicacid
DSB	Double-strand break
GFP	Green fluorescent protein
GM	Genetically modified
HR	Homologous Recombination
INCB	International Narcotics Control Board
MoClo	Modular Cloning
NHEJ	Non-Homologous End Joining
NptII	Neomycin phosphotransferase
OMT	O' Methyltransferase
pre-crRNA	Precursor cr-RNA
RNA	Ribonucleicacid
RNAi	RNA interferance
RNase	Ribonuclease
sgRNA	single guide-RNA
SNP	Single-Nucleotide Polymorphism
SpyCas9	*S.pyogenesis* Cas9
TALENs	Transciption activator-like endunucleases
Topo	Topo-homology domain
tracrRNA	transcirption activator CRISPR RNA
UN	United Nations
WGS	Whole Genome Sequence
ZFNs	Zinc-finger nucleases

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1. INTRODUCTION

1.1 Considerations On The Plant Genome Manipulations and Editing

Plants have been manipulating for varied types of reasons about from increasing the biosynthesis of some important metabolites to having more resistance against both some biotic and abiotic stress factors that are reducing the total yields every year in the world.

In the earliest times of farming till to the beginning of the last century, people have been using some basic crossing techniques in plant breeding to produce plants with some desirable traits. By the development of functional genetics techniques, we learned that it's possible to transfer any desired gene or trait from one organism to another and it's easy to use this technology to create some genetically modified (GM) plants that are not naturally existing in the world. But this caused arguments in public, due to having lack of understanding, knowledge and confidence of accepting scientific facts. Luckily, we have some another synthetic tools to manipulate genomes different from transgenic and conventional breeding techniques.

ZFNs (Zinc-finger nucleases) and TALENs (Transciption activator-like endunucleases) are the tools have been used to manipulate genome of desired organism in transcriptional level (Chen and Gao, 2015). Last few years have been passed by the superiority of those two systems. Both tools are protein-based endonuclease systems using the restriction enzyme called Fok-I to knock-out the gene of interest by forming double-strand breaks in DNA (Gaj et al., 2013). But these systems are hard to apply and quite troublesome in case of requirements like expertise necessity, and design of two different proteins that are flanking the target sequences in gene of interest.

By revolutionary scientific contributions of some elite scientific research groups to CRISPR studies, we now know that we can use CRISPR/Cas9 system as a tool to manipulate any gene of interest in eukaryotic systems just by using well-designed guide RNAs (Cho et al., 2013; Hwang et al., 2013; Jiang et al., 2013a; Jinek et al., 2013; Mali

et al., 2013c). Since the year of 2013, the time of first application in plant studies, functioning of Cas9-based CRISPR system in plants was reported in *Arabidopsis thaliana, Nicotiana benthamiana, Nicotiana tabaccum, Oriza sativa, Triticum aestivum, Sorghum bicolor, Zea mays, Marchantia polymorpha, Citrus sinensis and Populus tomentosa* (Feng et al., 2013; Jiang et al., 2013b; Li et al., 2013; Mao et al., 2013; Miao et al., 2013; Nekrasov et al., 2013; Upadhyay et al., 2013; Xie and Yang, 2013; Brooks et al., 2014; Gao and Zhao, 2014; Jia and Wang, 2014; Liang et al., 2014; Sugano et al., 2014; Xie et al., 2014; Xu et al., 2014b; Fan et al., 2015). Especially, *Marchantia* study with an unknown genome, encourage researchers that CRISPR/Cas9 system can be applied to any other plant species even with lack of genomic information necessary for determination of proper target sites. For these reasons, we decided to examine the applicability of this system in one of the mostly studying medicinal aromatic plants which is opium poppy (*Papaver somniferum* L.).

Papaver somniferum L., from the family of Papaveraceae, is one of the two plant species (with the other opium poppy, *Papaver setigerum* DC) known to biosynthesize morphine (Fairbairn and Wassel, 1964; La Valva et al., 1985). Despite of its importance as an oil plant, poppy is also synthesizing more than 30 alkaloids including codeine, thebaine, noscapine, papaverine and morphine that are used for medical purposes (Fairbairn and Wassel, 1964). Thanks to these properties, poppy has an important place in the world market. As an opposite situation to this, due to the presence of these anesthetic and addictive metabolites, poppy cultivation is subjected to the legal restrictions and regulations of UN (Tupper and Labate, 2012).

Morphine is particularly used for medicinal purposes due to its properties of being anesthetic and analgesic and it is mostly obtained from the latex of poppy but in very small amounts. Recent studies have shown that, the amount of morphine biosynthesis could be changed by manipulating several genes in the BIA pathway including (*R,S*)-reticuline 7-O-methyltransferase (*7OMT*), norcoclaurine-6-O-methyltransferase (*6OMT),* and 3'-hydroxy-N-methylcoclaurine 4'-O-methyltransferase (*4'OMT)* (Apuya et al., 2008; Desgagné-Penix and Facchini, 2012). These facts constitute the main

reason, 4OMT2 gene was subjected to be knocked out by CRISPR/Cas9 system in our study.

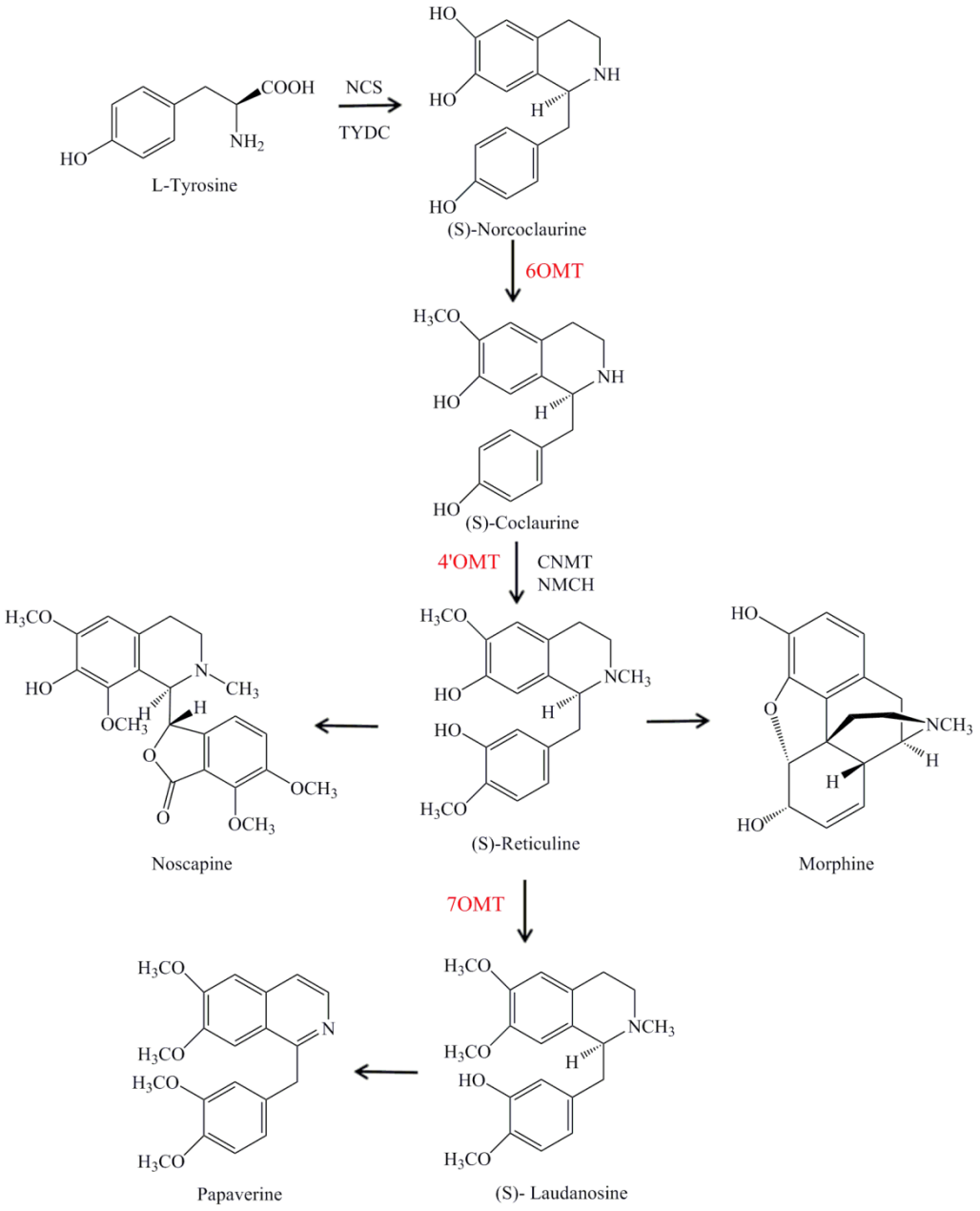


Figure 1.1 Morphine, noscapine and papaverine biosynthesis in opium poppy are catalyzed by essential enzymes of 4OMT, 6OMT and 7OMT in different steps. While 6OMT catalysis the methylation reaction of (S)-Norcoclaurine to form (S)-Coclaurine, 4OMT and 7OMT enzymes are catalysis the conversion of (S)-Norreticuline to (S)-Tetrahydropapaverine.

1.2 CRISPR/Cas System

1.2.1 The origins and working mechanism of CRISPR/Cas9 system

Our old world has been struggling with some huge problems about meeting the agricultural product demands of a growing population which is expected to be more than 9 billion in 2050 (Cohen, 2003). The global crop production needs to be doubled by the year of 2050 however the statistics about the projected yield trends are demonstrating that it's not possible to reach a desired range for now and this makes it the hardest conundrum that the world faced with (Ray et al., 2013). Due to the the rising population, increasing prosperity and dietary shifts of the people, especially in the developing countries, food demands and also their prices are inevitably rising.

Just by applying classical plant breeding techniques, it seems not reasonable to solve those problems. In order to address a biological question by classical breeding studies, more investment, labor and time are required. If the limited time taken into account, it's a necessity to find another solution which could be more fast, effective and less laborious.

Recently, new synthetic genome engineering tools have been discovered which enable us to edit plant genomes to obtain any desired traits. Clustered regularly-interspaced shorth palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9), a new synthetic plant breeding technique (NPBT), is revolutionized what we know about crop improvement so far. CRISPR is firstly reported by Ishino and his colleagues while studying on iap gene sequence in *E.coli* genome. Clustered sets of 29 nt repeats which are interspaced by 32 nt spacer sequences have been identified which are located just downstream of the iap gene and declared as well-conserved short repetitive sequences with an unknown biological significance (Ishino et al., 1987).

Improvements in genome sequencing technology have been provided more information to reveal this phenomenon present in the bacterial and archaeal genomes. Shorth

Regularly Spaced Repeats (SRSRs) are found in many of the sequenced genomes of prokaryotes and phylogenetic relevance of those peculiar elements are identified firstly by Mojica and his/her colleagues (Mojica et al., 2000). Afterwards, the acronym CRISPR is given by Jansen and Mojica to better symbolize and characterize this mobile repeatitive elements found in prokaryotic genome (Jansen et al., 2002). Jansen has identified the CRISPR locus is conserved within species but they are varied in between species. Furthermore, four of cas genes (cas1-4), which are adjacently located to the CRISPR loci, are discovered in the same study (Jansen et al., 2002). New findings made CRISPR studies more attractive and this gave rise to the emergence of the new informations. Presence of different Cas protein families and the existance of multiple CRISPR/Cas subtypes have been announced by Haft and his colleagues (Haft et al., 2005). Their findings would have contributed to the establisment of current classification of the CRISPR types as type I, II and III (Makarova et al., 2011a; Makarova et al., 2011c).

In the same period, CRISPR spacers are defined as sequences that are originated from any foreign, extrachromosomal parts (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). This brought together some parts of jigsaw and caused wide-spread speculations about the functions of CRISPR array and Cas genes in the microbial immunity against phage and virus DNAs. Due to the fact that, the spacers are known as the tracers coming from the previous invasions of foreign, extra-chromosomal elements (Bolotin et al., 2005). Therefore, CRISPR array could be modified by the spacers derived from pathogenic chromosomes and prokaryotic cells gains immunity by this way. Each spacer, which are integrated into host cell's genome, could be a candidate that assists the host cell to possess a new memory stick which contains the information to combat against the future-attacks of the same pathogen. Some shorth motifs which are adjacently located to the protospacer units of non-target DNA have been also identified. Later on, they will be referred as protospacer adjacent motifs (PAMs) (Bolotin et al., 2005).

A ground breaking report is published by Barrangou and his group while studying on type II CRISPR system of *S. thermophilus.* They brought out the first convincing proof

about the role and activity of CRISPR/Cas system in microbial adaptive immunity (Barrangou et al., 2007). The immunological importance of cas nuclease activiy againt phages attacts has shown significantly by inactivating the cas5, a protein which has been demonstrated as a potential-nuclease because of the HNH motif it possess (Barrangou et al., 2007).

In order to assemble and present all the generated knowledge about CRISPR system so far, CRISPRdb database has been created. This new platform was also providing some tools to identify new CRISPR spacers and repeats present in the microbial genomes (Grissa et al., 2007). According to the last update in early August of 2014, approximately 85% of all sequenced archeae and 45% of all known bacteria genomes contain varied types of CRIPSR systems.

Horvarth and Deveau gave more information about *S. thermophilus* type-II CRISPR system in terms of it's activity, diversity and response mechanisms against phages (Deveau et al., 2008; Horvath et al., 2008). Subsequent studies are focused more on the CRISPR systems working mechanism. The biogenesis mechanism of the CRISPR RNAs (crRNAs) from the type-I CRISPR array is firsly displayed in *E.coli* and this is followed by a study about the demonstration of the nuclease activity of cas on DNA targets in the guidance of crRNAs (Brouns et al., 2008). Unlike the known DNA-targeting mechanism, RNA-targeting nuclease activity of another type of CRISPR system is identified in an investigation on type-II CRISPR system in *P. furiosus* (Hale et al., 2009).

As understood from the previously published studies, intracellular working mechanism is changing according to CRISPR system type. *S. thermophilus* type-II Cas9 is firstly described as an only enzyme in the cas gene cluster that can cleave the DNA (Garneau et al., 2010). Thereafter, E. Charpentier and her colleagues brought to light the biogenesis and processing mechanism of tracrRNA and crRNA of type II CRISPR system (Deltcheva et al., 2011). What they pointed out is the necessity of tracrRNA in the biogenesis of crRNA from a precursor crRNA (pre-crRNA). Moreover, both

RNaseIII and Cas9 are required for crRNA preocessing and for a smooth-running CRISPR system. When pre-crRNAs are firslty transcribed from a CRISPR array, they form an RNA hybrid with tracrRNAs (trans-activating crRNAs) and an endogenious RNaseIII trims this intermediate complex. Further maturation results with the biogenesis of a mature crRNA (Carte et al., 2008; Makarova et al., 2011a; Nam et al., 2012). Apparently, Cas9, tracrRNA and crRNA are representing the cardinal units of the type II CRISPR/ Cas9 system (Deltcheva et al., 2011). CRISPR RNA was thought to be as a guide RNA which is specifically formed to select the target sequences. Most importantly, a key sequence within the crRNA, called as a seed sequence, is essential to govern the precise target recognition and interferance of CRISPR/Cas system (Semenova et al., 2011; Wiedenheft et al., 2011).

The first insight about transmission probability of CRISPR system from one organism to another is gained from Siksnys and his colleagues in their study which is verifiying the idea about transferring *S. thermophilus'*s type-II CRISPR loci into *E. coli*'s genome to reconstruct a new CRISPR system (Sapranauskas et al., 2011). In-vitro application of crRNA guided DNA-nuclease activity of SpCas9 (*S. pyogenesis*-Cas9) was firstly demonstrated by Jinek and her colleagues (Jinek et al., 2012) and they had gone one step forward with their astonishing design of a chimeric guide RNA which is formed by side by side fusion of targeting segment of crRNA with tracrRNA (Jinek et al., 2012).

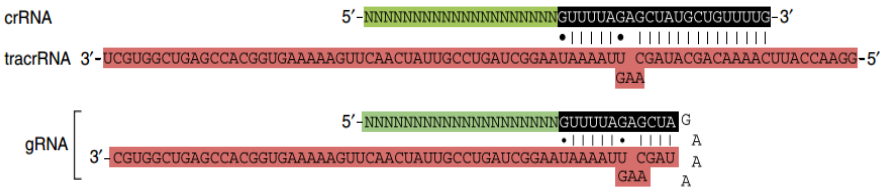


Figure 1.2. Chimeric sgRNA substitutes the tracrRNA&crRNA complex to ease the CRISPR system applications (Adopted figure from Sander and Joung (2014a)).

Adaptation of CRISPR/Cas system for servicing in mammalian genome editing is accomplished by two different research groups studying on *S. thermophilu*s and *S.*

pyogenesis type-II A CRISPR system in 2013 (Cong et al., 2013; Mali et al., 2013c). Multiplex CRISPR/Cas9 systems provide the opportunity to target several genes simultaneously with multiple sgRNAs (Cheng et al., 2013; Li et al., 2013). Since these pioneering studies, it has been realized by almost all the eminent research groups around the world that the robust and reliable genome editing in eukaryotic systems could be feasible by applying CRISPR/Cas9 system.

1.2.2 Structural properties and working mechanism of CRISPR/Cas9 system components

The type-II CRISPR/Cas systems is subdivided into three major groups (type-IIA-IIC) based on the architechture and organization of diverse types of Cas genes they possess. Interestingly, all type-II CRISPR systems are composed of a group of cas9,cas1 and cas2 genes in harmony with CRISPR array, tracrRNA encoding region, and RNaseIII (Deltcheva et al., 2011). Some variations are also present depending on the existance of csn2a, csn2b and cas4 genes (Chylinski et al., 2013; Chylinski et al., 2014).

Type-II A CRISPR systems have distinguished features like having repeats of 36 nt in length and possesing cas9, cas1 and cas2 genes in every members of the group. Whereas in the group of type II-B, the length of repeating segments is 37 and in type-IIC its' variable in between 36, 44 and 48 nt. Moreover type-II B group members contain a cas4 gene in their CRISPR locus as a prominent difference. In contrast to type-II A and B, type-II C group members have only the cas9,cas1 and cas2 genes (Chylinski et al., 2013; Chylinski et al., 2014).

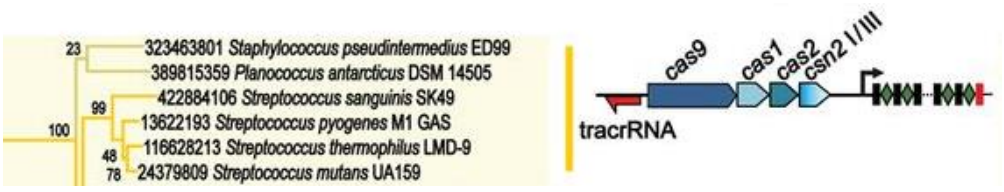


Figure 1.3 Phylogeny of Type II–A Cas9 and distributions and similarities of Cas sequences among the same group of Cas family (Adopted figure from Chylinski et al. (2014)).

For type I and II CRISPR/Cas systems, PAM within the target sequence, is a requisity. Surprisingly, there is no PAM present in the prokaryotic CRISPR array. This for to inhibit the self-targeting of prokaryotic genome by it's own immune (Marraffini and Sontheimer, 2010). Additionally, PAM is strictly required for obtaining a new spacer, a process called as spacer acquisition (Fineran and Charpentier, 2012), and also it's indispensible for an accurate and reliable target-DNA recognition and unwinding (Deveau et al., 2008; Garneau et al., 2010; Fonfara et al., 2013).

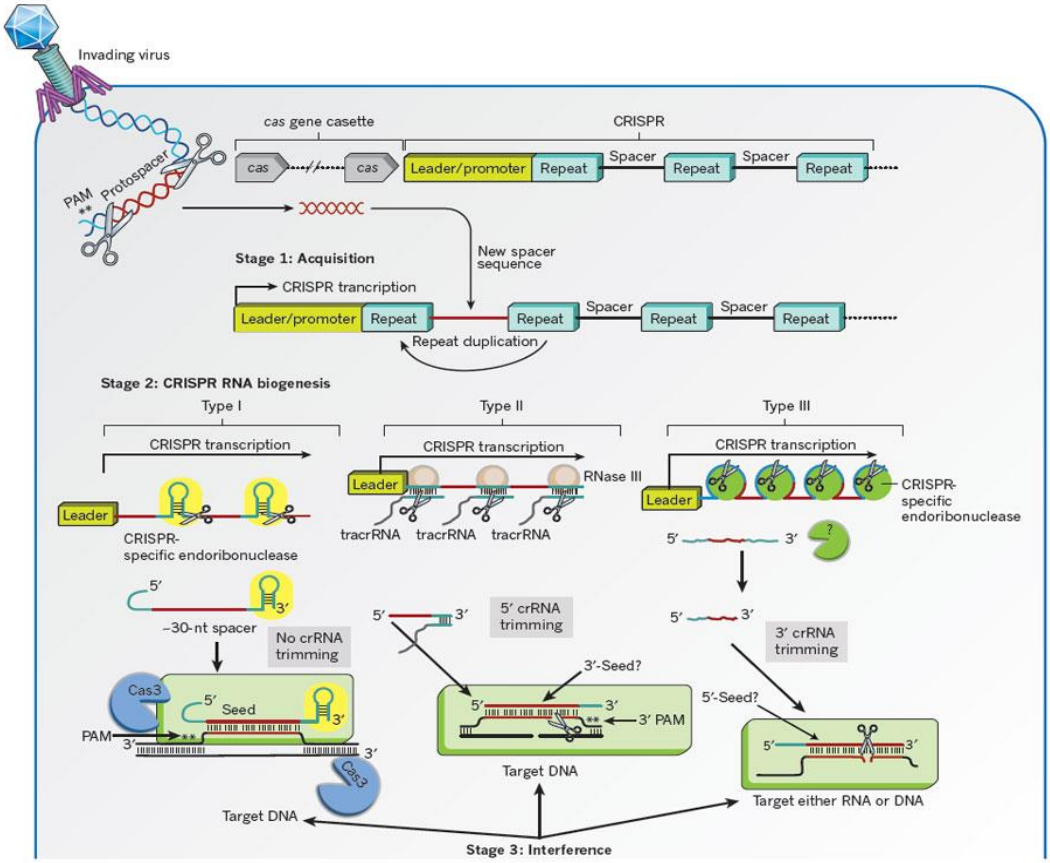


Figure 1.4 Demonstration of repetitive CRISPR locus and types (I: II: III) according to their trimming processes and binding patterns to the DNA (Wiedenheft et al., 2011).

Cas9 crystal structure-related studies revealed more information about the multidomains of Cas9 and their interaction with guide RNA and target DNA strands. Lately, Jinek and his colleagues are found that some conserved arginine residues (Arg 1333 and Arg 1335) in carboxy-terminal domain of SpCas9 have a role in major groove interactions to recognize the guanine-guanine (GG) dinucleotide in PAM of non-target DNA. Minor

groove interactions between the PAM and phosphodiester group of first nucleotide (+1) in the target DNA induces the target melting and unwinds the DNA strands. This makes available the Cas9-RNA complex to identify the nucleotides upstream of the PAM by base pairing between the seed sequence and the target DNA and causes generation of double-strand breaks (DSBs) (Anders et al., 2014). DSBs are formed by two specified individual nuclease domains of Cas9, namely HNH and RuvC-like nucleases. Cas9 performs a genome-wide PAM scan and once a proper match between the target DNA and sgRNA is formed, it cuts both strands of the DNA just as a scissors (Gasiunas et al., 2012; O'Connell et al., 2014).

The first example of the studies on the crystal structure of cas genes in type-II *S. pyogenesis* CRISPR/Cas system is the crystal sturucture of csn2 protein (Koo et al., 2012). Then, secondly, Jinek and his colleagues published the single-particle EM reconstructions of the *S. pyogenes* apo-Cas9, for the first time (Jinek et al., 2014). Thirdly, the high-resolution conformation of SpCas9 has been uncovered pretty much all the detailed structural mechanism related with the functional subdivisions and the domain organizations. Mainly, the SpCas9 is comprised of two major lobes which are a recognition lobe in alpha helical structure (REC), and a nuclease lobe (NUC) composed of HNH, RuvC and C-terminal domains (Nishimasu et al., 2014). In the intact structure Cas9, REC lobe is responsible for facilitating the contacting of RNA-DNA heteroduplex by it's arginine-rich bridge helix domain and NUC lobe is present to provide the nuclease activity to Cas9 as previously defined (Nishimasu et al., 2014).

