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Appendix 1. – Thesis topic application form

THESIS TOPIC*

APPLICATION FORM

Deadline: May 15., November 15.

Filled by the student!

Student Name: ___Owais Ahmad Kutty_____ Neptun ID: _SJQUHL

Course: _____M.Sc Agricultural Biotechnology_____

Level of Education: BA/BSc / MA/MSc* Grade: ____A_____

Specialisation*: _____Plant Biotechnology_____

Student e-mail address:__ owais.kuttay@gmail.com_____

Name of Host Institute / Department: _____Hungarian University of Agriculture and Life Sciences/ Institute of Genetics and Biotechnology/Department of Plant Biotechnology

Primary supervisor name and position:__ Dr. György Szittya (scientific advisor)_____

Independent Consultant name, position, workplace:_ Dr. Péter Gyula (Project leader, senior scientist)

Thesis topic: __ **Investigation of *SlDML2* promoter spatial and temporal expression pattern with reporter constructs in tomato**

Date: _____2024____ year _____04_____ month ___22____ day

Primary supervisor | Independent consultant | Student

Filled by the host course leader/coordinator and the Head of host institute/department!

Application for the thesis topic is accepted/not accepted*

Date: ___2024____year ____04_____ month __22___ date

_____
Course leader/coordinator

Student and thesis topic is admitted/not admitted by the Institute/Deparment*

Date: _____year _____ month _____ day

_____
Head of Institute/Department**

***Please undreline the relevant choice!**

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Appendix 4 – Declaration

STUDENT DECLARATION

Signed below, _____Owais Ahmad Kutty__, student of the Szent István Campus of the Hungarian University of Agriculture and Life Science, at the BSc/MSc Course of __Agricultural Biotechnology_____ declare that the present Thesis is my own work and I have used the cited and quoted literature in accordance with the relevant legal and ethical rules. I understand that the one-page-summary of my thesis will be uploaded on the website of the Campus/Institute/Course and my Thesis will be available at the Host Department/Institute and in the repository of the University in accordance with the relevant legal and ethical rules.

Confidential data are presented in the thesis: yes no*

Date: ____2024_____ month _04_____ day__22

Student

SUPERVISOR'S DECLARATION

As primary supervisor of the author of this thesis, I hereby declare that review of the thesis was done thoroughly; student was informed and guided on the method of citing literature sources in the dissertation, attention was drawn on the importance of using literature data in accordance with the relevant legal and ethical rules.

Confidential data are presented in the thesis: yes no *

Approval of thesis for oral defense on Final Examination: approved not approved *

Date: _____2024 ___ month _____04__ day___22

signature

***Please, underline the correct choice!**

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THESIS

OWAIS AHMAD KUTTY

M.Sc. Agricultural Biotechnology

Gödöllő
2024

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MAGYAR AGRÁR- ÉS ÉLETTUDOMÁNYI EGYETEM

Hungarian University of Agriculture and Life Sciences
Szent István Campus
Agricultural Biotechnology

Investigation of *SlDML2* promoter spatial and temporal expression pattern with reporter constructs in tomato

Primary supervisor: Dr. György Szittya
(Group leader, scientific advisor)

Secondary supervisor: Dr. Péter Gyula
(Project leader, senior scientist)

Author: **Owais Ahmad Kutty**
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Institute: Institute of Genetics and Biotechnology, Department of Plant Biotechnology

Gödöllő

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ABSTRACT OF THESIS

Thesis title: Investigation of *SlDML2* promoter spatial and temporal expression pattern with reporter constructs in tomato

Author name : Owais Ahmad Kutty

Course, level of education: M.Sc Agricultural Biotechnology (Plant Biotechnology)

Host Department/Institute: Institute of Genetics and Biotechnology/Department of Plant Biotechnology

***Primary thesis advisor**:* Dr. György Szittya (Group leader, scientific advisor)

***Secondary thesis supervisor*:** Dr. Péter Gyula (Project leader, senior scientist)

Epigenetics refers to heritable changes in gene expression that occur without modification of the underlying DNA sequence. DNA methylation and demethylation are two of the many mechanisms by which epigenetic changes can occur. These changes can affect gene expression and contribute to plant development. DNA demethylation can occur either passively or actively. Active DNA demethylation is regulated by DNA demethylases. There are four DNA demethylases in tomato (*SlDML1-4*). *SlDML2* controls fruit ripening. In line with this, its expression is induced during the breaker stage of ripening. Altering the *cis*-regulatory elements (promoters) of genes *in vivo* could result in phenotypes different from that of a null mutant. To map the regulatory elements that govern *SlDML2* expression, we created deletion series in the *SlDML2* promoter by multiplex CRISPR/Cas9 genome editing. One of our mutants contained a 2104 bp deletion and a 1 bp insertion in the proximal part of the *SlDML2* promoter which was shown to be differentially methylated during ripening. The mutant had severe developmental defects that could be a result of the deletion in its promoter. We measured the expression of *SlDML2* in the tomato shoots with RT-qPCR and found that the expression level was lower in the mutant. However, this method is not suitable to investigate the spatial and temporal expression in high detail. Therefore, we created two, a mutant and a wild-type *SlDML2* promoter driven GFP gene constructs (pICH86966 D2pro-GFP and pICH86966 A120pro-GFP) to investigate the expressional difference of *SlDML2* in a non-invasive way. The modules were assembled using the Golden Gate assembly method. We have started transformation of these gene constructs into tomato plants. We are expecting to observe a markedly different expression pattern using fluorescence microscopy in case of the two constructs that could help us explain the observed severe developmental phenotype.

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Appendix 6 – Thesis Review Report

DIPLOMADOLGOZAT/SZAKDOLGOZAT BÍRÁLATI LAP
THESIS REVIEW REPORT

A dolgozat készítőjének neve, Neptun kód / Candidate's name, neptun code:

_____Owais Ahmad Kutty, SJQUHL_____

A dolgozat készítőjének szakja, tagozata, képzési helye / Candidate's department, training place:

____ Institute of Genetics and Biotechnology/Department of Plant Biotehnology_____

A dolgozat címe / Title of the thesis:

Investigation of *SlDML2* promoter spatial and temporal expression pattern with reporter constructs in tomato _____

A bíráló neve, beosztása, szervezeti egység / Thesis evaluator's name, title, department:

_____

A diplomadolgozat nem fogadható el/ The thesis cannot be evaluated if:

- súlyos szakmai tévedéseket tartalmaz / it contains major technical errors,
- szegényes a felhasznált forrásmunkák köre, / the amount of sources used is not efficient,
- súlyosan megsérti a tartalmi formai követelményeket / or it severly violates the formal requirements.

