UNIVERSITÀ DEGLI STUDI DI PADOVA
Dipartimento di Biomedicina comparata e alimentazione

Corso di laurea magistrale
Biotecnologie Per L'Alimentazione

Preparazione di costrutti genetici per modificare i genomi di piante orticole

Relatore
Prof. Livio Trainotti
Co-supervisore
Prof. Fabio Vianello

Laureanda
Ronak Samadpour
Matricola n.
2046877

ANNO ACCADEMICO 2023/2024

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Department of Comparative Biomedicine and Food Science

Second Cycle Degree (MSc)
in Biotechnologies for food science

Preparation of genetic constructs to modify the genomes of horticultural plants

Supervisor
Prof. Livio Trainotti
Co-supervisor
Prof. Fabio Vianello

Submitted by
Ronak Samadpour
Student n.
2046877

ACADEMIC YEAR 2023/2024

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Abstract in English
Cell-to-cell signaling in plant depends also on peptide hormones. Hundreds of genes in plant genomes encode these small peptide signals, but only a tiny fraction of these genes have been functionally characterized. Complete identification of these peptide-encoding genes is challenging due to their small size and significant sequence diversity. The CLAVATA (CLV)/EMBRYO SURROUNDING REGION-RELATED (CLE) gene family encodes short peptides involved in plant meristem maintenance, cell differentiation, the defense from pathogens, and environmental responses. Recent findings brought the tomato (*Solanum Lycopersicum*) CLE gene family from 15 to 52 members, including 37 newly identified genes that had not been annotated before. Although we now know 52 CLE peptide genes in *S. lycopersicum*, their functions and roles remain unknown for most of them. We have identified CLE peptides that are highly expressed in flower, suggesting they may be involved in the flowering process. The overall goal of the project is understanding how these peptides influence flowering. Therefore, we have employed a multiplexing strategy using CRISPR/Cas9-mediated mutagenesis to knockout specific combinations of those genes. The genes that are involved in flowering are (*CLE6*, *CLE19*, *CLE30*, *CLE31*, *CLE34*) and for the ripening is *CLE11*. To assemble the vectors for the generation of the KO-lines we took advantage of *pDIRECT_22C*, a vector system based on Golden Gate Assembly techniques. CRISPR/Cas9-designed constructs will be delivered into *S. lycopersicum* with a transformation technique based on the infection of young cotyledon sections by *Agrobacterium tumefaciens*.

Keywords: *Solanum lycopersicum*, CRISPR/Cas9, CLE Peptides

Abstract in Italian
I peptidi ormonali rappresentano una delle principali classi di molecole coinvolte nella comunicazione cellula-cellula negli organismi vegetali. Nei genomi delle piante, centinaia di geni codificano per questi piccoli peptidi ormonali, ma solo una frazione di essi è stata caratterizzata funzionalmente. L'identificazione completa di tali geni costituisce una sfida rilevante per la ricerca odierna, a causa delle dimensioni ridotte e dell'alta variabilità di sequenza che caratterizzano queste molecole. La famiglia di geni peptidici CLAVATA (CLV)/EMBRYO SURROUNDING REGION-RELATED (CLE) codifica brevi peptidi implicati in molteplici processi molecolari negli organismi vegetali, come il mantenimento del meristema, la differenziazione cellulare, la difesa contro i patogeni e la risposta agli stress ambientali. Studi recenti hanno approfondito il ruolo dei peptidi CLE in *Solanum lycopersicum*, ampliando il numero di peptidi noti appartenenti a questa famiglia con la scoperta di 37 nuovi geni, portando così a 52 il totale dei geni *CLE* conosciuti in questa specie. Tuttavia, il ruolo di questi messaggeri molecolari è ancora poco chiaro, in particolare per quanto riguarda i geni recentemente annotati. In questo studio, è stata condotta un'analisi di una selezione di peptidi CLE, concentrandosi in particolare sui geni con un'espressione elevata nel corso della fioritura (*CLE6*, *CLE19*, *CLE30*, *CLE31*, *CLE34*), e della maturazione dei frutti (*CLE11*) al fine di approfondire il loro ruolo nella regolazione di tali processi. A tal fine, è stata adottata una strategia di *knock-out* multiplo mediante la mutagenesi basata sul sistema CRISPR/Cas9, eliminando combinazioni specifiche di questi geni. Per assemblare i vettori destinati alla generazione delle linee knock-out è stato impiegato *pDIRECT_22C*, un sistema di vettori basato sulla tecnologia Golden Gate Assembly. I costrutti ottenuti verranno quindi utilizzati per trasformare linee di *S. lycopersicum* tramite *Agrobacterium tumefaciens*.

Parole chiave: *Solanum lycopersicum*, CRISPR/Cas9, peptidi CLE

Chapter 1

1. Introduction

1.1 *Solanum lycopersicum* cultivar *Florida petite*

The tomato (*Solanum lycopersicum L.*) originating in the Andes, arrived in Europe in the 16th century, sparking a worldwide interest that turned this plant into a common agricultural mainstay. As one of the most commercially important crops in the world today, it thrives on all continents and is a major paradigm for genetically engineering agronomically important features in dicotyledonous crops (Paduchuri et al., 2010).

Tomato fruit contains so many bioactive substances such as carotenoids (lycopene, β-carotene, and lutein), phenolics (flavonoids and tannins), and vitamins (C, E, K, and B) (Ilahy et al., 2011),(Raiola et al., 2014). These compounds have positive effect on health for having anti-inflammatory and anticancer effects and preventing chronic diseases (obesity, diabetes, coronary heart disease, and hypertension) (Kris-Etherton et al., 2002);(Bharti et al., 2014). Also, the peculiar flavor of tomato is due to presence of acetaldehyde and a number of volatile compounds (Mathieu et al., 2009).

Both production and consumption are steadily increasing, and this nutritious fruit is consumed both fresh and in a variety of processed forms, including soups, sauces, juices, and even powder concentrates (Mathieu et al., 2009). The tomato has an enormous impact; in 2011 alone, it reached a stunning 160 million tons grown on nearly 4.8 million hectares of land, placing it seventh in the world in terms of output, behind staples like maize, rice, and wheat. It is an old crop that has stood the test of time, and its importance only grows every year (SHASHI, 2021).

According to toxonomy, this diploid plant (2n = 2x =24) with twelve pairs of chromosome pairs is a member of the enormous *Solanaceae* family, a group of over 3,000 species that includes commercially important crops like peppers, potatoes, eggplants, and even tobacco. In the recent years, the tomato was placed in the same genus as the potato, *Solanum lycopersicum* (Bai & Lindhout, 2007).

The tomato has been the focus of scientific attention lately and is now regarded as a top model organism, particularly after its genome was sequenced (Tomato Genome Consortium, 2012).

S. lycopersicum is important in the genetically modify biotechnology, because this includes a short life cycle, efficient seed production, low levels of gene duplication, a compact genome of just 950 Mb, homozygosity and a high degree of self-fertility, and its capacity to adapt to a variety of culture settings (Bai and Lindhout 2007). Furthermore, it is easy to manage pollination and hybridization, can be propagated asexually through grafting, and can regenerate entire plants from different explants (Tomato Genome Consortium, 2012). Because of its special and beneficial characteristics, cv. *Florida petite* has become the gold standard for study among the many tomato genotypes (Kobayashi et al., 2014). The Florida Basket and Ohio 4013-3 cultivars were crossed to create this dwarf variation, which was originally bred for the genetic modification purposes (SHASHI, 2021). Florida petite cultivar has dark-green, rugose leaves and small, matured fruits. Mutations in important genes like SELF-PRUNING (SP), DWARF (D), and MINATURE (mnt) are responsible for its compact phenotype (Marti et al., 2006)**.**

High levels of conserved gene content and order were found in this family and other families of cultivated crops, according to molecular comparative mapping studies (Wu & Tanksley, 2010). Novel techniques for creating new genotypes that could satisfy new grower, consumer, and processor criteria have been implemented by utilizing the tomato genome sequence and potent genetic resources (Mueller et al., 2009).

The International Solanaceae Genome Project (SOL) was founded to coordinate the research activities of many international groups and to create a network of knowledge about the *Solanaceae* family (Mueller, Solow, et al., 2005). In contrast, the Solanaceae Genomics Network website (SGN; http://www.sgn.cornell.edu) was developed to make it easier to distribute genomic data in a comparative genomic context for *Solanaceous* species in general and tomatoes in particular (Mueller, Tanksley, et al., 2005).

Notably, the ripening process in tomatoes also plays a significant role in determining the phytochemical content because it causes significant physiological, biochemical, and structural changes, such as the breakdown of chlorophylls and the synthesis of carotenoids, primarily lycopene and β-carotene, and other phytochemicals. These changes in ripening lead to changes in tomato fruit color, flavor, firmness, phytochemical content, and ultimately their market quality (Melendez-Martinez et al., 2010). Because tomato fruits are picked at various ripening stages based on customer and market desire, it is crucial to comprehend how variations in the contents of these phytochemicals are influenced by the ripening process.

The two intrinsic attributes of tomato fruits are their nutritional value and their organoleptic characteristics. The fruit texture, flavor, and scent are examples of its organoleptic characteristics. In terms of nutrients, tomato fruits are a low-fat, high-fiber, low-calorie source of several vitamins and minerals as well as a variety of other compounds, including carotenoids, sugars, flavonoids, ascorbic acids, and folate. The color, shape, firmness, and shelf life of tomato fruits are other crucial characteristics. Table 1 provides instances of effective genetic engineering to improve fruit quality characteristics (Gerszberg et al., 2014).

Fruit trait	Targeted gene
Firmness	b-galactosidase, EXP1A (expansine)
Flavonoid content	CHI, CHS, CHI, F3H, FLS, STS, CHR, FNSII, MYB12, S1MYB12, Del, Ros, ANT1, AN2
Carboxylic acids	SlAco3b

Table1. Examples of successful genetic engineering of tomato

The tomato has a variety of biological characteristics that set it apart from other model organisms like *Arabidopsis thaliana* or *Oryza sativa*. Additionally, a huge reservoir of mutants, both naturally occurring and those brought about by chemicals or radiation, are preserved and made accessible through repositories such as the Tomato Genetic Resource Center, LycoTill platforms, and TOMATOMA base, enriching the tomato genetic landscape (Lozano et al., 2009);(Minoia et al., 2010); (Saito et al., 2011). Discovery the molecular complexities behind the tomato numerous features is still a struggle, even though these genetic resources set the stage for incredible developments (Gerszberg et al., 2014).

1.2 Peptide hormones in the plant

The conceptual framework for comprehending the complex regulatory mechanisms that influence plant development and mediate responses to environmental stresses has been dominated by phytohormones such as auxin. Peptide hormones (PHs) are used as complex signaling molecules that can function in both localized and systemic communication. These peptide signals highlight a complex regulatory network that was unknown since recently in plant biology (Olsson V., et al 2018).

Plant peptide hormones (PHs) are complexly divided into various classes, mostly based on whether they are subjected to post-translational processing. Transcripts may encode functional or supposedly nonfunctional precursors for those produced from precursor proteins. In the latter case these hormones usually start off as nonfunctional prepropeptides and are then finely processed and modified to produce mature and active versions. These are classified based on distinct characteristics of the mature molecules (Tavormina et al., 2015):

(a) those that are strongly packed with cysteine residues.

(b) those that show post-translational modifications (PTMs), like tyrosine (Tyr) sulfation or proline (Pro) hydroxylation, with the latter occasionally further embellished with glycosyl groups.

(c) peptides that have not a lot of Cys or PTMs but are distinguished by particular amino acid sequences that are crucial to their bioactivity.

In the case of non-precursor proteins, new studies have recognized peptide hormones derived from open reading frames (ORFs) encoded in non-traditional genomic locations, including microRNA sequences, the 5' untranslated sections of mRNAs, and different intergenic transcript fragments (Lauressergues et al., 2015).

1.2.1 Post-Translational Processing of Peptide Hormones

1. The encoded genes for nonfunctional precursor peptides are expressed under certain developmental or environmental stimuli. Also, for controlling the ensuing signaling cascade complex, post-translational modifications can further control the final maturation of these peptides. The production of biologically active peptides in response to various internal and external stimuli is highly tuned by this additional layer of regulation, which deftly integrates into the overall control (Olsson et al., 2019).

The plant peptide hormones are translated into prepropeptides, which are immature molecules with an N-terminal sorting sequence that guides their passage along the secretory pathway. The prepropeptides go through a selective post-translational modification (PTMs) and planned sequence of proteolytic cleavages as they pass through this pathway and beyond into the extracellular matrix. As a result, the mature and biologically active peptides would be produced. Prepropeptides are all distinguished by the exact removal of the sorting sequence,

which is carried out by a signal peptidase located in the endoplasmic reticulum and results in a more refined propeptide. Though, the ensuing structural and proteolytic improvements show significant variation between peptide families and even between ligands in the same family. A precise peptide length, particular folding configurations, and the presence of PTMs are essential for efficient receptor binding, according to insights from the crystal structures of peptide receptor complexes (Butenko et al., 2014).

1.2.2 CLAVATA3 or CLE peptides in the plant

A complex network of tiny peptide hormones that control division cycles, start differentiation pathways, and precisely modify hormonal balance and defense against pathogens and abiotic sensing are all part of the complex network of plant signaling (Chen et al., 2020). Among these, the short peptide, CLAVATA3/EMBRYO-SURROUNDING REGION-RELATED (CLE) group, is essential for the development of the root protophloem, aid in vascular patterning throughout secondary growth pathway, and determine crucial control over cellular division in the apical meristems of shoots and roots (Xu et al., 2015). CLE peptides block nodulation processes in leguminous species (Okamoto et al., 2013). CLE genes are small, with a central variable segment, a highly conserved C-terminal CLE domain, and an N-terminal signal sequence. These genes encode non-functional prepropeptides that are about 100 amino acids long (Stuhrwohldt et al., 2020).

After translation, these peptides go through a number of further processing stages to attain biological activity, frequently including cleavage by subtilases in addition to modifications including proline hydroxylation and glycosylation (Imin et al., 2018). The Class XI leucine-rich repeat receptor-like kinases (LRR-RLKs), a specialized subset of receptors essential to peptide signaling, come into contact with and detect CLE peptides after they have matured and been secreted into the apoplast (Hazak et al., 2017).

The perception of mature CLE peptides in Arabidopsis has been related to three receptors, referred to as BARELY ANY MERISTEM (BAM) receptors, in addition to the traditional CLAVATA1 receptor-like kinase (CLV1). An extracellular ligand-binding domain that selectively identifies CLE peptides, a transmembrane domain that binds the receptor to the plasma membrane, and an intracellular kinase domain that phosphorylates downstream signaling cascades are the three functional domains to make up these receptors. Additionally,

CLV3 INSENSITIVE KINASES (CIKs), a family of receptor-like kinases, mediate CLV3 signaling in the shoot apical meristem and CLE signaling in root tissues, making them crucial co-receptors in CLE peptide detection (Hu et al., 2018). The leucine-rich repeat receptor-like protein CLAVATA2 (CLV2) creates a dimer with the pseudokinase CORYNE (CRN), producing a special complex that allows for the detection of the complete range of root-active CLE peptides (Hazak et al., 2017).

Numerous plant species, including important crops like tomato, rice, wheat, maize, soybean, grape, potato, and cucumber, have been the subject of extensive genome-wide investigations of CLE gene families (Gancheva et al., 2021). However, CLE gene annotation is a difficult task due to the small gene size and significant sequence diversity. The tomato (*S.lycopersicum*) is a crucial model organism for molecular research to enable in-depth investigations into developmental processes and genetic modification (Sato S et al., 2012). Discovery new regulators that promote growth and physiological adaptability is critical since these findings could meaningfully increase tomato yield and strengthen its resistance to environmental stresses. According to the reports, the tomato genome contained only 15 SlCLE genes (F. Zhang et al., 2014), which was comparatively little when compared to other plant species but recently, using powerful bioinformatic tools, the family has been expanded to 52 SlCLE genes, which is illustrated in Figure 1 (Carbonnel et al., 2022).