1.2.3 The recovery mechanism of genome determines the type of the manipulation

After the induction of DSBs by Cas9 nuclease activity, unwinding of the DNA strands alert the nonhomologous end joining (NHEJ) and homology directed repair (HDR) mechanisms for genome repair. NHEJ mediated repair causes the introduction of new nucleic acid units in various lengths, a process known as InDel formation, and induces the formation of a frame-shift mutation in any coding sequence which actually means gene knock-out (Gorbunova and Levy, 1997). The HDR mechanism normally uses the homogenious sister chromatins to repair distrupted sequences, so the same mechanism

can be used to ligate limited length of desired nucleic acid segments to the target sequences by courtesy of exogenous DNA donor templates (Capecchi, 1989; Bibikova et al., 2001; Sander and Joung, 2014b). Cas9 nickase (Cas9n), which is a Cas9 derivative DNA nuclease, cuts only from one side of DNA either from sense or anti-sense strands depending on the selection of the target sequences. To achieve this, RuvC and HNH mutant versions of Cas9n are created by introducing the mutations in D10A and H840A respectively (Ran et al., 2013; Essletzbichler et al., 2014). The only nuclease or multiplex-nickase activiy of Cas9 induce the NHEJ mechanism, contrarily single DNA nicks stimulate the HDR. Obviously, introducing single nicks in DNA targets with single guide-RNAs can be more appropriate for donor DNA integration studies (Hsu et al., 2014).

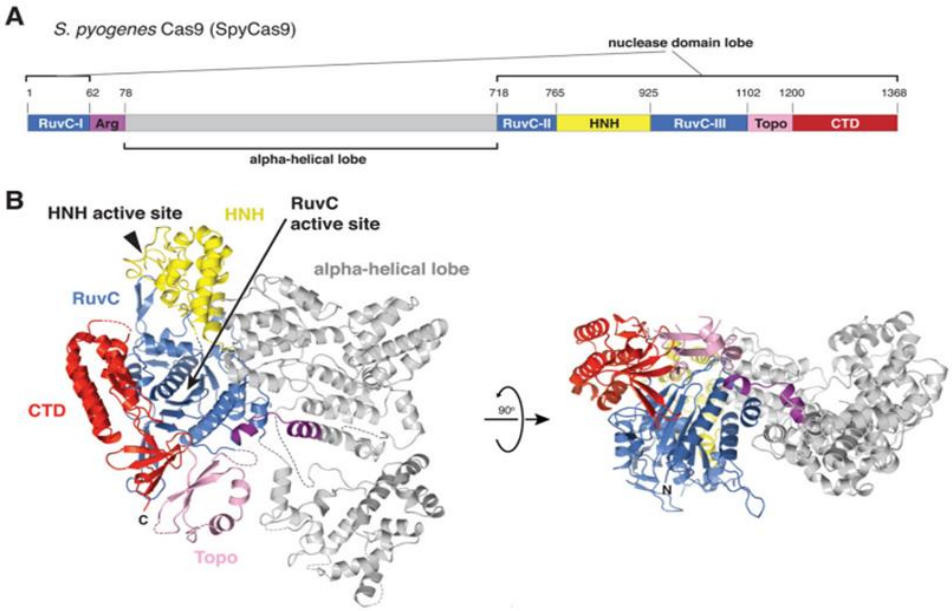


Figure 1.5 (A) The schematic representation of the major functional domains of the SpyCas9 enzyme. (B) The enzyme is made up of two different lobes which are an enzymatic and alpha helical lobes as introduced in ribbon & surface demonstration (Adopted figure from Jinek et al. (2014)).

CRISPR/Cas9 system makes possible even the excision of large chromosomal segments. Yang and his colleagues have been reported that they formed some large heritable chromosomal deletions in rice genome in range of 115–245 kb in length by

CRISPR/Cas9 system (Zhou et al., 2014). Moreover, fully haploid human cell lines can be generated by introducing megabase-scale deletions by this excellant system (Essletzbichler et al., 2014). Optimization of Cas9 codon is also important to achieve the maximum efficiency in genome editing. In plants, the plant codon-optimized Cas9 demonstrates higher performance in comparison with the human codon-optimized Cas9 due to their relatively differential expression profiles in plant tissues (Xu et al., 2014a).

1.2.4 Recent Improvements in genome editing by applying CRISPR/Cas9

There are some web-based tools to design more precise CRISPR assays. These tools offer their users to design a better guide-RNA which has low potential off target-sites in number. Randomly designed guide-RNAs have a potential to engine any other gene/genes that is different from the gene of interest and it's impossible to anticipate their influence on the genome. This makes all the new genome editing technologies, including the CRISPR/Cas9, poor methods to apply for target-specific genome editing.
In order to eliminate those unexpected effects, designing a well-defined guide RNA for Cas9 editing is essential. The number of on-line tools is incresing day by day by the contributions from different universities from varied sides of the world. Each designed tool has its own WGS database to blast the designed guide RNA in to see if there is any undesired complementarity that is different from the targeted region inside the genome.
CRISPR/Cas9 system allows manipulation of multiple genes via well-designed sgRNAs specific to each targeted gene or genes (Belhaj et al., 2013; Hsu et al., 2014; Sakuma et al., 2014; Li et al., 2015).

By further understanding the Cas9 structure and functioning mechanism, it's much easier to develop new strategies to edit desired genomes with improved derivatives of this elegant tool. Recently, new split-Cas9 tools was developed for inducible genome editing and transcription by rapamycin (Wright et al., 2015; Zetsche et al., 2015). When split forms of Cas9 are transferred into cells, they reassemble by the induction of rapamycin and introduce a DSB into the targeted region inside the genome. Moreover, newly identified Cas9 proteins can provide us a wider platform for genome editing in therapeutic applications like 1/3 sized (in compare with SpCas9) *Staphylococcus aureus* Cas9 protein (Ran et al., 2015).

Table 1.1 On-line tools servising to design CRISPR tools for some certain organisms with known genome information.

Name of the Software	Web -site	Designer/Author & Year	Publication	Specificity&Function
CasOT	http://eendb.zfgenetics.org/casot/	Xiao et al. (2014)	Bioinformatics	Cas9 off-target searching
E-CRISP	http://www.e-crisp.org/E-CRISP/	Heigwer et al. (2014)	Nat. Methods	Cas9 off-target searching
Cas-OFFinder	http://www.rgenome.net/cas-offinder/	Bae et al. (2014)	Bioinformatics	CRISPR off-target analysis
ZiFit Targeter	http://zifit.partners.org/ZiFiT/	Sander et al. (2010)	Nucleic Acids Research	ZFN,TALEN and CRISPR design tools
The CRISPR Design Tool	http://www.genome-engineering.org/	Hsu et al. (2013)	Nat. Biotechnology	Cas9 off-target searching
CRISPR-PLANT Database	http://www.genome.arizona.edu/crispr/	Xie et al. (2014)	Molecular Plant	Cas9 off-target searching special for plants
Cas9 guide RNA design	http://cas9.cbi.pku.edu.cn	Ma et al. (2013)	Hindawi BioMed Research International	gRNA design for model systems
flyCRISPR Optimal Target Finder	http://flycrispr.molbio.wisc.edu/tools	Gratz et al. (2014)	Genetics	gRNA design for fly studies
flyCas9	http://www.shigen.nig.ac.jp/fly/nigfly/cas9/cas9TargetFinder.jsp	Kondo and Ueda (2013)	Genetics	Promotor & gRNA design for fly studies
DRSC Find CRISPRs	http://www.flyrnai.org/crispr2/	Ren et al. (2013)	Proc Natl Acad Sci USA	Cas9 off-target searching in Drosophila
CRISPRI	http://crispi.genouest.org	Rousseau et al. (2009)	Bioinformatics	CRISPR databese

Table 1.2 The table[1] is demonstrating the summary of recent CRISPR/Cas9 studies in plants (Belhaj et al., 2013).

Plant species	Target gene(s)	Promoter for Cas9	Cas9 version	Promoter for sgRNA	Delivery method	Reference
Arabidopsis thaliana	*AtPDS3*, *AtFLS2*, *AtRACK1b* and *AtRACK1c*	35DPPDK	Plant codon-optimized Cas9	AtU6	Protoplast co-transfection and *Agrobacterium* infiltration	Li *et al.* (2013)
	GFP	35S	*Chlamydomonas reinhardtii* codon-optimized Cas9	OsU6	*Agrobacterium*-mediated transformation	Jiang *et al.* (2013*b*)
	CHL1, *CHL2*, and *TT4i*	OsUBQ1	Human codon-optimized Cas9	OsU3	*Agrobacterium*-mediated transformation	Mao *et al.* (2013)
	BRI1, *JAZ1*, and *YFP*	2×35S	Human codon-optimized Cas9	AtU6-26	*Agrobacterium*-mediated transformation	Feng *et al.* (2013)
Nicotiana benthamiana	*NbPDS3*	35DPPDK	Plant codon-optimized SpCas9	AtU6	*Agrobacterium* infiltration	Li *et al.* (2013)
	NbPDS	35S	Human codon-optimized SpCas9	AtU6	*Agrobacterium* infiltration	Nekrasov *et al.* (2013)
	Nbpds	CaMVE35S	Human codon-optimized SpCas9	CaMVE35S	*Agrobacterium* infiltration	Upadhyay *et al.* (2013)
	NbPDS	2×35S	Human codon-optimized Cas9	AtU6	*Agrobacterium* infiltration	Belhaj *et al.* (2013)
	GFP	35S	*Chlamydomonas reinhardtii* codon-optimized Cas9	OsU6	*Agrobacterium* infiltration	Jiang *et al.* (2013*b*)
Oryza sativa	*OsPDS*,*OsBADH2*, *Os02g23823* and *OsMPK2*	2×35S	Rice codon-optimized Cas9	OsU3	Transformation using particle bombardment	Shan *et al.* (2013*b*)
	OsSWEET11 and *OsSWEET14*	CaMV 35S	Wild-type SpCas9 and rice codon-optimized Cas9	OsU6	*Agrobacterium*-mediated transformation	Jiang *et al.* (2013*b*)
	OsMYB1	OsUBQ1	Human codon-optimized Cas9	OsU3	*Agrobacterium*-mediated transformation	Mao *et al.* (2013)
	ROC5, *SPP* and *YSA*	35S	Human codon-optimized Cas9	OsU6-2	*Agrobacterium*-mediated transformation	Feng *et al.* (2013)
	OsMPK5	35S	Human codon-optimized Cas9	OsU6	*Agrobacterium*-mediated transformation	Xie and Yang (2013)
	CAO1 and *LAZY1*	Ub1	Rice codon-optimized Cas9	OsU3	*Agrobacterium*-mediated	Miao *et al.* (2013)
Triticum aestivum	*TaMLO*	2X35S	Rice codon-optimized Cas9	TaU6	Protoplast transformation	Shan *et al.* (2013*b*)
	Tainox and *TaPDS*	CaMVE35S	Human codon-optimized SpCas9	CaMVE35S	*Agrobacterium*-mediated transformation	Upadhyay *et al.* (2013)
	TaMLO-A1	Ub1	Plant codon-optimized Cas9	TaU6	Transformation using particle bombardment	Wang *et al.* (2014)
Sorghum bicolor	*DsRED2*	Rice Actin1	Monocot codon-optimized synthetic Cas9	OsU6	*Agrobacterium*-mediated transformation	Jiang *et al.* (2013*b*)
Marchantia polymorpha L.	*MpARF1*	CaMV 35s and MpEF1α	Human codon-optimized Cas9	MpU6-1	*Agrobacterium*-mediated transformation	Sugano *et al.* (2014)
Citrus sinensis	*CsPDS*	CaMV 35S	Human codon-optimized Cas9	CaMV 35S	*Agrobacterium* infiltration	Jiang and Wang (2014)

[1]As an addition to this table, there are also two more reports were published about the CRISPR/Cas9 application on the plants of *Zea mays* L., *Marchantia polymorpha, Citrus sinensis and Populus tomentosa.*

1.2.5 Limitations of CRISPR/Cas9 System

CRISPR is a sequence specific genome editing technology. Due to this high specificity, the system has some limitations in practice. One of the encountered difficulties in CRISPR applications is the presence and the necessity of PAM, which is a specific three-nucleotide sequence that is adjacently located to the 20 nucleotides of target region on DNA (Jinek et al., 2012). Another restriction is the low-number of tolerable mismatches in the seed sequence (8-12 bp) of crRNA that is complementary to its target. Particularly, the mismatches adjacent to the PAM are more effectively inhibit the formation of RNA-DNA heteroduplex to unwind DNA strands for homology-dependent cleavage (DiCarlo et al., 2013).

In type II CRISPR/SpyCas9 system, PAM has to be in the orientation of 5'-NGG-3'. Any changes in the PAM sequence, even a single base-change or a SNP, causes the failure in the Cas9-DNA binding and so does the enzymatic activity (Sternberg et al., 2014). Due to the fact that, the nuclease activity is triggered by the homology-dependent binding of Cas9 to PAM region just as an allosteric regulator (Sternberg et al., 2014). Although the PAM region seems to be a strict restriction for the system, interestingly there is no PAM-flanking region in the CRISPR array (Marraffini and Sontheimer, 2010). This is for to inhibit the self-targeting of the prokaryotic genome by itself. It's also essential to know the WGS of the organism that is subjected, due to eliminate the possible manipulations at the off-target sites (Kuscu et al., 2014). Once the sgRNA is designed it's critically important to analyze whether the sgRNA is targeting a desired gene or any other. Cas9/sgRNA ratio is also one of the major limitation factor that can affect the mutagenesis frequency when it's lower than 1:1 in the system of interest (Li et al., 2013).

One more restriction of the CRISPR system is the target low-binding efficiency of secondary structures of designed guide RNA that possibly interferes the recognition of target sequence (Bassett et al., 2013; Chiu et al., 2013; Mali et al., 2013a; Cho et al., 2014).

1.3 pTV00: a tobravirus vector for plant expression

pTRV plant expression system is based on binary genome, which are pTRV1 (RNA1) and pTRV2 (RNA2) (Senthil-Kumar and Mysore, 2014). This system is especially used for post-transcriptional gene silencing (PTGS) in plants (Depicker and Van Montagu, 1997). Whereas pTRV1 expression provides the viral movement proteins for systemic silencing, pTRV2 encodes the coat protein and any inserted gene or gene segments as desired (Ratcliff et al., 1999; Ratcliff et al., 2001).

pTV00 is a type of tobravirus vector that express RNA2. As a limiting factor, pTV00 requires RNA1 (pBINTRA6) proteins for it's replication and movement and also contains an intron that provides the replication and expression in bacteria (Hamilton and Baulcombe, 1989). Obviously, the co-expression of RNA1 and RNA2 is necessary for proper operation of the system in plants (Ratcliff et al., 2001). For co-expression, 1:1 ratio of Agrobacterium that contains RNA1 and RNA2 can be used for agor-infiltration. Less amount of RNA1, inhibits the encoding of RNA2 in plant tissues (http://www.plantsci.cam.ac.uk) (Ratcliff et al., 2001). As an additional information, it's important to keep plants below 24ºC prior to pTRV infection to promote the T-DNA transfer (Salas et al., 2001).

In this study, we used pTRV2 vector to encode sgRNA_4OMT2 and sgRNA_PDS with AtU6p. Due to the systemic in-planta expression of TRV system, the co-infiltration of TRV1, TRV2 and hCas9 coding plasmids in plant leaves provided a further developed system for CRISPR/Cas9 applications in plants (Ali et al., 2015).

2. MATERIALS AND METHODS

2.1. Plant Material

P. somniferum L. (cv. **Office-95**) *and N. benthamiana* L. seeds were obtained from TMO, Ankara and grown in the mixture of 1/2 perlite, 1/2 peat in an environment of 24/20°C – 16/8 hours light/dark and 60% humidity in plant growth cabinets (PolEko).

Figure 2.1 Plant species used in this study were grown in the controlled plant growth cabinets.

2.2. Basic Information about Plasmids

In this study, the commercial Addgene vectors of 46965 (pK7WGF2::hCas9), 46966 (pICH86966::AtU6p::sgRNA_PDS), 46968 (pICSL01009::AtU6p), 48002 (pICH47751), 49771 (pICH47742::2X35S-5'UTR-hCas9(STOP)-NOST) and manually synthesized plasmids of pICH86966::AtU6p::sgRNA_4OMT2, pTRV2::AtU6p::sgRNA_4OMT2, pTRV2::AtU6p::sgRNA_PDS and pTRV1 (pBINTRA6) were used. Commercial plasmids of Addgene were supplied as a stab of pre-transformed *E. coli* in their specific strains of DH5α and DH10B. Bacterial cells are

cultured in the LB medium containing the appropriate amounts of their specific antibiotics (Table 1.)

Table 2.1 Characteristic features of used plasmids.

	Plasmid code	Bacterial Resistance	Low / High Copy	*E. coli* Bacterial Strain
1	46965 (pK7WGF2::hCas9)	Spec.	Low	DH5α
2	46966 (pICH86966::AtU6p::sgRNA_PDS)	Kan.	Low	DH5α
3	46968 (pICSL01009::AtU6p)	Spec.	High	DH5α
4	48002 (pICH47751)	Amp.	High	DH10B
5	49771	Amp.	High	DH5α
6	pICH47751::AtU6p::sgRNA_4OMT2	Amp.	High	DH10B
7	pTRV2::AtU6p::sgRNA_PDS	Kan.	Non	DH5α
8	pTRV2::AtU6p::sgRNA_4OMT2	Kan.	Non	DH5α
9	pTRV1	Kan.	Non	DH5α

2.3 Verification of Plasmids

Both the commercial and synthesized plasmids must be verified before applying agro-infiltration to the plants. The verification was done by enzymatic digestion with a pre-defined restriction endonucleases, classical PCR and colony PCR just after transfecting inside the bacterial cells either *E. coli* or *A. tumefaciens*. Lastly, plasmids were controlled whether the all sequences were correct or not via Sanger dideoxy sequencing. After fulfilling all the requirements for plasmid verification, they can be transferred inside the plant cells with the assistance of natural genome engineer, *A. tumefaciens*.

2.3.1 Verification by enzymatic digestion assay

All the plasmids were isolated by using the GeneJET Plasmid Miniprep Kit (Fermentas) according to the manufacturer's protocol. 5 mL and 10 mL of bacterial cultures were used for the high and low-copy-number-plasmids respectively. Isolated plasmids were quantified in NanoDrop 2000c.

All the plasmids were verified by enzymatic digestion with restriction endonucleases (Thermo Scientific). The recognition sites for the enzymatic digestions were selected according to the individual maps of plasmids in the Addgene's website (https://www.addgene.org/) (Appendixes). A general enzymatic digestion assay for each individual plasmid was processed by combining ~250 ng of plasmid DNA, 5 units of restriction enzyme and 2 µL of 10 X specific enzyme buffer in a single 1.5 mL microcentrifuge tube with sterile dH_2O in a final volume of 20 µL. The tubes were incubated in a thermal block for 1-3 hours at 37°C.

Digested pDNAs were run on the 0.8% agarose gel by electrophoresis for an hour. The agarose gel was prepared by homogenizing the mixture of 100 mL 1 X TBE, 0.8 g agarose powder and 1,8 µL ethidium bromide. For electrophoresis, 1 X TBE buffer was used. To prepare 1 L 10 X TBE, compounds of 108 g TRIS, 55 g boric acid, and 40 mL of 0.5 M EDTA(pH: 8.0) were mixed in a single bottle filled up to 1 L with distilled water, and sterilized by autoclave.

2.3.2 Verification by classical PCR

Plasmids coded by numbers of 46965 and 49771 encode synthetic SpCas9 protein. Specifically designed flCas9F02 and flCas9R03 primers were used to amplify about 685 bp from inside the hCas9 insert of the plasmid. Plasmid 49771 was used as a positive control in the reaction. The PCR mixture was prepared by combining 2.5 µL 10 X Taq polymerase buffer, 1 µL dNTP (10 mM), 2 µL $MgCl_2$ (10 mM), 0.25µL Taq DNA polymerase (5 U/µL), 1 µL/each of flCas9F02 and flCas9R03 primers (Appendixes) (10 µM/µL), 1 µL from each of the plasmids (~100 ng/µL) as templates and dH_2O up to 25 µL for each sample. Thermal-cycler adjustments was: 5 min 95°C pre-denaturation followed by 35 cycles of 1 min at 95°C, 1 min at 52°C 1 min at 72°C and 5 min. for final extension at 72°C. The end-product was run on the 2% agarose gel in 1 X TBE buffer solution.

Plasmid 46966 was verified by a classical PCR reaction which was done in the mixture of 2.5 µL 10 X Taq polymerase buffer, 2 µL $MgCl_2$ (10 mM), 1 µL dNTP (10 mM), 1 µL from each of sgRNApaIF and sgRNApaIR primers (Appendixes) (10 µM/µL), 0.25 µL Taq DNA polymerase (5 U/µL), 1 µL (~100 ng/µL) pDNA template and distilled water up to 25 µl. Thermal-cycler protocol was 5 min 95°C pre-denaturation, 35 cycles of 1 min at 95°C, 1 min at 45°C 1 min at 72°C and lastly 5 min for final extension at 72°C. The amplified segments were run on the 2% agarose gel.

2.3 Construction of Plasmids

In order to express the desired DNA segments inside the plant tissue, a proper assembly of those expressing regions was required. For instance; specific promoters, terminators and UTRs were indispensable parts required to express any specific CDS regions inside the desired cell type. Therefore, it's necessary to select proper segments to control the expression time and level. In this study, two different types of vectors were constructed as viral and synthetic.

2.3.1 Construction of synthetic plasmids

The sgRNAs targeting the *PDS* and *4OMT2* genes were expressed both from synthetic and virus based vectors. For *PDS*, the targeting region is directly cloned from plasmid 46966 together with U6 promoter of *A. thaliana* into pTRV2. For *4OMT2*, firstly the sgRNA_4OMT2 insert is formed by classic PCR and then U6p was ligated to the synthesized RNA by MoClo L-1 reaction. The insert, AtU6p::sgRNA_4OMT2, was cloned and ligated into pTRV2.

2.3.1.1 In-vitro synthesis of sgRNA encoding synthetic plasmids

The *PDS* gene targeting RNA (sgRNA_PDS) was encoded by the plasmid numbered by 46966, where the sgRNA_PDS was placed under an AtU6 promoter.

4OMT2 targeting sgRNA was synthesized by using a classical PCR by using 2.5 µL 10 X Taq polymerase buffer, 1 µL dNTP (10 mM), 2 µL $MgCl_2$ (10 mM), 0.25 µL Taq DNA polymerase (5 U/µL), 1 µL/each of F & R (4OMT2sgRNA_1F and 4OMT2sgRNA_1R) primers (10 µM/µL), 1 µl (~100 ng/µL) plasmid 46966 as a template and distilled water up to 25 µL. The thermal-cycler conditions were adjusted to 5 min 95°C pre-denaturation followed by 35 cycles of 1 min at 95°C, 1 min at 53°C 1 min at 72°C and lastly 5 min for final extension at 72°C.

2.3.1.2 Assembly of AtU6p with sgRNA_4OMT2 by MoClo L1 reaction

Then the PCR product was purified by using Roche's High Pure PCR Product Purification Kit. The AtU6p and a synthesized sgRNA were assembled by MoClo (Modular Clonning) method (Weber et al., 2011). The single-step reaction was done by the proper combinations of 40 fmoles of each vectors (46968: an AtU6p encoding L0 plasmid, plasmid 48002: empty backbone plasmid used as MoClo L1 destination vector) and 100 ng purified sgRNA_4OMT2 amplicon with 0.25 µL of Bsa I (10 U/µL) (New England Biolabs) and 200 CEU of Thermo Scientific T4 Ligase (5 Weiss U/µL), 1.5 µL of 10 X T4 ligase buffer, and 1.5 µL 10 X BSA up to the final volume of 15 µL.

The reaction mix was incubated in thermal-cycler for 1 min at 37°C, followed by 30 cycles of 3 min at 37°C and 4 min at 16, followed by one cycle of 5 min 50°C; 5 min at 80°C. The end-product was transferred into chemically competent *E.coli* (strain DH10B) cells by heat-shock reaction for blue-white colony screening.