Plágium, hivatkozás nélküli jelentős szövegfelhasználás esetén a dolgozat összpontszáma: 0! / In case of plagiarism, when the sources of quotations are not indicated, the total score of the thesis is 0. Kérjük jelölje az értékelésének megfelelő pontszámokat az 1-től 5-ig terjedő pontskálán. Please, evaluate the reviewing aspects below on scale 1 to 5.

I. <u>Témaválasztás / Choice of topic</u>

1. Célkitűzések, logikai ív, koherens gondolatmenet / Objectives, logical and coherent train of thoughts:

1 2 3 4 5

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II. Szakirodalmi feldolgozás / Use of literature

2. Az elméletek, fogalmak, modellek ismerete, alkalmazása / The knowledge and application of theories, concepts and models:

1 2 3 4 5

3. Elemző, értékelő, összehasonlító, kritikai észrevételek / Analytic, evaluative, comparative and critical observations:

1 2 3 4 5

4. Szakirodalmi hivatkozások / Literature references:

1 2 3 4 5

III. Egyéni vizsgálat (amennyiben a dolgozat szakirodalmi áttekintés témájú, az itt szereplő kérdéseket a szakirodalom feldolgozásának színvonala alapján szükséges értékelni) / Individual research (if the thesis is a literature review, these questions should be evaluated according to the quality of reviewing)

5. A kutatási kérdések/hipotézisek / Stating research questions/hypotheses:

1 2 3 4 5

6. Az adatgyűjtés és adatfeldolgozás módszertana/ The method of data collection and processing:

1 2 3 4 5

7. Elemzőkészség / Analytical skills:

1 2 3 4 5

8. Következtetések és javaslatok / Conclusions and suggestions:

1 2 3 4 5

IV. Formai követelmények / Formal requirements

9. A dolgozat stílusa / The style of the thesis:

1 2 3 4 5

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10. A dolgozat struktúrája / The structure of the thesis:

1 2 3 4 5

A DOLGOZAT ÖSSZPONTSZÁMA / TOTAL SCORE OF THESIS: _____

Plágium / Plagiarism: Elfogadható színvonalú forráskezelés / Acceptable use of literature*

Érdemjegy /Final grade:
0-25 pont: elégtelen/insufficient (1)
26-31 pont: elégséges/sufficient (2)
32-38 pont: közepes/satisfactory (3)
39-44 pont: jó/good (4)
45-50 pont: jeles/excellent (5)

Védésre javaslom / I recommend it for final examination*: igen nem

ÉRDEMJEGY / GRADE: _____

Általános összefoglaló vélemény a dolgozatról / General, summarizing opinion about the thesis:

_____
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A bíráló szakmai kérdései / Questions of the thesis referee:

_____
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_____

Kelt: _____ év _____ hó _____ nap

_____
Bíráló neve és beosztása/Referee name and position
Bíráló munkahelye/Referee place of work

THESIS

OWAIS AHMAD KUTTY

M.Sc. Agricultural Biotechnology

Gödöllő

2024

MAGYAR AGRÁR- ÉS ÉLETTUDOMÁNYI EGYETEM

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Gödöllő

2024

1. TABLE OF CONTENTS

2. ABBREVIATIONS

bp (base pairs)

nt (nucleotide)

rpm (rotation per minute)

DNMTs (DNA methyltransferases)

RdDM (RNA-directed DNA methylation)

DMRs (differentially methylated regions)

DRM (DOMAINS REARRANGED METHYLTRANSFERASES)

dsRNA (double-stranded RNAs)

siRNAs (small interfering RNAs)

SHH 1(Sawadee Homeodomain Homolog 1)

DCL3 (DICER-LIKE 3)

DML (DEMETER-LIKE)

DME (DEMETER)

ROS1 (REPRESSOR OF SILENCING 1)

Pol (POLYMERASE)

RNAi (RNA interference)

rdd (ros1/dml2/dml3)

EPF2 (EPIDERMAL PATTERNING FACTOR 2)

LMWg (low-molecular-weight glutenins)

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)

PCR (polymerase chain reaction)

TEs (transposable elements)

PAM (protospacer adjacent motif)

tracrRNA (trans-activating CRISPR RNA)

crRNA (CRISPR RNA)

pre-crRNA (precursor crRNA)

sgRNA (single-guide RNA)

HR (homologous recombination)

DSBs (double-stranded breaks)

NHEJ (non-homologous end joining)

HDR (homologous direct repair)

MMEJ (microhomology-mediated end joining)

WDV (wheat dwarf virus)

ToLCV (tomato leaf curl virus)

TRV (tobacco rattle virus)

TMV (tobacco mosaic virus)

PEBV (pea early browning virus)

BNYVV (beet necrotic yellow vein virus)

BSMV (barley stripe mosaic virus)

DRs (developmental regulators)

WUS (WUSCHEL)

CLV (CLAVATA)

STM (SHOOT MERISTEMLESS)

MP (MONOPTEROS)

PDS (PHYTOENE DESATURASE)

QTLs (Quantitative trait locus)

PL (PECTATE LYASE)

3. INTRODUCTION

In the realm of agriculture, the tomato (*Solanum lycopersicum*) stands as a paramount vegetable crop, playing a crucial role in human diets by providing essential compounds such as vitamins, minerals, and antioxidants. Renowned globally, it holds a prominent position as one of the most popular vegetables. Beyond its economic and nutritional significance, the tomato serves as a primary model for studying fleshy fruit development and ripening. Investigation into the molecular bases of traits like fruit set, color, flavor, size, and nutritional quality is pivotal (Seymour *et al.*, 2013; Azzi *et al.*, 2015). The quality and quantity of tomato yields are subject to various environmental factors. Tomato plants in natural environments face a range of stresses, including temperature fluctuations, water scarcity, light variations, and attacks by pests and pathogens. Additionally, nutrient availability plays a significant role in fruit formation. Light and temperature are particularly crucial, as unfavorable conditions can lead to delays in fruiting or hinder ripening. Different tomato varieties exhibit distinct responses to environmental cues, underscoring the importance of inherited traits in both growth and stress management. Traditional genetic inheritance is augmented by the involvement of epigenetic mechanisms in plant stress responses and fruit development. Epigenetics denotes heritable alterations in gene expression that occur independently of changes to the underlying DNA sequence. These modifications encompass histone post-translational modifications and DNA methylation, which are passed on through DNA replication and cellular propagation. Consequently, they play a pivotal role in establishing and preserving cell-type-specific gene expression profiles (Gallusci *et al.*, 2016). DNA methylation at cytosine residues is a well-preserved epigenetic modification observed in plants. It is linked to the suppression of transposable elements, the maintenance of chromatin structure, and the regulation of gene expression (Zhu, 2009; Zhang and Zhu, 2012; Zhang, Lang and Zhu, 2018). The dynamic nature of DNA methylation, coupled with instances of stable patterns, can lead to transgenerational epigenetic inheritance, influencing traits such as hybrid vigor or heterosis in agriculture (Groszmann *et al.*, 2011, 2014). In plants, DNA methylation occurs in three distinct sequence contexts: CG, CHG, and CHH. *Arabidopsis thaliana* maintains CG and CHG methylation via DNA METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3), respectively. CHH methylation, contingent upon chromatin context, is upheld by either CMT2 or DOMAINS REARRANGED