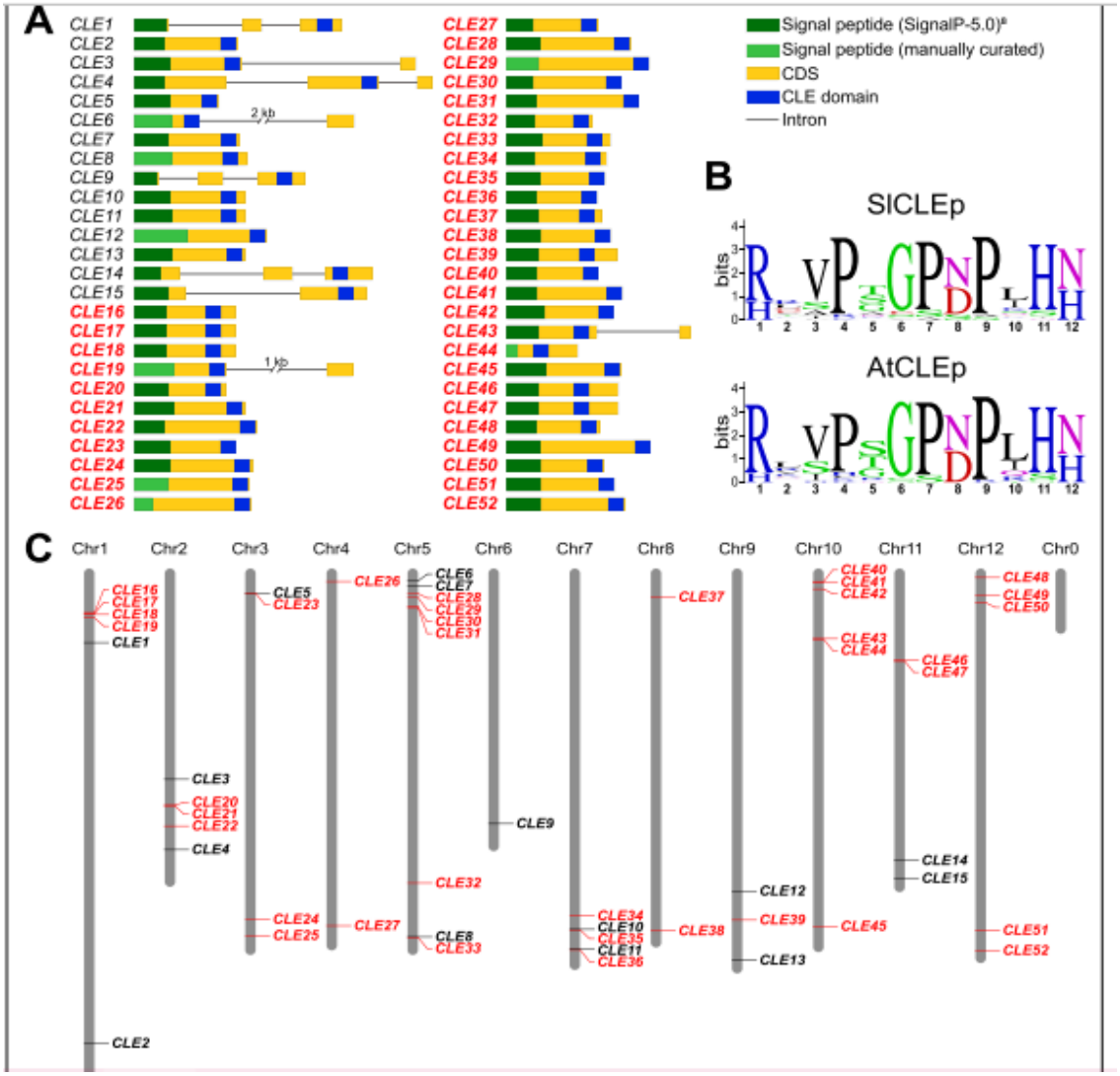


Fig.1. *CLE* genes identified in the tomato genome **A.** The tomato *CLE* genes' structure; the red-colored names denote the new *CLE* genes discovered recently **B.** WebLogo's (https://weblogo.berkeley.edu/logo.cgi) sequence logo of the conserved *CLE* domain in tomato and Arabidopsis; the height of the bars indicates the conservation value of each amino acid at the specified position **C.** The tomato *CLE* genes' chromosomal position (Carbonnel et al., 2022).

1.3 CRISPR/Cas9 and Gene Editing

Two essential components make up the CRISPR-Cas9 system, which modifies DNA:

1) The enzyme called Cas9: This functions as a set of "molecular scissors" that can split the two DNA strands (phosphodiester bonds) at a particular spot in the genome, allowing for the addition or removal of DNA fragments (www.yourgenome.org).

2) RNA fragment known as guide RNA (gRNA) which is made up of a little, roughly 20-base-long segment of a pre-designed RNA sequence embedded in a larger RNA scaffold. The spacer sequence "guides" Cas9 to the correct region of the genome while the conserved portion binds to the Cas9 protein as shown in Figure 2. This guarantees that the Cas9 enzyme performs its cut at the appropriate location inside the genome (www.yourgenome.org).

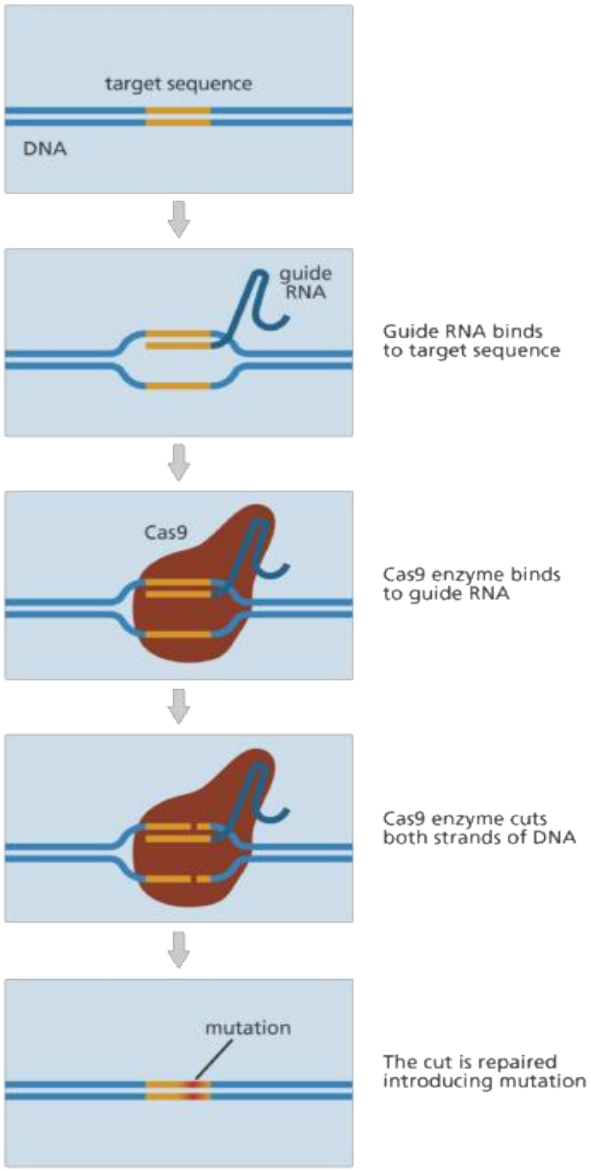


Fig.2. Diagram showing how the CRISPR-Cas9 editing tool works. Image obtained from www.yourgenome.org.

The cleavage produced by the CRISPR/Cas system prepares the way for endogenous repair mechanisms in order to implement the intended DNA sequence alterations (F. Zhang et al., 2014; Y. Zhang et al., 2014). To restore DSBs the nonhomologous end joining (NHEJ) DNA repair system is used which is a repair technique that regularly results in short insertion or deletion (indel) alterations at the break site and commonly leads to a functional knockout of the

target gene (Brooks et al., 2014). Gene knockouts have been accomplished across a variety of plant species that are transformable because of the accuracy of CRISPR/Cas9 technology.

As an alternative, homology-dependent repair (HDR), which uses genome information from a "donor" DNA template to direct exact sequence correction, can be used to fix double-strand breaks (DSBs). A further difficulty with HDR is that it requires the co-delivery of the donor DNA template and the SSN (sequence-specific nuclease) expression cassette into the plant cell, which is significantly less common than (NHEJ) in somatic cells (Baltes et al., 2014).

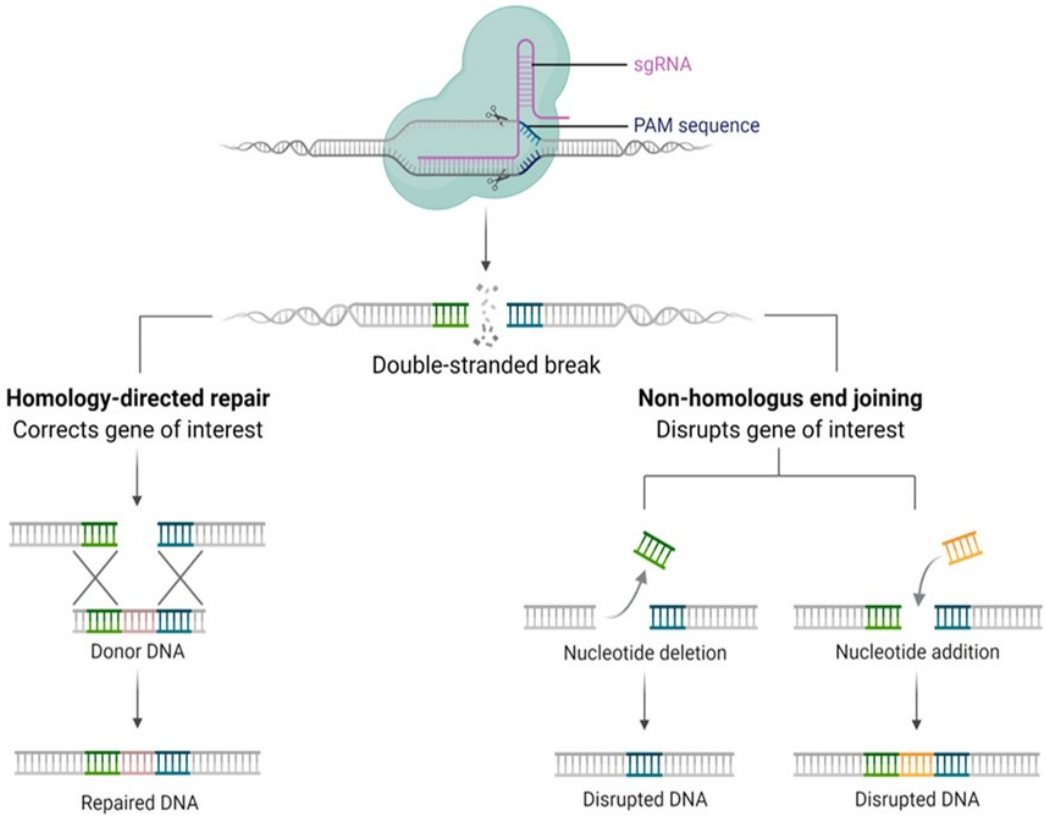


Fig.3 General methodology of CRISPR-Cas9 mediated gene knockout and gene knockin. In gene KO the CRISPR-associated (cas) enzyme cleaves the DNA creating a double strand break (DSB). The cas enzyme is guided by single guide RNA (sgRNA) in the presence of protospacer adjacent motif (PAM) sequence which is essential for the nuclease activity of cas. In case of gene KI, the gene of interest gets incorporated into the DSB by non-homologous end joining (NHEJ) (Wani, A et al., 2023).

Plant genome editing using CRISPR/Cas9 was first reported in 2013, and since then, this approach has been widely utilized to generate loss-of-function alleles in various plant species. The effectiveness of this method was assessed in a diversity of crop species, including *Oryza sativa* (F. Zhang et al., 2014), *Sorghum bicolor* (Jiang et al., 2013), *Triticum aestivum* (Wang et al., 2018), and *S.lycopersicum* (Brooks et al., 2014), as well as model plants, such as *Arabidopsis thaliana* and *Nicotiana benthamiana* (Li et al., 2014). CRISPR/Cas9 is quickly emerging as the preferred tool for gene editing in plants due to the rise in stable transgenic lines and gene alterations observed in primary transformants of crops. Tomato is the perfect organism of solanaceous crops for testing CRISPR/Cas genome editing because of its economic significance, ploidy level of the genome, high-quality genome sequence, and availability of effective transformation techniques (Wang, 2006).

Streptococcus pyogenes CRISPR/Cas9 system is a powerful tool for modifying the genomes of both monocot and dicot plants, which have effectively converted a non-fictional mutant GFP in tobacco and *A. thaliana* into a functioning GFP gene (Jiang et al., 2013).

The CRISPR/Cas9 system can be effectively used to modify the tomato genome. The tomato RIN gene, which controls fruit ripening, serves as the primary target for this modification. They have targeted three areas of the RIN gene, resulting in alterations that either involve the deletion of more than three bases or the insertion of a single base. Heterologous mutants expressing the remaining wild type allele achieved full ripening red color, showing the critical role of RIN in ripening, whereas the RIN protein-deficient mutants produced incompletely ripe fruits with red color pigmentation that was much lower than that of the wild type. The T1 progeny inherited a number of alterations produced at three different target sites, demonstrating the CRISPR/Cas9 system's suitability for tomato (Ito et al., 2015).

Because its target specificity depends on short guide RNAs (gRNAs) rather than proteins that need to be carefully re-engineered for every new locus, CRISPR/Cas9 is particularly beneficial for multiplexed gene editing (Baltes & Voytas, 2015). Many methods for co-expressing

numerous gRNAs have been developed; these usually entail creating separate gRNA expression cassettes, each of which is driven by a different RNA polymerase III (Pol III) promoter (Xing et al., 2014). As an alternative, a single polycistronic transcript can create several gRNAs, which are then separated into distinct gRNAs by RNA-processing enzymes. These include natural tRNA-processing enzymes in the host cells (Xie et al., 2015), self-cleaving ribozymes (Gao & Zhao, 2014), and the CRISPR-associated endoribonuclease Csy4 from *Pseudomonas aeruginosa* (Tsai et al., 2014). When combined, these techniques increase CRISPR systems' capacity for highly coordinated, multi-target gene editing.

The use of CRISPR-Cas9 to knock out a gene in *S. lycopersicum* that codes for a peptide provides an intriguing glimpse into the diverse functions of plant peptides, clarifying their influence on tomato physiology, fruit ripening and flowering, developmental processes, and adaptive stress reactions (Olsson V., et al 2018). Peptide hormones are important signaling molecules in plants, coordinating intricate networks that react to internal growth cues or external stimuli (Jaganathan et al., 2018). Through the exact disruption of these particular genes, we could investigate resulting changes in phenotypic, developmental and flowering pathways or revealing layers of functional relevance and interdependencies within the biological framework of the *S. lycopersicum* plant.

1.4 Golden Gate Assembly

Carola Engler and associates developed the one-step cloning process known as Golden Gate assembly, in 2008. The technique makes use of restriction enzymes of Type IIS (such as BsaI), which break DNA outside of their recognition sequences. An orderly assembly of a vector and one or more DNA fragments is the end outcome (https://www.snapgene.com).