2.3.2 Construction of viral vectors

Apa I recognition sites were added to flanking sites of AtU6p::sgRNA_4OMT2 and AtU6p::sgRNA_PDS inserts by using sgRNApaIF and sgRNApaIR primers as previously mentioned. The PCR products were desalted by using High Pure PCR Product Purification Kit (Roche). After desalting the amplicons, they were ligated into pTZ57R/T (Thermo Scientific) or pGEM-T (Promega), T-A cloning vectors. 5 µL from

each end-product were transfected to chemically competent DH5α cells by using heat-shock transfection method as described in below section 4 and confirmed by colony-PCR (section 5) by using sgRNApaIF and sgRNApaIR primers and Sanger dideoxy sequencing.

The verified inserts were used to ligate in pTRV (Tobacco Rattle Virus) expression vectors for in-planta expression. An empty backbone of pTV:00 (or pTRV2, a tobravirus vector based on RNA2) plasmid and both two inserts were digested with Apa I restriction endonuclease and run on the 2% agarose gel to separate the digested and undigested parts apart from each other.

After gel electrophoresis, digested amplicons and pTRV2 vector were removed from the agarose by using sterile scalpel and purified with QIAquick Gel Extraction Kit (Qiagen). For each insert, the ligation reaction was performed by using T4 DNA ligase. The reaction mixture of 0.5 µL T4 DNA ligase (5 U/µL), 1.5 µL 10 X T4 ligase buffer, 1 µL ~50 ng/µL Apa I digested pTRV2 plasmid, 5 µL digested insert (10 ng/µL) and dH_2O up to 15 µL of final volume in a single PCR tube. The mixtures were incubated for an overnight at 4°C and 5 µL from each of the ligation products were used to transfect into chemically competent *E. coli* DH5α cells.

2.4 Plasmid Transfection to *E. coli* Cells

Chemically competent *E.coli* cells were prepared by RbCl buffers (Hanahan, 1985). First, a single colony of bacteria was inoculated to a 3 mL volume of fresh Hi-salt LB broth and leaved for an overnight incubation at 37°C at 250 rpm. After bacterial culture reached to the stationary phase, 3 mL of a seed culture is inoculated to 200 mL of fresh Hi-salt LB broth and placed in a shaker-incubator at 37 °C at 200 rpm till the OD_{600} value reaches to 0.5. After incubation, the bulk bacterial culture was chilled in ice for 15 min. and centrifuged for 5 min at 3500 rpm at 4 °C. The pellet was re-suspended in 10-20 mL of ice-cold Buffer-1 (Appendixes) and re-centrifuged in the same conditions. And lastly, the pellet was re-suspended in 6-8 mL of ice-cold Buffer-2 (Appendixes) gently by pipetting up and down until all the suspension was homogenized. The final

suspension was aliquoted to 1.5 mL sterile micro-centrifuge tubes each containing 100 µL of competent bacterial cells which could be stored at -80°C for longer periods.

100 µL chemically competent *E.coli*, DH10B cells were thawed on ice for 10 minutes and then mixed with 10 µL of the cut-ligation reaction end product. The mixture was incubated on ice for 30 min then heat-shocked at 42°C for 90 sec. and re-incubated 5 min more on ice. 900 µL of liquid LB broth (Hi-Salt) is added to the mixture and incubated at 37°C for 80-90 min to recover the cells. Transfected cells were spun down by centrifugation at 3000 rpm for 10 min at RT (Room Temp). After harvesting cells, pellet part was re-suspended by adding 100 µL LB broth and plated on specific medium. For conventional bacterial selection, antibiotic markers were used for the positive colony selection and for blue-white colony selections 100 µL of IPTG (0.5 mM), 200 µL of Xgal (100 mg/mL) and 100 µL specific antibiotics (50-100 mg/mL Spec, Kan, Amp, Carb etc.) were used simultaneously in 1:2:1 volumetric ratio per 100 mL Sigma Aldrich LB agar in respect.

2.5 Confirmation of Positive Colonies

The positive colonies containing the true copy of desired plasmids were confirmed by colony PCR with its specific primers and Sanger dideoxy sequencing.

Selected white colonies were re-suspended in 5 µL of high-pure dH_2O and store as a template in -20°C. The colony-PCR reaction was done by using the mixture of 2.5 µL 10 X Taq polymerase buffer, 2 µL $MgCl_2$ (10 mM), 1 µL dNTP (10 mM), 1 µL from each of sgRNApaIF and sgRNApaIR primers (10 µM/µL), 0.25 µl Taq DNA polymerase (5 U/µL), 5 µl of white colony suspension as template and distilled water up to 25 µL. Thermal-cycler protocol was 5 min 95°C pre-denaturation, 35 cycles of 1 min at 95°C, 1 min at 45°C 1 min at 72°C and lastly 5 min for final extension at 72°C.

For further verification, the confirmed plasmid samples in colony PCR were sequenced by a commercial company (RefGen, Ankara, Turkey) by Sanger dideoxy sequencing

method. Plasmids validated with sequencing were used to transfect in *A. tumefaciens* cells.

2.6 Transfection of Plasmids to *A. tumefaciens* Cells

Cas9 and sgRNA encoding vectors were transfected to plant cells by using A. tumefaciens mediated transfection method. To transfect the plasmid into Agrobacterium cells, we used two different methods as electro-transformation and a classic freeze-thaw transformation method.

Verified plasmids, containing the synthetic 35Sp::h*Cas9*, AtU6p:sgRNA_PDS and AtU6p:sgRNA_4OMT2 inserts were transferred into the electro-competent *A. tumefaciens* cells by using electroporator (Invitrogen, Neon Transfection System). Electro-competent EHA105 cells were prepared as follows. A single colony of bacteria was inoculated into a 100 mL of fresh LB broth and incubated at 28°C at 250 rpm in a shaker-incubator until the OD_{600} value reaches the early log phase (0.7-1.00). Then the bacterial culture was aliquoted into 15 mL conical centrifuge tubes and placed in ice for 10 min and then centrifuged at 4000 rpm for 10 min. The pellet was re-suspended in the same volume of ice-cold sterile distilled water and re-centrifuged at 4000 rpm for 10 min. This step should be repeated at least 3-4 times to remove the access amount of salt from the bacteria. The pellets from each tube were combined in a single tube to increase the bacterial cells if necessary. Finally at the last step, the pellet was re-suspended in 8 mL of ice-cold sterile 10% glycerol solution and aliquoted into the 1.5 mL micro-centrifuge tubes in desired volumes.

Invitrogen Neon Transfection System was used to transfect the desired plasmids into the electro-competent cells. For each reaction, 1 µL pDNA (100 ng/µL), 10 µL of electro-competent *Agrobacterium*, and 11 µL of R buffer were mixed in a single PCR tube and mixed well. 10 µL from this mixture was used to treat an electrical impulse. The electroporator was adjusted to 1800 V, 10 milliseconds for 4 pulses. The transfected cells were transferred in a sterile 900 µL of low-salt LB broth and incubated in a

shaker-incubator for 3-4 hours at 28°C, 100 rpm. After recovering the cells, they were plated in LB agar medium containing the appropriate amounts of antibiotics.

Alternatively, chemically competent *Agrobacterium* cells were prepared by using $CaCl_2$ according to Holsters's protocol (Holsters et al., 1978). A single colony of desired *Agrobacterium* strain was transferred from the freshly cultured LB agar plate to 5 mL of low-salt LB broth medium containing the appropriate amounts of antibiotics specific for that strain. For strain EHA105, 20 mg/L rifampicin was used in the growth medium due to the natural resistivity it possess. After an overnight incubation at 28°C at 250 rpm, 500 µL of bacterial suspension were used as a seed culture to re-cultivate in 50 mL of LB broth medium and growth for an overnight at 28°C in a shaker-incubator. The bacterial culture was chilled on ice for 30 min and 45 mL of this bacterial culture was transferred into the pre-chilled, 50 mL falcon tubes and centrifuged for 10 min. at 4000 rpm at 4°C. Then the supernatant part was decanted and the pellet was re-suspended in 5 mL of ice cold 20 mM $CaCl_2$. The tubes were re-centrifuged for 5 min at 4000 rpm, 4°C and the pellet was re-suspended in 1 mL of ice cold 20 mM $CaCl_2$. 100 µL of aliquots were distributed in 1.5 mL pre-chilled microcentrifuge tubes and directly frozen in the liquid nitrogen and stored at -80°C.

Table 2.2 Used Agrobacterium strains and their natural resistivity against antibiotics.

	***A. tumefaciens* strain**	**Natural antibiotic resistance (Chrom. or Ti plasmid)**
1	GV3101	Rifampicin and Gentamycin
2	EHA105	Rifampicin

For chemically competent *Agrobacterium* transfection, freeze-thaw transformation protocol was applied. 500 ng of pDNA was added for the each tube containing 100 µL of competent *Agrobacterium* and incubated on ice for 15-30 min. All the tubes were frozen in the liquid nitrogen for 5 min then heat-shocked in a heat-block at 37°C for 5 min. After the heat-shock, the tubes were thawed on ice for 5 min. 1 mL of low-salt LB broth (without any antibiotics) was added to the tubes and incubated in a shaker

incubator for 3-4 h at 100 rpm, 28°C. The bacterial suspensions were plated on the LB agar plates containing the specific antibiotic in appropriate amounts.

2.7 Agro-infiltration to plants

Agro-infiltration of plants was performed by using a needleless syringe. A plasmid containing overnight grown culture of Agrobacterium cells (OD_{600}: 2.00-2.5) were harvested by centrifugation at 3000 rpm for 10 minutes, and harvested bacterial pellets were re-suspended in induction buffer mixture containing 10 mM $MgCl_2$, with 100 µM acetosyringone and 1 mM MES (2-(*N* morpholino) ethanesulfonic acid) at pH 5.6 (OD_{600}: 0.8). The prepared mixture were incubated for 4 hours in dark at room temperature and infiltrated into leaves by a needleless syringe.

Inoculations of synthetic plasmids were performed by using 1:1 ratio mixed bacteria containing pICH86966::AtU6p::sgRNA_4OMT2, 46965, 46966 plasmids for sgRNA and hCas9 in-planta expression. There are two sets of application as pICH86966::AtU6p::sgRNA_4OMT2 + 46965 and 46966 + 46965 respectively. The first set is prepared for *P. somniferum* and the second one is for *N. benthamiana* only.

Figure 2.2 Agro-inoculation of plant leaves with a needleless syringe at lower-face.

For pTRV2 inoculations, 1:1:1 volumetric ration of pTRV1, RNA encoding pTRV2, and hCas9 encoding 46965 plasmids were mixed just before the agro-inoculation or 4

hours of incubation and co-infiltrated into the plant leaf tissues. The reason of co-infiltration of pTRV1 with pTRV2 is due to; ptv00 needs pTRV2 to sustain for replication and movement proteins.

Table 2.3 Plasmids with their host strains and plant species that will be subjected to inoculation.

	Co-infiltration and *A. tumefaciens* strain	**Plant Species**
1	pTRV1 (GV3101) + pTRV2::AtU6p::sgRNA_PDS (EHA105) + 46965 (EHA105)	*N. benthamiana*
2	46966 (EHA105) + 46965 (EHA105)	*N. benthamiana*
3	pTRV1 (GV3101) + pTRV2::AtU6p::sgRNA_4OMT2 (EHA105) + 46965 (EHA105)	*P. somniferum*
4	pICH47751::AtU6p::sgRNA_4OMT2 (EHA105) + 46965 (EHA105)	*P. somniferum*

Table 2.4 Used plasmids and their specific properties in detail.

Bacteria & Strain	Plasmid	Description	Bacterial Resistance	Reference
E.coli (DH5α) & *A. tumefaciens* (AGL1, EHA105)	46965 (pK7WGF2::hCas9)	Expresses human codon optimized Cas9 in plant tissues.	Spec.	Nekrasov et al., 2013
E.coli (DH5α) & *A. tumefaciens* (AGL1)	46966 (pICH86966::AtU6p::sgRNA_PDS)	Expresses sgRNA specifically designed for PDS gene under AtU6 promoter.	Kan.	Nekrasov et al., 2013
E.coli (DH5α)	46968 (pICSL01009::AtU6p)	Contains A. thaliana U6 promoter used for MoClo applications	Spec.	Nekrasov et al., 2013
E.coli (DH10B)	48002 (pICH47751)	L1 clonning vector in MoClo, binary vector for T-DNA delivery into plants	Amp.	Weber et al., 2011
E.coli (DH5α)	49771 (pICH47742::2x35S-5'UTR-hCas9(STOP)-NOST)	hCas9 expressor plasmid with 2 X 35S promoter and NOST terminator	Amp.	Belhaj et al., 2013
E.coli (DH10B) & *A. tumefaciens* (EHA105)	pICH47751::AtU6p::sgRNA_4OMT2	Synthetic vector, contains a transcription unit for sgRNA_4OMT2	Amp.	This work
E.coli (DH5α) & *A. tumefaciens* (AGL1)	pTRV2::AtU6p::sgRNA_PDS	Viral vector, contains a transcription unit for sgRNA_PDS	Kan.	This work
E.coli (DH5α) & *A. tumefaciens* (AGL1)	pTRV2::AtU6p::sgRNA_4OMT2	Viral vector, contains a transcription unit for sgRNA_4OMT2	Kan.	This work
E.coli (DH5α) & *A. tumefaciens* (GV3101)	pTRV1 (pBINTRA6)	Viral vector transcribes movement proteins for systemic silencing with TRV2	Kan.	Hamilton and Baulcombe, 1989

2.8 Harvesting of the sgRNA_Cas9 treated plant tissues

After 2 days subsequent to agro-infiltration, the leaves were collected by using sterile scalpels, sterilized by using 70% ethanol, directly frozen in the liquid nitrogen and kept at -80°C for gDNA isolation step.

2.9 Isolation of the gDNA from the treated leaves

The plant gDNAs from both control group and treated plants were extracted by using standardized CTAB & sucrose plant DNA extraction protocol (Sahu et al., 2012). Firstly, both suspension and extraction buffers (Appendixes) were preheated for 20 min. at 60°C. Approximately 400 mg of frozen leaves are grounded in liquid nitrogen by the help of pre-chilled mortar and pestle or by using TissueLyser II system (QIAGEN, Valencia, CA) in the presence 150 mg of PVP. The mixture is transferred into 2 mL microcentrifuge tubes and suspended in 2 volumes of suspension buffer, preferentially 1.2 mL for each tube. All the content was homogenized by vortex and tubes were incubated for the following 40 minutes at 60°C. During this incubation, all the tubes were inverted after each 5 min. After the incubation, suspensions are centrifuged at 10000 rpm for 15 min at RT. 1.5 mL of extraction buffer (CTAB Buffer) was added to the each tube and 6-8 times inverted to distribute the buffer homogeneously inside the tube and let to incubate at 60°C for 30 min more. The tubes were centrifuged at 12000 rpm for 15 min at RT and the aqueous phase was carefully transferred into a new 2 mL microcentrifuge tubes. Double volume of chloroform: isoamyl alcohol (24:1 volumetric ratio) was added to the each tube and mixed by inverting the tube 15-20 times. The tubes were centrifuged for 15 min at 12500 rpm and the aqueous transparent phase was transferred into a new 2 mL microcentrifuge tubes again. Double volumes of pre-chilled isopropanol was added and kept at -20°C for an overnight incubation to precipitate all the DNA content.

After an overnight incubation, the precipitated DNA was collected by centrifuging at 12000 rpm for 15 min. The supernatant part was discarded and the pellet is washed with 100% ethanol, 70% ethanol by applying sequential centrifugation steps at 12000 rpm

for 15 min. The supernatants were discarded and the pellets were air dried in a sterile laminar flow cabinet. Lastly, 50-100 µL of sterile distilled water was added to dissolve gDNA.

After gDNA isolation, each sample was treated with RNase A by adding 3 µl of 100 mg/mL RNase A (Qiagen) to the tubes and incubating at 37°C for 20 min. The isolates were enriched by enzymatic digestion.

2.10 PCR amplification of the *PDS* and *4OMT2* targeted regions

The 100 ng gDNA isolated from sgRNA & hCas9 expressing plant leaves were used as templates to clone the targeted regions of *PDS* and *4OMT2* genes.

For the amplification of *PDS* region, 100 ng of gDNA, 2.5 µL 10 X Taq polymerase buffer, 2 µL $MgCl_2$ (10 mM), 1 µL dNTP (10 mM), 1 µL from each PDS_MlyIF and PDS_MlyIR primers (10 µM/µl) (Nekrasov et al., 2013), 0.25 µL Taq DNA polymerase (5 U/µL), and distilled water up to 25 µL were incubated at thermal-cycler conditions of 5 min 95°C pre-denaturation, 35 cycles of 1 min at 95°C, 1 min at 46°C 1 min at 72°C and lastly 5 min for final extension at 72°C.

For *4OMT2* targeted region, again 100 ng of gDNA, 2.5 µL 10 X Taq polymerase buffer, 2 µL $MgCl_2$ (10 mM), 1 µL dNTP (10 mM), 1 µL from each 4OMT2_AgeIF and 4OMT2_AgeIR primers (10 µM/µL) (Appendixes), 0.25 µL Taq DNA polymerase (5 U/µL), and distilled water up to 25 µL were mixed for each of replicate and tubes were incubated at thermal-cycler conditions of 5 min 95°C pre-denaturation, 35 cycles of 1 min at 95°C, 1 min at 48°C 1 min at 72°C and lastly 5 min for final extension at 72°C.

2.11 Enrichment of the samples

The enrichment of the samples was achieved in two independent steps (Nekrasov et al., 2013). The first enrichment of the CRISPRed samples was performed by digesting the sgRNA/Cas9 treated gDNA of plants with enrichment restriction enzymes of Mly I and Apa I for *PDS* and *4OMT2* targeted samples respectively, whom recognition sites were specifically localized on the 3' end of the 20 bp target region, just before the PAM (NGG).

After harvesting the sgRNA_PDS/Cas9 treated *N. benthamiana* leaves, they were treated with Mly I restriction enzyme. 1 µL (10 U/µl) of Mly I (Thermo scientific), 2 µL of 10 X Tango buffer, 1 µg of isolated gDNA and sterile dH_2O were mixed in a single 1.5 mL microcentrifuge tube up to the final volume of 20 µL and incubated for 3 hours at 37°C. The same protocol was followed for the *P. somniferum* gDNA samples with Apa I enzyme. 1 µg of CRISPRed poppy gDNA was treated with 1 µL (10 U/µL) of Apa I enzyme (Jena Biosciences) in the mixture of 2 µL of 10 X Apa I specific buffer and sterile dH_2O in a single 1.5 mL microcentrifuge tube up to the final volume of 20 µL. The mixture was incubated for 2 hours at 25°C.

The second enrichment was performed with the PCR products obtained from amplification of the targeted regions. The amplified regions of the *PDS* and *4OMT2* genes were enriched by direct treatment of the previously mentioned enzymes to 25 µL of whole desalted PCR products.

2.12 Cloning of the amplicons into the vectors (p-GEMT)

After mutating targeted sites inside PDS and 4OMT2 genes with CRISPR/Cas9 system, the following step is to identify the InDels in those exact regions. Firstly, the targeted gene segment should be amplified from the external flanking sites with a specific primer and ligated into sequencing vectors like pGEM-T. Then, ligation product should be transferred into bacteria to select the positive colonies that contain a cloned full copy of

InDel formed target regions. After verifying with M13 colony PCR, isolated plasmids were further analyzed with Sanger dideoxy sequencing system.

2.12.1 Ligation of CRISPRed sites of 4OMT and PDS genes

For each insert, the ligation reaction was performed by using T4 DNA ligase. The reaction mixture of 0.5 µL (5 U/µL) T4 DNA ligase, 1.5 µL 10 X T4 ligase buffer, 2 µL 1/10 diluted pGEMT plasmid, 5 µL (10 ng/µL) digested insert and dH_2O up to 15 µL of final volume in a single PCR tube. The mixtures were incubated for an overnight at 4°C and 5 µL from each of the ligation products were used to transfect into chemically competent *E. coli* DH5α cells for blue-white colony selection as previously described before. The selected white colonies were firstly confirmed with M13 colony PCR and then Sanger sequencing.

2.12.2 M13 colony PCR

M13 colony-PCR reaction was done by using the mixture of 2.5 µL 10 X Taq polymerase buffer, 2 µL $MgCl_2$ (10 mM), 1 µL dNTP (10 mM), 1 µL from each of M13F and M13R primers (10 µM/µl), 0.25 µL Taq DNA polymerase (5 U/µL), 5 µL of white bacterial colony suspension as template and distilled water up to 25 µl for each single tube. Thermal-cycler protocol was 5 min 95°C pre-denaturation, 35 cycles of 30 sec at 95°C, 30 sec at 46°C, 1 min at 72°C and lastly 5 min for final extension at 72°C. The PCR products were loaded on a 2% agarose gel for electrophoresis.

2.13. Validation of InDels via sequencing

According to the result of M13 colony-PCR, positive colonies were selected and transferred into Hi LB broth medium containing appropriate amount of ampicillin and incubated for an overnight at 37°C, 250 rpm. pGEM-T or pTZ57R/T plasmids containing the inserts of *4OMT2* and *PDS* gene partial sequences were isolated from the grown bacterial culture with GeneJET Plasmid Miniprep Kit (Thermo Scientific) as

described in the manufacturer's protocol. The purified plasmids were sequenced by using Sanger dideoxy sequencing method from M13 sites by RefGen (Ankara, Turkey). The sequenced gene fragments were examined for InDel (Insertion & Deletion) analysis to see the double-nickase effect of CRISPR/Cas9 system.

3. RESULTS

3.1 Verification by Enzymatic Digestion Assay

All plasmids were verified by enzymatic digestion by single or double digesting them from different restriction endonuclease recognition sites. Agarose jel electrophoresis of the digested plasmid DNA was done on the 0.8% Agarose jel (100ml 1X TBE, 1.8 µl EtBr) for an hour (as seen in Figure 3.3).

3.2 Construction of Plasmids

3.2.1 Construction of Synthetic Plasmids

There were two synthetic plasmids were used in this study to trancribe sgRNAs in plant tissues. Plasmid #46966 is a commercial plasmid and plasmid 47751::AtU6p::sgRNA_4OMT2 was synthesized in this study by using MoClo moduler clonning technique.

3.2.1.1 Assembly of AtU6p with sgRNA_4OMT2 by MoClo L1 reaction

In order to construct 47751::AtU6p::sgRNA_4OMT2 plasmid, firstly sgRNA_4OMT2 was synthesized by a single step PCR by using plasmid 46966 as a template. The amplicon size for sgRNA_4OMT2 was about 164 bp (Figure 3.3). The amplified region was cloned into pGEM-T vector and sequenced with Sanger dideoxy method for the furthering reactions (Figure 3.2).