METHYLTRANSFERASES (DRM1 and DRM2) through the RNA-directed DNA methylation (RdDM) pathway, which is responsible for initiating *de novo* DNA methylation. RdDM depends on internally generated 24-nt small interfering RNAs (siRNAs) produced by DNA-DEPENDENT RNA POLYMERASE IV and V (PolIV/V), RNA-DEPENDENT RNA POLYMERASE 2 (RDR2), and DICER-LIKE 3 (DCL3). This process involves the synthesis of short RNA precursors, their conversion into double-stranded RNA, and subsequent cleavage into 24-nt siRNAs, ultimately leading to DNA methylation (Zhai *et al.*, 2015). In plants, DNA methylation can be diminished through either passive loss due to the lack of maintenance or active removal facilitated by DNA demethylases. Active DNA demethylation entails the action of 5-methylcytosine DNA glycosylase enzymes, which instigate the removal of methylated cytosine residues. These removed methyl groups are then replaced by unmethylated cytosines via a series of sequential steps. Arabidopsis harbors four genes encoding DNA demethylases: *REPRESSOR OF SILENCING 1* (*ROS1*), *DEMETER* (*DME*), *DEMETER-LIKE 2* (*DML2*), and *DML3* (Penterman, Uzawa and Fischer, 2007). The tomato genome contains four presumed *DML* genes: *SlDML1*, *SlDML2*, *SlDML3*, and *SlDML4*, each displaying distinct expression patterns. Notably, the targeted deletion of *SlDML2* using CRISPR/Cas9 technology impedes the progression of fruit ripening, indicating its pivotal role in this process. However, comprehensive understanding of the functions of other *SlDML* genes is lacking, underscoring the necessity for further investigation. While the regulatory mechanisms governing the transcription of tomato *SlDML* genes beyond developmental contexts remain unexplored, the involvement of active DNA demethylation in plant stress responses is well-established (Yu *et al.*, 2013; Pandey *et al.*, 2016).

To investigate the spatial and temporal expressions of *SlDML2* in high detail we used a mutant (A120) created using CRISPR/Cas9-based genome editing technology and a wild-type *SlDML2* promoter driven GFP gene constructs to investigate the expressional difference of *SlDML2 in planta* in a non-invasive way. The mutant plants exhibited altered growth and developmental patterns compared to wild-type plants. The findings provide insights into the potential role of *SlDML2* in plant development, which could be useful in developing strategies for crop improvement. Addressing these questions will contribute valuable knowledge to the understanding of epigenetic regulation in tomato plants.

4. OBJECTIVES

DNA demethylation has been extensively studied in *Arabidopsis thaliana*, but there is limited information available for other plant species, especially those of agricultural significance. In tomato, for instance, there are four known DNA demethylases, but only SlDML2 has been thoroughly investigated thus far. This is because SlDML2 was found to be significantly upregulated during fruit ripening, prompting researchers to explore its functional role. Using CRISPR/Cas9-based genome editing technology, the involvement of SlDML2 in fruit ripening regulation was experimentally confirmed. In contrast, the other three SlDMLs did not exhibit a similar expression pattern during fruit ripening, which may have led to less focus on their investigation. However, given that some of these demethylases are expressed widely throughout development and in response to stress, it is likely that they serve other important functions, such as regulating developmental processes or stress responses. There is also a possibility that they have overlapping functions with SlDML2 in fruit ripening regulation.

To elucidate the function of SIDML2 gene in tomato our main objective is to compare the expression patterns of a null mutant (A120) and Wild type tomato. To achieve that goal we have set these objectives:

A. We propose the creation of a null mutant for *SlDML2* (A120), which exhibits the highest and most ubiquitous expression among the identified *DMLs*.

B. Create a wild type(D2-pro) and mutant *SIDML2* (A120) promoter driven GFP reporter constructs. (Assembled using Golden Gate assembly method)

C. Agrobacterium-mediated transformation of these reporter modules.

D. Characterization of gene expression patterns of GFP driven by the wild type and mutant *SIDML2* promoters.

5. LITERATURE REVIEW

5.1. Epigenetics

Epigenetics, originating in the 1940s and 1950s with the identification of DNA methylation and the concept of chromosomal imprinting, was first termed by Waddington in 1942 (Waddington, 1942). It encompasses the examination of how genes interact with environmental factors to influence an organism's development and characteristics. Over the ensuing decades, the field of epigenetics has experienced significant growth, with mounting evidence indicating its pivotal role in governing gene expression regulation and contributing to the onset of various diseases (Bird, 2007). Essentially, epigenetics entails the study of heritable alterations in gene expression that occur independent of changes to the DNA sequence, encompassing structural modifications like DNA methylation and histone modification, as well as the involvement of non-coding RNAs (ncRNAs). In the realm of plants, epigenetic mechanisms are crucial for developmental processes and facilitate rapid adaptation to shifting environmental conditions (Chang *et al.*, 2020).