Within a single reaction, Golden Gate assembly can be divided into two separate steps:

1) Type IIS digestion by restriction enzymes

2) DNA ligation

1.4.1 Restriction Digestion by Type IIS Enzymes

There are compatible Type IIS restriction sites in the insert fragment(s) and destination vector.

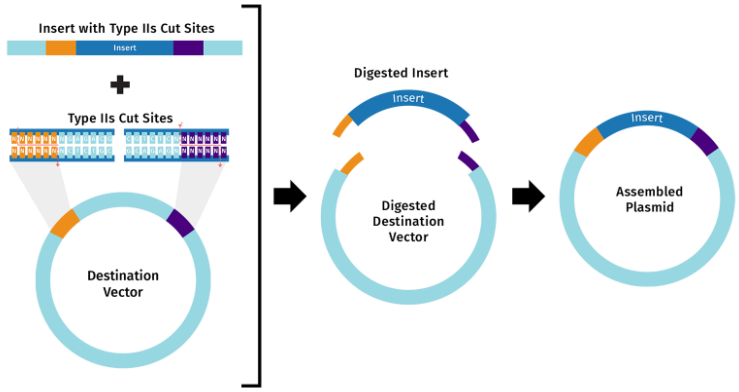


Fig.4. Golden Gate single insertion Cloning (https://www.snapgene.com).

Golden Gate assembly uses Type IIS restriction enzymes, as opposed to Type IIP restriction enzymes, which recognize a palindromic sequence and cleave outside the recognition site, as in traditional restriction cloning. Golden Gate assembly is made feasible by a number of special characteristics of type IIS restriction enzymes (https://www.snapgene.com).

For the non-palindromic recognition site, there is no palindromic aspect to the recognition site and typically contain four to seven nucleotides. This type of cleavage takes place away from the location of recognition. Sequences can be digested for cloning without damaging crucial sequences due to the relocated cleavage location (https://www.snapgene.com).

Moreover, each strand has staggered cleavage, which produces distinct overhangs (1–5 nucleotides) connected to a single recognition site. A 4-base overhang, for instance, can have up to 256 distinct overhang sequences, allowing several DNA fragments to be put together in a single process. Fusion Sites are a common term used to describe the distinctive four overhangs of four base pairs. The length of the recognition site, the offset of the cleavage site from the recognition site, and the type of overhang are all very varied throughout type IIS restriction enzymes (https://www.snapgene.com).

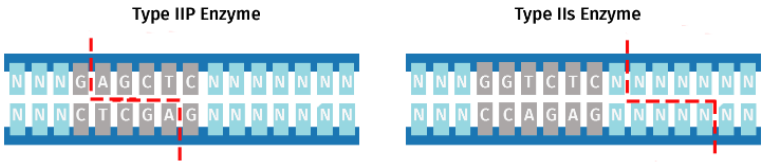


Fig.5. Type IIP v. Type IIS restriction enzymes. Image obtained from https://www.snapgene.com

1.4.2 DNA Ligation

Following digestion, DNA ligase joins the complementary overhangs of the target vector and DNA insert(s) to form an orderly assembly. Because the restriction sites are removed from the final construct and undesirable nucleotides are not introduced between the DNA segments, the procedure is frequently referred to as "scarless" or "seamless" (https://www.snapgene.com/guides/golden-gate-assembly).

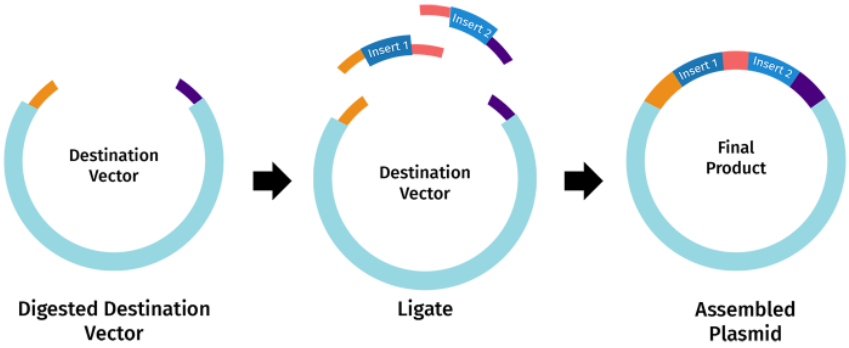


Fig.6. Multi-insert golden gate cloning. Image obtained from https://www.snapgene.com

Aim of this study

The goal of this thesis is to explore the possible roles of CLE peptides (CLAVATA3/EMBRYO SURROUNDING REGION) genes in flowering and fruit ripening in *S. lycopersicum*. While CLE peptides are known to be significant for plant development and signaling, little is recognized about how exactly they affect different growth processes, like flower formation and fruit ripening.

We used a multiplex gene-editing strategy utilizing CRISPR/Cas9 technology, which enabled the simultaneous deletion of numerous CLE genes, to examine these functions. By removing particular combinations of CLE genes which showed high expression in the flowering and ripening stages, we want to uncover their roles in these developmental processes.

In order to deliver the gene-editing components, the experimental design for the creation of CRISPR/Cas9 vector was carried out in this thesis, trough the employment of the *pDIRECT_22C* plasmid. This vector is characterized by a plant-optimized Cas9 gene, kanamycin resistance (kanR), and ccdB gene for selection, a cloning site for multiple gRNAs, making it ideal for our investigation.

To employ the CRISPR/Cas9 technique toward the targeted CLE genes, the Golden Gate Assembly method was used, which was ideal for creating complex constructs such as those required for multiplex editing. Golden Gate Assembly, using the *SapI* restriction enzyme, allows for precise insertion of multiple guide RNAs (gRNAs) that would guide the Cas9 protein to the specific CLE gene loci in *S. lycopersicum* cv. *Florida Petite*.

For gRNAs expression, the CmYLCV promoter was chosen, known for its effectiveness in dicot plants. Verification of successful gene knockout was achieved by using specific primers (Rep as the forward primer and Csy as the reverse primer), which helped to confirm whether the targeted CLE genes have been effectively inactivated.

Through this approach, the study seeks to develop the genetic constructs aimed to deepen the understanding of CLE peptides and their roles in tomato plant development. The findings from this research could offer new insights into CLE function and contribute to broader applications

in crop improvement, particularly in enhancing traits related to flowering and ripening in tomatoes and other related crops.

Chapter 2

3. Material and method

3.1 Plant Material: *Solanum lycopersicum* cultivar *Florida petite*

The *Solanum lycopersicum* plant was moved to soil and kept in the growth chamber of the Plant Genome Editing facility at 25°C with a 16-hour light and 8-hour dark photoperiod. After that, they were transferred to the greenhouse of the Department of Biology in Padova, where they were raised in controlled environments with a light/dark cycle of 10/14 hours and temperatures varying from 23 to 27°C according on the season.

The dwarf tomato *S. lycopersicum* cv. *Florida Petite* was used because we had a well-tested transformation protocol in the laboratory. Moreover, it was the model organism for fruit-research and we were interested in the fruit development and ripening. Six new identified CLE (CLAVATA3) genes (*CLE6, CLE11, CLE19, CLE30, CLE31, CLE34*) which are located on three different chromosomal locations in the plant *S. lycopersicum* were examined. Each of these CLE genes, would express the peptide hormones that are crucial to the complicated regulatory signaling that coordinate physiological activities, fruit ripening and flowering in the plant tomato *(Solanum lycopersicum)*. The CRISPR-Cas9 technique was used to create the knockout genes, while Golden Gate Assembly was utilized to clone the gene-editing constructs.

3.1.1 The peptide sequences of Solanum lycopersicum CLE genes of interest: CLE6, CLE11, CLE19, CLE30, CLE31, CLE34

The chromosome locations and peptide sequences of SlCLEs are described in this table (Carbonnel, S et al., 2022):

Name	Predicted CLEp	Chromosome	Start	End
CLE6	RRVPNGPDPIHN	5	1311175	1313680
CLE19	RRVPNGPDPIHN	1	7704510	7706013
CLE30	RSIPSGPNPLHN	5	5853919	5854191
CLE31	RLVPSGPNPLHN	5	5856133	5856446

CLE34	RRVPTGPNPLHN	7	59399859	59400098
CLE11	RVAPQGPDAQHH	7	65194792	65195058

Table 2. The chromosomal locations and peptide sequences of CLEp

3.2 Bacteria strains and vector

3.2.1 Strains of *Escherichia coli*

In this thesis, two strains of *Escherichia coli* were used: electrocompetent *E. coli* DH10β and *E. coli* DB3.1 were used for cloning and plasmid amplification.

E. coli strain DH10β is a critical bacterial host for plasmid replication and cloning procedures that are necessary for the Golden Gate Assembly. DH10β genetic alterations gives it stability, efficiency and adaptability which makes it suitable for working with *pDIRECT_22C* plasmid that is used for Golden Gate cloning.

The recA1 mutation avoids homologous recombination and this protection preserves the structural integrity of cloned plasmid. High yields of intact plasmid DNA is ensured by the endA1 mutation's inactivation of endonuclease I and could increase the quality of plasmid preparation (Kawashima, H et al., 1984). The genome of *E. coli* strain DB3.1 contains the gyrA462 gene, which confers resistance to the ccdB toxicity gene.

This strain is especially well-suited for the development or growth of a plasmid vector that contains a ccdB gene (such as our Golden Gate System Vector) and possesses the chain kanamycin resistance. Both DB3.1 and DH10β were stored at -80°C in glycerol 30% of solution and had to be cultivated at an aerobic condition temperature of 37°C. (www.geneuniversal.com).

3.2.2 Vector *pDIRECT_22C* (Addgene No. 91135)

The *pDIRECT_22C* plasmid was a well-designed toolkit for cloning and expressing genes in *E. coli*. The important components of *pDIRECT_22C* were the *kanR* gene that allowed *E. coli* to resist to kanamycin. This selection mechanism enabled us to identify their successful clones.

The second interesting component of *pDIRECT_22C* was *ccdB* gene. This gene produced a protein that was toxic to *E. coli* and, if it was expressed, it could kill the strain of DH10β. During the Golden Gate cloning, the *ccdB* gene, which was harmful to the selection process was cleaved by *SapI* enzyme.

This indicated that the selection process only results in the survival of the bacteria carrying the cloned plasmid. The *ccdB* gene produced its lethal protein if the bacteria were transformed with a plasmid that was not cloned, and those bacteria was unable to survive. The *pDIRECT_22C* map is presented in Figure 1.

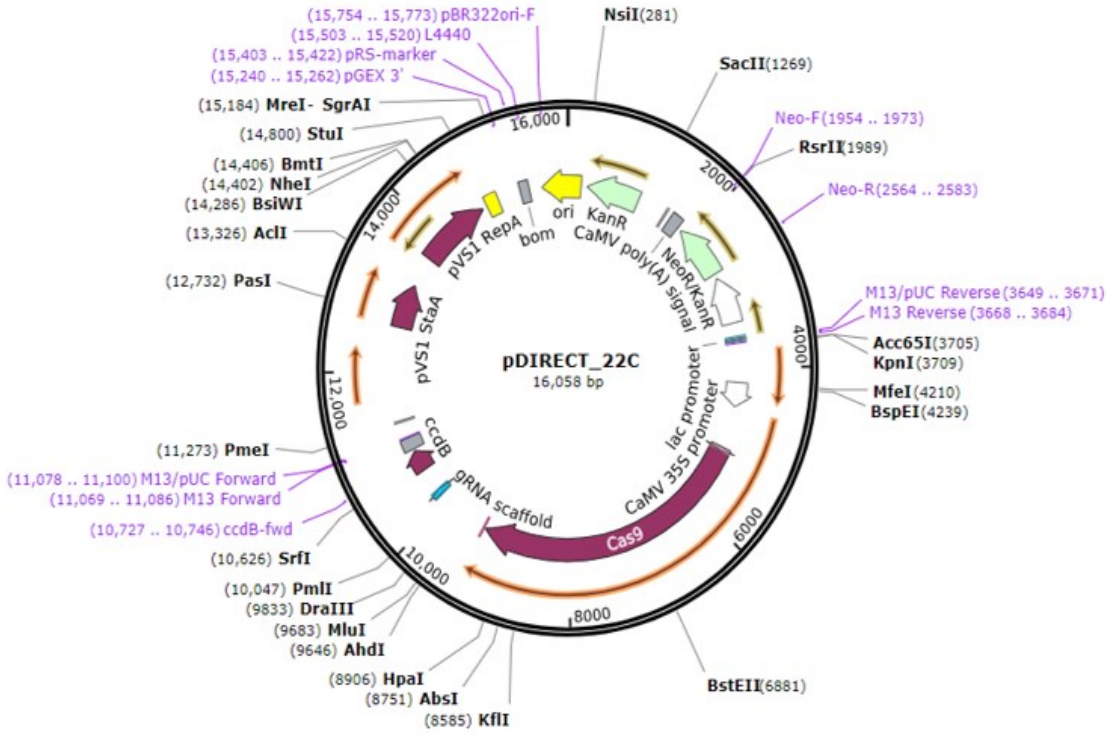


Figure.7. Vector map. The map of pDIRECT_22C. Image obtained through Addgene.

3.3 Golden Gate Assembly

This set of protocol described cloning of four gRNA spacers into Csy4 array controlled by an RNA Pol II promoter in *pDIRECT_22C* vector and based on the PCR products contained the processing elements, gRNA repeats, and segments of target-specific gRNA spacers, which provided distinct sequence stretches for designing the Golden Gate junctions (Čermák et al., 2017).

First, primers were designed to amplify both the promoter and each gRNA parts. For designing the primers, the online primer design tool was used, which accepted the list of 20 bp gRNA spacer sequences in FASTA format as input:

http://cfans-pmorrell.oit.umn.edu/CRISPR_Multiplex/.

Also, the design of the gRNAs was performed with two different tools:

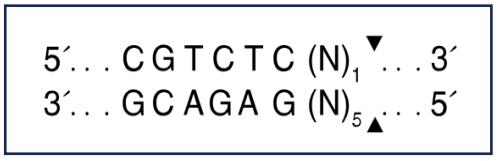
Chopchop (https://chopchop.cbu.uib.no/)

Crispor (http://crispor.tefor.net/crispor.py)

CHOP-CHOP is a web-based tool to design gRNA for CRISPR, it can simplify the sorting process for a CRISPR guide sequence in a DNA sequence input. With the exception of the PAM sequence (5'-NGG), the ideal gRNA sequence was 21 base pairs (bp), however it could be as few as 18–25 bp. Since smaller gRNAs had a higher off-target chance and larger gRNAs (25 bp) had lower efficiency, 20 bp gRNA was created.

The *pDIRECT_22C* vector was used as template for PCR amplification of specific component as well as the cloning backbone. Golden Gate assembly was used to combine n+1 (n = number of gRNA spacers) components (PCR products) into the vector using two type restriction enzymes (*Esp3I* and *SapI*) and *T7 DNA ligase*.