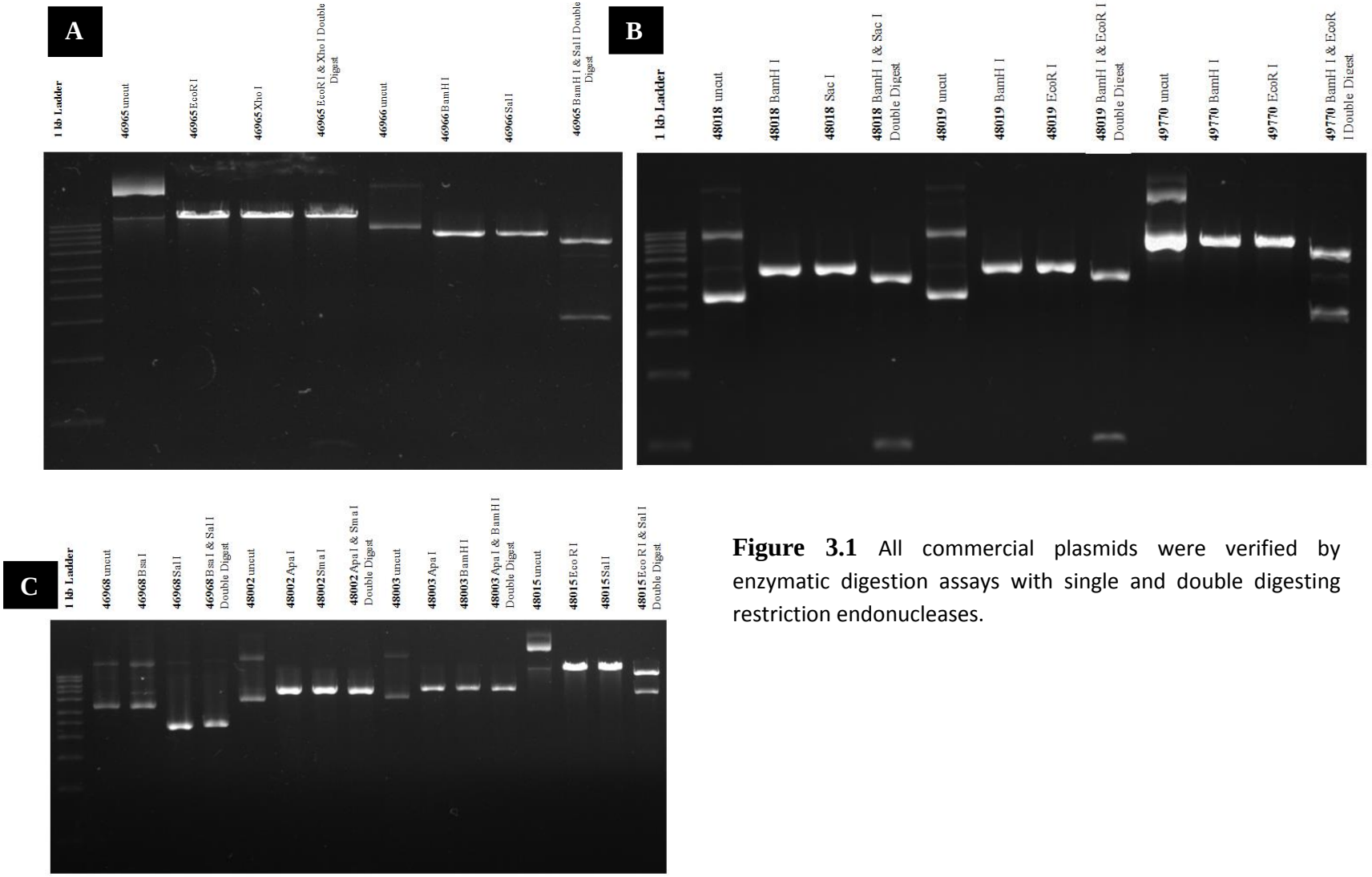


Figure 3.1 All commercial plasmids were verified by enzymatic digestion assays with single and double digesting restriction endonucleases.

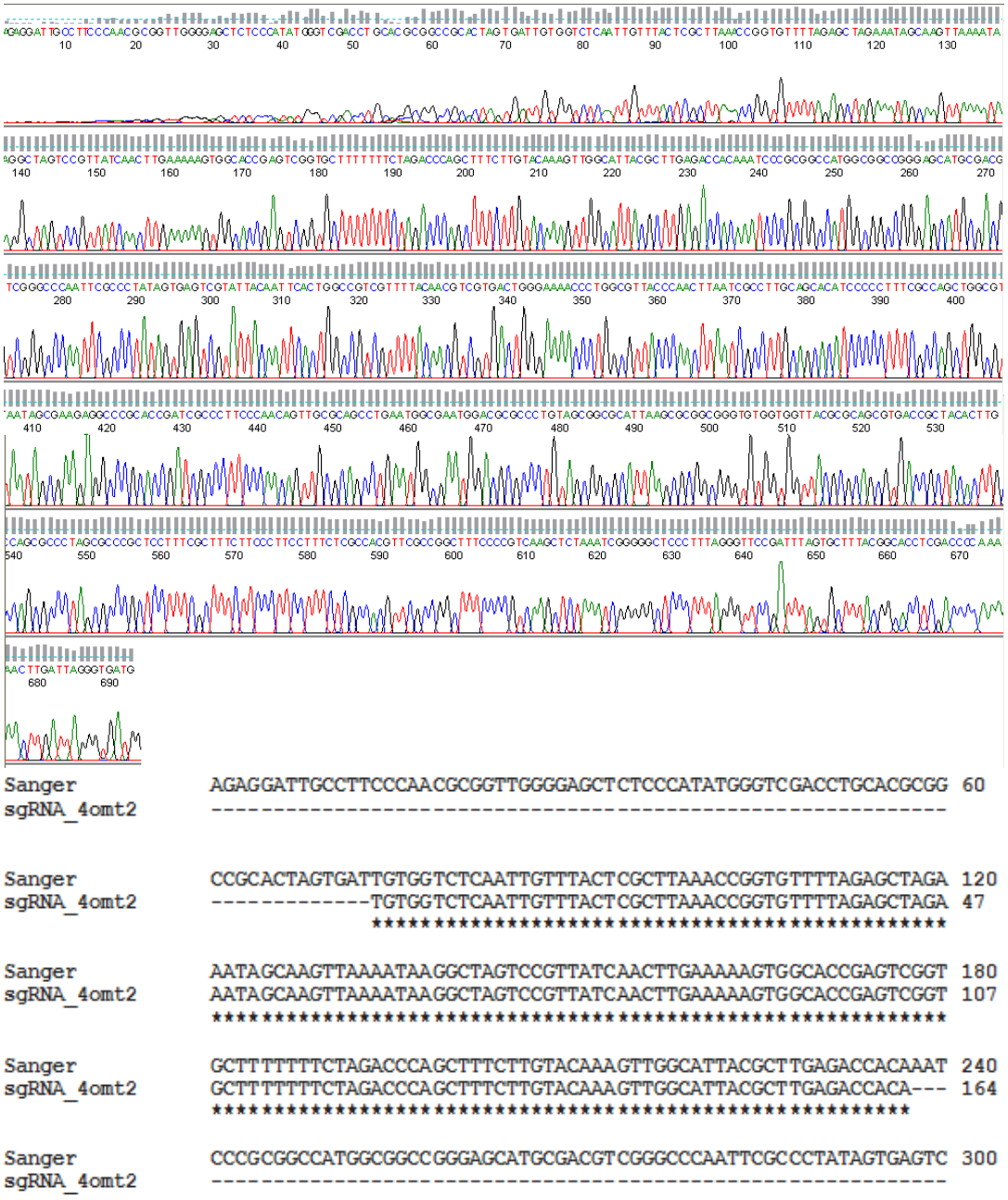


Figure 3.2 pGEM-T::sgRNA_4OMT2 was sequenced with M13 primers and sgRNA_4OMT2 insert was verified by Sanger dideoxy method.

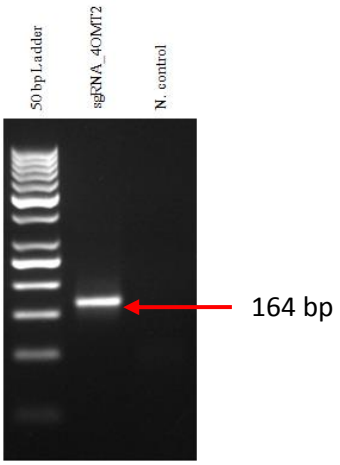


Figure 3.3 *4OMT2* targeting sgRNA was synthesized by using PCR.

Plasmids 46968 and 48002 were used digested with BsaI type IIS restriction enzyme to see whether the enzyme and plasmids work or not in MoClo L1 reaction. As seen in Figure 3.4, three replicates of plasmid 46968 and plasmid 48002 were perfectly digested with enzyme and verified for using in further reactions.

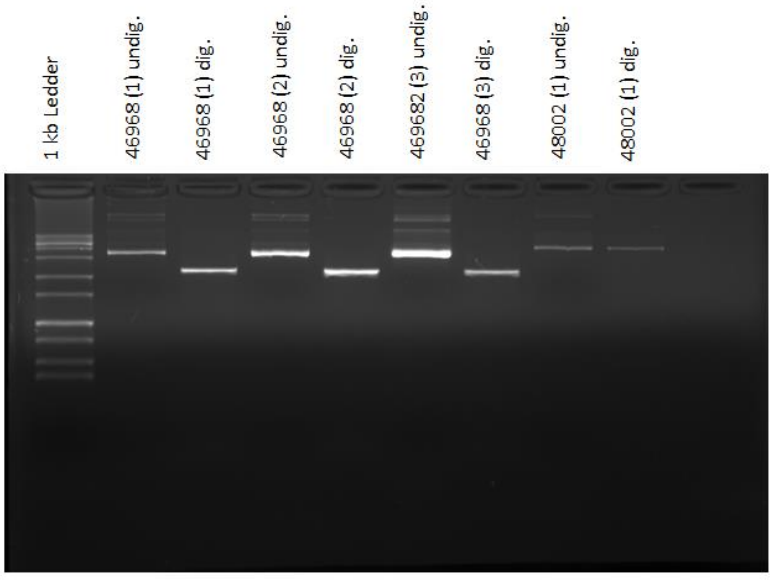


Figure 3.4 Before MoClo L1 reaction, each components of the reaction was verified by enzymatic digestion with BsaI restriction enzyme.

3.2.1.2 Confirmation of positive colonies by PCR

After *4OMT2* MoClo L1 reaction, the reaction product was transfected into bacteria and colonies were scanned with colony PCR. Bacterial colony samples of 1-1 to1-12 and 2-1 to 2-3 were screened by colony PCR and positive results obtained, except sample 1-7.

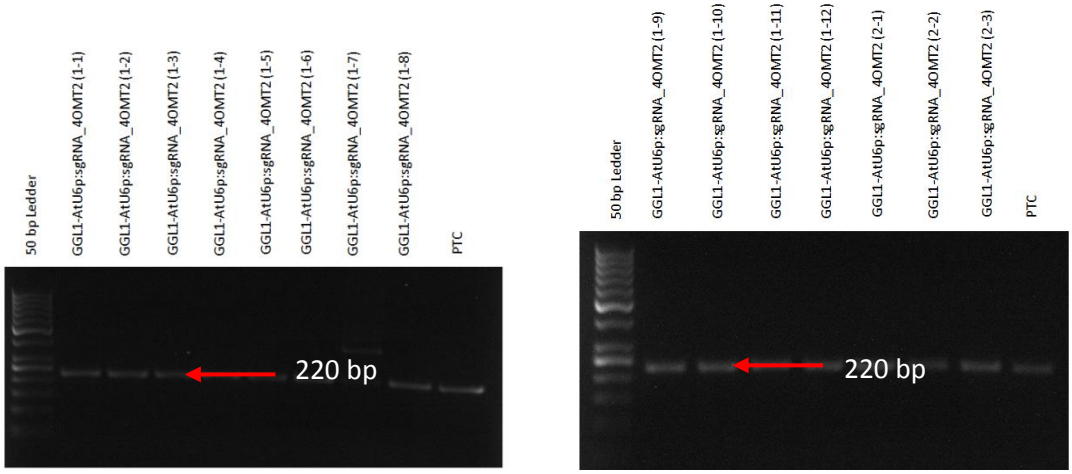


Figure 3.5 Agarose gel electrophoresis results of GGL1_4OMT2 colony PCR products.

Moreover, four of selected plasmid isolates were confirmed with classical PCR with specific primers. Sample 1-1, 1-2 and 1-3 were confirmed, but sample 1-7 was negative as expected from the result of colony PCR.

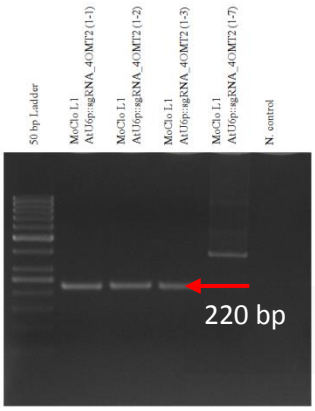


Figure 3.6 Agarose gel electrophoresis results of GGL1_4OMT2 classic PCR products.

Additionally, isolated plasmids were trated with BsaI and BpiI restriction endonucleases. As seen below in Figure 3.7, plasmid pICH47751(48002)::AtU6p_sgRNA::4OMT2 was not digested with BsaI, but digested with BpiI. This is due to the removal of BsaI recognition sites after L1 reaction, but BpiI recognition sites were conserved for the futher reactions. This property of plasmid faccilitates the enzymatic verification itself with ease.

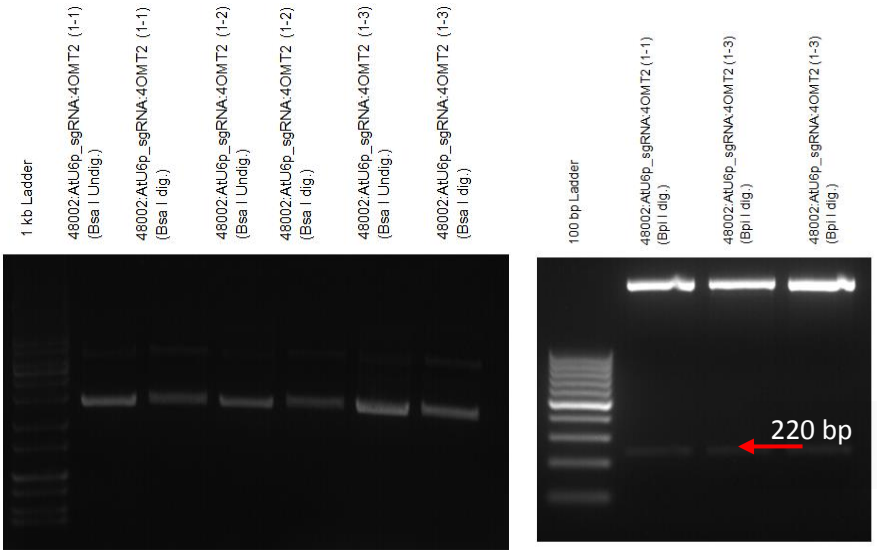
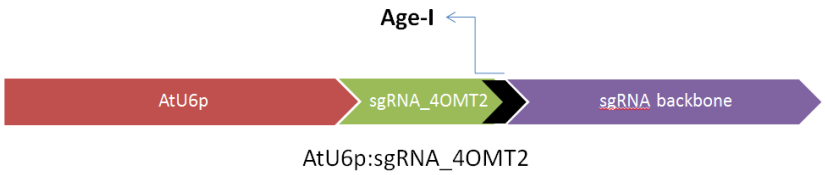


Figure 3.7 Enzymatic verification of pICH47751(48002)::AtU6p_sgRNA::4OMT2 by digesting Bsa I and Bpi I Type II-S restriction endonucleases.

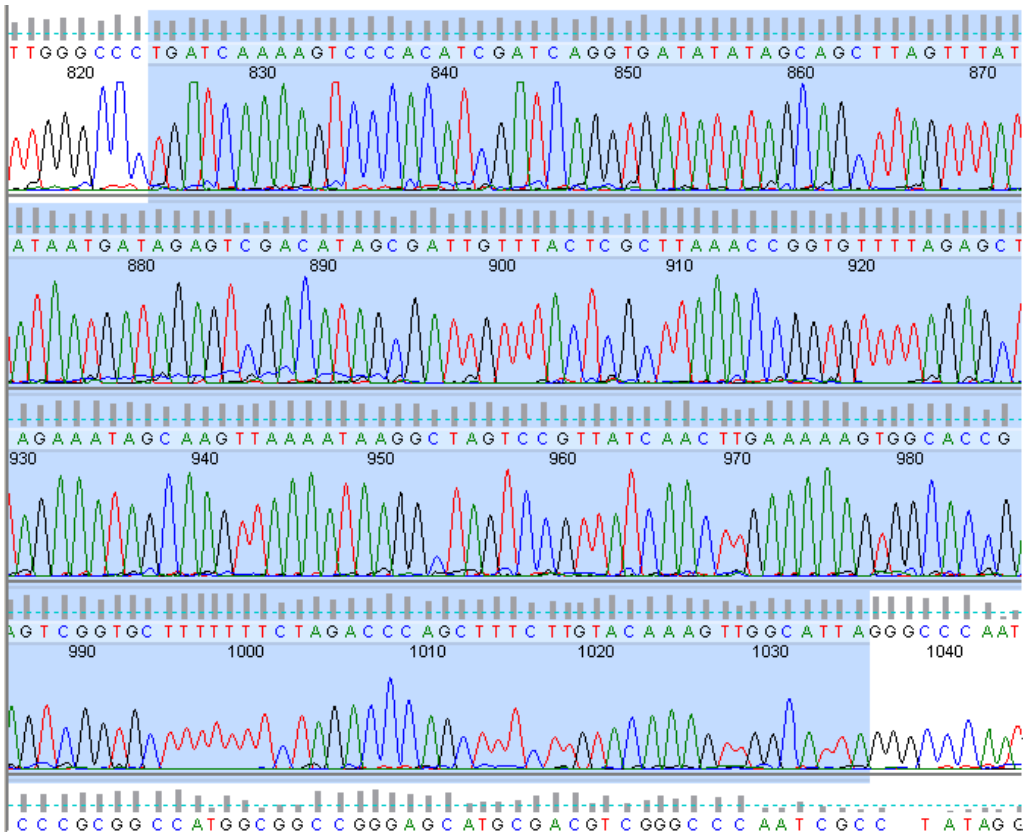


AtU6p:sgRNA_4OMT2

GGAGTGATCAAAAGTCCCACATCGATCAGGTGATATATAGCAGCTTAGTTTATATAATGATAGAG
TCGACATAGCGATTGTTTACTCGCTTAAACCGGTGTTTTAGAGCTAGAAATAGCAAGTTAAAAT
AAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTCTAGACCCAG
CTTTCTTGTACAAAGTTGGCATTACGCTT

Figure 3.8 Schematic representation of AtU6p::sgRNA_4OMT2. Nucleotides in red demostrates the *A. thaliana* U6 promoter. While greens were representing the 20 bp target sequence of *4OMT2* gene loci (black region in between green and purple demonstrated AgeI recognition site) and purple ones for tracrRNA-crRNA chimeric RNA.

AtU6p::sgRNA_4OMT2 insert in pICH47751 plasmid backbone was sequenced by using Sanger dideoxy method again and *4OMT2* MoClo reaction was verified.



Sequence ID: lcl|Query_22729 **Length:** 212 **Number of Matches:** 1

Range 1: 1 to 212 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
392 bits(212)	1e-113	212/212(100%)	0/212(0%)	Plus/Plus

```
Query  824   TGATCAAAAGTCCCACATCGATCAGGTGATATATAGCAGCTTAGTTTATATAATGATAGA  883
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     TGATCAAAAGTCCCACATCGATCAGGTGATATATAGCAGCTTAGTTTATATAATGATAGA  60

Query  884   GTCGACATAGCGATTGTTTACTCGCTTAAACCGGTGTTTTAGAGCTAGAAATAGCAAGTT  943
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    GTCGACATAGCGATTGTTTACTCGCTTAAACCGGTGTTTTAGAGCTAGAAATAGCAAGTT  120

Query  944   AAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCttttttttCT  1003
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   AAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTCT  180

Query  1004  AGACCCAGCTTTCTTGTACAAAGTTGGCATTA  1035
             ||||||||||||||||||||||||||||||||
Sbjct  181   AGACCCAGCTTTCTTGTACAAAGTTGGCATTA  212
```

Figure 3.9 The results of Sanger sequencing for AtU6p::sgRNA_4OMT2 reads.

3.2.2 Construction of viral plasmids

Viral-based plasmids were designed firstly by amplfying AtU6p::sgRNA_PDS and AtU6p::sgRNA_4OMT2 inserts from the commercial plasmid of 46965 (Addgene) and plasmid pICH47751::AtUp::sgRNA_4OMT2. Then, amplified regions were cloned into pGEM-T vector and sequenced with Sanger dideoxy method in order to guarantee the integrity of the sequence.

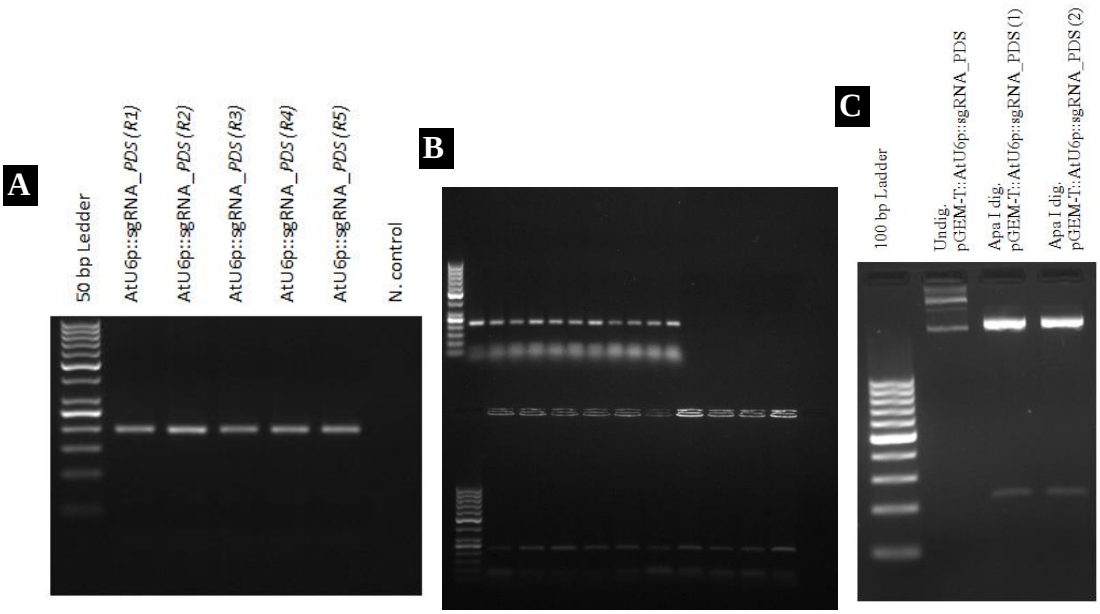
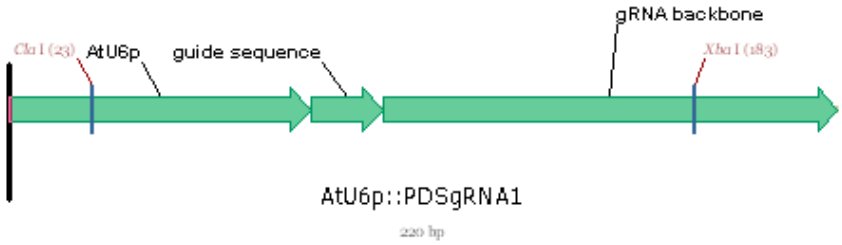


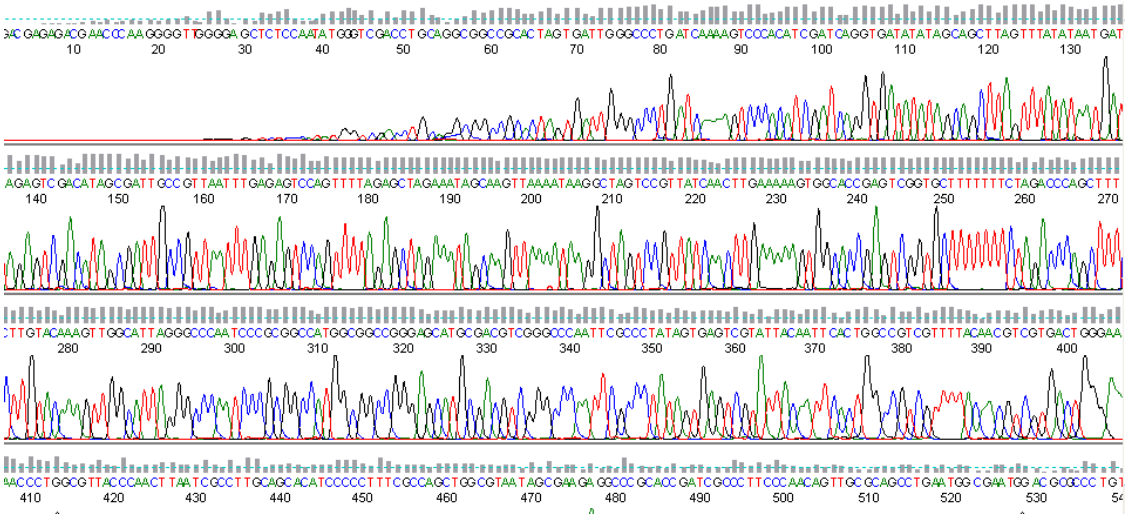
Figure 3.10 (A) Agarose gel electrophoresis results of AtU6p-sgRNA-PDS PCR by using 46966 plasmid as a template. **(**B) Agarose gel electrophoresis results of pGEM-T::AtU6p::sgRNA_PDS ligation colony PCR. **(C)** Agarose gel electrophoresis results of pGEM-T::AtU6p::sgRNA_PDS Apa-I digestion.