In agricultural contexts, epigenetic alterations can significantly influence hybrid vigor, also known as heterosis. This phenomenon refers to the heightened growth and yield observed in the progeny resulting from the crossbreeding of two distinct varieties, surpassing the performance of either parent individually (Groszmann *et al.*, 2013). Within the context of heterosis, the amalgamation of two disparate genomes instigates epigenetic modifications that augment heterozygosity and diversity, consequently enhancing the overall performance of hybrid plants. For instance, inter-specific hybridization has been observed to elevate crop yields in species like maize and rice (Groszmann *et al.*, 2011, 2013). Likewise, investigations into hybrid *Arabidopsis* plants have revealed the presence of heterosis across multiple traits, including vegetative biomass, size, cell count, and seed yield (Groszmann *et al.*, 2014). Heterosis has been linked to a range of interactive attributes, including alterations in gene expression, metabolic processes, and epigenetic regulation. However, the precise molecular mechanisms underpinning heterosis continue to be an area of ongoing investigation and are not yet comprehensively elucidated. Transcriptomic analyses of hybrids and their parental plants have unveiled instances where gene expression levels deviate from the average levels observed in the parent plants, a

phenomenon termed non additive expression (Kapazoglou *et al.*, 2018). In *Arabidopsis*, alterations in DNA methylation patterns, which influence gene expression, have been detected in hybrid plants (Shen *et al.*, 2012). Likewise, alterations in overall epigenetic profiles and transcriptional activity have been recorded in hybrid rice and maize plants in comparison to their parental counterparts (He *et al.*, 2013). Environmental fluctuations can induce heritable alterations in epigenetic patterns, which in turn correlate with shifts in gene expression and phenotypic diversity (Kim *et al.*, 2019). These phenotypic variances are instrumental in epibreeding efforts aimed at enhancing diverse crop production traits. Use of epialleles for improvement of various agronomical traits (Figure 1).

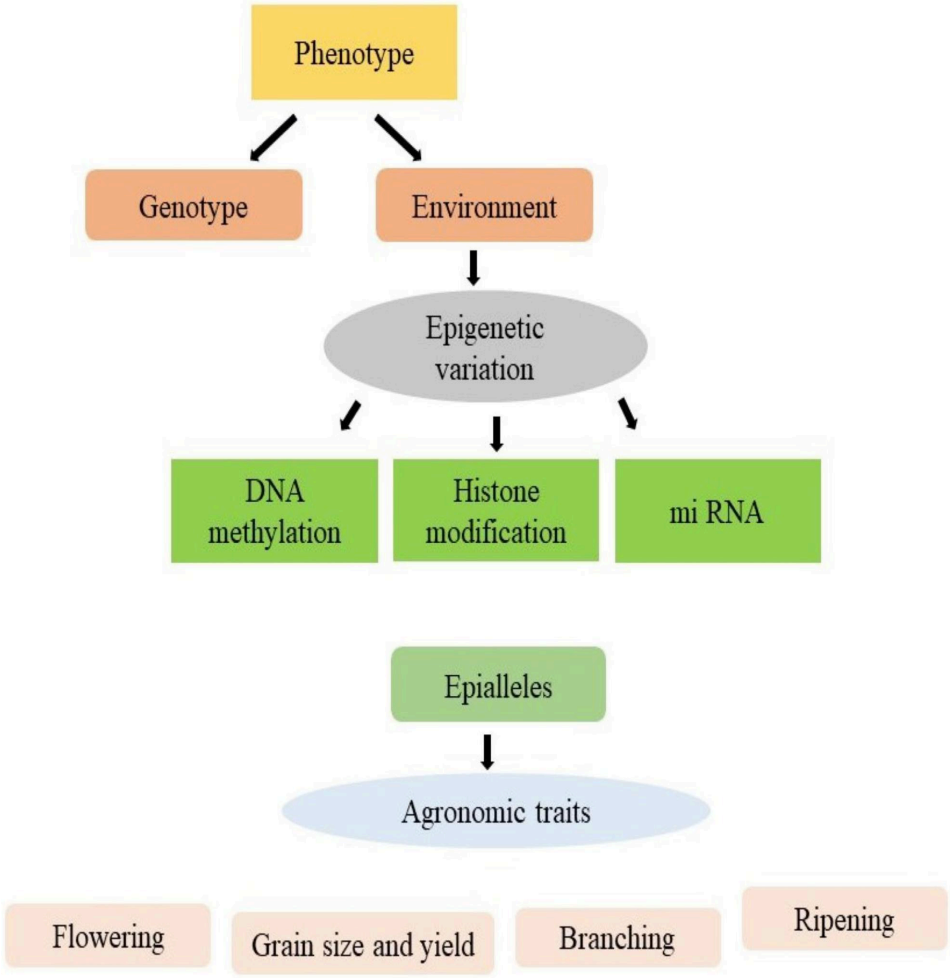

Figure 1. Use of epialleles for improvement of various agronomical traits (Gupta and Salgotra, 2022).

Plants extensively employ cytosine methylation within their genomes to tightly regulate the expression of both transposable elements (TEs) and genes. Despite the rigorous control

mechanisms in place, there can be instances of spontaneous methylation loss or acquisition at individual cytosines or clusters of cytosines (van der Graaf *et al.*, 2015).

Intense environmental stress, such as drought, has been shown to elevate the rate of epimutations. A study conducted with rice demonstrated that exposure to drought stress across 11 successive generations resulted in the accumulation of transgenerational epimutations, thereby enhancing the adaptability of the progeny in natural settings. Furthermore, the study revealed that genes involved in stress response pathways exhibited increased transgenerational epimutations, and the DNA methylation patterns of drought-responsive genes were altered over multiple generations of drought exposure. Notably, approximately 30% of the methylation alterations observed were found to be stable and heritable (Zheng *et al.*, 2017). In a study conducted by Wang et al., it was discovered that 29% of the alterations in DNA methylation induced by drought stress persisted even after the conditions returned to normal (Wang *et al.*, 2011). In a separate investigation, Kumar and Singh noted that 25% of the heightened methylation observed in the rice genotype IR-64-DTY1.1 persisted even after the conclusion of the drought period (Kumar, 2016). Likewise, in *Arabidopsis thaliana*, a single characteristic associated with seed dormancy exhibits transgenerational epiallelic stability when subjected to drought conditions (Ganguly *et al.*, 2017). Hence, epigenetics emerges as a crucial determinant in plants' capacity for long-term adaptation and evolution under challenging environmental conditions (Zheng *et al.*, 2017).

5.2. DNA methylation in plants

DNA methylation is an inheritable mechanism entailing the addition of a methyl group (CH3) to the fifth carbon of a cytosine base. It plays a pivotal role in regulating numerous functions, including gene expression, genomic integrity, gene imprinting, and the suppression of transposable elements. Disruption of DNA methylation can lead to developmental irregularities, such as the impairment of tomato fruit ripening (Zhang, Lang and Zhu, 2018). Novel epialleles can arise from alterations in DNA methylation patterns, potentially facilitating plant enhancement and adaptation. During a comprehensive genome-wide DNA methylation analysis in *Arabidopsis thaliana*, substantial methylation of transposable elements and other repetitive DNA sequences in heterochromatin was documented (Gupta and Salgotra, 2022). DNA

methylation serves pivotal functions in governing gene expression, growth, development, and resilience to environmental stresses, while also contributing to genome stability (Zilberman *et al.*, 2007).