The recognition sequence of *Esp3I* enzyme →

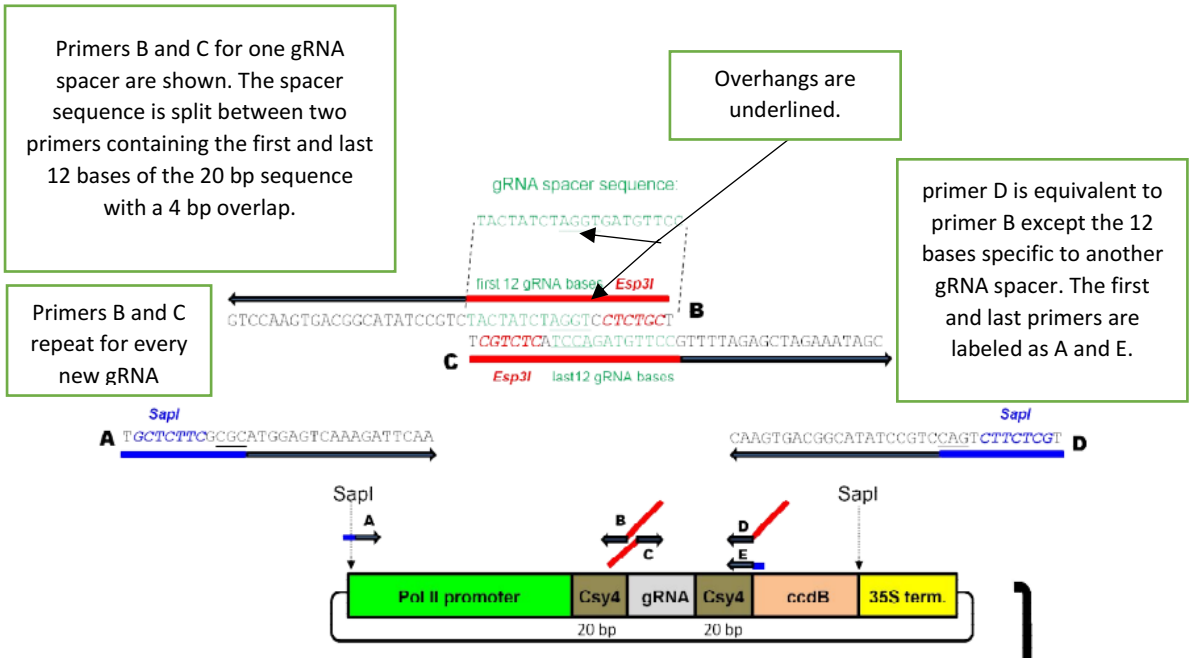


The recognition sequence of *SapI* enzyme → 5′...GCTCTTC(N)$_1$▼...3′
3′...CGAGAAG(N)$_4$▲...5′

The *ccdB* cassette, together with the promoter and gRNA scaffold, would be released from the vector by *SapI* cleavage, opening the backbone for the assembly of the generated PCR products by generating certain 3 bp overhangs.

Additionally, *SapI* produced overhangs on the 5' end of the first PCR product and the 3' end of the last PCR product that were complementary to the plasmid backbone. Specific 4 bp overhangs were produced on the 3' end of the first, the 5' end of the last, and each end of every other PCR product by *Esp3I*.

Each distinct gRNA spacer sequence of 20 bp was divided between two primers to produce successive segments.



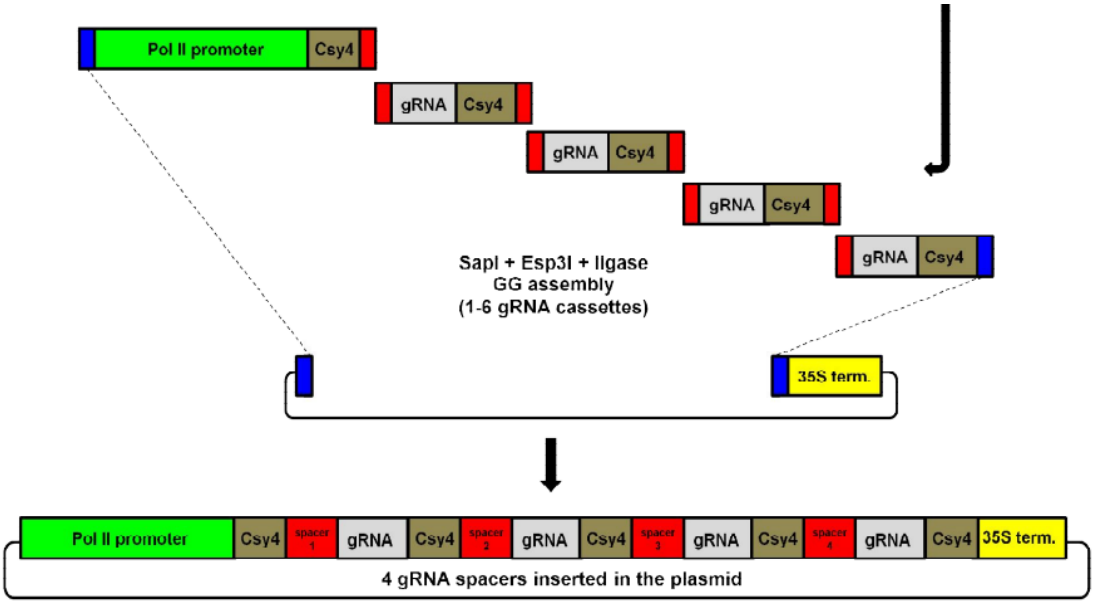


Fig.8. Golden Gate assembly of four gRNA spacers: PCR products containing the promoter and gRNA cassettes are assembled into an array in a single step Golden Gate reaction (in this example using *SapI* and *Esp3I*) to yield the final expression cassette (Čermák et al., 2017).

The 3' end of the first PCR product, which also contained the promoter, would contain the first half of the first gRNA spacer, and the 5' end of the second PCR product contained the second half of the first gRNA.

The midsection (positions 9–12) of each gRNA spacer had the 4 bp overhangs produced by the second type IIs enzyme, resulting in a full length (20 bp) gRNA spacer upon ligation. Because these 4 bp overhangs in positions 9–12 of each gRNA dictated the order in which the individual pieces were formed, bases in these 4 positions shouldn't be the same in any two gRNA spacers that were being constructed.

The efficiency of this cloning technique permitted the assembly of up to six gRNA spacers in a single phase, despite the fact that theoretically any number of gRNA spacers might be constructed.

3.3.1 Design primers for each gRNA being assembled as follows

CSY

TCGTCTCCxxxxxxxxxCTGCCTATACGGCAGTGAAC: we replaced the red Xs for the first 12 bases of the first gRNA spacer being put together. Also, we used this primer for each new gRNA starting from the second to the second last.

REP

TCGTCTCAxxxxxxxxxGTTTTAGAGCTAGAAATAGC: we substituted the final 12 bases of the first gRNA being assembled for the red Xs. We used this primer for each additional gRNA, starting with the second one.

3.3.2 The primer design and Csy4 gRNA array assembly

The primer sequences with the CmYLCV promoter and PCR fragments that were used to put together a Csy4 array of four gRNAs of *CRcle*[30,31] and two gRNAs of *CRcle*[34] are shown in the below table.

>CmYLCV	*TGCTCTTCGGCGCTGGCAGACATACTGTCCCAC*
>CSY_CLE30 gRNA2	*TCGTCTCCTGATCTTTTCTCCTGCCTATACGGCAGTGAAC*
>REP_CLE30 gRNA2	*TCGTCTCAATCAATCCCTTCGTTTTAGAGCTAGAAATAGC*
>CSY_CLE31 gRNA2	*TCGTCTCCCGCCTCCGTCATCCTGCCTATACGGCAGTGAAC*
>REP_CLE31 gRNA2	*TCGTCTCAAGGCGGTAGAAAGTTTTAGAGCTAGAAATAGC*
>CSY_CLE30 gRNA1	*TCGTCTCCGCCACTGTCCACCTGCCTATACGGCAGTGAAC*
>REP_CLE30 gRNA1	*TCGTCTCATGGCGAATTCTTGTTTTAGAGCTAGAAATAGC*
>CSY_CLE31 gRNA1	*TCGTCTCCTGCTATCAACTCCTGCCTATACGGCAGTGAAC*
>REP_CLE31 gRNA1	*TCGTCTCAAGCAGATTACAGGTTTTAGAGCTAGAAATAGC*
>CSY_CLE34 gRNA2	*TCGTCTCCTGCCGGATGGCGCTGCCTATACGGCAGTGAAC*

>REP_CLE34 gRNA2	*TCGTCTCAGGCAACCACCACGTTTTAGAGCTAGAAATAGC*
>CSY_CLE34 gRNA1	*TCGTCTCCTCTCCTCATGTCCTGCCTATACGGCAGTGAAC*
>REP_CLE34 gRNA1	*TCGTCTCAGAGAGTTCCTACGTTTTAGAGCTAGAAATAGC*
>CSY_TERM	*TGCTCTTCTGACCTGCCTATACGGCAGTGAAC*

Table 3. Primers (type IIS sites are highlighted in turquoise, gRNA spacer sequences in green and overhangs underlined)

gRNA spacer sequences of CRcle[30,31]	
>CLE30 gRNA2	*GAGAAAAGATCAATCCCTTC*
>CLE31 gRNA2	*GATGACGGAGGCGGTAGAAA*
>CLE30 gRNA1	*GTGGACAGTGGCGAATTCTT*
>CLE31 gRNA1	*GAGTTGATAGCAGATTACAG*

Table 4. four guide RNA (gRNA) spacer sequences of *CRcle[30,31]* in the Golden Gate cassette.

gRNA spacer sequences of CRcle[34]	
>CLE34 gRNA2	*CGCCATCCGGCAACCACCAC*
>CLE34 gRNA1	*GACATGAGGAGAGTTCCTAC*

Table 5. Two guide RNA (gRNA) spacer sequences of *CRcle[34]* in the Golden Gate cassette.

The primer sequences with the CmYLCV promoter and PCR fragments that were used to put together a Csy4 array of four gRNAs of *CRcle[6,19]* and three gRNAs of *CRcle[11]* are shown below.

>oCmYLCV	*TGCTCTTCGGCGCTGGCAGACATACTGTCCCAC*
>CSY_CLE19gRNA1	*TCGTCTCCAGACCCTATCCACTGCCTATACGGCAGTGAAC*
>REP_CLE19gRNA1	*TCGTCTCAGTCTGGGCCATTGTTTTAGAGCTAGAAATAGC*
>CSY_CLE6gRNA1	*TCGTCTCCTGACCCAATCCACTGCCTATACGGCAGTGAAC*
>REP_CLE6gRNA1	*TCGTCTCAGTCAGGACCATTGTTTTAGAGCTAGAAATAGC*
>CSY_CLE11gRNA1	*TCGTCTCCAAGTTTACGCGACTGCCTATACGGCAGTGAAC*
>REP_CLE11gRNA1	*TCGTCTCAACTTAGACCATGGTTTTAGAGCTAGAAATAGC*
>CSY_CLE11gRNA2	*TCGTCTCCAACCCTCATGGTCTGCCTATACGGCAGTGAAC*
>REP_CLE11gRNA2	*TCGTCTCAGGTTGCTCCACAGTTTTAGAGCTAGAAATAGC*
>CSY_CLE11gRNA3	*TCGTCTCCTAAACTTAGACCCTGCCTATACGGCAGTGAAC*
>REP_CLE11gRNA3	*TCGTCTCATTTACGCGAATTGTTTTAGAGCTAGAAATAGC*
> oCsy-TERM	*TGCTCTTCTGACCTGCCTATACGGCAGTGAAC*

Table 6. Primers (type IIS sites are highlighted in turquoise, gRNA spacer sequences in green and overhangs underlined)

gRNA spacer sequences of CRcle^{6,19}	
>CLE19gRNA1	*TGGATAGGGTCTGGGCCATT*
>CLE6gRNA1	*TGGATTGGGTCAGGACCATT*
>CLE19gRNA2	*AGGTTGTACAAGAGGTTATG*
>CLE6gRNA2	*TTGATTGAAGTACTTGAAAA*

Table 7. four guide RNA (gRNA) spacer sequences of *CRcle^{6,19}* in the Golden Gate cassette.

gRNA spacer sequences of CRcle^{6,19,30,31,34}	
>CLE19gRNA1	*TGGATAGGGTCTGGGCCATT*
>CLE6gRNA1	*TGGATTGGGTCAGGACCATT*
>CLE30 gRNA1	*GTGGACAGTGGCGAATTCTT*
>CLE31 gRNA1	*GAGTTGATAGCAGATTACAG*
>CLE34 gRNA1	*GACATGAGGAGAGTTCCTAC*

Table 8. five guide RNA (gRNA) spacer sequences of *CRcle^{6,19,30,31,34}* in the Golden Gate cassette.

gRNA spacer sequences of CRcle^{11}	
>CLE11gRNA1	*TCGCGTAAACTTAGACCATG*
>CLE11gRNA2	*ACCATGAGGGTTGCTCCACA*
>CLE11 gRNA3	*GGTCTAAGTTTACGCGAATT*

Table 9. five guide RNA (gRNA) spacer sequences of *CRcle^{11}* in the Golden Gate cassette.

3.3.3 Restriction enzymes for the digestion of *pDIRECT_22C*

We linearized the plasmid *pDIRECT _22C* with *BanI* enzyme in order to avoid the generation of an undesired amplicon in the promoter amplification step, due to the fact that the amplification of the promoter region with the non-digested plasmid involve the production of two PCR products.

This step was essential to be cut in one place and made a straight piece of DNA by adding enzyme *BanI* and rCutSmart™ buffer. The enzyme *BanI* cuts in a manner that produce

overhanged ends in the *pDIRECT_22C* sequence context. The recognition sequence was GGYRCC, where Y = C or T and R = A or G.

Incubation: For 1:30 hours, the plasmid was digested at 37°C. This was the ideal temperature for this enzyme, as its activity was typically temperature-dependent.

Verification: whole amounts of digested plasmid were loaded on the Agarose gel electrophoresis 1% to verify the correct digestion.

Compounds		**amount**
Buffer	rCutSmart™	1 µl
enzyme	BanI	1 µl
MIDI	pDIRECT_22C (60 ng/µL)	8 µl

Table 10. Restriction enzymes for the digestion of *pDIRECT_22C* (60 ng/µL)

3.3.4 Taq hifi polymerase for plasmid DNA

We set up a hifi-PCR reaction for every gRNA cassette. We utilized the designed primer combinations with the target cloning vector as a template BanI digested pDIRECT_22C for the promoter and non-digested pDIRECT_22C for the fragments.

The hifi-PCR Condition:

The hifi-PCR should be done in 25uL as described in the SuperFi protocol:

Primer forward (Rep) - 1,25 uL for each well

Primer reverse (Csy) - 1,25 uL for each well

Buffer - 5 uL

dNTPs - 0,5 uL

Taq Superfi - 0,25 uL

Autoclave MilliQ water - 16,25 uL

DNA - 0,5 uL (7,1 water + 0,9 pDirect_22C 5ng/uL)

The conditions of hifi-PCR are described in the below table:

Step	Temperature	Time	Cycles
Initial denaturation	98°C	1 min	1x
Denaturation	98°C	10 sec	30x
Annealing	60°C	15 sec	
Extension	72°C	15 sec	
Final Extension	72°C	2 mins	
Hold	4°C	∞ (infinity)	1x

Table 11. Taq hifi polymerase (hifi-PCR) condition

To verify the size and quality, we loaded 5 µl of each PCR product on the Agarose gel electrophoresis 1% to verify that the amplification was successful. The first product would be 524 bp and all the other products would be +/- 136 bp. After that, each PCR product was diluted 10 times to be used in the Golden Gate reaction.

3.3.5 Set up a Golden Gate reaction

Up to six fragments could be assembled in a pDIRECT_22C plasmid at once using Golden Gate cloning. The plasmid donor, the target cloning vector, the type IIS restriction enzymes, and ligase were pipetted into a single tube for cloning, and the mixture was then incubated in a heat cycler. The order of the reactions is described below:

a) mQ H2O up to 20 µl

b) 10 µl 2x T7 DNA ligase buffer

c) 1 µl T7 DNA ligase

d) 0.5 µl Esp3I

e) 0.5 µl SapI

f) 60 ng of the target vector

g) 0.5 µl of each 10 times diluted PCR product

All the reactions were pipette up and down multiple times to mix properly. Also, it was important, before adding the *SapI* enzyme to the reaction, it had to be combined by pipetting up and down multiple times.