GGAGTGATCAAAAGTCCCACATCGATCAGGTGATATATAGCAGCTTAGTTTATATAATGATAG
AGTCGACATAGCGATTGCCGTTAATTTGAGAGTCCAGTTTTAGAGCTAGAAATAGCAAGTTAA
AATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTCTAGACC
CAGCTTTCTTGTACAAAGTTGGCATTACGCT

Figure 3.11 Schematic presentation of AtU6p::sgRNA_PDS. In figure, nucleotides in blue demostrates the A. thaliana U6 promoter. While reds were representing the 20 bp target sequence in PDS loci, greens for chimera of tracrRNA and crRNA.

pGEM-T::AtU6p::sgRNA_PDS plasmids were sequenced with Sanger dideoxy sequencing method and seen as totally matched with the pre-designed nucleic acid sequence (as seen in Figure 3.12).



```
Sanger              CGCACTAGTGATTGGGCCCTGATCAAAAGTCCCACATCGATCAGGTGATATATAGCAGCT 120

AtU6p_sgRNA_PDS     ------------GGGCCCTGATCAAAAGTCCCACATCGATCAGGTGATATATAGCAGCT 47
                               **********************************************

Sanger              TAGTTTATATAATGATAGAGTCGACATAGCGATTGCCGTTAATTTGAGAGTCCAGTTTTA 180

AtU6p_sgRNA_PDS     TAGTTTATATAATGATAGAGTCGACATAGCGATTGCCGTTAATTTGAGAGTCCAGTTTTA 107
                    ************************************************************

Sanger              GAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACC 240

AtU6p_sgRNA_PDS     GAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACC 167
                    ************************************************************

Sanger              GAGTCGGTGCTTTTTTTCTAGACCCAGCTTTCTTGTACAAAGTTGGCATTAGGGCCCAAT 300

AtU6p_sgRNA_PDS     GAGTCGGTGCTTTTTTTCTAGACCCAGCTTTCTTGTACAAAGTTGGCATTAGGGCCC--- 224
                    ************************************************************
```

Figure 3.12 The results of Sanger sequencing for AtU6p::sgRNA_PDS reads.

Isolated AtU6p::sgRNA_PDS and AtU6p::sgRNA_4OMT2 inserts (possess ApaI restriction sites) were ligated into ptv::00 (TRV2) viral vector and transfected into *E. coli*. Transfected cells were scanned for positive clones with colony PCR. Later, verified ones were used to transfect into plant tissues via agro-infiltration.

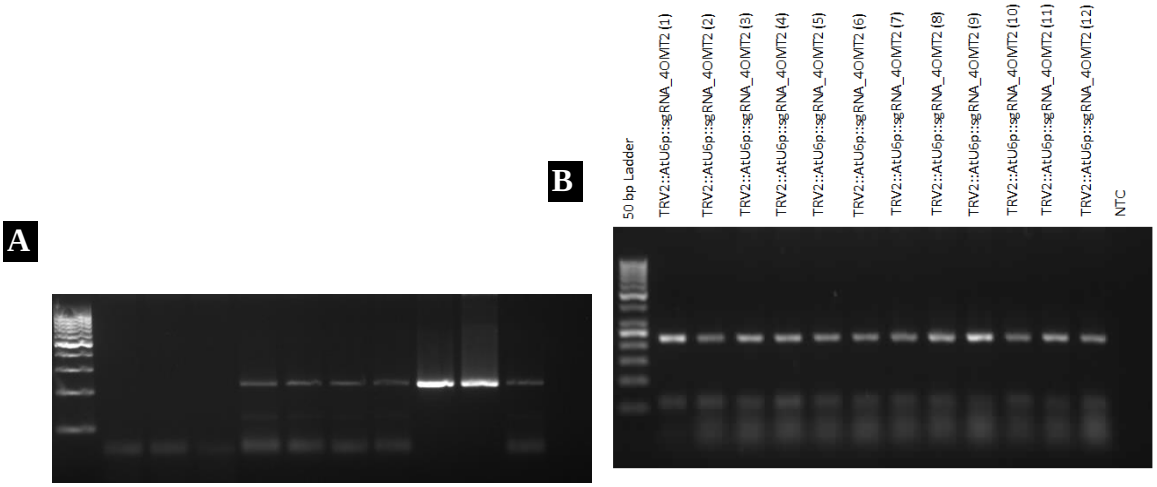


Figure 3.13 Colony PCR results of TRV::AtU6p::sgRNA_PDS (A) and TRV::AtU6p::sgRNA_4OMT2 (B).

3.3 AFLP analysis for validation of InDel containing DNA frames

~450 bp from PDS and 95 bp of 4OMT2 gene regions were amplified and digested with enrichment enzymes of MlyI and AgeI respectively. AFLP results were demontrated that CRISPR/Cas9 system was worked in *P. somniferum* and *N. benthamiana*. Undigested bands, which were resistant to digestive activity of restrcition endonucleases in seperate lanes were isolated and cloned into pGEM-T plasmid for Sanger sequencing.

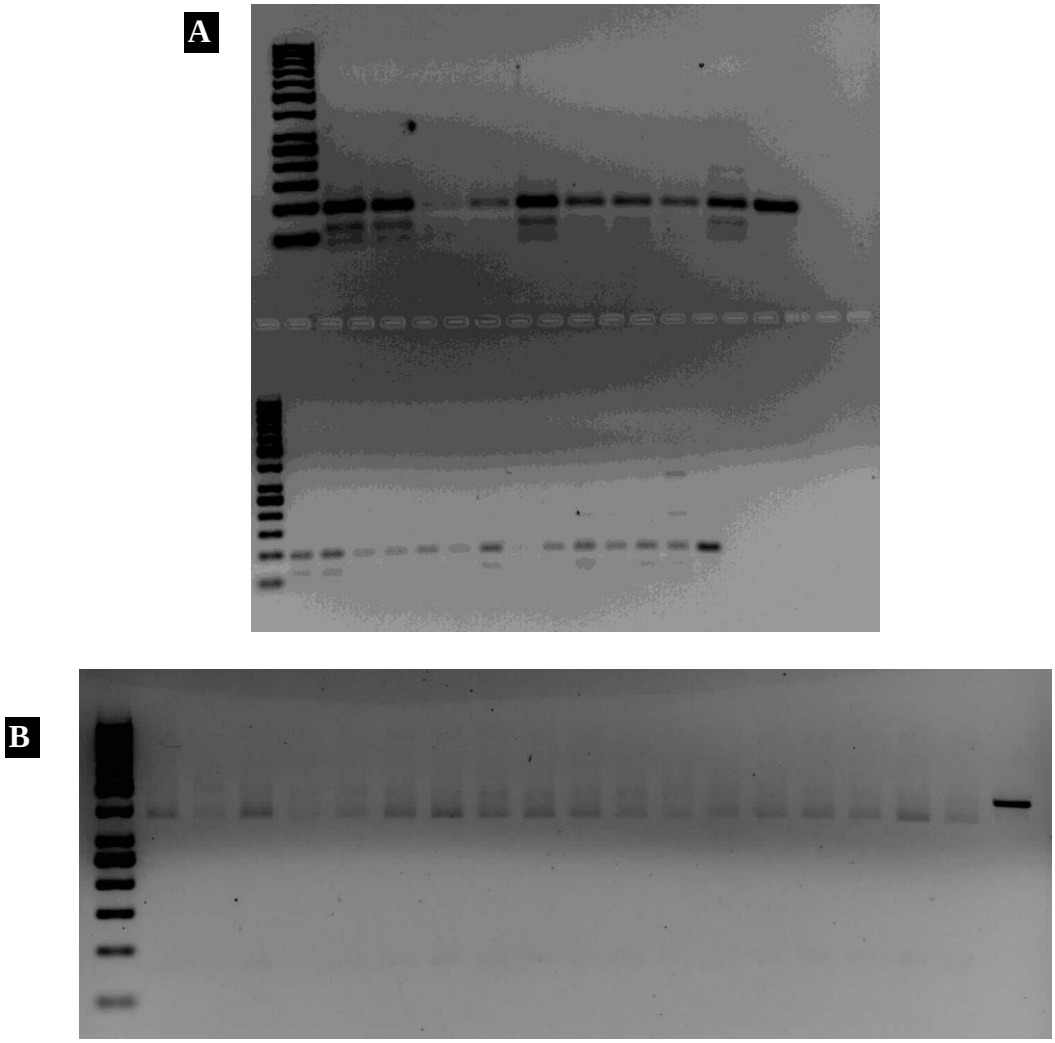


Figure 3.14 AFLP results of CRISPRed Pap4OMT2 and NbPDS genes. (A) Digested PCR product of *Pap4OMT2* gene frame with AgeI. Lanes at the top were belonged to virus mediated (TRV) application and the bottom ones were belong to synthetic based application. (B) Digested PCR product of *NbPDS* gene frame with MlyI. The first 10 lanes (from left to right) were belong to synthetic based application and samples from 11-18 were belonged to virus mediated (TRV) application. Both for A, B, bands at the last lanes (at the rightmost) were positive control.

3.4 InDel Analysis

3.4.1 InDel analysis for synthetic application

For synthetic application, 16 of 20 sequenced plasmid samples were found to possess InDels as a result of endonuclease activity of CRISPR/Cas9 system.

```
SangerS1          GAGTTGAGAAGGTTTACTCGCTTAAAATAGTCGGTACTCTACTTTTA    (-3/+3)
SangerS2          GAGTTGAGAAGGTTTACTTGCTTAAATCGGTCGGTACTCTACTTTAA    (-2/+2)
SangerS3          GAGTTGAGAAGGTTTACTTGCTTAAATCGGTCGGTACTCTACTTTTA    (-2/+2)
SangerS4          GAGTTGAGAAGGTTTACTTGCTTAAATCGGTCGGTACTCTACTTTTA    (-2/+2)
SangerS5          GAGTTGAGAAGGTTTACTCGCTTAAATCGGTCGGTACTCTACTTTAA    (-1/+1)
SangerS6          GAGTTGAGAAGGTTTACTCATTTAAGTCGGTCGGTACTCTACTTTTA    (-4/+4)
SangerS7          GAGTTGAGAAGGTTTACTCATTTAAGTCGGTCGGTACTCTACTTTTA    (-4/+4)
SangerS8          GAGTTGAGAAGGTTTACTTGCTTAAATCGGTCGGTACTCTACTTTTA    (-2/+2)
SangerS9          GAGTTGAGAAGGTTTACTCGCTTAAAATAGTCGGTACTCTACTTTTA    (-3/+3)
SangerS10         GAGTTGAGAAGGTTTACTCGCTTAAAATAGTCGGTACTCTACTTTTA    (-3/+3)
SangerS13         GAGTTGAGAAGGTTTACTCATTTAAGTCGGTCGGTACTCTACTTTTA    (-4/+4)
SangerS14         GAGTTGAGAAGGTTTACTTGCTTAAATCGGTCGGTACTCTACTTTTA    (-2/+2)
SangerS15         GAGTTGAGAAGGTTTACTTGCTTAAATCGGTCGGTACTCTACTTTTA    (-2/+2)
SangerS16         GAGTTGAGAAGGTTTACTCATTTAAGTCGGTCGGTACTCTACTTTTA    (-4/+4)
SangerS18         GAGTTGAGAAGGTTTACTCGCTTAAAATAGTCGGTACTCTACTTTTA    (-3/+3)
SangerS19         GAGTTGAGAAGGTTTACTCGCTTAAAATAGTCGGTACTCTACTTTTA    (-3/+3)
AY217334.1        GAGTTGAGAAGGTTTACTCGCTTAAACCGGTCGGTACTCTACTTTTA
4OMT2TrgSeq       -----------GTTTACTCGCTTAAACCGGT----------------
```

*Cyanide: AgeI recognition seq. (for enrichment); Yellow: PAM; Grey: Target seq.

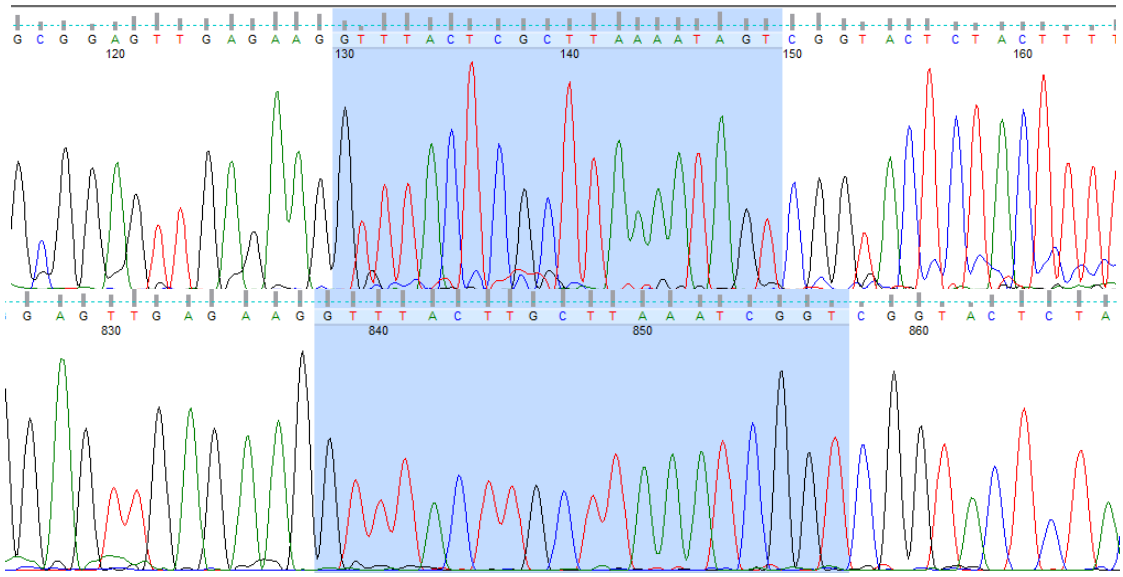


Figure 3.15 Representative demonstration of InDels formed by synthetically expressed sgRNA4OMT2.

3.4.2 InDel analysis for viral application

For viral application, 15 of 19 sequenced plasmid samples were found to possess InDels as a result of endonuclease activity of CRISPR/Cas9 system.

```
SangerV1        AGTTGAGAAGGTTTACTCGCTTAAAATAGTCGGTACTCTACTTTTA    (-3/+3)
SangerV2        AGTTGAGAAGGTTTACTTGCTTAAATCGGTCGGTACTCTACTTTTA    (-2/+2)
SangerV3        AGTTGAGAAGGTTTACTTGCTTAAATCGGTCGGTACTCTACTTTTA    (-2/+2)
SangerV4        AGTTGAGAAGGTTTACTTGCTTAAATCGGTCGGTACTCTACTTTTA    (-2/+2)
SangerV5        AGTTGAGAAGGTTTACTTGCTTAAATCGGTCGGTACTCTACTTTTA    (-2/+2)
SangerV7        AGTTGAGAAGGTTTACTCGCTTAAAATAGTCGGTACTCTACTTTTA    (-3/+3)
SangerV8        AGTTGAGAAGGTTTACTCATTTAAGTCGGTCGGTACTCTACTTTTA    (-4/+4)
SangerV9        AGTTGAGAAGGTTTACTCATTTAAGTCGGTCGGTACTCTACTTTTA    (-4/+4)
SangerV10       AGTTGAGAAGGTTTACTCGCTTAAAATAGTCGGTACTCTACTTTTA    (-3/+3)
SangerV11       AGTTGAGAAGGTTTACTTGCTTAAATCGGTCGGTACTCTACTTTTA    (-2/+2)
SangerV13       AGTTGAGAAGGTTTACTTGCTTAAATCGGTCGGTACTCTACTTTTA    (-2/+2)
SangerV14       AGTTGAGAAGGTTTACTTGCTTAAATCGGTCGGTACTCTACTTTTA    (-2/+2)
SangerV16       AGTTGAGAAGGTTTACTCGCTTAAAATAGTCGGTACTCTACTTTTA    (-3/+3)
SangerV30       AGTTGAGAAGGTTTACTCGCTTAAAATAGTCGGTACTCTACTTTAA    (-3/+3)
SangerV32       AGTTGATAAGGTTTACTCGCTTA-ACCGGTCGGCACTCTACTTTTA    (-1)
AY217334.1      AGTTGAGAAGGTTTACTCGCTTAAACCGGTCGGTACTCTACTTTTA
4OMT2TrgSeq     ----------GTTTACTCGCTTAAACCGGT----------------
```

*Cyanide: AgeI recognition seq. (for enrichment); Yellow: PAM; Grey: Target seq.

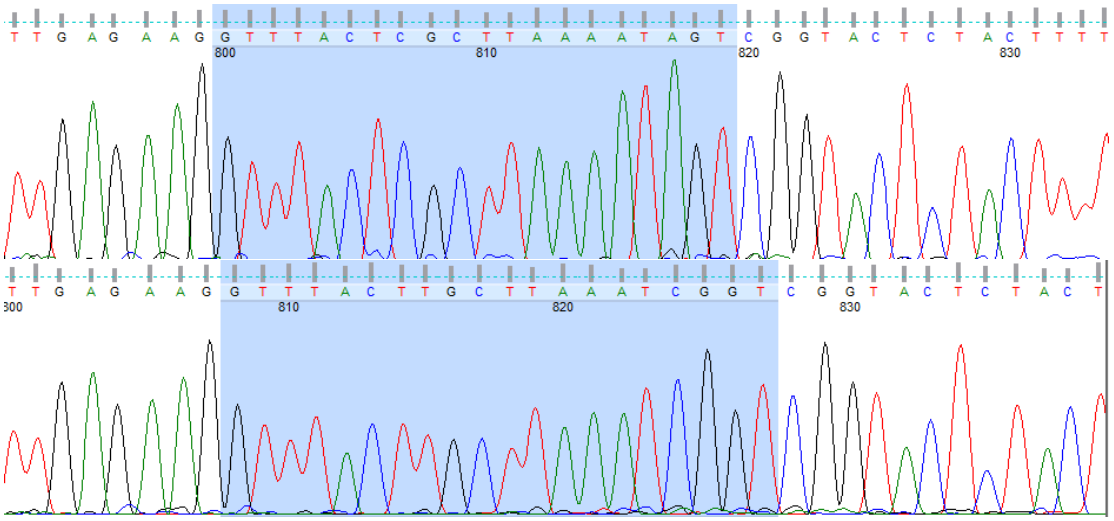


Figure 3.15 Representative demonstration of InDels formed by virally expressed sgRNA4OMT2.

4 DISCUSSION

TRV system has been used in VIGS studies in *P. somniferum* (Hileman et al., 2005; Wijekoon and Facchini, 2012). By taking account these studies, we decided to use TRV vector to express our sgRNAs to improve the CRISPR/Cas9 system efficiency. Due to the fact that, in the literature, all used olasmids were synthetic in plants and in order to form an InDel inside genomes with this system both Cas9 and sgRNA plasmids should be transcribed in the same individual cell. With an advantage of being a viral vector, TRV can be systemically distributes sgRNAs from an individual to neigbouring cells inside plant tissues. Additionally, there was a published report about the usage of TRV vectors for sgRNA transcription in Cas9 encoding tissues of *N. benthamiana* (Ali et al., 2015). As a preliminary research, to show the effectiveness of CRISPR/Cas9 system in *P. somniferum*, we designed both synhtetic and virally encoding sgRNA plasmids and used them to knock-out a special gene, *4OMT2*, which having roles in production of valuable medicinal aromatic alkaloids like noscapine and morphine.

In our AFLP result, the relative mutagenesis rate of 4OMT2 gene was too high in compare to the other well-known studies in the field. Additionally, it's the first study in the literature so the best way to identify InDel regions in genome is using genome sequencing techniques. In Sanger dideoxy sequencing results, InDels were detected and this is an obvious indication for operatibility of CRISPR/Cas9 system in *P. somniferum*. This is the first report for plant CRISPR application studies in the literature. Also, multiple numbers of sgRNAs should be examined to check the effectivity of the system. Some sgRNAs can work better than the others this is due to the position of targeting gene segment or the secondary structure of sgRNAs itself.

In order to eliminate those unexpected effects, designing a well-defined guide RNA for Cas9 editing is essential. The number of on-line tools are incresing day by day by the contributions from different universities from varied sides of the world. Each designed tool has it's own WGS (whole genome sequence) database to blast the designed guide RNA in to see if there is any undesired complementarity that is different from the targeted region inside the genome. Even the transcriptome data and important ESTs are

present in databases, the WGS of *P. somniferum* is still not known. That is one of the biggest disadvantage that we were faced with during our studies. One more restriction of the CRISPR system is the target low-binding efficiency of secondary structures of designed guide RNA that possibly interferes the recognition of target sequence.

Particularly, screening of CRISPRed genome with NGS platforms can be more beneficialy to eliminate off-target effects of designed systems. sgRNAs with low toxicity can be used to establish sgRNA libraries to specific to each targeted organisms. As a "transgene clean" technology, CRISPR/Cas9 system can be used in varied types of studies in the field of non-GM functional genetics studies (Xu et al., 2015). CRISPR/Cas9 with other known synthetic genome editing tools of ZFN and TALENs, denoted as Non-GMO tools many time in literature (Kanchiswamy et al., 2015).

After this study, by the assistance of increasing amount of bioinformatics data about P. somniferum, it's now easier to use CRISPR/Cas9 system to manipulate other BIA-related gene or genes in *P. somniferum* or other species of genus Papaver. In this study, *Pap4OMT2* and *NbPDS* genes were knocked-out by using sgRNAs both transcribed from viral (TRV) and synthetic vectors.

Transfering newly-acquired properties to next progenies is also possible with CRISPR/Cas9 system (Friedland et al., 2013; Gao and Zhao, 2014; Xing et al., 2014; Belhaj et al., 2015). For the furthering research, this possibility can be examined in *P. somniferum* and also in other species of Papaver, beacuse this study constitues a pioneering research for the applications of CRISPR system in genus Papaver. In the close future, with an increased knowledge on Papaver genome, it could be more easies to target any gene or gene groups with multiplex applications. Even elimination of large chromosomal segments could be possible with CRISPR/Cas9 system. CRISPR/Cas9 system provides a chance to change any part of the genome without any borders so, UTRs, lnRNA or any type of RNA coding DNA sequence can be manipulated (Ho et al., 2014). Regaining some certain genes, which are previously lost in modern crops but

still present in the wild types, is also possible with HDR dominated CRISPR applications (Wang et al., 2014).

One of the most important detail in CRISPR studies is Cas9/sgRNA ratio as one of the major limitation factor that can affect the mutagenesis frequency when it's lower than 1:1 in the system of interest (Li et al., 2013). This is also valid for TRV1/TRV2 ratio. Furthermore, selection of agrobacterium strain is important for the transfer of CRISPR system parts from bacteria to plant tissues in health. In literature, strain EHA105 and GV3101 were used for gene silencing in *P. somniferum* (Hileman et al., 2005). Also in our study, same strains were used.

In the applications of CRISPR/Cas9 in plants, its not possible to express both Cas9 and sgRNA from a single viral vector. This is due to the low-cargo capacity of plant viral vectors. In new recent papers, there were strategies that can be used to overcome this huge obstacles. One of them is split Cas9, a divided Cas9 nucleic acid sequence is transferred in organisms and triggered to re-assemble for functioning (Zetsche et al., 2015). The other one is 1/3 sized Staphylococcus aureus Cas9 protein (when it's compared to SpCas9) (Ran et al., 2015). Theoretically, those two new strategies can be used to express Cas9 endonuclease in plant tissues by using virus-based vectors

Consequently, our study demonstrated that CRISPR/Cas9 sytem can work in *P. somniferum* genome. This preliminary research would be used as a model to target any other gene or genes inside Papaver genomes. The advantage of studying with a known WGS should be also taken into account because the more knowledge we get, the more freedome we have to design new genomically edited plant species.

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APPENDIXES

Appendix-1

Map of used plasmids in this study.