DNA methylation is facilitated by various DNA methyltransferases, utilizing S-adenosyl-L-methionine as a methyl group donor. These enzymes catalyze DNA base modifications in a manner that is specific to both the sequence context and genomic region. Methylcytosine (5-mC), recognized as the fifth DNA base, was identified prior to the recognition of DNA as the genetic material in living cells. While conventional 5-mC has historically received greater attention, recent discoveries of additional base modifications such as hydroxymethylcytosine (5-hmC), formylcytosine (5-fC), carboxylcytosine (5-caC), and N6-methyladenine (6-mA) have sparked significant interest in epigenomic research. In plants, cytosine methylation can occur in all sequence contexts (CG, CHG, and CHH, where H=A, C, or T). Heterochromatic regions in plants, including *Arabidopsis* and other species, are typically enriched with methylcytosines, particularly within repetitive sequences and transposable elements (TEs). However, transposable elements and 5-mC can also be interspersed within euchromatic regions (Holligan *et al.*, 2006; Rathore *et al.*, 2020). The dynamics of DNA base methylation is contingent upon the reversibility of associated processes, which in turn governs the activation or suppression of gene expression. Ongoing exploration reveals an ever-expanding diversity and intricacy of epigenetic modifications (including DNA and histone modifications, as well as non-coding RNA biogenesis) across various organisms. The potential for combinatorial interactions among these epigenetic marks suggests that the complexity of epigenetic codes may be substantially greater than currently understood (Kumar, Chinnusamy and Mohapatra, 2018).

DNA methylation patterns typically consist of CG (symmetrical), CHG (symmetrical), or CHH (asymmetrical) motifs, with a notable abundance observed in heterochromatic transposable elements (TEs) and repetitive sequences. Methylation occurring within gene regulatory regions can lead to transcriptional gene silencing. Interestingly, it has been found that the DNA methyl-reader proteins SU(VAR)3-9 homologs SUVH1 and SUVH3 can sometimes promote gene expression (Harris et al., 2018; Xiao et al., 2019). Methylation of cytosine within hemimethylated CG dinucleotides, formed during DNA replication, is catalyzed by methyltransferase 1 (MET1), analogous to DNA methyltransferase 1 in mammals. MET1 adds a

methyl (CH3) group to the fifth carbon of cytosine in the daughter strand of replicated DNA. The recruitment of MET1 to hemimethylated CG sites is facilitated by methylation protein variants, which are orthologs of UHRF1 (Woo, Dittmer and Richards, 2008). Methylation occurring at CHG sites within the daughter DNA strand is primarily facilitated by CHROMOMETHYLASE 3 (CMT3), with a secondary contribution from CHROMOMETHYLASE 2 (CMT2) (Stroud *et al.*, 2014). The dynamics of DNA methylation in plants is illustrated in Figure 2.

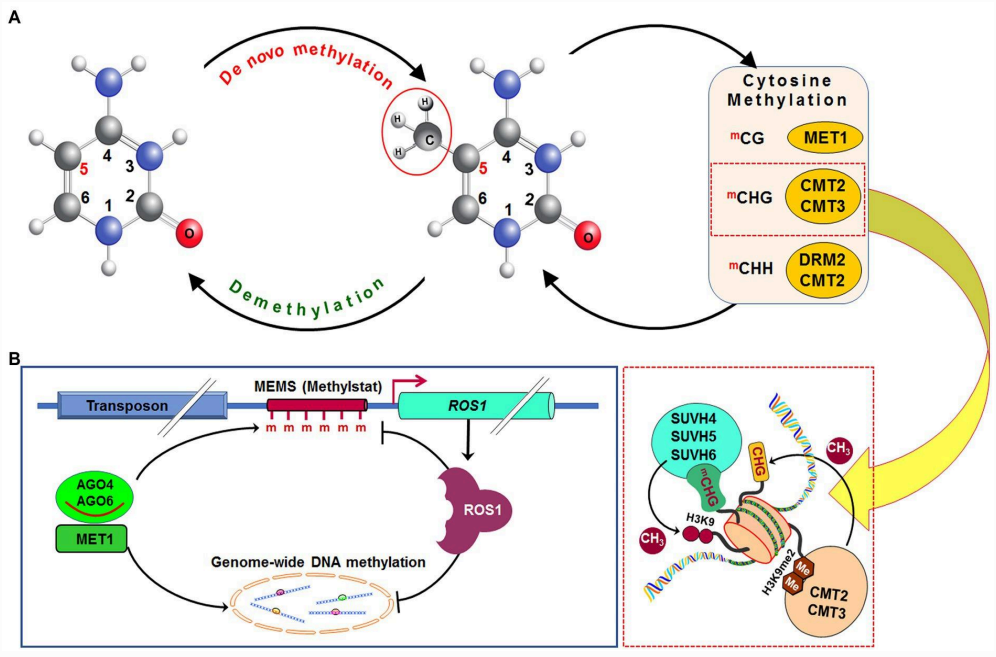

Figure 2: Dynamics of DNA methylation in plants

(A). *De novo* DNA methylation occurs across all cytosine contexts (CG, CHG, and CHH, where H=A, C, or T). Following DNA replication, maintenance of methylation in the CG context is overseen by methyltransferase 1 (MET1). In the CHG context, methylation is sustained primarily by chromomethylase 2 (CMT2) or CMT3, while in the CHH context, maintenance is orchestrated by CMT2 or by DRM2 through the RdDM pathway. Methylated CHG (mCHG) sites attract histone H3 lysine 9 (H3K9)-specific suppressor of variegation 3-9 homolog proteins 4, 5, and 6 (SUVH4, SUVH5, and SUVH6), leading to the generation of dimethylated H3K9 (H3K9me2), which facilitates the activity of CMT2 and CMT3. **(B).** Methylation of the methylation monitoring sequence (MEMS), also known as the "methylstat," located in the promoter region of the *REPRESSOR OF SILENCING 1* (*ROS1*) gene, is essential for *ROS1* transcription. The cytosine methylation status at MEMS is regulated by MET1/RdDM and *ROS1* itself, thereby enabling the sensing and regulation of DNA (de)methylation homeostasis (Kumar and Mohapatra, 2021).