We put the 20 µl of reactions in the PCR machine and proceed with the cycle: (37˚C/5min + 25˚C/10min) + 4°C hold 12 times.

3 µl of the Golden Gate reaction was transformed into *E. coli* (DH10β which was sensitive to the presence of the ccdB gene) by the electroporation method. This technique helped foreign plasmid DNA to enter electrocompetent cells by delivering an electric shock. An 'MicroPulser Electroporator' (BioRad) with a 25 µF capacity was used to apply an electric discharge of 2 kV after the Clonase reaction products were added to DH10β *E. coli* electrocompetent cells. Therefore, the transformed *E. coli* was added to 950 µl of LB medium directly to be incubated at 37˚C for 1 hour. Then 700 µl and 300 µl of incubated one, transformed to each plate on LB + 50 µg/ml of the kanamycin antibiotic and incubated at 37˚C for one night.

3.3.6 Inoculum Preparation

For selection, the transformed strains of E. coli were inoculated in Luria-Bertani (LB) in table broth supplemented with kanamycin (1:1000). A single colony of transformed E. coli was picked from the agar plate (LB + kanamycin) by using a sterile tip, and inoculated into 7 mL of the LB broth with kanamycin 50 µg/ml in a 15mL tube. We screened the colonies to recognize the correct clones. The culture was incubated at 37°C for 16–18 hours under agitation conditions. following the overnight incubation, we evaluated the culture growth and turbidity.

Compounds	Luria Bertani solid Medium (LB-Agar) for 1L	Luria Bertani Liquid Medium (LB) for 1L
NaCl	10 g	10 g
Yeast extract	5 g	5 g
Tryptone	10 g	10 g
Agar	15 g	-

Table 12. Bacterial media for E. coli growth. 1L medium was used in the recipes. Every medium was autoclaved according to normal protocol at 121°C before to use. these media were added with antibiotic: Kanamycin 50 ug/mL.

3.3.7 Mini-prep of preparation of plasmid DNA

3 mL of bacterial culture (LB with kanamycin) was added into a microcentrifuge tube and centrifuge for 3 minutes at 16,000 x g. The supernatant was discarded and 200 µL of cold P1 resuspension buffer (containing 100 µg/mL RNAse A, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0) was added to the pellet, and vortex until fully mixed. After that, 200 µL of P2 lysis buffer (0.2 M NaOH, 1% SDS) was added and the tube inverted 10 times gently to mix. After incubating the tube for 1 minute at the room temperature, 200 µL of cold P3 neutralization buffer (3.0 M potassium acetate, pH 5.5) was added. The tube was vortex briefly, then it was spin at 16,000 x g in a cooled centrifuge (4°C) for 5 minutes.

The clear supernatant was transferred to a new microcentrifuge tube. 600 µL of chloroform/3-methyl-1-butanol (24:1, v/v) was added and the mixture was vortex thoroughly and centrifuge for 5 minutes. We transferred the upper (aqueous) phase to another tube and 600 µL of chloroform/3-methyl-1-butanol (24:1, v/v) was added before vortex and centrifuge for 5 minutes. After centrifugation, the aqueous phase was removed and for the DNA precipitation, 1 mL of 100% ethanol was added. After that, the tube was placed in the freezer -80°C for 10 minutes.

For the step of DNA pellet, the tube was centrifuge at 16,000x g at 4°C for 15 minutes. the ethanol supernatant was discarded and 500 µL of 70% ethanol was added to wash the pellet. We did the centrifugation again at 16,000 x g at 4°C for 5 minutes. The ethanol wash was removed to allow the pellet to air dry. Finally, the DNA was re-suspended in 50 µL of molecular-grade water (mQ H2O) and stored at -20°C.

3.3.8 PCR screening of the cloning

PCR amplified DNA products were electrophoresed on 1% agarose gel. Electrophoresis unit filled with 1 X TAE buffer, gel along with casting tray was submerged in 1 X TAE buffer and comb was removed. Loading dye was added to each sample, mixed by pipetting up and down and loaded in every well without forming any bubbles. Agarose gel was loaded in following order: 1,500 bp ladder, PCR products of plasmid DNA, and in last well non template control (NTC), in that no DNA template was added. From the 20µl of PCR product, we loaded 10µl into each well and electrophoresed at 120 volts constant current till tracking dye reached the other end.

For screening of the cloning, 'PCRBIO *Taq DNA Polymerase* (PCRBIOSYSTEM) was used in the following reaction mix:

Forward Primer (10 µg/µL) (CmYLCY): 0,4 uL

Reverse Primer (10 µg/µL) (CsyTERM): 0,4 uL

PCR Buffer 10X: 2 uL

dNTPs (10 mM): 0,4 uL

PCRBIO Classic Taq (5 U/µL): 0,2 uL

DNA: 2 uL

mQ H2O to a total volume of 20 uL

Step	Temperature	Time	Cycles
Initial denaturation	95°C	2 min	1x
Denaturation	95°C	30 sec	40x
Annealing	60°C	30 sec	
Extension	72°C	1 min	
Final Extension	72°C	3 min	
Hold	4°C	∞ (infinity)	1x

Table 13. The condition of PCR for the cloned plasmid DNA

3.3.9 Sequencing of the cloned plasmid

To verify that the cloned plasmid was accurate the Sanger sequencing was used by BMR Genomics. to set up the sequencing reactions using plasmid-specific forward and reverse primers were applied. The final chromatograms were examined, and DNASTAR software was used to evaluated calling and sequence quality. We made a comparison between the obtained sequences and the reference sequence to be sure that the plasmid was correctly cloned.

promoter	**primer name**	**primer sequence**
CmYLCV	TC320	CTAGAAGTAGTCAAGGCGGC
	M13F	GTAAAACGACGGCCAGT

Table 14. Primers for colony PCR and sequencing of *pDIRECT_22C* plasmid (5' to 3')

Chapter 3

4. Results

4.1 Vector Design

The *pDIRECT_22C* vector enables multiplex CRISPR mutagenesis in plants through the expression of the Cas9 protein and a customizable array of multiple gRNAs (Čermák et al., 2017). It features a kanamycin resistance gene for selection and includes the ccdB gene within the cloning site. According to the material and method chapter in section 3.2.2 (Vector *pDIRECT_22C*, Addgene No. 91135), selecting transformed *Escherichia. coli* DH10β colonies on kanamycin-supplemented media is made possible by the *kanR* gene, which gives kanamycin resistance.

The identification of viable clones was made easier by the ability of successfully transformed *E. coli* bearing the *pDIRECT_22C* vector to survive on kanamycin. Also, the ccdB gene served as a negative selection signal in the original vector design since bacterial growth would be inhibited by its expression, as it produces a toxin that stops *E. coli* DH10β from growing. This gene was cleaved by the *SapI* enzyme during the Golden Gate Assembly procedure in order to allow the development of effectively modified bacterial cells containing the gRNA cassettes insert.

As promoter to drive the transcription of polycistronic gRNA sequences we used the CmYLCV promoter, already included inside the *pDIRECT_22C* vector, in order to ensure the effectiveness of gRNA expression in *S. lycopersicum*. This vector system was designed to carry out the knock out of the CLE genes in tomato plant by using CRISPR/Cas9.

This Cas9-sgRNA construct was first cloned and transformed into *E. coli* DH10β which was employed for multiplexing strategy using CRISPR/Cas9-mediated knockout of the PHs genes at three different chromosomal loci in *S. lycopersicum*. This arrangement made use of a

pDIRECT_22C vector system to deliver gRNAs that specifically targeted the loci, resulting in accurate modifications in the target sites.

4.2 Construction of the gRNAs

We set up multiple hifi-PCR reactions for all of the gRNA cassettes by using the primer combinations described in Table 15. As template we used the digested *pDIRECT_22C* for the promoter and non-digested *pDIRECT_22C* for the gRNA fragments as represented in the following table (CmYLCV as a promoter, Csy as a reverse primer and Rep as a forward primer).

The multiplexing strategy, recapitulated in table 15 shows a combination of the CLEs for simultaneous targeting; such as (CRISPRcle number 30,31 as $CRcle^{30,31}$), (CRISPRcle number 6,19 as $CRcle^{6,191}$), (CRISPRcle number 34 as $CRcle^{34}$) and (CRISPRcle number 6,19,30,31,34 as $CRcle^{6,19,30,31,34}$).

This multiplexing strategy was determined by reasons like phylogenetic proximity and functional similarity. Based on the (table 2) in chapter 2, both CLE30 and CLE31 genes are encoded on the same chromosome (number 5), with their loci at 5:5853919–5854191 and 5:5856133–5856446, respectively. By reducing the possibility of ineffective multiplexing brought on by positional effects, targeting genes that are physically adjacent to each other in the genome would make cloning and editing procedures easier.

Additionally, due to the functional resemblance within the CLEs gene family, the encoded peptides exhibit similar sequences and potential similar activities. This indicates they might have complementary roles in the flowering process. So, by knocking out these genes all at once, it would be possible to examine their combined impact and functions.

Title: **Example**	**CLE30** **CLE31** **gRNA,** ***CRcle*^{30,31}**	**CLE34** **gRNA,** ***CRcle*^{34}**	***CLE19*** **CLE6** **gRNA,** ***CRcle*^{6,19}**	**CLE19** **CLE6** **CLE30** **CLE31** **CLE34** **gRNA,** ***CRcle*^{6,19,30,31,34}**	**CLE11** **gRNA,** ***CRcle*^{11}**
PCR1 >CmYLCV >csy-A (digested plasmid)	CmYLCV Csy 30.1	CmYLCV Csy 34.1	CmYLCV Csy 19.1	CmYLCV Csy 19.1	CmYLCV Csy11.1
PCR2 >rep-A >csy-B (non-digested plasmid)	Rep 30.1 Csy 30.2	Rep 34.1 Csy 34.2	Rep 19.1 Csy 6.1	Rep 19.1 Csy 6.1	Rep11.1 Csy11.2
PCR3 >rep-B >csy-C (non-digested plasmid)	Rep 30.2 Csy 31.1	Rep 34.2 Csy TERM	Rep 6.1 Csy 19.2	Rep 6.1 Csy 30.1	Rep11.2 Csy11.3
PCR4 >rep-C >csy-D (non-digested plasmid)	Rep 31.1 Csy 31.2		Rep 19.2 Csy 6.2	Rep 30.1 Csy 31.1	Rep11.3 Csy TERM
PCR5 >rep-D >csy-E (non-digested plasmid)	Rep 31.2 Csy TERM		Rep 6.2 Csy TERM	Rep 31.1 Csy 34.1	
PCR6 >rep-E >csy-F (non-digested plasmid)				Rep 34.1 Csy TERM	

Table 15. Crisper Golden Gate for *CRcle*^{30,31}, *CRcle*^{34}, *CRcle*^{6,19}, *CRcle*^{6,19,30,31,34}, *CRcle*^{11} gRNAs with their designed primers.

4.2.1 Expected DNA sequence of the gRNA cassette for *CRcle*[30,31]

In the following sequences, that are the results of the in silico design of the different gRNAs for each gene target, it is possible to recognize the type IIS restriction sites highlighted in turquoise, CmYLCV promoter in yellow, Csy4 sites in pink, gRNA spacer sequences in green, gRNA repeat sequences in grey and type IIs overhangs underlined:

>reaction#1
TGCTCTTCGCGCTGGCAGACATACTGTCCCACAAATGAAGATGGAATCTGTAAAAGAAAACGCGTGAAATAATGCGTCTGAC
AAAGGTTAGGTCGGCTGCCTTTAATCAATACCAAAGTGGTCCCTACCACGATGGAAAAACTGTGCAGTCGGTTTGGCTTTTTC
TGACGAACAAATAAGATTCGTGGCCGACAGGTGGGGGTCCACCATGTGAAGGCATCTTCAGACTCCAATAATGGAGCAATG
ACGTAAGGGCTTACGAAATAAGTAAGGGTAGTTTGGGAAATGTCCACTCACCCGTCAGTCTATAAATACTTAGCCCCTCCCT
CATTGTTAAGGGAGCAAAATCTCAGAGAGATAGTCCTAGAGAGAGAAAGAGAGCAAGTAGCCTAGAAGTAGTCAAGGCGGC
GAAGTATTCAGGCACGTGGCCAGGAAGAAGAAAAGCCAAGACGACGAAAACAGGTAAGAGCTAAGCTTCCTGCAGGTTCAC
TGCCGTATAGGCAGGAGAAAAGATCAGGAGACGA

>reaction#2
TCGTCTCAATCAATCCCTTCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGC
ACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGATGACGGAGGCTGAGACGA

>reaction#3
TCGTCTCAAGGCGGTAGAAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGTGGACAGTGGCTGAGACGA

>reaction#4
TCGTCTCATGGCGAATTCTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGAGTTGATAGCATGAGACGA

>reaction#5
TCGTCTCAAGCAGATTACAGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGTCAGAGACGA

4.2.2 Expected DNA sequence of the gRNA cassette for *CRcle*[34]

>reaction#1
TGCTCTTCGCGCTGGCAGACATACTGTCCCACAAATGAAGATGGAATCTGTAAAAGAAAACGCGTGAAATAATGCGTCTGAC
AAAGGTTAGGTCGGCTGCCTTTAATCAATACCAAAGTGGTCCCTACCACGATGGAAAAACTGTGCAGTCGGTTTGGCTTTTTC
TGACGAACAAATAAGATTCGTGGCCGACAGGTGGGGGTCCACCATGTGAAGGCATCTTCAGACTCCAATAATGGAGCAATG
ACGTAAGGGCTTACGAAATAAGTAAGGGTAGTTTGGGAAATGTCCACTCACCCGTCAGTCTATAAATACTTAGCCCCTCCCT
CATTGTTAAGGGAGCAAAATCTCAGAGAGATAGTCCTAGAGAGAGAAAGAGAGCAAGTAGCCTAGAAGTAGTCAAGGCGGC
GAAGTATTCAGGCACGTGGCCAGGAAGAAGAAAAGCCAAGACGACGAAAACAGGTAAGAGCTAAGCTTCCTGCAGGTTCAC
TGCCGTATAGGCAGCGCCATCCGGCAGGAGACGA

>reaction#2
TCGTCTCAGGCAACCACCACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGACATGAGGAGATGAGACGA

>reaction#3

TCGTCTCAGAGAGTTCCTACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGTCAGAGACGA

4.2.3 Expected DNA sequence of the gRNA cassette for *CRcle*$^{6.19}$

>reaction#1

TGCTCTTCGCGCTGGCAGACATACTGTCCCACAAATGAAGATGGAATCTGTAAAAGAAAACGCGTGAAATAATGCGTCTGAC
AAAGGTTAGGTCGGCTGCCTTTAATCAATACCAAAGTGGTCCCTACCACGATGGAAAAACTGTGCAGTCGGTTTGGCTTTTTC
TGACGAACAAATAAGATTCGTGGCCGACAGGTGGGGGTCCACCATGTGAAGGCATCTTCAGACTCCAATAATGGAGCAATG
ACGTAAGGGCTTACGAAATAAGTAAGGGTAGTTTGGGAAATGTCCACTCACCCGTCAGTCTATAAATACTTAGCCCCTCCCT
CATTGTTAAGGGAGCAAAATCTCAGAGAGATAGTCCTAGAGAGAGAAAGAGAGCAAGTAGCCTAGAAGTAGTCAAGGCGGC
GAAGTATTCAGGCACGTGGCCAGGAAGAAGAAAAGCCAAGACGACGAAAACAGGTAAGAGCTAAGCTTCCTGCAGGTTCAC
TGCCGTATAGGCAGTGGATAGGGTCTGGAGACGA