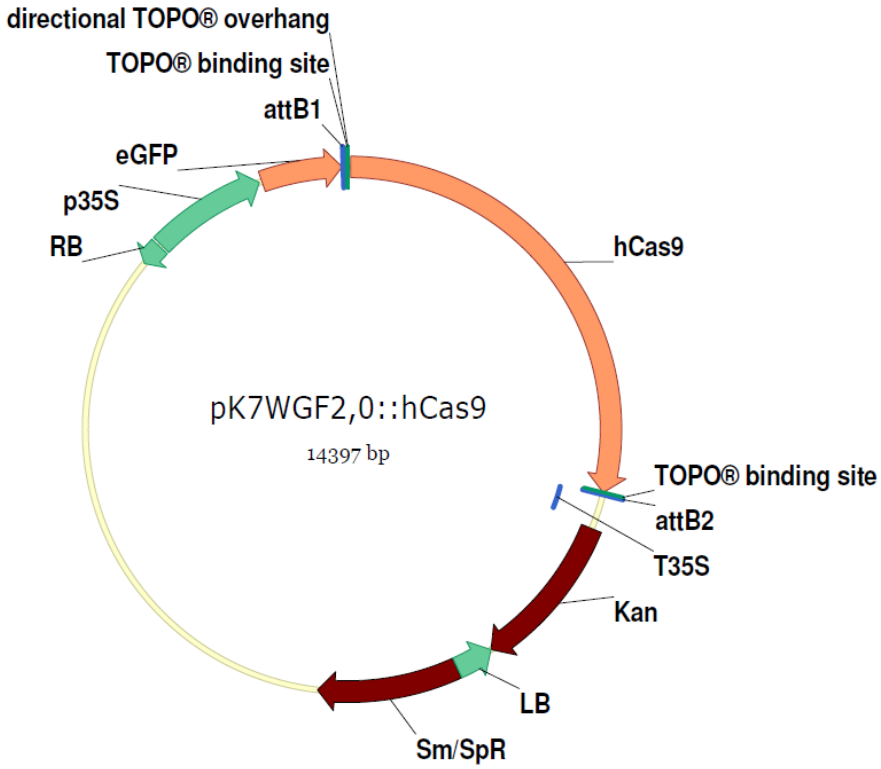


Figure 1. Human codon optimized Cas9 expressing vector. Addgene ID# 46965.

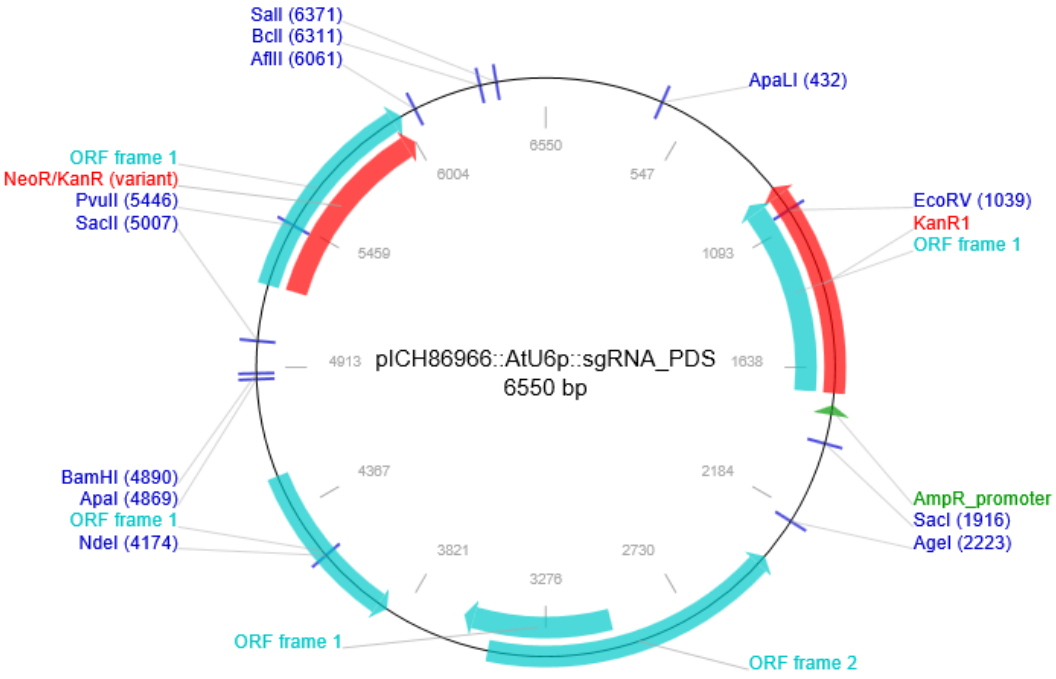


Figure 2. sgRNA_PDS expressor (Under *A. thaliana* U6 promoter) vector. Addgene ID# 46966.

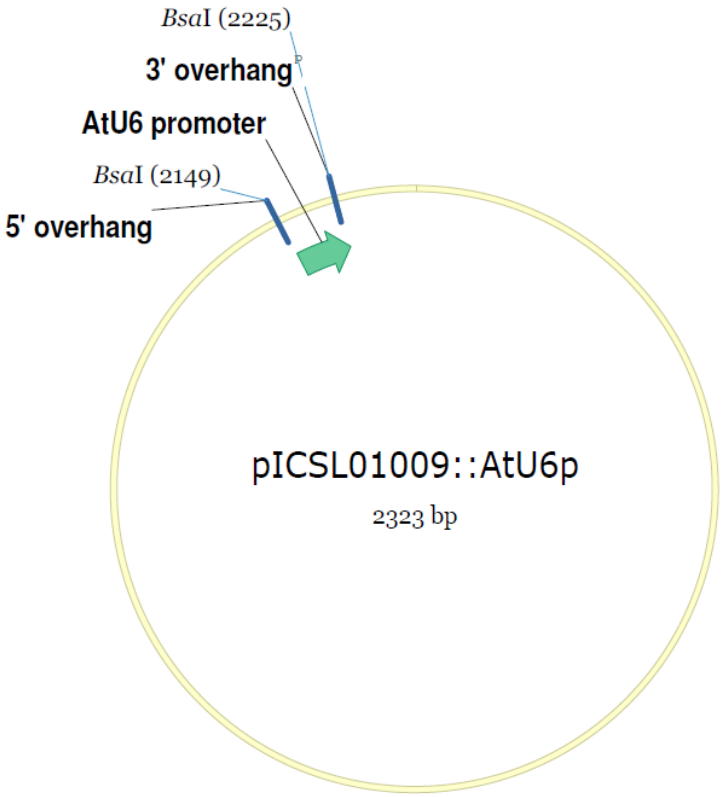


Figure 3. *A. thaliana* U6 promoter containing vector for MoClo reactions with Addgene ID# 46968.

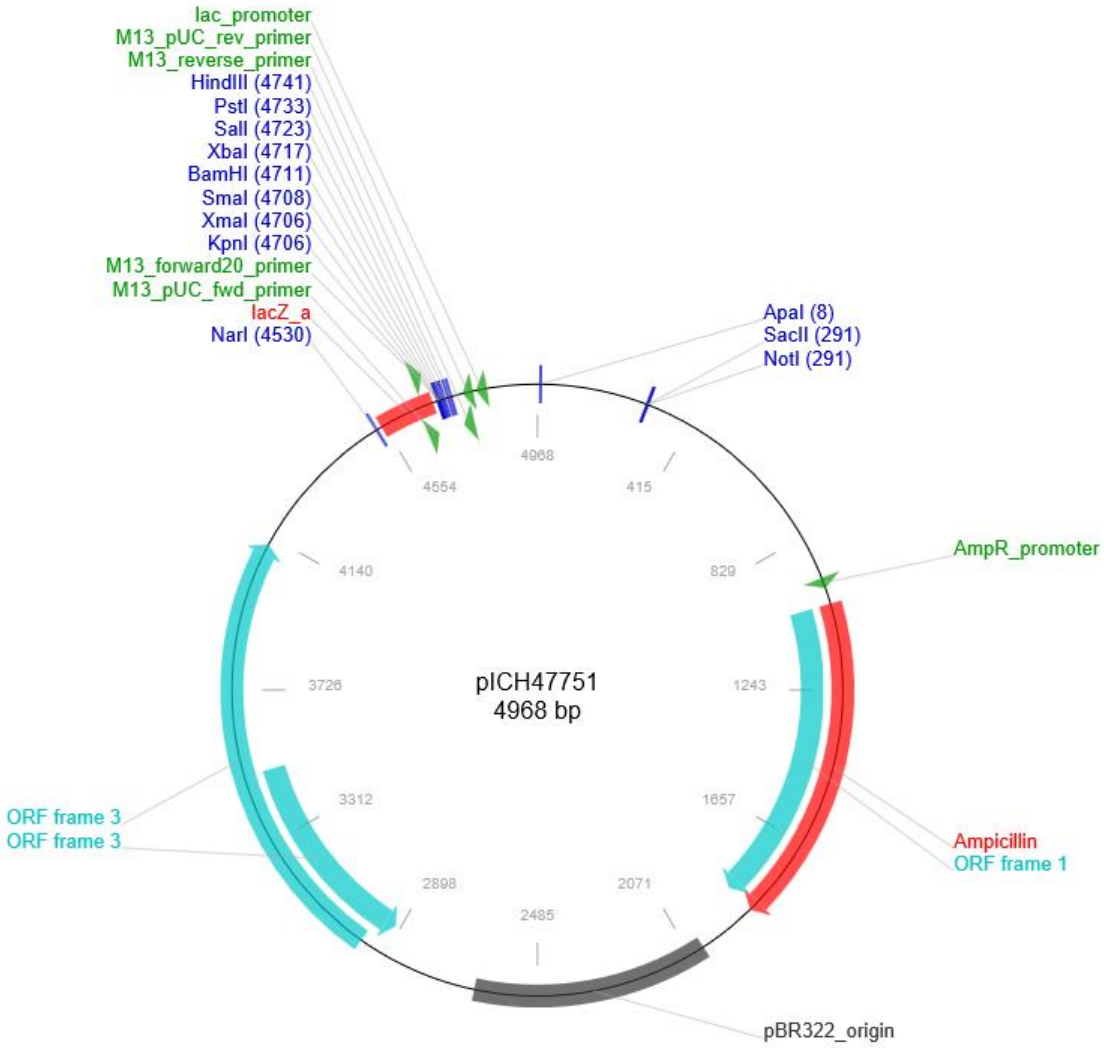


Figure 4. Golden Gate L1 clonning vector for MoClo reactions with Addgene ID# 48002.

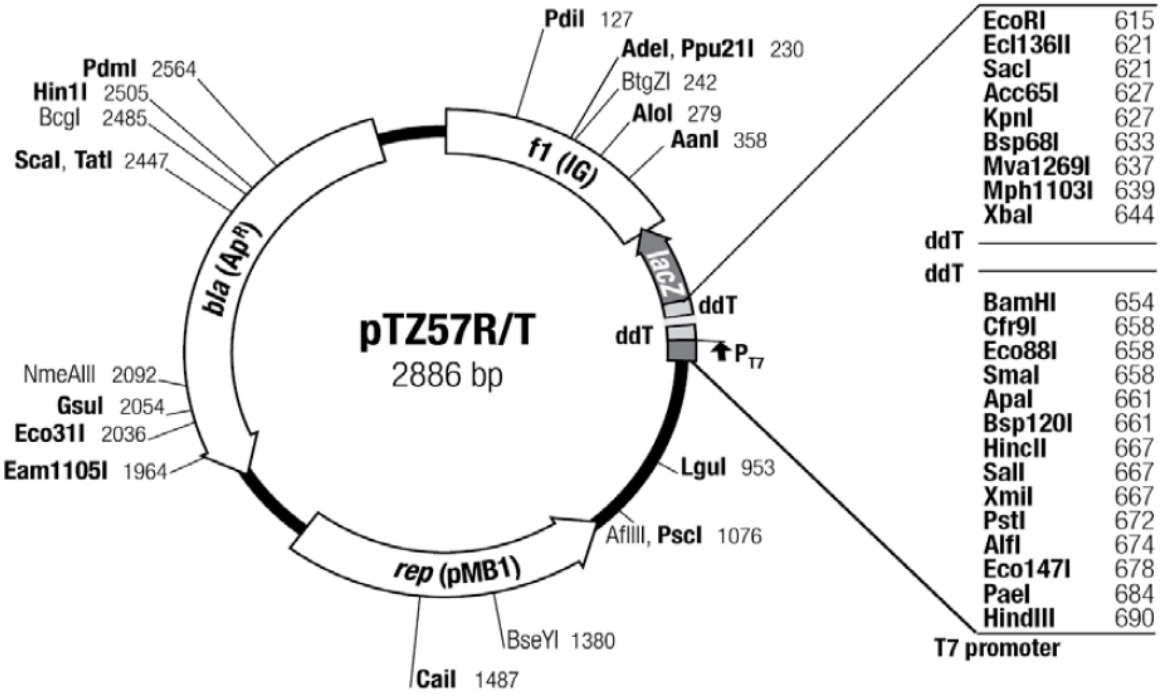


Figure 5. T-A clonning vector for conventional clonning reactions.

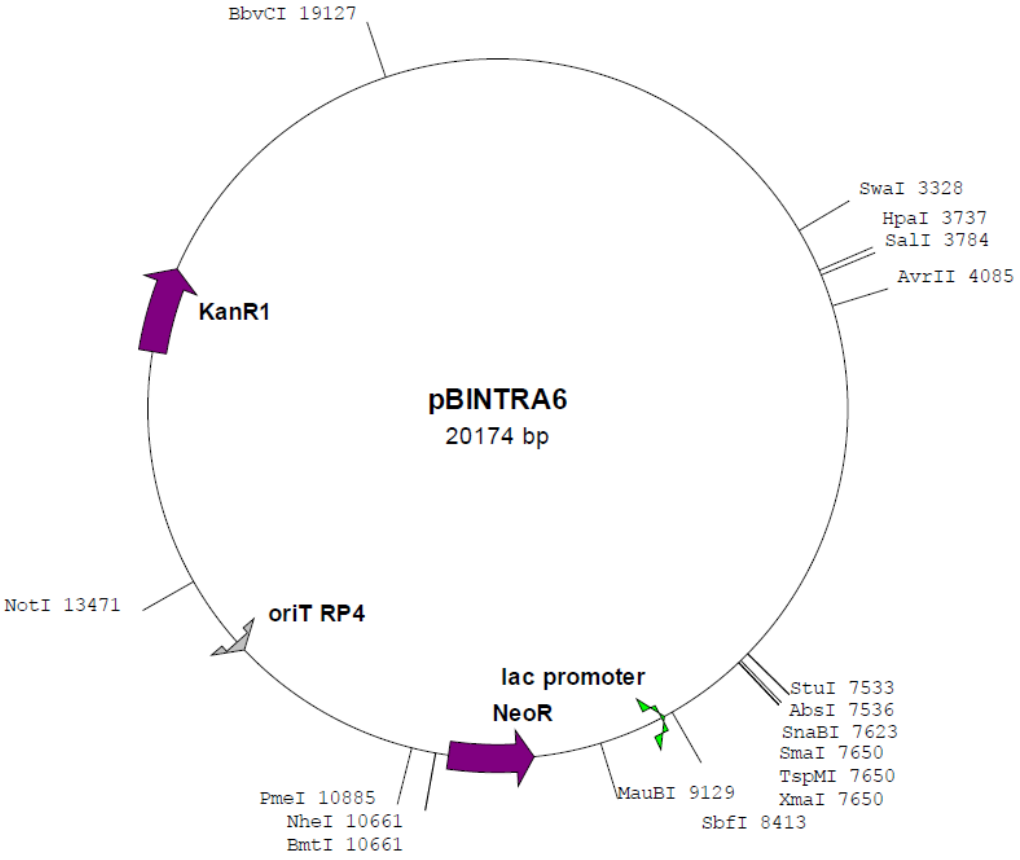


Figure 6. TRV1 vector for TRV mediated genomic manipulation reactions.

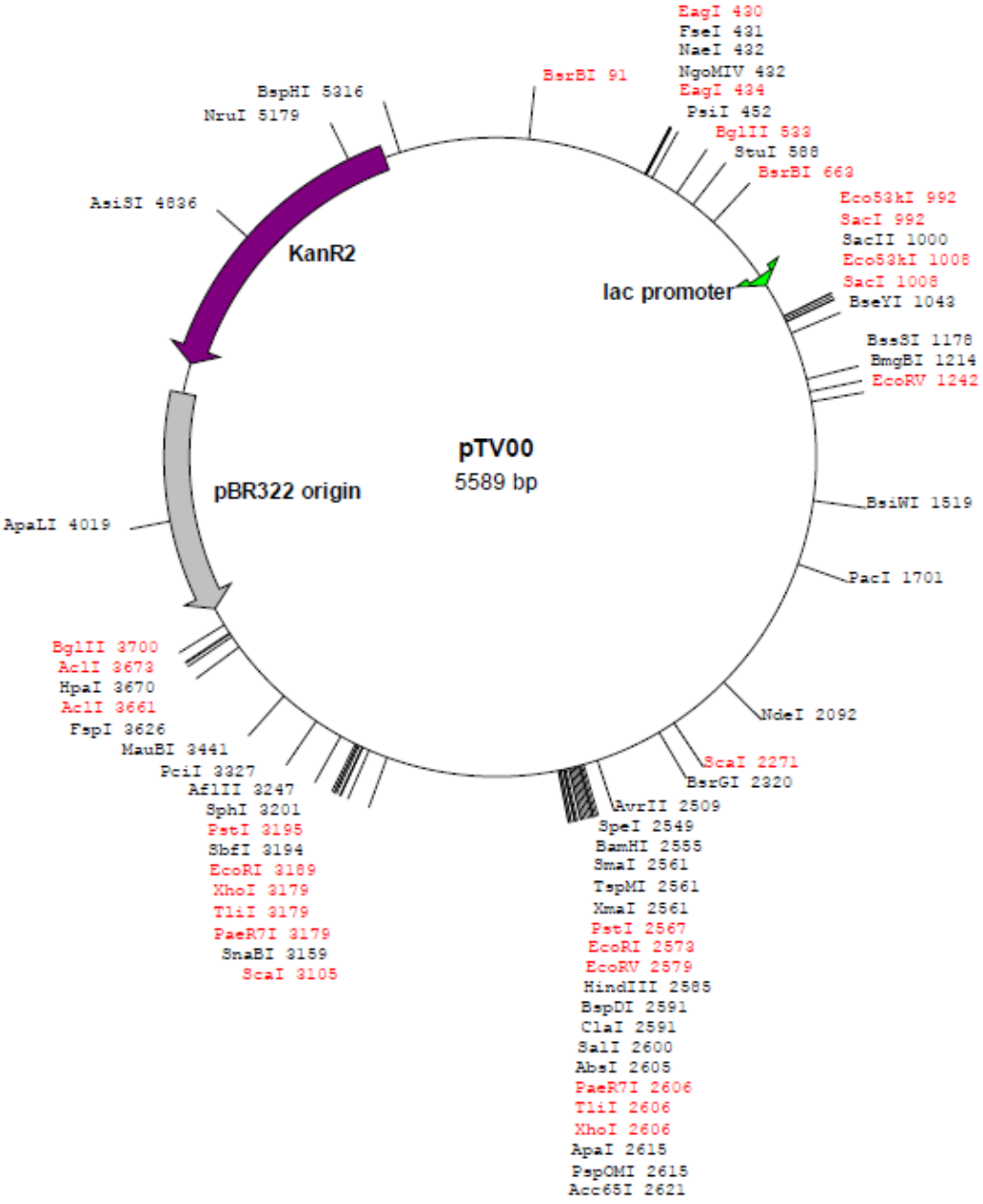


Figure 7. TRV2 vector for TRV mediated genomic manipulation reactions.

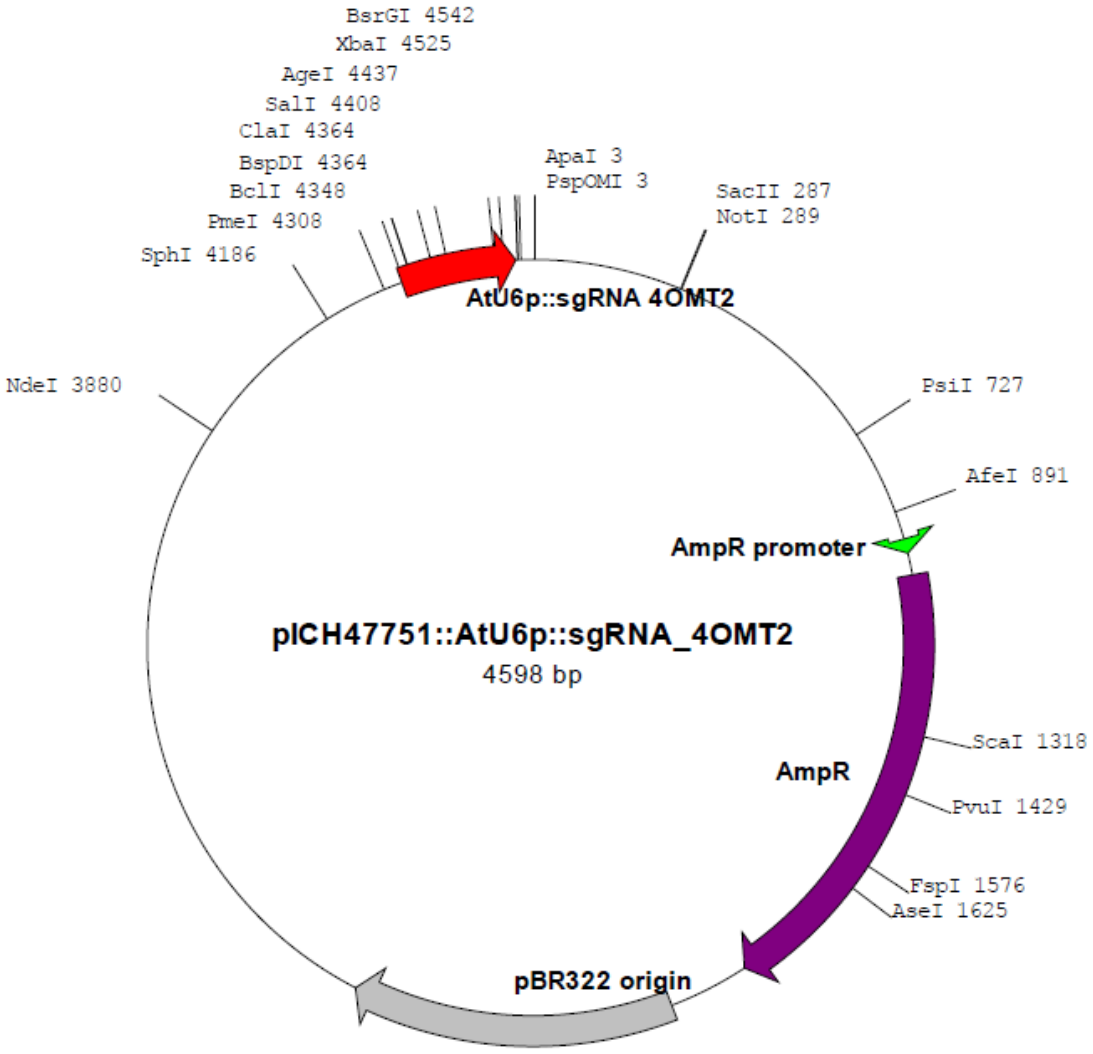


Figure 8. Synthetic sgRNA_4OMT2 expressor (Under *A. thaliana* U6 promoter) vector (Synthesized in this study).