5.3. DNA demethylation in plants

Passive and active DNA demethylation are two separate mechanisms that can lead to the loss or elimination of DNA methylation. The former is known as passive DNA demethylation, while the latter is termed active DNA demethylation. Passive DNA demethylation occurs as a

result of DNA replication, wherein insufficient DNA methylation by DNA methyltransferases causes a decrease in the overall level of DNA methylation (Zhang, Lang and Zhu, 2018). Consequently, this leads to a diminished level of DNA methylation, ultimately resulting in the depletion of DNA methylation on the newly synthesized DNA strand, a phenomenon referred to as passive DNA demethylation (Liu and Lang, 2020a).

In mammals, the process of active DNA demethylation commences with the deamination and/or oxidation of 5-methylcytosine (5-mC). In contrast, in plants, active DNA demethylation involves the direct excision of 5-methylcytosine (5-mC) through the action of methylcytosine DNA glycosylases (Penterman *et al.*, 2007; Kumar, Chinnusamy and Mohapatra, 2018; Li *et al.*, 2018). These enzymes are capable of initiating active DNA demethylation by excising methylcytosine from the DNA backbone, thereby generating a single-nucleotide gap. Subsequently, this gap is filled with an unmethylated cytosine via the base-excision repair (BER) pathway (Liu and Lang, 2020a). REPRESSOR OF SILENCING 1 (ROS1), was the first identified DNA demethylase in *Arabidopsis* and is exclusive to plant species (Gong *et al.*, 2002). The ROS1 protein family, comprising AtROS1, AtDME, AtDML2, and AtDML3, are enzymes that exhibit dual functionality as DNA glycosylase/lyases, possessing both DNA glycosylase and apurinic/apyrimidinic (AP) lyase activities. The active and passive DNA demethylation (Liu and Lang, 2020b) are summarized in Figure 3.

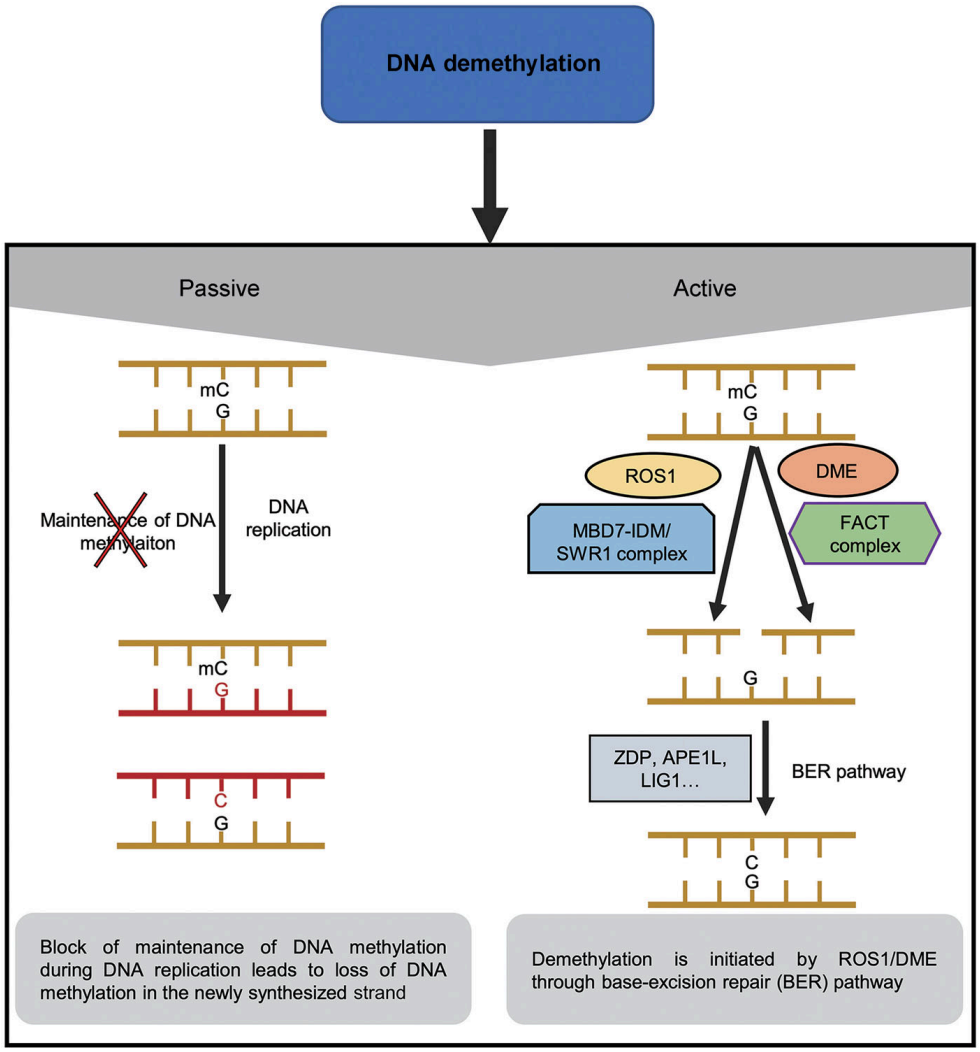

Figure 3: Mechanisms of Active and Passive DNA demethylation (Liu and Lang, 2020b).

Passive DNA demethylation occurs during DNA replication when the DNA methylation pathway is inactive, resulting in the dilution of DNA methylation in the newly synthesized DNA. Active DNA demethylation, on the other hand, is facilitated by the plant-specific enzyme family ROS1/DME. Recruitment of ROS1 and DME to target loci is mediated by the MBD7-IDM or SWR1 complex and the Facilitates Chromatin Transactions (FACT) complex, respectively. Upon recruitment to the target loci, ROS1/DME enzymes remove the 5-methylcytosine (5-mC) from the DNA backbone. Subsequently, the single-nucleotide gap is filled with an unmethylated cytosine through the base-excision repair pathway, involving enzymes such as apurinic or apyrimidinic site lyase (ARP, APE1L), zinc finger DNA 3′-phosphatase (ZDP), and DNA ligase I (LIG1).

5.4. Role of active DNA demethylation in physiological processes

Active demethylation is believed to be significantly involved in diverse physiological phenomena, encompassing plant and growth development, fruit ripening, genomic stability, and responses to stress (Zhu, 2009). In plants, DNA methylation is directed towards transposable elements (TEs) and other repetitive DNA sequences. While long transposable elements are predominantly situated within heterochromatin and methylated by CMT2 and CMT3, regions rich in genes and euchromatin contain a substantial proportion of shorter transposable elements or sequences derived from transposable elements, which are targeted by the RNA-directed DNA methylation (RdDM) pathway (Zilberman *et al.*, 2007; Stroud *et al.*, 2014).