>reaction#2

TCGTCTCAGTCTGGGCCATTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGTGGATTGGGTCATGAGACGA

>reaction#3

TCGTCTCAGTCAGGACCATTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGAGGTTGTACAAGTGAGACGA

>reaction#4

TCGTCTCACAAGAGGTTATGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGTTGATTGAAGTATGAGACGA

>reaction#5

TCGTCTCAAGTACTTGAAAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGTCAGAGACGA

4.2.4 Expected DNA sequence of the gRNA cassette for *CRcle*^{6,9,30,31,34}

>reaction#1

TGCTCTTCGCGCTGGCAGACATACTGTCCCACAAATGAAGATGGAATCTGTAAAAGAAAACGCGTGAAATAATGCGTCTGAC
AAAGGTTAGGTCGGCTGCCTTTAATCAATACCAAAGTGGTCCCTACCACGATGGAAAAACTGTGCAGTCGGTTTGGCTTTTTC
TGACGAACAAATAAGATTCGTGGCCGACAGGTGGGGGTCCACCATGTGAAGGCATCTTCAGACTCCAATAATGGAGCAATG
ACGTAAGGGCTTACGAAATAAGTAAGGGTAGTTTGGGAAATGTCCACTCACCCGTCAGTCTATAAATACTTAGCCCCTCCCT
CATTGTTAAGGGAGCAAAATCTCAGAGAGATAGTCCTAGAGAGAGAAAGAGAGCAAGTAGCCTAGAAGTAGTCAAGGCGGC
GAAGTATTCAGGCACGTGGCCAGGAAGAAGAAAAGCCAAGACGACGAAAACAGGTAAGAGCTAAGCTTCCTGCAGGTTCAC
TGCCGTATAGGCAGTGGATAGGGTCTGGAGACGA

>reaction#2

TCGTCTCAGTCTGGGCCATTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGTGGATTGGGTCATGAGACGA

>reaction#3

TCGTCTCAGTCAGGACCATTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGTGGACAGTGGCTGAGACGA

>reaction#4

TCGTCTCATGGCGAATTCTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGAGTTGATAGCATGAGACGA

>reaction#5

TCGTCTCAAGCAGATTACAGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGACATGAGGAGATGAGACGA

>reaction #6

TCGTCTCAGAGAGTTCCTACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGTCAGAGACGA

4.2.5 Expected DNA sequence of the gRNA cassette for *CRcle*[11]

>reaction#1

TGCTCTTCGCGCTGGCAGACATACTGTCCCACAAATGAAGATGGAATCTGTAAAAGAAAACGCGTGAAATAATGCGTCTGAC
AAAGGTTAGGTCGGCTGCCTTTAATCAATACCAAAGTGGTCCCTACCACGATGGAAAAACTGTGCAGTCGGTTTGGCTTTTTC
TGACGAACAAATAAGATTCGTGGCCGACAGGTGGGGGTCCACCATGTGAAGGCATCTTCAGACTCCAATAATGGAGCAATG
ACGTAAGGGCTTACGAAATAAGTAAGGGTAGTTTGGGAAATGTCCACTCACCCGTCAGTCTATAAATACTTAGCCCCTCCCT
CATTGTTAAGGGAGCAAAATCTCAGAGAGATAGTCCTAGAGAGAGAAAGAGAGCAAGTAGCCTAGAAGTAGTCAAGGCGGC
GAAGTATTCAGGCACGTGGCCAGGAAGAAGAAAAGCCAAGACGACGAAAACAGGTAAGAGCTAAGCTTCCTGCAGGTTCAC
TGCCGTATAGGCAGAAGTTTACGCGAGGAGACGA

>reaction#2

TCGTCTCAACTTAGACCATGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGAACCCTCATGGTTTGAGACGA

>reaction#3

TCGTCTCAGGTTGCTCCACAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGTAAACTTAGACCTGAGACGA

>reaction#4

TCGTCTCATTTACGCGAATTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGTCAGAGACGA

4.3 Validation of resulting gRNA cassettes by PCR

To confirm the successful assembly and accuracy of gRNA cassettes into the *pDIRECT_22C* plasmid, PCR amplifications were conducted by using Taq HiFi DNA polymerase. In order to confirm the presence of guide RNA (gRNA) sequences intended to target particular loci within the CLE genes, which were essential for the multiplex editing strategy, PCR amplification produced a distinct band on the Agarose Gel Electrophoresis that matched the anticipated size of the target area.

4.3.1 PCR-mediated synthesis of gRNAs for *CRcle*^{30,31} and *CRcle*^{34} genes (Digested and non-Digested pDIRECT_22C)

The results of hifi-PCR are explained as:

A) For the Digested *pDIRECT_22C* Plasmids (*CRcle*^{30,31} and *CRcle*^{34} genes): Since the expected size for the digested plasmid products were 524 bp, the hifi-PCR reactions correctly amplified the target regions as anticipated and the plasmids were properly digested (see Figure 9).

B) For the non-digested *pDIRECT_22C* Plasmids (*CRcle*^{30,31} and *CRcle*^{34} genes): The 136 bp bands for all the *CRcle*^{30,31} and *CRcle*^{34} genes indicated that all the non-digested plasmids' target area was successfully amplified. The band size shows that the sequences were correctly amplified by the hifi-PCR. Moreover, the 127 bp bands for *CRcle*^{30,31} and *CRcle*^{34} genes with the primers (Rep 31.2+ Csy TERM) and (Rep 34.2+ Csy TERM) show the terminator sequences were also amplified correctly.

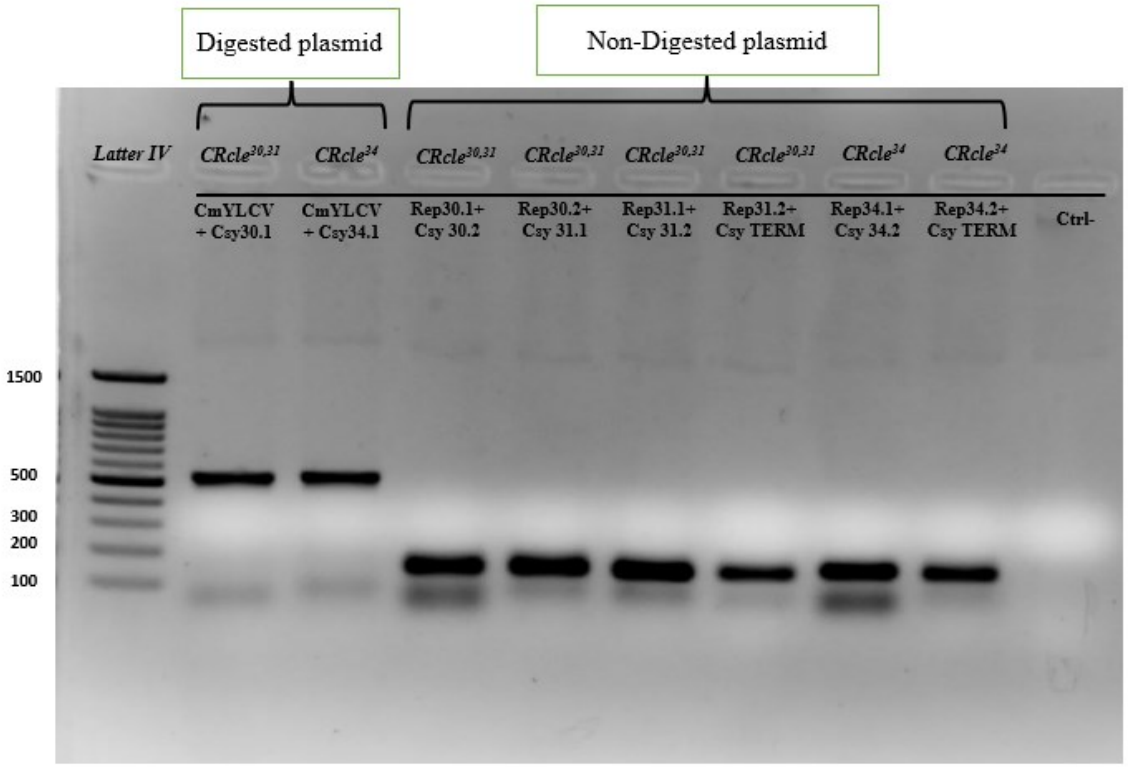


Fig.9. Gel Electrophoresis of PCR products for *CRcle*^{30,31} and *CRcle*^{34} genes with their specific primers (Digested and Non-Digested *pDIRECT_22C* plasmid)

4.3.2 PCR-mediated synthesis for *CRcle*^{6,19} and *CRcle*^{6,19,30,31,34} genes (Digested and non-Digested pDIRECT_22C)

The following is an explanation of the hifi-PCR results:

A) For the Digested *pDIRECT_22C* Plasmids of *CRcle*^{6,19} and *CRcle*^{6,19,30,31,34}: The hifi-PCR amplified the target regions as expected, and the plasmids were digested appropriately, since the expected size for the digested plasmid product were 524 bp.

B) For the non-digested *pDIRECT_22C* Plasmids of *CRcle*^{6,19} and *CRcle*^{6,19,30,31,34}: The 136 bp bands in the non-digested plasmids in Figure 10 and Figure 11 showed that, the target regions had been amplified successfully. Furthermore, the terminator sequences were amplified appropriately, as seen by the 127 bp bands in *CRcle*^{6,19} and

CRcle^{6,19,30,31,34} genes with the primers (Rep 6.2+ Csy TERM) and (Rep 34.1, Csy TERM) respectively.

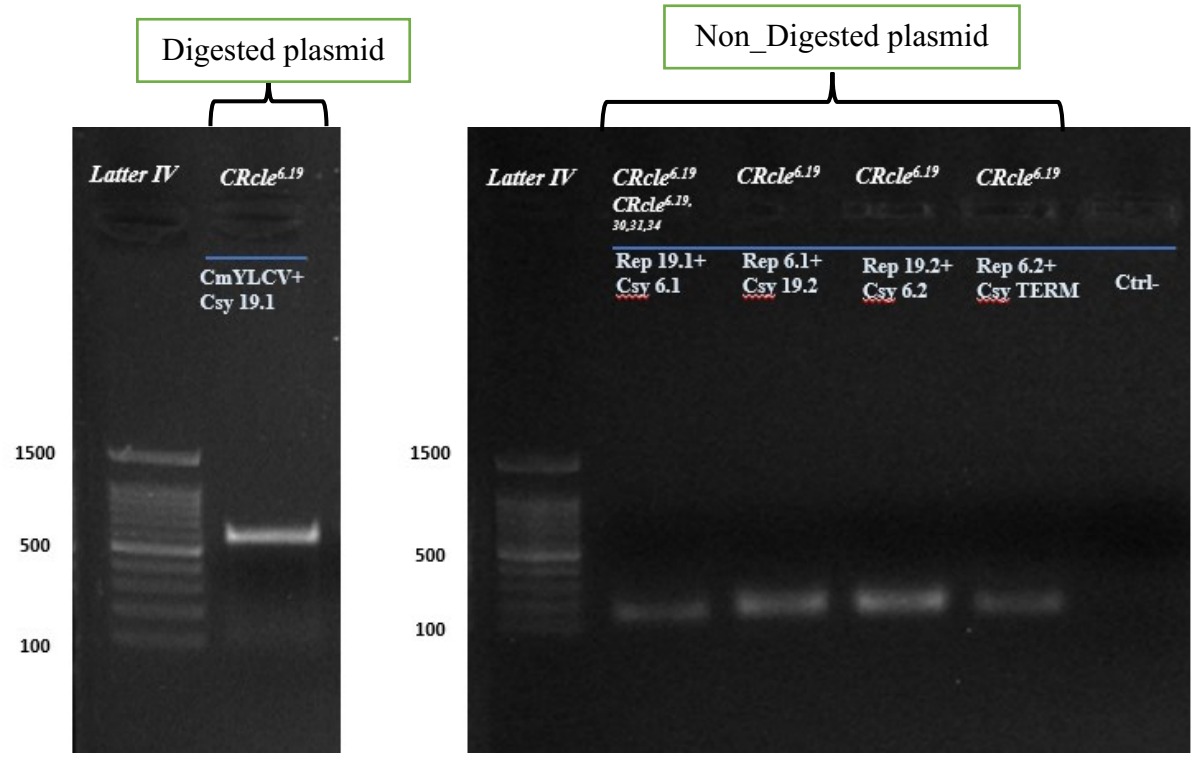


Fig.10. Gel Electrophoresis of PCR products for *CRcle*$^{6.19}$ genes with their specific primers (Digested and Non-Digested *pDIRECT_22C* plasmid)

The primers (Rep 19.1, Csy 6.1) used for the non-digested *pDIRECT_22C* plasmid of *CRcle*$^{6.19}$ and *CRcle*$^{6.19,\ 30,31,34}$ genes which is shown in fig10.

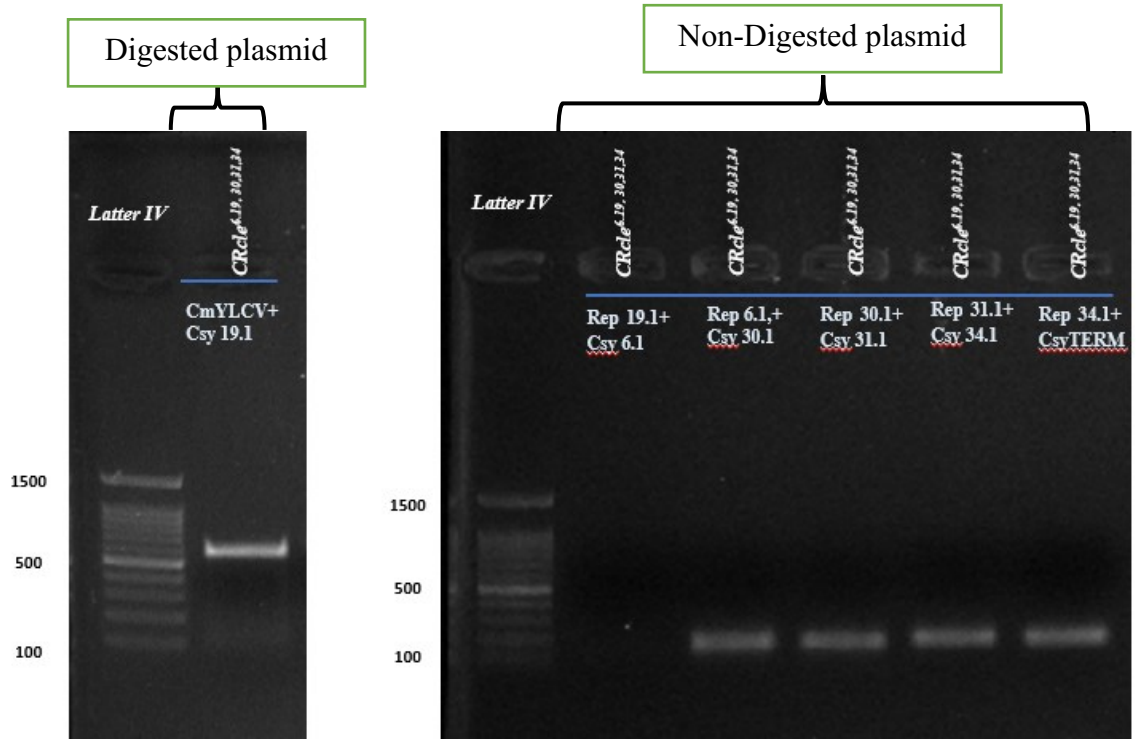


Fig.11. Gel Electrophoresis of PCR products for *CRcle*^{6,19,30,31,34} genes with their specific primers (Digested and Non-Digested *pDIRECT_22C* plasmid)

4.3.3 PCR-mediated synthesis for *CRcle*^{11} gene (Digested and non-Digested pDIRECT_22C)

The expected size for the digested plasmid *pDIRECT_22C* plasmid of *CRcle*^{11} with the primers (CmYLCV, Csy11.1) was 524 bp, which illustrates the hifi-PCR amplified was successful and the plasmid was properly digested. Based on the material and method chapter in section 2.4.4 (Taq hifi polymerase for plasmid DNA), the hifi-PCR products for the the non-digested *pDIRECT_22C* Plasmid of *CRcle*^{11} should be 136 bp and for the terminator sequence of *CRcle*^{11} with the primers (Rep11.3, Csy TERM) would be 127 bp.