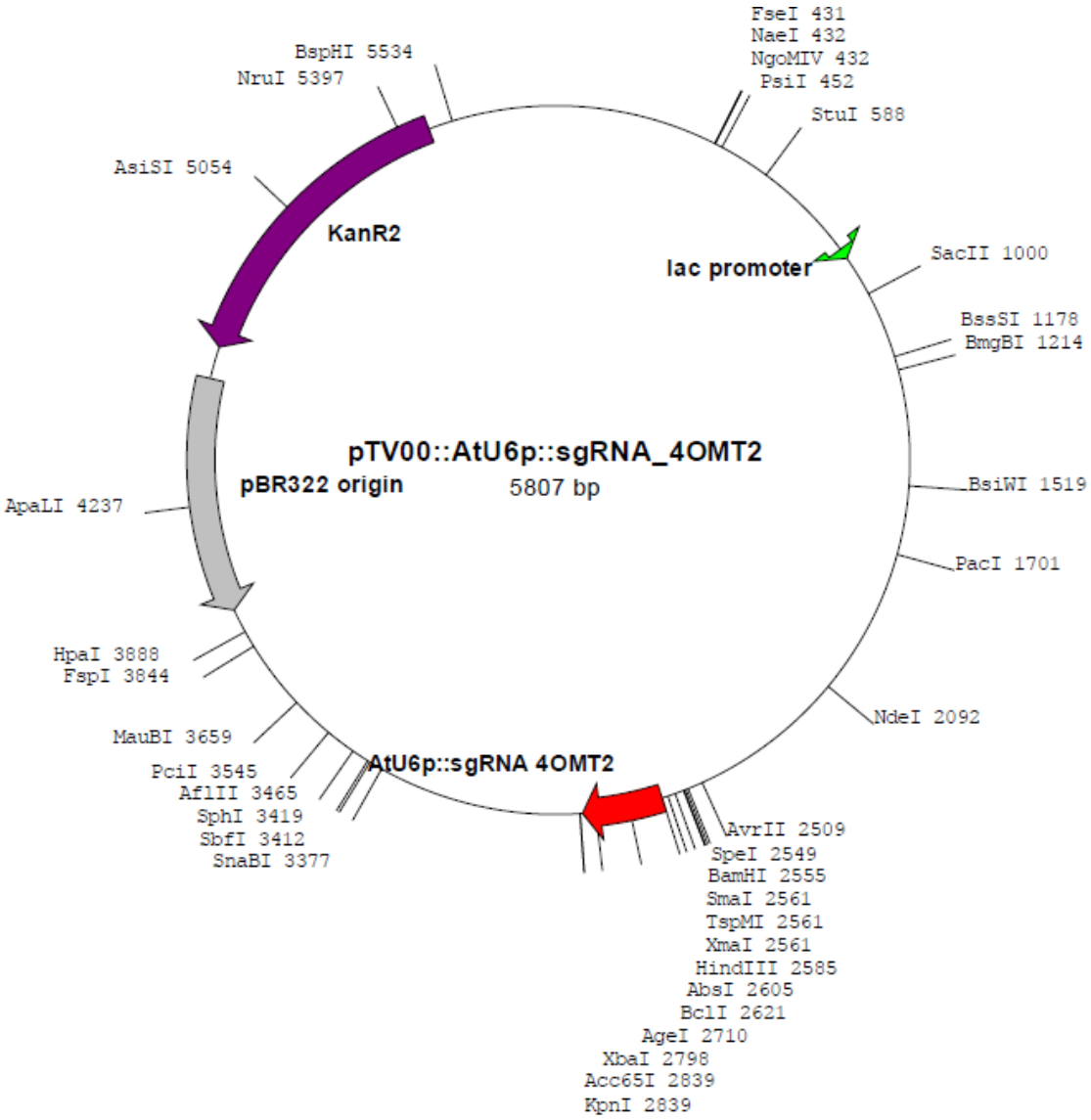


Figure 9. Viral (TRV based) sgRNA_4OMT2 expressor (Under *A. thaliana* U6 promoter) vector (Synthesized in this study).

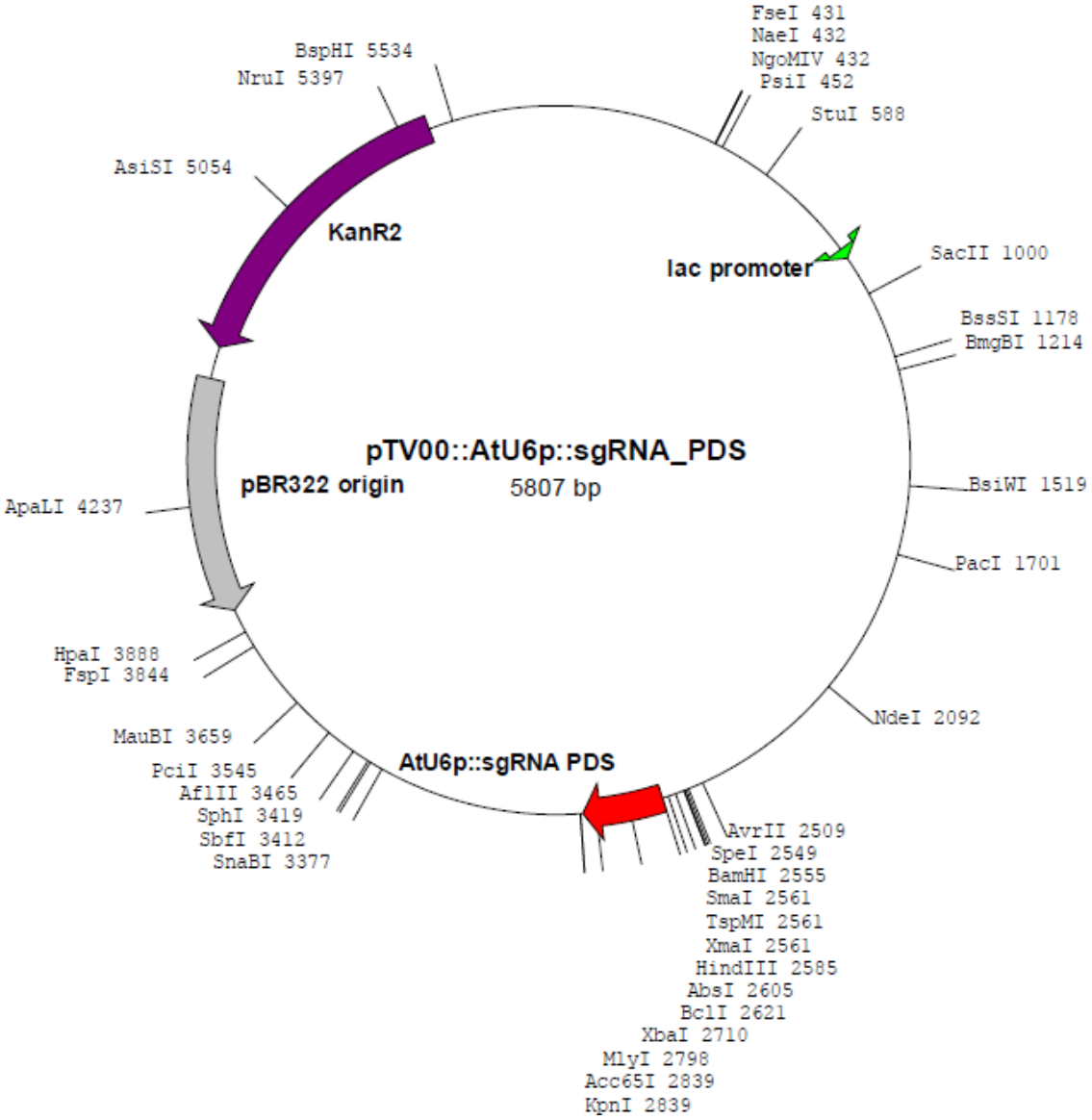


Figure 10. Viral (TRV based) sgRNA_PDS expressor (Under *A. thaliana* U6 promoter) vector (Synthesized in this study).

Appendix-2

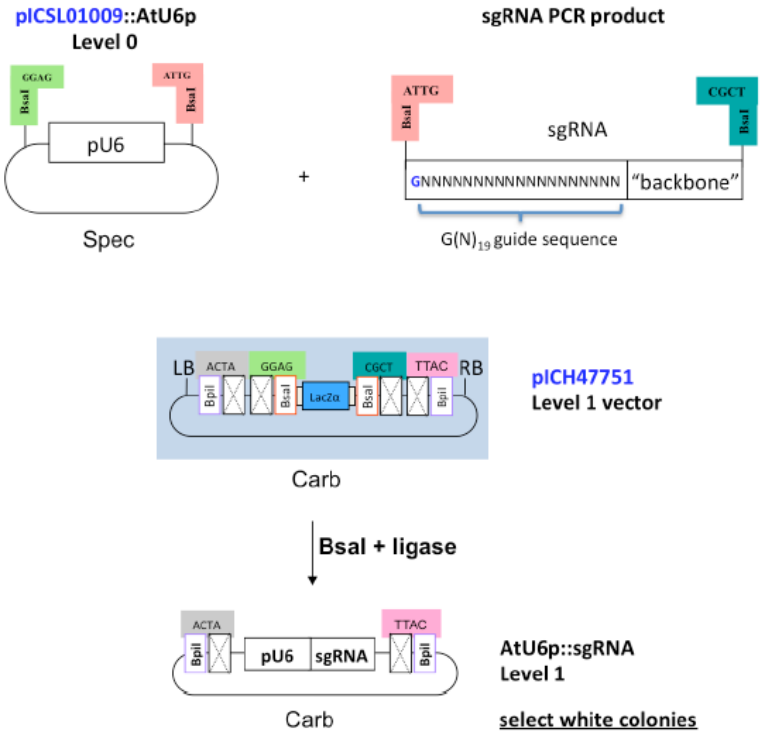


Figure 1. Demonstration of MoClo Level 1 reaction used for clonning of pICH47751::AtU6p::sgRNA_4OMT2, pTV00:: AtU6p::sgRNA_4OMT2 and Ptv00:: AtU6p::sgRNA_PDS

Appendix-3

List of primers used in this study.

Primer Name	Sequence
PDS_MlyIF	GCTTTGCTTGAGAAAAGCTCTC
PDS_MlyIR	ACATAACAAATTCCTTTGCAAGC
4OMT2_AgeIF	GTAACGGCGGAGTTGAGAAG
4OMT2_AgeIR	GCCTAGTATCATCGGCACCA
4OMT2sgRNA_1F	tgtggtctcaATTGTTTACTCGCTTAAACCGGT gttttagagctagaaatagcaag
4OMT2sgRNA_1R	tgtggtctcaAGCGTAATGCCAACTTTGTAC
M13F	GTAAAACGACGGCCAGTG
M13R	GGAAACAGCTATGACCATG
flCas9F02	CGAAGAGAACCCGATCAACG
flCas9R03	CCTGAGGATAGCGTGCAGTT
sgRNApaIF	GGGCCCTGATCAAAAGTCCCACA
sgRNApaIR	GGGCCCTAATGCCAACTTTGTACAAG
4OMT2ConF	TCCCAGTGCTCAGGCTATC
4OMT2ConR	CGGCCAGTAAAGGAAAAAGA

Appendix-4

Used buffers for chemically compotent cell preparation and plant gDNA isolation.

A 4.1 Chemically Competent *E. coli* Preparation

Buffer 1 (pH: 5.8 with dilute acetic acid filter sterilized.)

- CaCl2 10 mM
- Glycerol 15%
- KAc 30 mM
- RuCl 100 mM

Buffer 2 (pH: 6.5 with 0.2 M KOH, filter sterilized.)

- CaCl2 75 mM
- Glycerol 15%
- MOPS 10 mM
- RuCl 10 mM

A 4.2 Plant gDNA Isolation

Sucrose Buffer (Suspension Buffer) (pH: 8)

- 50mM EDTA
- 120 mM Tris-HCI
- 1 M NACI
- 0.5 M sucrose
- %2 TRİTON-X 100
- %0.2 β-mercaptoethanol (add just before use!)

CTAB Buffer (Extraction Buffer)

- 20 mM EDTA
- 100 mM TRİS-HCI
- 1.5 M NACI
- %2 CTAB
- %1 β-mercaptoethanol (add just before use!)

Appendix-5

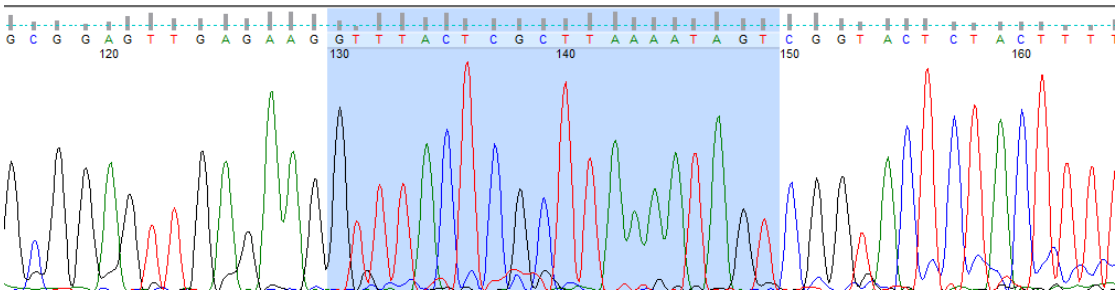
Yellow: F and R primers

Red: Target seq. (20 bp) in *4OMT2* gene (GenBank: AY217334.1)

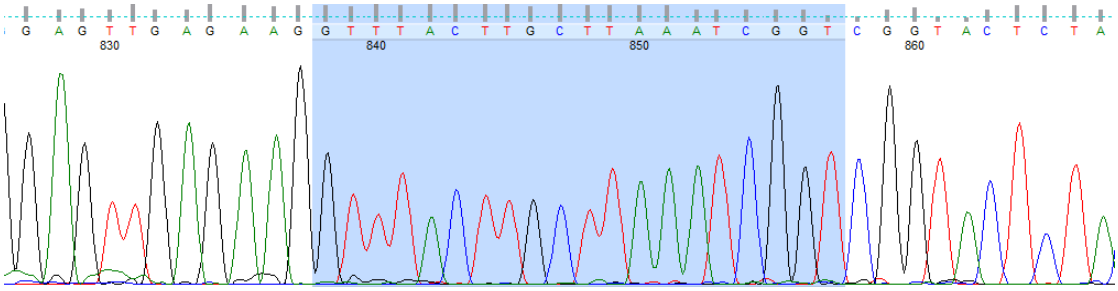
```
4OMT2CDS      AAAGCCAAAGCAAAAAACTGTCTTCTCTTCTTGACAATTATATACAGCAAAAATCGCATA
R             ------------------------------------------------------------

4OMT2CDS      TCTAATGGGTAGTTTAGATGCAAAACCAGCTGCTGCAACACAAGAAGTTTCCATCAAAGA
R             ------------------------------------------------------------

4OMT2CDS      TCAAGCTCAACTATGGAATATAATCTATGGTTTTGCTGATTCTCTTGTTCTTCGCTGCGC
R             ------------------------------------------------------------

4OMT2CDS      GGTAGAGATTGGAATCGCGGATATTATCAAAAACAATGATGGGGCAATCACACTTGCACA
R             ------------------------------------------------------------

4OMT2CDS      ACTTGCGGCGAAACTCCCAATTACAAATGTCAGTTCTGATTACTTGTACAGAATGGTAAG
R             ------------------------------------------------------------

4OMT2CDS      ATACTTGGTACACCTGAATATCATAGAACAAGAAACTTGTAACGGCGGAGTTGAGAAGGT
R             ------------------------------------------------------------

4OMT2CDS      TTACTCGCTTAAACCGGTCGGTACTCTACTTTTAAGAGATGCAGAAAGAAGTATGGTGCC
R             ----------------------------------------------------TGGTGCC
                                                                  *******

4OMT2CDS      GATGATACTAGGCATGACTCAAAAAGATTTCATGGTTTCATGGCATTTCATGAAAGAAGG
R             GATGATACTAGGC-------------------------------------------------
              *************
```

Appendix-6

SangerS1



SangerS2



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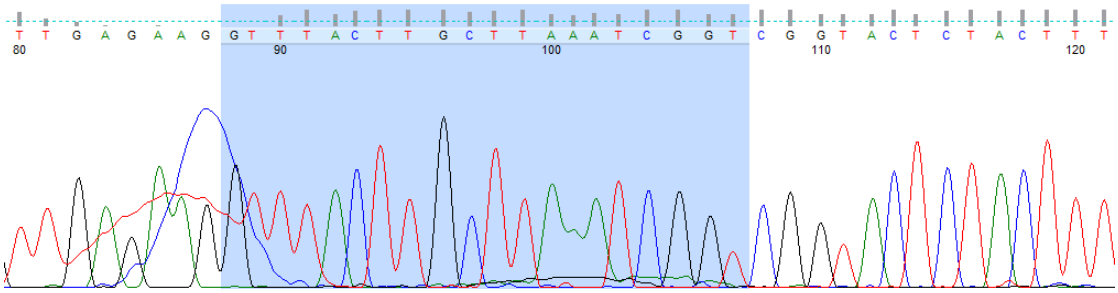

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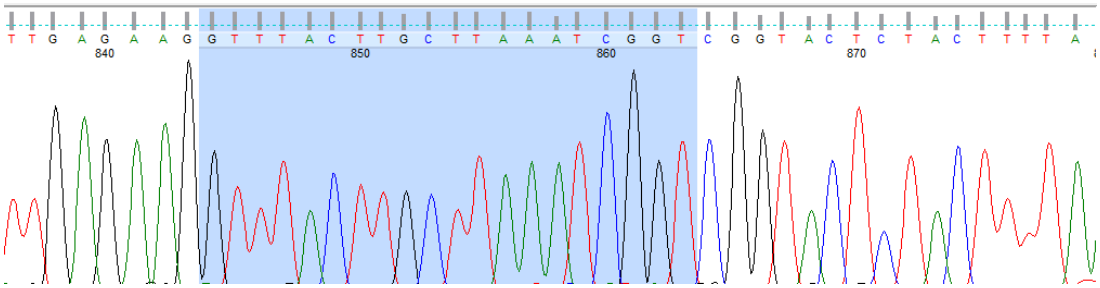
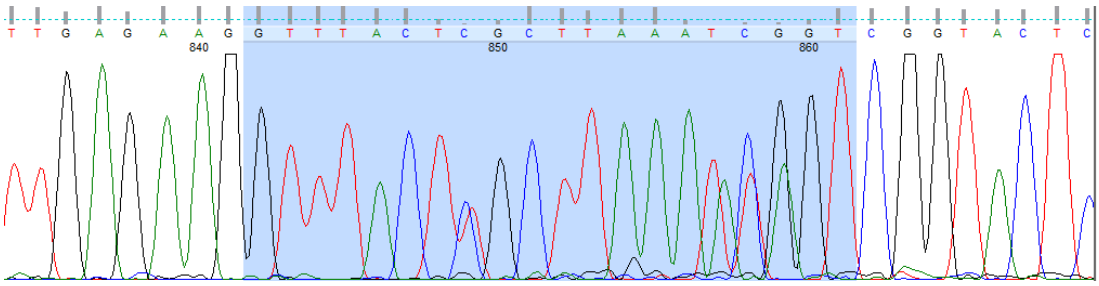


SangerS5



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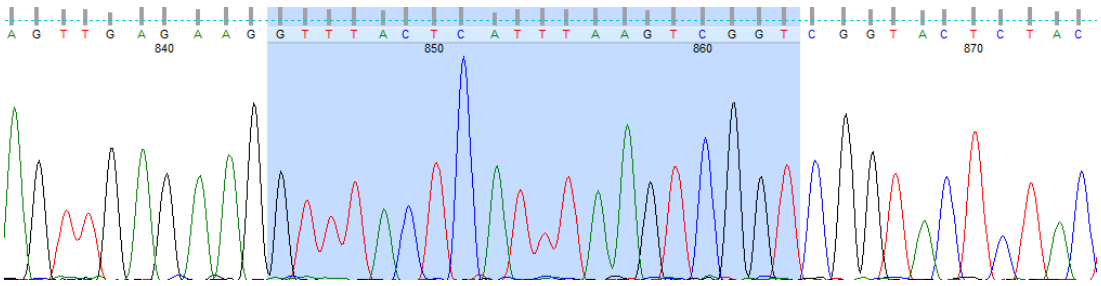

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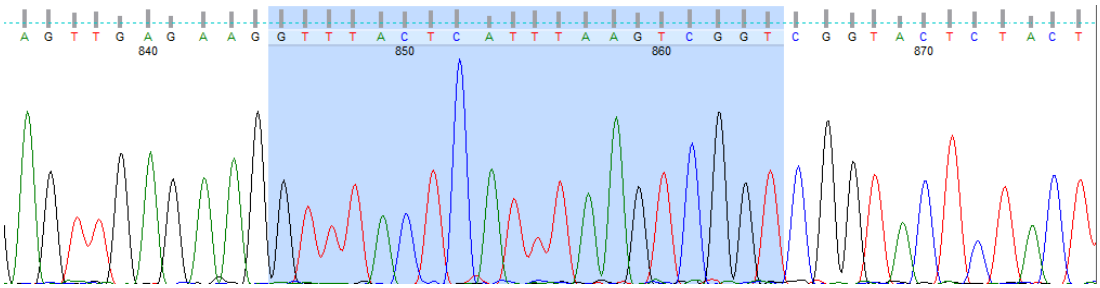

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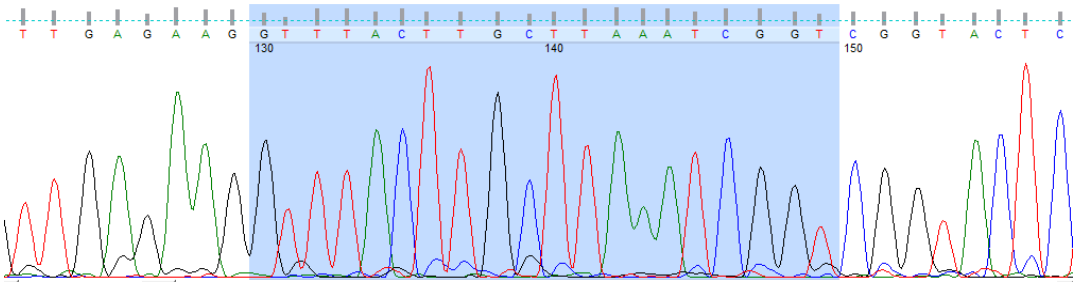

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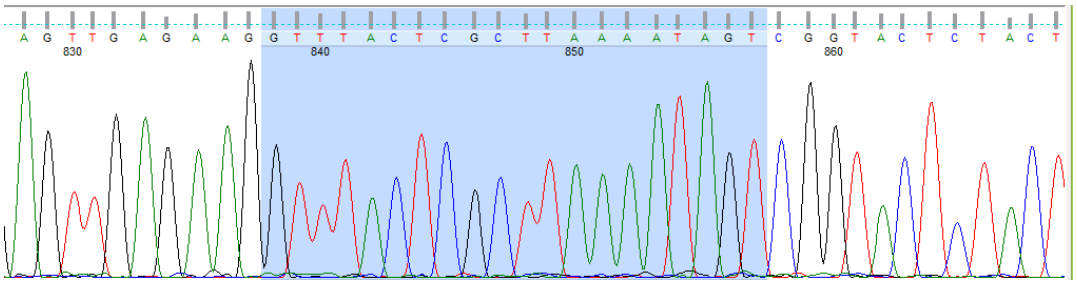

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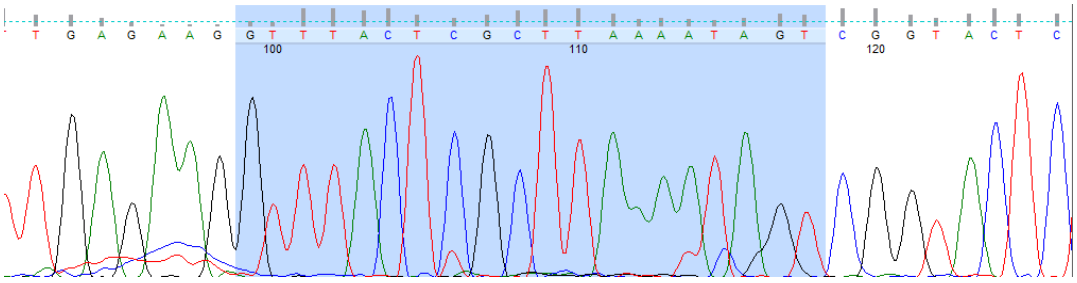

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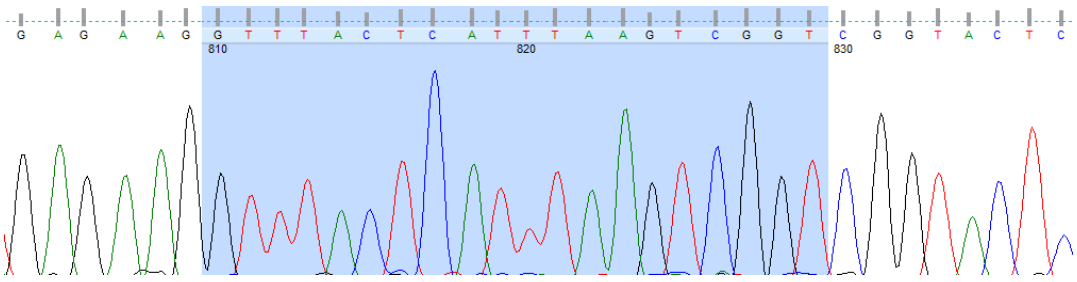