A fundamental role of DNA demethylation in plants is to uphold genome stability by counteracting RNA-directed DNA methylation (RdDM), thereby averting the spread of methylation to adjacent genes. Additionally, active DNA demethylation is implicated in genome-wide alterations in methylation patterns during reproductive development, which occur in both female and male gametophytes. Demethylation in gametophytes, predominantly occurring in companion cells and primarily targeting euchromatic transposable element sequences, may serve to reinforce methylation patterns in gametes. One consequence of this process is the phenomenon of gene imprinting in the endosperm (Calarco *et al.*, 2012). During reproductive development, further demethylation occurs during seed maturation and germination, along with the process of fruit ripening. The *Arabidopsis* ROS1, DML2, and DML3 exhibit broad expression across various vegetative tissues in plants and appear to play a role in maintaining the stability and flexibility of the plant epigenome, safeguarding the genome from excessive methylation. *Arabidopsis* ROS1, identified as the primary 5-methylcytosine (5-meC) DNA glycosylase in vegetative tissues, was discovered through a screening process aimed at identifying mutants displaying aberrant expression of the repetitive RD29A-LUC transgene (Gong *et al.*, 2002). In wild-type plants, both the transgene and the corresponding endogenous gene are actively expressed. However, in *ros1* mutant plants, there is a notable occurrence of transcriptional silencing and increased methylation observed at both loci. This observation underscores the protective role of active DNA demethylation in preventing genes from undergoing incorrect silencing. Additionally, comparative analysis between the *ros1* mutant and wild-type plants reveals nearly 5000 regions exhibiting hypermethylation in the mutant,

predominantly situated within transposable elements (TEs) and intergenic regions. ROS1 primarily targets transposons located in close proximity to protein-coding genes. In *ros1* mutant plants, there is an observed spread of hypermethylation from the edges of transposable elements to neighboring sequences. This indicates that DNA demethylation by ROS1 serves to delineate transposons and genes, preventing the spread of DNA methylation from transposable elements and thereby safeguarding nearby genes from transcriptional repression. Analysis of hypermethylated regions in *ros1* mutants revealed numerous targets of the RdDM pathway, suggesting that ROS1 acts to counteract DNA methylation established by RdDM. However, it has been noted that ROS1 also opposes RdDM-independent DNA methylation at certain loci (Tang *et al.*, 2016).

Another significant function of DNA demethylation in plants is the activation of genes in response to various environmental stresses or biological stimuli, often achieved by targeting transposable element sequences located at the 5' regions of these genes. For example, moderate differences in global methylation levels caused by phosphate (Pi) deficiency were demonstrated in *Arabidopsis* involving both hypo- and hyper-methylation, and they are mainly manifested in TEs, promoters, and genes in the CG and CHG contexts. Methylated regions are predominantly enriched in proximity to genes that are induced by transcriptional stimuli. Furthermore, Pi deficiency causes lower Pi uptake by the *ros1* mutant plants compared to the wild-type. This implies that DNA demethylation is vital for Pi starvation tolerance (Yong-Villalobos *et al.*, 2015). Furthermore, active DNA demethylation is involved in the improvement of salt tolerance and the response to salt stress. Specifically, methylcytosine demethylation was reduced after salt stress exposure in the salt-tolerant rice variant Pokkali, while the salt-sensitive IR29 variant showed no change in demethylation levels. This loss of methylation in the salt-tolerant rice variant showed a spike in the expression levels for DNG701 and DNG710, which encode 5-methylcytosine DNA glycosylases. Demethylation of the transposon Ty3-gypsy and a telomeric repetitive sequence was also observed (Ferreira *et al.*, 2015).

5.5. Genome editing with CRISPR/Cas 9 system

The CRISPR-Cas9 (clustered regularly interspaced short palindromic repeat and CRISPR-associated nuclease 9) is an adaptive bacterial immune system evolved against

bacteriophages that was tailored for genome engineering. Using a guide RNA, the Cas9 enzyme is targeted to a particular genomic sequence. RNA-guided genome-editing is a simple and promising new technique for biology and biological aspects involving a variety of creative applications across domains (Ding *et al.*, 2016). The advanced CRISPR/Cas9 methodology is not limited to being a molecular mediator that gives in-depth answers to biological inquiries but includes a wide variety of new and potential applications within the field of biology (Ding *et al.*, 2016).

The bacterial CRISPR/Cas system executes the degradation of the invading phage genome through three sequential steps: acquisition or adaptation, biogenesis or expression of CRISPR RNA (crRNA), and interference or targeting (Figure 4).

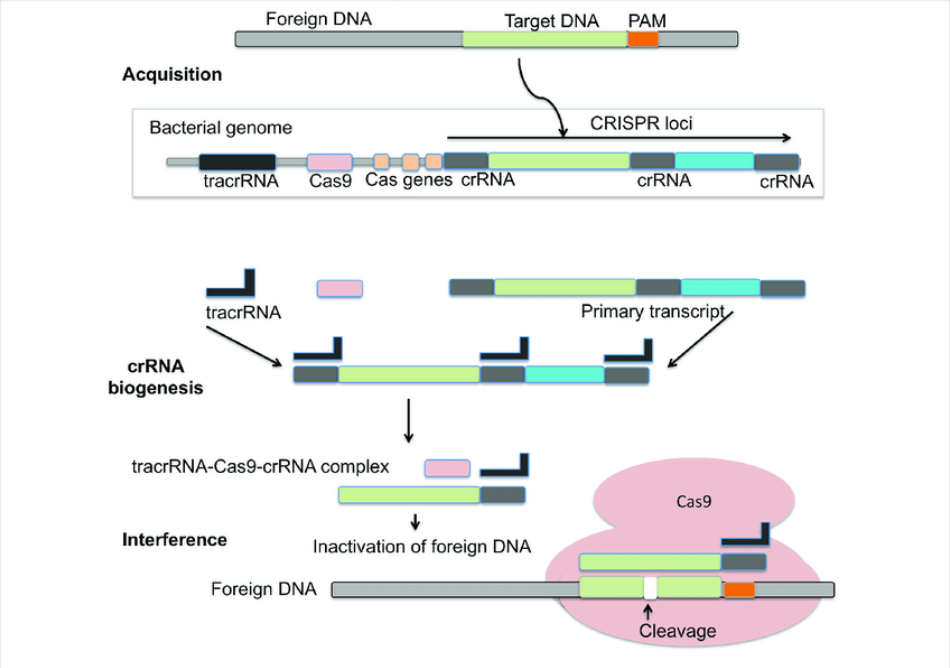

Figure 4: Mechanism of CRISPR/cas9 action (Arora and Narula, 2017)

During the acquisition phase, foreign DNA fragments are integrated into the CRISPR loci within the bacterial genome. These CRISPR loci are subsequently transcribed into primary transcripts, which are then processed into crRNA molecules with the assistance of tracrRNA during crRNA biogenesis. In the interference step, the Cas9 endonuclease, along with a crRNA, targets and cleaves foreign DNA sequences near the PAM region.