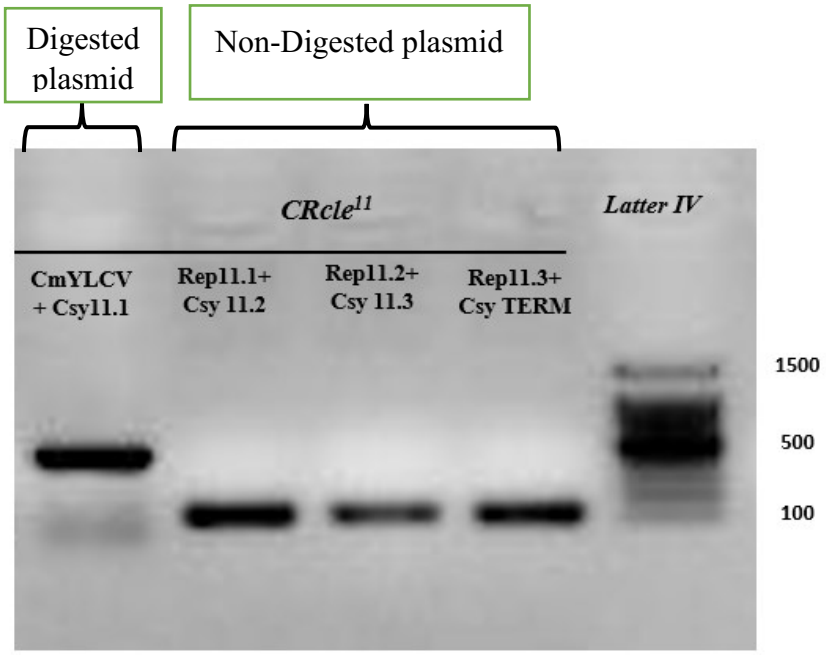


Fig.12. Gel Electrophoresis of PCR products for *CRcle*[11] genes with their specific primers (Digested and Non-Digested *pDIRECT_22C* plasmid)

4.4 Assembly of the final vector

The PCR products obtained as described above included the processing elements, gRNA repeats, and the fragments of target-specific gRNA spacers that provided distinct sequence stretches for designing the Golden Gate junctions. Based on the part 2.4 (Golden Gate Assembly), to assemble the final vector by the Golden Gate reaction, the cloning of gRNA spacers into Csy4 array controlled by an RNA Pol II promoter in *pDIRECT_22C* vector was needed. T7 DNA ligase and two type restriction enzymes (*Esp3I and SapI*) were utilized in Golden Gate assembly to integrate the components of gRNA spacers into the *pDIRECT_22C* vector.

4.4.1 Expected DNA sequence of the complete gRNA array of *CRcle*[30,31] assembled into *pDIRECT_22C* plasmid

In the following sequences it is feasible to recognize the CmYLCV promoter highlighted in yellow, Csy4 sites in pink, gRNA spacer sequences in green, gRNA repeat sequences in grey and 35S terminator in the plasmid backbone in red:

TGGCAGACATACTGTCCCACAAATGAAGATGGAATCTGTAAAAGAAAACGCGTGAAATAATGCGTCTGACAAAGGTTAGGT
CGGCTGCCTTTAATCAATACCAAAGTGGTCCCTACCACGATGGAAAAACTGTGCAGTCGGTTTGGCTTTTTCTGACGAACAAA
TAAGATTCGTGGCCGACAGGTGGGGGTCCACCATGTGAAGGCATCTTCAGACTCCAATAATGGAGCAATGACGTAAGGGCTT
ACGAAATAAGTAAGGGTAGTTTGGGAAATGTCCACTCACCCGTCAGTCTATAAATACTTAGCCCCTCCCTCATTGTTAAGGG
AGCAAAATCTCAGAGAGATAGTCCTAGAGAGAGAAAGAGAGCAAGTAGCCTAGAAGTAGTCAAGGCGGCGAAGTATTCAG
GCACGTGGCCAGGAAGAAGAAAAGCCAAGACGACGAAAACAGGTAAGAGCTAAGCTTCCTGCAGGTTCACTGCCGTATAGG
CAGGAGAAAAGATCAATCCCTTCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAG
TGGCACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGATGACGGAGGCGGTAGAAAGTTTTAGAGCTAGAAATAGCAAGT
TAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGTGGACAGTG
GCGAATTCTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCG
GTGCGTTCACTGCCGTATAGGCAGGAGTTGATAGCAGATTACAGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAG
TCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGTCGATCGACAAGCTCGAGTTTCT
CCATAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCCTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAA
CCCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAAATCCAGTACTAAAATCC
AGATCCCCCGAATTA

4.4.2 Expected DNA sequence of the complete gRNA array of *CRcle*^{34} assembled into *pDIRECT_22C* plasmid

TGGCAGACATACTGTCCCACAAATGAAGATGGAATCTGTAAAAGAAAACGCGTGAAATAATGCGTCTGACAAAGGTTAGGT
CGGCTGCCTTTAATCAATACCAAAGTGGTCCCTACCACGATGGAAAAACTGTGCAGTCGGTTTGGCTTTTTCTGACGAACAAA
TAAGATTCGTGGCCGACAGGTGGGGGTCCACCATGTGAAGGCATCTTCAGACTCCAATAATGGAGCAATGACGTAAGGGCTT
ACGAAATAAGTAAGGGTAGTTTGGGAAATGTCCACTCACCCGTCAGTCTATAAATACTTAGCCCCTCCCTCATTGTTAAGGG
AGCAAAATCTCAGAGAGATAGTCCTAGAGAGAGAAAGAGAGCAAGTAGCCTAGAAGTAGTCAAGGCGGCGAAGTATTCAG
GCACGTGGCCAGGAAGAAGAAAAGCCAAGACGACGAAAACAGGTAAGAGCTAAGCTTCCTGCAGGTTCACTGCCGTATAGG
CAGCGCCATCCGGCAACCACCACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAG
TGGCACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGACATGAGGAGAGTTCCTACGTTTTAGAGCTAGAAATAGCAAGTT
AAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGTCGATCGACA
AGCTCGAGTTTCTCCATAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCCTATAGGGTTTCGCTCATGTGTTGA
GCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAAATCCA
GTACTAAAATCCAGATCCCCCGAATTA

4.4.3 Expected DNA sequence of the complete gRNA array of *CRcle*^{6,19} assembled into *pDIRECT_22C* plasmid

TGGCAGACATACTGTCCCACAAATGAAGATGGAATCTGTAAAAGAAAACGCGTGAAATAATGCGTCTGACAAAGGTTAGGT
CGGCTGCCTTTAATCAATACCAAAGTGGTCCCTACCACGATGGAAAAACTGTGCAGTCGGTTTGGCTTTTTCTGACGAACAAA
TAAGATTCGTGGCCGACAGGTGGGGGTCCACCATGTGAAGGCATCTTCAGACTCCAATAATGGAGCAATGACGTAAGGGCTT
ACGAAATAAGTAAGGGTAGTTTGGGAAATGTCCACTCACCCGTCAGTCTATAAATACTTAGCCCCTCCCTCATTGTTAAGGG
AGCAAAATCTCAGAGAGATAGTCCTAGAGAGAGAAAGAGAGCAAGTAGCCTAGAAGTAGTCAAGGCGGCGAAGTATTCAG
GCACGTGGCCAGGAAGAAGAAAAGCCAAGACGACGAAAACAGGTAAGAGCTAAGCTTCCTGCAGGTTCACTGCCGTATAGG
CAGTGGATAGGGTCTGGGCCATTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAG
TGGCACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGTGGATTGGGTCAGGACCATTGTTTTAGAGCTAGAAATAGCAAGTT
AAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGAGGTTGTACAA
GAGGTTATGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGG
TGCGTTCACTGCCGTATAGGCAGTTGATTGAAGTACTTGAAAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGT
CCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGTCGATCGACAAGCTCGAGTTTCTC
CATAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCCTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAAC
CCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAAATCCAGTACTAAAATCCA
GATCCCCCGAATTA

4.4.4 Expected DNA sequence of the complete gRNA array of *CRcle*^{6,19,30,31,34} assembled into *pDIRECT_22C* plasmid

TGGCAGACATACTGTCCCACAAATGAAGATGGAATCTGTAAAAGAAAACGCGTGAAATAATGCGTCTGACAAAGGTTAGGT
CGGCTGCCTTTAATCAATACCAAAGTGGTCCCTACCACGATGGAAAAACTGTGCAGTCGGTTTGGCTTTTTCTGACGAACAAA
TAAGATTCGTGGCCGACAGGTGGGGGTCCACCATGTGAAGGCATCTTCAGACTCCAATAATGGAGCAATGACGTAAGGGCTT
ACGAAATAAGTAAGGGTAGTTTGGGAAATGTCCACTCACCCGTCAGTCTATAAATACTTAGCCCCTCCCTCATTGTTAAGGG
AGCAAAATCTCAGAGAGATAGTCCTAGAGAGAGAAAGAGAGCAAGTAGCCTAGAAGTAGTCAAGGCGGCGAAGTATTCAG
GCACGTGGCCAGGAAGAAGAAAAGCCAAGACGACGAAAACAGGTAAGAGCTAAGCTTCCTGCAGGTTCACTGCCGTATAGG
CAGTGGATAGGGTCTGGGCCATTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAG
TGGCACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGTGGATTGGGTCAGGACCATTGTTTTAGAGCTAGAAATAGCAAGTT
AAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGTGGACAGTGG
CGAATTCTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGG
TGCGTTCACTGCCGTATAGGCAGGAGTTGATAGCAGATTACAGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGT
CCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGACATGAGGAGAGTTCCTACGTTTT
AGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGTTCACTGCCG
TATAGGCAGGTCGATCGACAAGCTCGAGTTTCTCCATAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCCTAT
AGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTA
ATTCCTAAAACCAAAATCCAGTACTAAAATCCAGATCCCCCGAATTA

4.4.5 Expected DNA sequence of the complete gRNA array of *CRcle*^{11} assembled into *pDIRECT_22C* plasmid

TGGCAGACATACTGTCCCACAAATGAAGATGGAATCTGTAAAAGAAAACGCGTGAAATAATGCGTCTGACAAAGGTTAGGT
CGGCTGCCTTTAATCAATACCAAAGTGGTCCCTACCACGATGGAAAAACTGTGCAGTCGGTTTGGCTTTTTCTGACGAACAAA
TAAGATTCGTGGCCGACAGGTGGGGGTCCACCATGTGAAGGCATCTTCAGACTCCAATAATGGAGCAATGACGTAAGGGCTT
ACGAAATAAGTAAGGGTAGTTTGGGAAATGTCCACTCACCCGTCAGTCTATAAATACTTAGCCCCTCCCTCATTGTTAAGGG
AGCAAAATCTCAGAGAGATAGTCCTAGAGAGAGAAAGAGAGCAAGTAGCCTAGAAGTAGTCAAGGCGGCGAAGTATTCAG
GCACGTGGCCAGGAAGAAGAAAAGCCAAGACGACGAAAACAGGTAAGAGCTAAGCTTCCTGCAGGTTCACTGCCGTATAGG
CAGTCGCGTAAACTTAGACCATGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAG
TGGCACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGACCATGAGGGTTGCTCCACAGTTTTAGAGCTAGAAATAGCAAGTT
AAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGGTCTAAGTTT
ACGCGAATTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGG
TGCGTTCACTGCCGTATAGGCAGGTCGATCGACAAGCTCGAGTTTCTCCATAATAATGTGTGAGTAGTTCCCAGATAAGGGA
ATTAGGGTTCCTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTAT
CAATAAAATTTCTAATTCCTAAAACCAAAATCCAGTACTAAAATCCAGATCCCCCGAATTA

4.5 Results for the cloning of *CRcle*^{6,19}, *CRcle*^{30,31}, *CRcle*^{34} and *CRcle*^{6,19,30,31,34} genes

To confirm that the target genes had been successfully cloned, PCR screening was carried out after the preparation of plasmid DNA. CmYLCV and CsyTERM were used as the forward and reverse primers respectively, Agarose Gel Electrophoresis was used to evaluate the PCR results. Unfortunately, no amplification bands matching the anticipated insert size were seen, from which we concluded that, the cloning attempts for *CRcle*^{6,19}, *CRcle*^{30,31}, *CRcle*^{34} and *CRcle*^{6,19,30,31,34} were unsuccessful (figure 13a and 13b).

The expected number of bands on the 1% Agarose Gel Electrophoresis is described below:

A) Targeting *CRcle*^{6,19} and *CRcle*^{30,31} genes five bands expected;
B) Targeting *CRcle*^{34} gene three bands expected;
C) Targeting *CRcle*^{6,19,30,31,34} genes six bands expected.

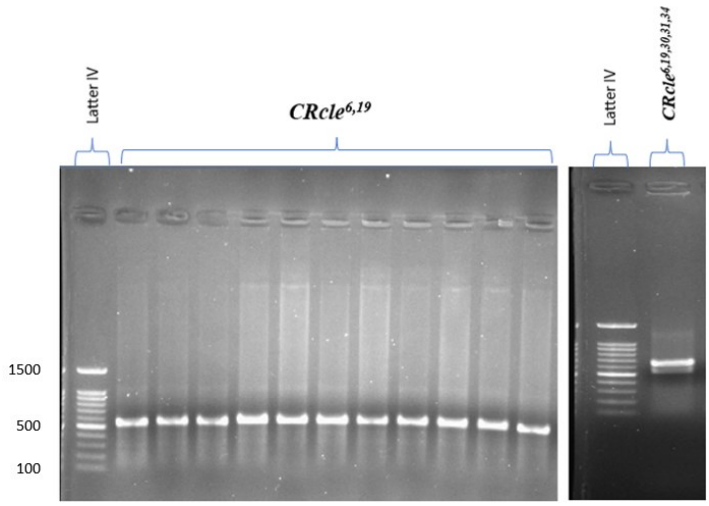


Fig.13a. Results for the cloning of *CRcle*^{6,19} and *CRcle*^{6,19,30,31,34}. In the first case 5 bands were expected, while in the latter six.

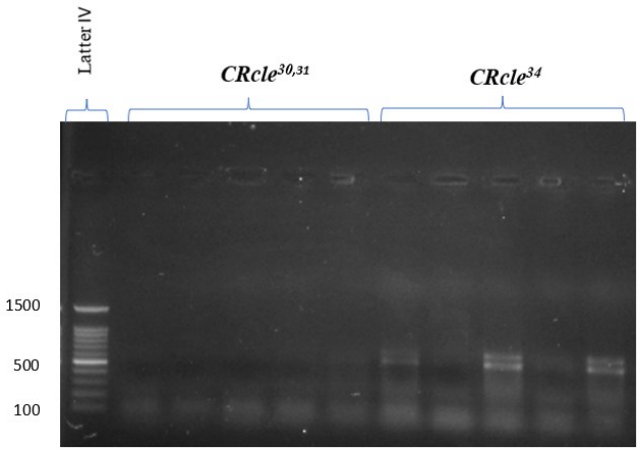


Fig.13b. Results for the cloning of *CRcle*^{30,31} and *CRcle*^{34}. In the first case 5 bands were expected, while in the latter three.