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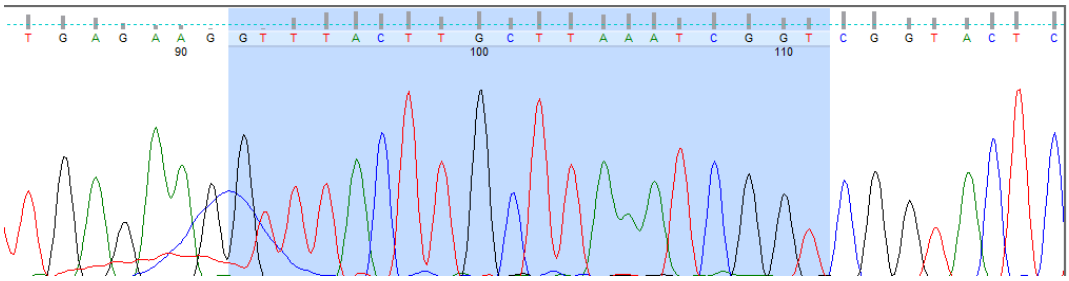

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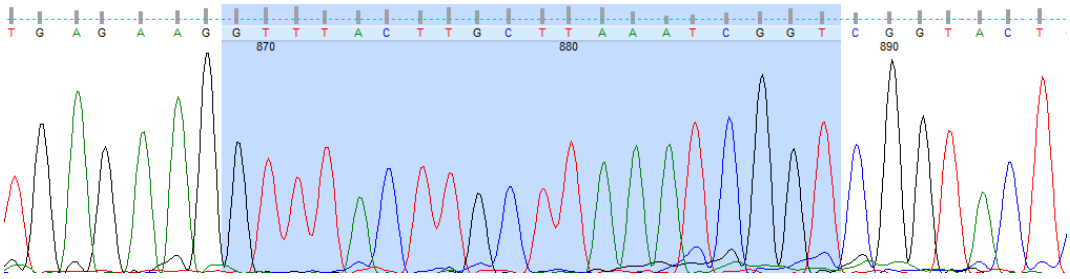

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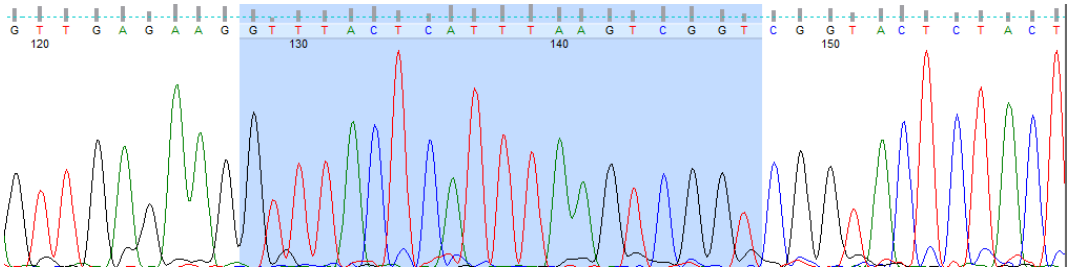

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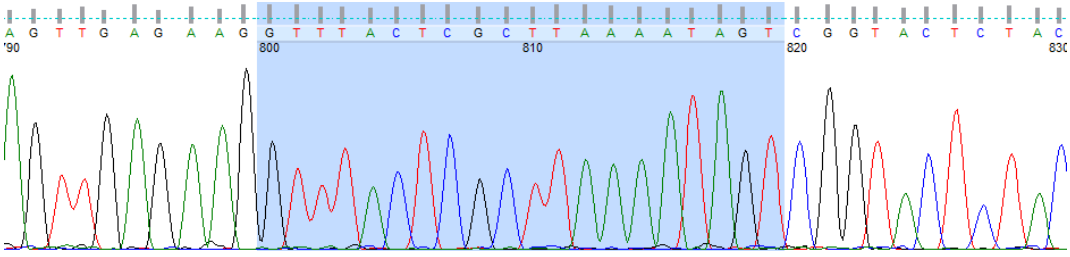

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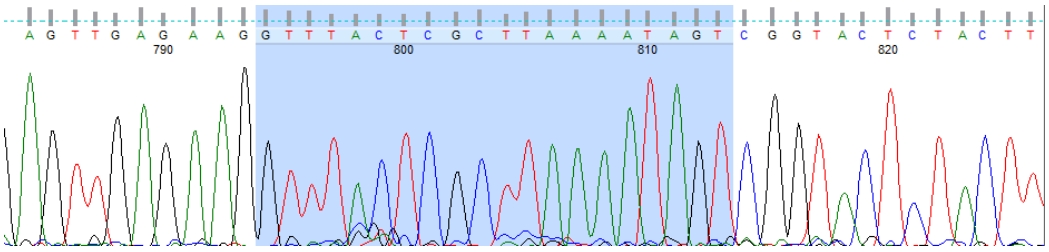

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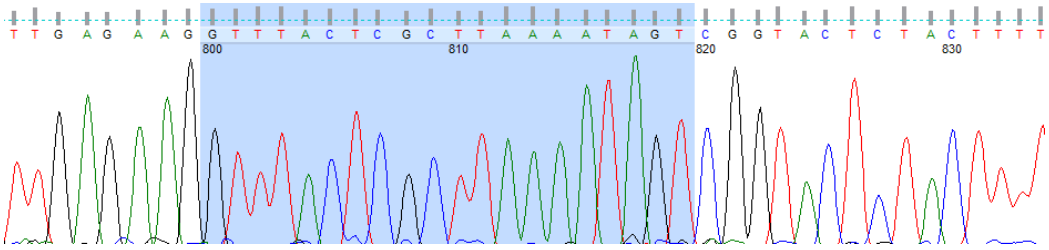

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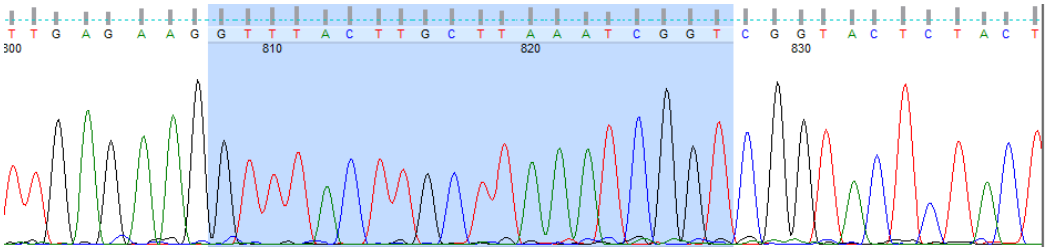

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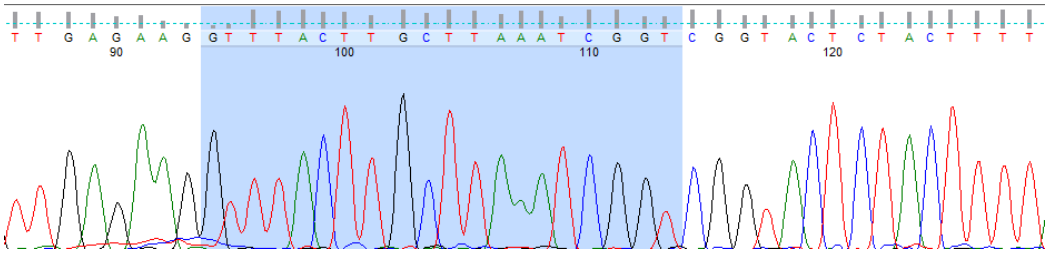

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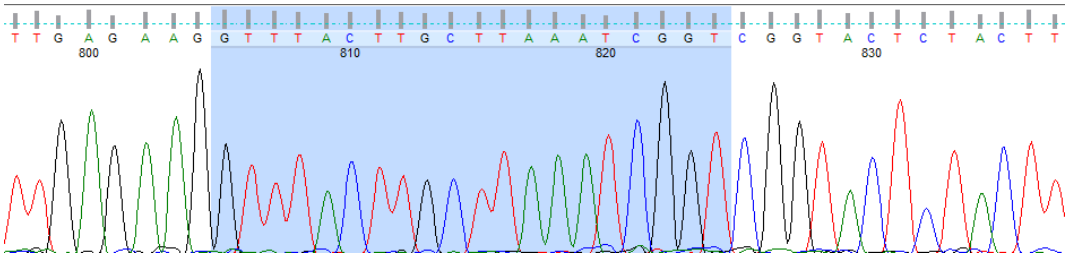

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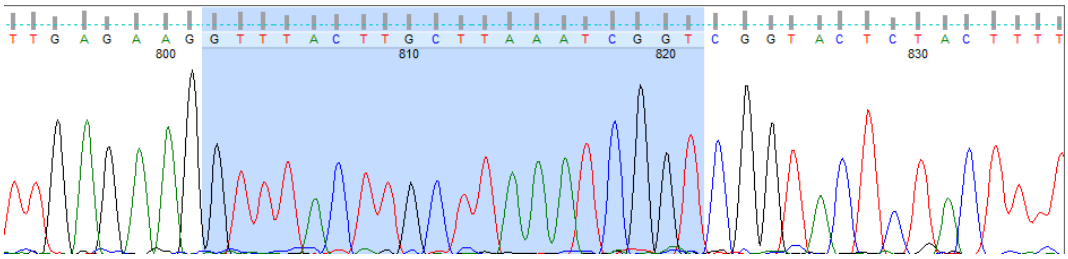

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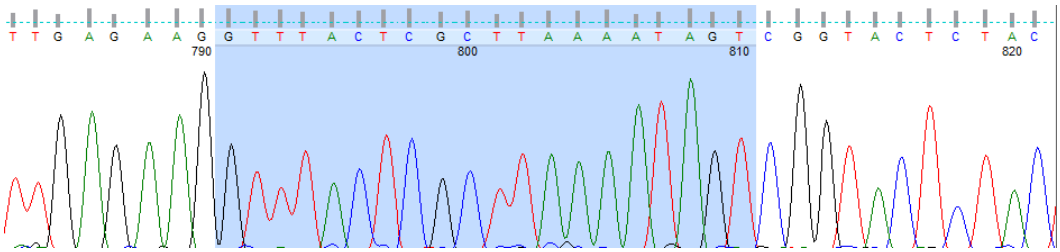

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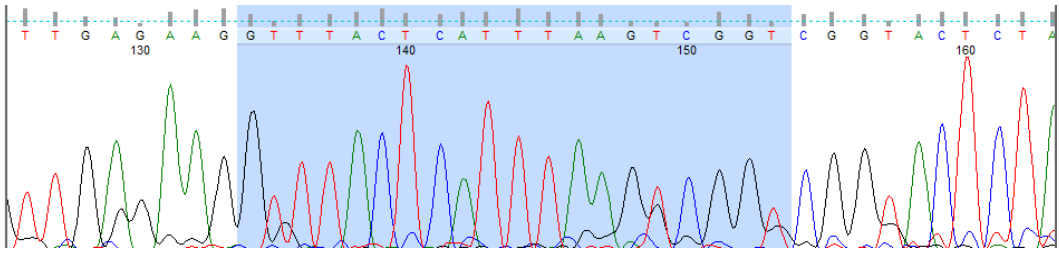

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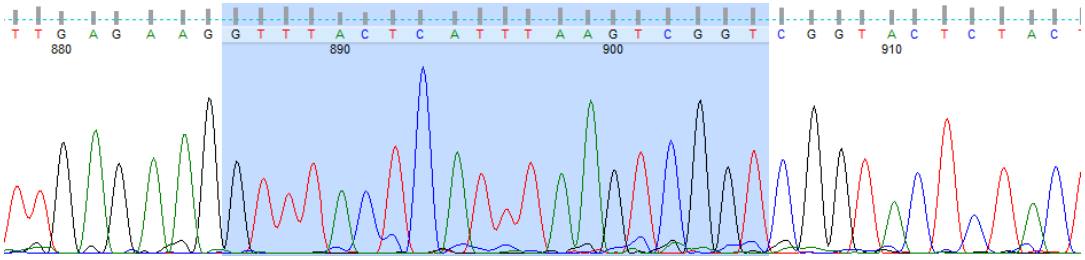

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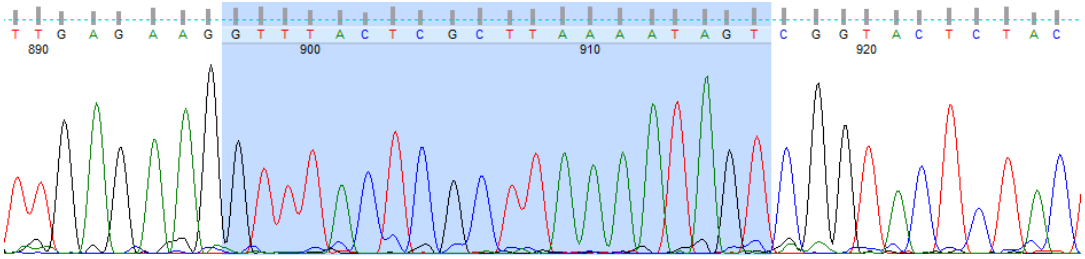

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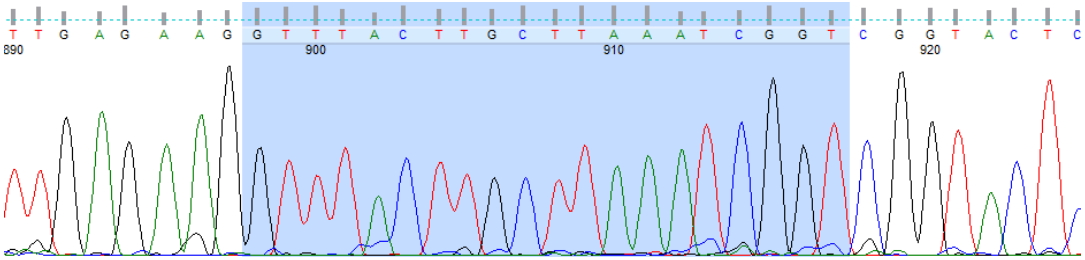

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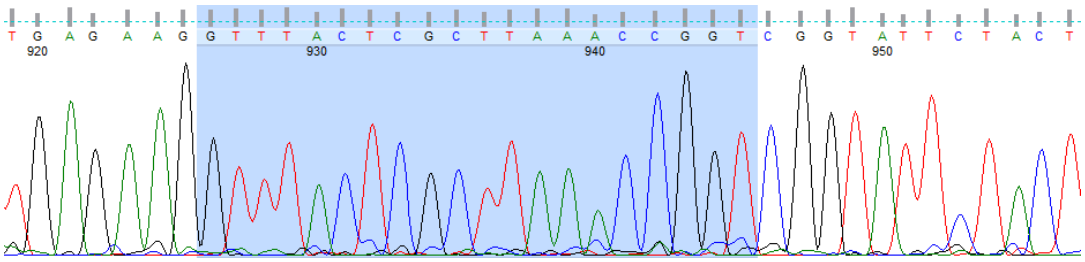

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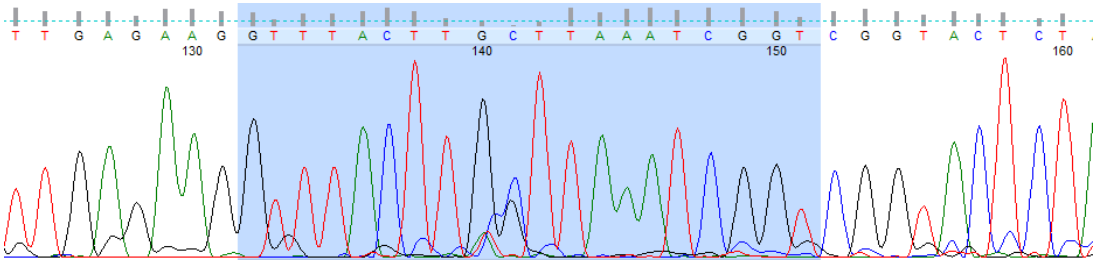

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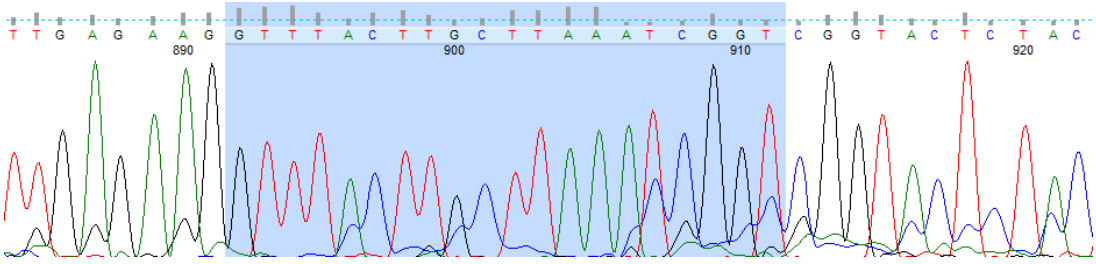

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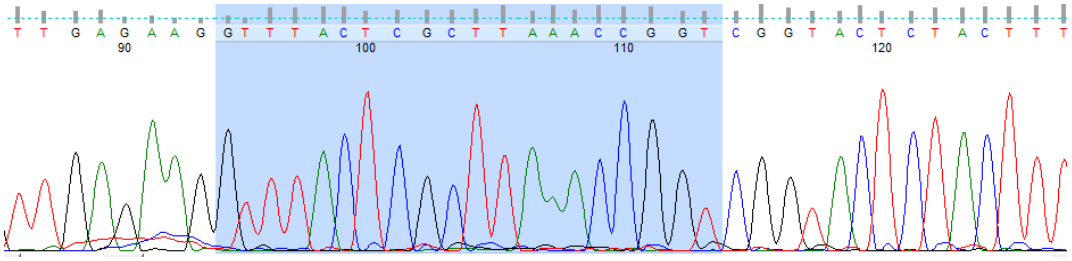

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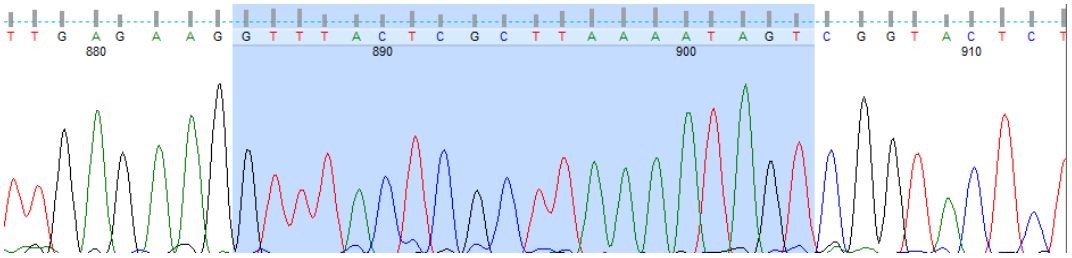

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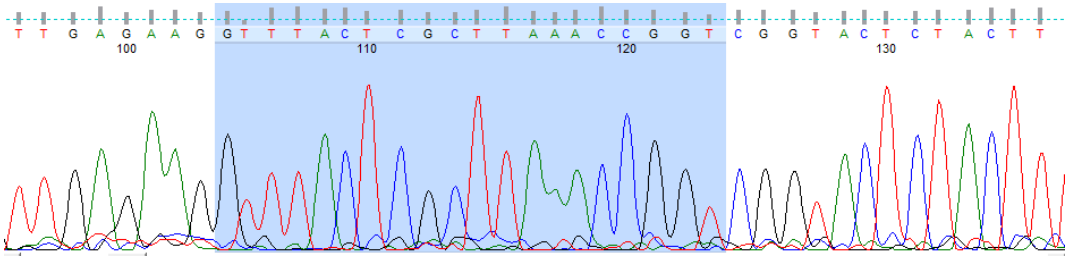

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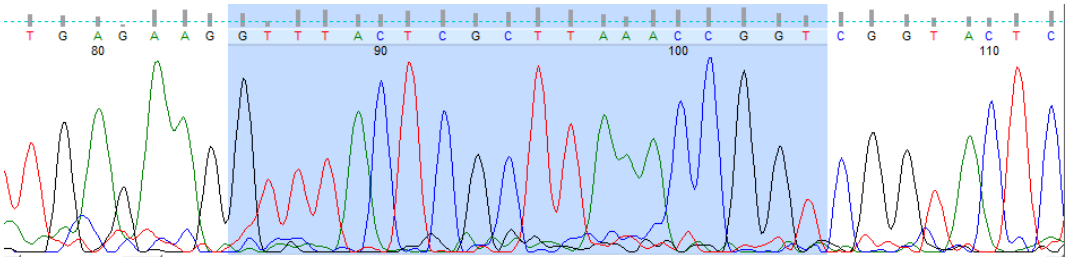

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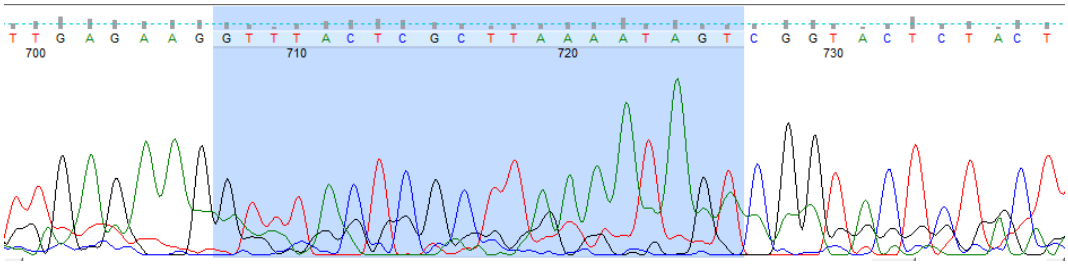

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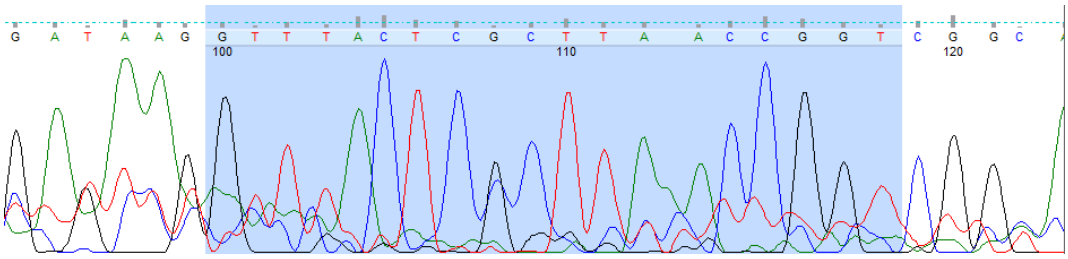

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Yagiz Alagoz, Tugba Gurkok, Iskender Parmaksiz & Turgay Unver. 2015. Identification and Sequence Analysis of Alkaloid Biosynthesis Genes in Papaver Section Oxytona, Turkish Journal of Biology (Accepted, DOI: 10.3906/biy-1505-22)

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Posters & Presentations

▪Ünver T., Türktaş M., İnal B., Gürkök T., Özhüner E., Derelli Tüfekçi E., Alagöz Y., Pekmezci Y., Zıplar Tanman Ü., Babaoğlu G., Açar E., Özden Y., Genomic and Transcriptomic Approaches for Plant Biotechnology, National Congress in Molecular Biology and Biotechnology, Afyon / Turkey, oral presentation (Aug 21-24, 2015).

▪Alagöz Y. , Gürkök T., Ünver T, Synthetic RNA-guided CRISPR/Cas9 System-mediated Gene Knock-Out in Genus Papaver, National Congress in Molecular Biology and Biotechnology, Afyon / Turkey, oral presentation (Aug 21-24, 2015).

▪Alagöz Y. , Demirhan S., 'TRV Mediated Virus Induced Gene Silencing and Its Methodology', 2nd Place Award in Biotech2011 National Biotechnology Student Conferance, Istanbul / Turkey, for the best poster presentation (Oct 24-26, 2011).

▪Demirhan S., Alagöz Y., 'Agrobacterium mediated gene transfer to the plants', in Biotech2011 National Biotechnology Student Conferance, Istanbul / Turkey, poster presentation (Oct 24-26, 2011).