4.6 Result for the cloning of *CRcle*^{11} gene

The cloning of *CRcle*^{11} gene was verified by PCR screening after preparation of plasmid DNA. CsyTERM as the reverse primer and CmYLCV as the forward primer were used to amplify the target regions. Agarose Gel Electrophoresis was used to examine the resultant PCR products. As a result, four distinct bands from 500 bp to 800 bp within the expected size range were seen, signifying that *CRcle*^{11} was cloned succesfully and sequences were correctly assembled to the *pDIRECT_22C* vector during the cloning procedure because it matches the expected fragment sizes (Figure 14).

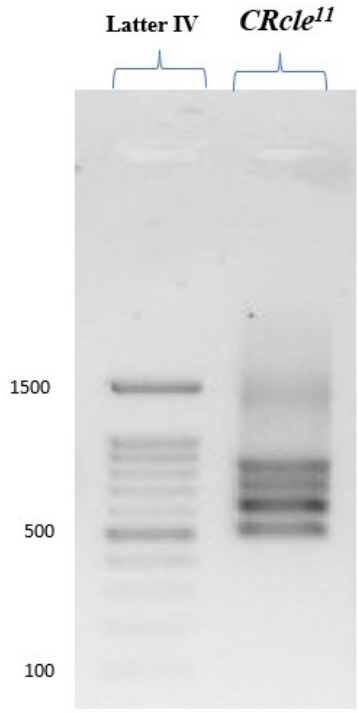


Fig.14. Result for the cloning of *CRcle*^{11}. In this case the 4 observed bands matched the expected ones.

The *CRcle*^{11} sample was sent for sequencing to confirm the correctness of the cloned sequences. The cloned vector would be used to transform into *S. lycopersicum* by Agrobacterium tumefaciens to incorporate the CRISPR/Cas9-designed constructs into the plant genome after sequencing confirmation.

4.7 Validation of the *CRcle*^{11} construct by DNA Sanger Sequencing

The sequencing proceeded exactly as planned and fit our predicted structure perfectly (Figure 15). Despite the inherent ambiguity of the first bases, the chromatogram of Sanger sequencing revealed an almost perfect matching within the designed sequence. The chromatogram's highlighted focus segment was especially important since it coincides with the gRNA insertion location, where the fragments ligated during the complex Golden Gate Assembly process. The assembled of two gRNA pieces highlighted the accuracy and efficacy of the Golden Gate reaction in accomplishing precise molecular fusion.

Score	Expect	Identities	Gaps	Strand
1110 bits(601)	0.0	624/634(98%)	6/634(0%)	Plus/Plus

```
Query  10    CGTGGCC-GG-AG-AG-AAAGCC-AGACGATCATAATCAGGTAAGCGCTAAGCTTCCTGC  64
             ||||||| || || || |||||| |||||| | || |||||||| ||||||||||||||
Sbjct  412   CGTGGCCAGGAAGAAGAAAAGCCAAGACGACGA-AAACAGGTAAGAGCTAAGCTTCCTGC  470

Query  65    AGGTTCACTGCCGTATAGGCAGTCGCGTAAACTTAGACCATGGTTTTAGAGCTAGAAATA  124
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  471   AGGTTCACTGCCGTATAGGCAGTCGCGTAAACTTAGACCATGGTTTTAGAGCTAGAAATA  530

Query  125   GCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGT  184
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  531   GCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGT  590

Query  185   TCACTGCCGTATAGGCAGACCATGAGGGTTGCTCCACAGTTTTAGAGCTAGAAATAGCAA  244
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  591   TCACTGCCGTATAGGCAGACCATGAGGGTTGCTCCACAGTTTTAGAGCTAGAAATAGCAA  650

Query  245   GTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGTTCAC  304
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  651   GTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGTTCAC  710

Query  305   TGCCGTATAGGCAGGGTCTAAGTTTACGCGAATTGTTTTAGAGCTAGAAATAGCAAGTTA  364
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  711   TGCCGTATAGGCAGGGTCTAAGTTTACGCGAATTGTTTTAGAGCTAGAAATAGCAAGTTA  770

Query  365   AAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGTTCACTGCC  424
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  771   AAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGTTCACTGCC  830

Query  425   GTATAGGCAGGTCGATCGACAAGCTCGAGTTTCTCCATAATAATGTGTGAGTAGTTCCCA  484
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  831   GTATAGGCAGGTCGATCGACAAGCTCGAGTTTCTCCATAATAATGTGTGAGTAGTTCCCA  890

Query  485   GATAAGGGAATTAGGGTTCCTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCT  544
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  891   GATAAGGGAATTAGGGTTCCTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCT  950

Query  545   TAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATTCCTAAAACCA  604
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  951   TAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATTCCTAAAACCA  1010

Query  605   AAATCCAGTACTAAAATCCAGATCCCCCGAATTA  638
             |||||||||||||||||||||||||||||||||
Sbjct  1011  AAATCCAGTACTAAAATCCAGATCCCCCGAATTA  1044
```

Fig.15. Alignment of DNA sequences obtained after sequencing of the *CRcle*^{11} construct ("*Query*") with that of the designed one ("*Sbjct*").

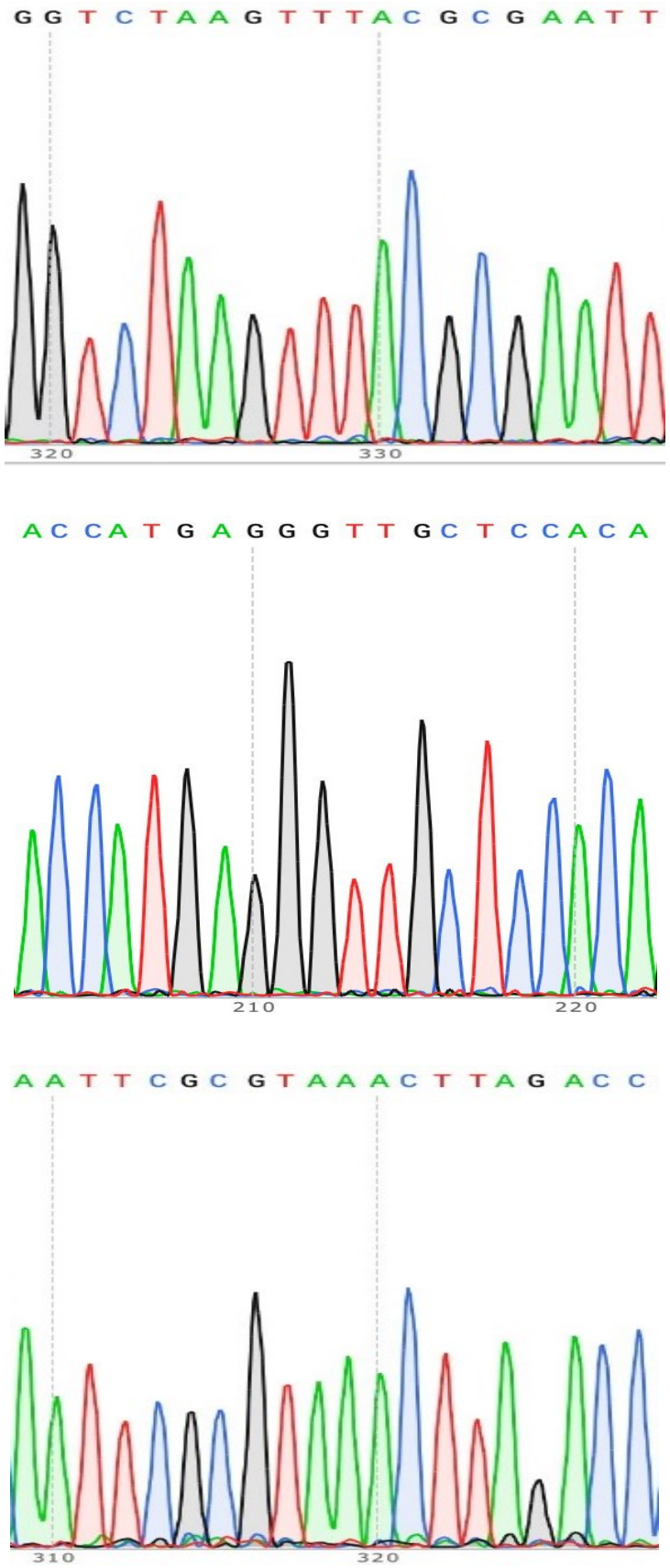


Fig.16. The sequencing chromatograms of gRNAs in *CRcle*^{11}

5. DISCUSSION

The CRISPR/Cas9 multiplexing experiment aimed to build the genetic constructs to knock out several CLE genes involved in flowering and ripening processes in *S. lycopersicum*. These included *CLE6, CLE19, CLE30, CLE31, CLE34* (targeted for flowering) and *CLE11* (targeted for ripening). The results, as observed from Agarose Gel Electrophoresis of PCR amplifications, yielded varying outcomes, with negative results for $CRcle^{6,19}$, $CRcle^{30,31}$, $CRcle^{34}$, $CRcle^{6,19,30,31,34}$ but positive results for $CRcle^{11}$.

During the cloning into *E. coli* DH10β of the target genes which are involved in the flowering, some technical difficulties occurred which caused the unsuccessful generation of the desired plasmid. Some of the possible reasons are described below.

If the *ccdB* gene was not successfully removed from the *pDIRECT_22C* plasmid used in the cloning process, it would have a fatally toxic effect on *E. coli* strain DH10β. The goal was to carefully remove the *ccdB* sequence using the *SapI* enzyme in a Golden Gate Assembly technique, guaranteeing that only plasmids that successfully recombined would survive. Also, we found that some bacteria were sometimes resistant to the *ccdB* gene and growing with the original plasmid, so some mutations in the *ccdB* gene or expression of its suppressor(s) might have occurred that lowered its lethality.

Based on the number of gRNAs in each cassette (Table 15), the expected number of bands for each cloned *pDIRECT_22C* plasmid were 5 bands for $CRcle^{6,19}$, 3 bands for $CRcle^{34}$, 5 bands for $CRcle^{30,31}$ and 6 bands for $CRcle^{6,19,30,31,34}$. The negative results for certain combinations might also underscore the considerable difficulties in assembling and amplifying complex constructs within multiplex CRISPR/Cas9 systems.

On the Agarose Gel Electrophoresis, the screened colonies did not yield the anticipated amplification bands (Figures 13a, 13b), indicating possible problems with PCR efficiency. It may also suggest inefficient cleavage or repair mechanisms at the targeted loci. Even after using the CmYLCV and CsyTERM primers to screen the putatively cloned *pDIRECT_22C* plasmid, this result did not change. Low-quality template DNA, primer mismatches, or less-

than-ideal PCR conditions are all potential contributory factors that need to be optimized for each construct and that we had not the possibilities to pursue due to time constrains.

According to the material and method section 3.3, the Golden Gate Assembly method, employing *Esp3I* and *SapI* enzymes, was integral to constructing the gRNA cassettes for CRISPR/Cas9 targeting of CLE genes. These Type IIS restriction enzymes were chosen for their ability to cut DNA outside their recognition sequences, generating precise overhangs essential for the accurate assembly of gRNA cassettes. The creation of four unique overhangs ensured that each fragment should have been ligated in the correct orientation to form the desired plasmid.

However, inconsistencies in the enzymatic reactions might have posed challenges during the assembly process. Factors such as suboptimal reaction conditions leading to incomplete digestion could have disrupted the generation of accurate overhangs, leading to unsuccessful ligation of the target gene sequences into the *pDIRECT_22C* plasmid. These disruptions might account for the negative results observed on Agarose Gel Electrophoresis of the screened colonies.

Introducing the cloned *pDIRECT_22C* plasmid into *E. coli* DH10β via electroporation method (section 3.3.5) is a pivotal step in achieving the successful construction of CRISPR/Cas9 constructs. This technique, which requires careful adjustment of several parameters to optimize effectiveness, used an electric pulse to help competent bacterial cells absorb cloned plasmid DNA.

One possible explanation for the low transformation efficiency observed in result part is related to the electric pulse settings. Applying either insufficient or excessive voltage can adversely affect the permeability of the *E. coli* membrane, limiting the successful uptake of the desired plasmid, in this case *pDIRECT_22C*. Additionally, inadequate recovery conditions after electroporation, such as using nutrient-deficient media (LB) or providing insufficient recovery time (1 hour at 37˚C), may impair the cells' ability to repair their membranes and replicate, thereby reducing the yield of transformation.

Moreover, the quality of the *pDIRECT_22C* plasmid and the competency of *E. coli* DH10β cells are critical factors in ensuring transformation success. Low-quality plasmid DNA can

hinder the process, while poorly prepared electrocompetent cells, due to inadequate washing or improper storage, may exhibit reduced DNA uptake.

Despite these challenges, the successful amplification of the gRNA fragments were validated for the targeted combinations of CLE genes involved in flowering and ripening process. As evidenced by the Agarose Gel Electrophoresis (Figure 9, 10, 11, 12), the digested plasmids and non-digested plasmids demonstrated clear amplification bands corresponding to the expected fragment sizes for several constructs. These results confirm the proper preparation of gRNA fragments to clone into the *pDIRECT_22C* plasmid for all the combinations.

Also, the successful generation of the correct vector was achieved in $CRcle^{11}$. The presence of distinct bands on Agarose Gel Electrophoresis 1% (Fig 14) corresponding to the expected size of PCR products confirms successful preparation of the vector $CRcle^{11}$, furtherly proved by means of Sanger sequencing (see par. 4.7). This vector has already been tested in plant and it showed editing at the level of the target gene CLE11, indicating the proper functioning of the designed construct (Marco Boschin et al., unpublished data).

One of the factors contributing to positive results is guide RNA efficiency. The gRNAs targeting CLE11 were likely well-designed, with high specificity, leading to effective Cas9-mediated cleavage. As a result, the multiplexing cassette targeting CLE11 in three different sites have been assembled correctly, ensuring proper delivery and expression of the gRNAs and Cas9 components (Marco Boschin et al., unpublished data).

Completing the sequencing process will also be a critical step to ensure the accuracy and fidelity of the genetic modifications introduced. This validation is essential for confirming that the desired modifications have been achieved. Importantly, initial experiments have already employed the functional $CRcle^{11}$ plasmid, which has successfully produced some transgenic plants. These preliminary results provide a robust platform for further studies, paving the way for deeper insights and more refined applications in this field (Marco Boschin et al., unpublished data).

Chapter 5

6. Conclusion

This thesis represents a significant advancement in creating genetic tools for precise genome editing in *Solanum lycopersicum* by using the CRISPR-Cas9 system. Through the efficient application of Golden Gate Assembly, we designed and constructed gRNA arrays targeting CLE genes with potential roles during flowering and fruit ripening. These gRNAs were successfully incorporated into the *pDIRECT_22C* plasmid, a vector tailored for effective use in dicot plants, establishing a strong foundation for future genetic editing experiments in *S. lycopersicum*.

Although challenges arose during cloning and validation, the project achieved a key milestone with the successful assembly of the *CRcle*^{11} plasmid. This construct is a critical resource for investigating the CLE11 gene's potential role in fruit ripening, offering a targeted approach to uncovering its functional significance.

In conclusion, this work makes a meaningful contribution to plant biotechnology by providing a flexible, reusable framework for exploring complex genetic traits in tomatoes. While the functions of CLE peptides require further study, this thesis has successfully established essential tools and methods to facilitate ongoing research, achieving its intended objectives.

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