In the initial phase, known as adaptation or acquisition, the CRISPR system's Cas proteins discern between self and foreign DNA by recognizing the 3–4-nt-long protospacer adjacent motif (PAM) located next to the protospacer in the phage genome. This PAM recognition is crucial for both adaptation and interference processes (Mojica *et al.*, 2009; Zetsche *et al.*, 2015). When exposed to foreign DNA, a few nucleotide base pairs are directly adjacent to the PAM motif incorporated into the CRISPR array in the bacterial host. This incorporation leads the host to create memory of the foreign DNA (Hille and Charpentier, 2016). The foreign sequence, once integrated, is transcribed into RNA and associates with tracrRNA (trans-activating CRISPR RNA), forming a precursor molecule known as premature crRNA (pre-crRNA). Through processing, pre-crRNA matures into active crRNA, guiding the Cas endonuclease to induce double-stranded breaks in the target DNA sequence. CrRNAs, also referred to as guide RNAs, exert their specific function by guiding the Cas endonuclease to the target site. The incoming genome, which is complementary to the fully mature crRNA and located adjacent to the PAM motif, becomes susceptible to these double-strand breaks (Rath *et al.*, 2015). The final stage, interference, involves the recognition by crRNAs and the formation of complementary base pairs with the foreign genome. This recognition leads to the cleavage and destruction of the complex formed between the invading genome and the crRNA (Bhattacharjee *et al.*, 2020).

The Cas protein possesses dual functionalities, serving as both an endonuclease and a helicase. Specifically, the Cas nuclease within Cas9 cleaves the foreign genome at a precise DNA site located upstream of the PAM (Zheng *et al.*, 2020). If there is a mutation in the PAM or if there is a mismatch between the spacer and the invader's DNA, cleavage is prevented, rendering the organism susceptible to infection (Hille and Charpentier, 2016). The RNA-guided Cas9 enzyme is a commonly employed CRISPR nuclease in DNA editing. Its mechanism involves inducing double-stranded breaks (DSBs) at a precise location in the genome, facilitated by a single-guide RNA (sgRNA) molecule (Anzalone, Koblan and Liu, 2020). By the coming of the CRISPR-Cas system, genome editing has been revolutionized and offers a very accurate way to change DNA sequences. A major milestone in this technology was the creation of single-guide RNA (sgRNA) that came through linking tracrRNA and crRNA thus making it readily available

for researchers (Bhatia, Pooja and Yadav, 2023). The single-guide RNA (sgRNA) is an engineered version of the native crRNA-tracrRNA complex, streamlined into a single continuous sequence. Acting as a molecular beacon, the sgRNA directs the Cas9 protein to precisely bind and cleave both strands of the target DNA sequence, inducing double-stranded breaks (DSBs). Subsequently, the DNA repair mechanisms are activated, with the error-prone non-homologous end joining (NHEJ) pathway or the more precise homologous direct repair (HDR) pathway mending the DSBs (Boti *et al.*, 2023). The application of CRISPR/Cas9 for genome editing in plants commonly leads to mutations characterized by short deletions typically of 10 base pairs or less, along with single nucleotide insertions, particularly favoring A/T nucleotides across various plant species. While single nucleotide substitutions are rare, they have been observed, notably in soybean protoplasts, exhibiting the highest mutation frequency among the tested species. It seems that gene-specific factors might influence the repair of double-stranded breaks (DSBs) through microhomology-mediated end joining (MMEJ), as longer deletions are less frequently observed. In rice, the mutation patterns vary depending on the target gene (Bortesi et al. 2016). Consistent mutations have been detected in different sets of soybean hairy roots and somatic embryos, implying the presence of unidentified regulations dictating the preferred mutation types at specific targets. The research findings also indicated that the seven most potent single-guide RNAs (sgRNAs) predominantly induced short deletions, whereas less efficient sgRNAs led to more insertions and substitutions (Jacobs *et al.*, 2015). Notably, in rice, all identified off-target mutations involved 1-base pair insertions, indicating that the interaction between the single-guide RNA (sgRNA) and the target sequence may influence the specific type of mutation that arises (Li *et al.*, 2016). In higher plants, off-target effects are generally rare, and when they do occur, only a small subset of single-guide RNAs (sgRNAs) are typically impacted. Whole-genome sequencing has verified that unintended mutations, known as off-targets, are uncommon. Research conducted on rice and wheat has demonstrated that off-target effects can be reduced by designing sgRNAs with mismatches located outside the seed sequence. Thus, the development of more accurate sgRNAs represents an effective approach for mitigating such occurrences (Bortesi *et al.*, 2016). In some plants like *Arabidopsis* (Sauer *et al.*, 2016), barley (Lawrenson *et al.*, 2015), soybean (Jacobs *et al.*, 2015), and rice (Xie and Yang, 2013), unexpected cutting has been observed at sites with mismatches within the seed region (Bortesi *et al.*, 2016). The likelihood of such events happening might increase in target sequences exhibiting a high GC content (above

70%), as noted in studies by (Li *et al.*, 2016), but this trend was not observed in studies involving sequences with lower GC content. Nevertheless, off-target mutations are less common compared to on-target mutations, indicating the possibility of consistently obtaining only on-target mutations in all experimental trials. Additionally, it was observed that off-target mutations exclusively appeared in T1 rice plants containing Cas9/sgRNA transgenes, while their absence in plants with segregated CRISPR components suggests that careful selection of T1 offspring could mitigate or eliminate off-target effects (Xu *et al.*, 2015). The probability of unintended mutations is contingent upon the concentration of the Cas9/sgRNA ribonucleoprotein (RNP) complex. Opting for transient expression of these components may decrease the risk of off-target effects; however, this could potentially compromise on-target efficiency (Tsai *et al.*, 2015; Bortesi *et al.*, 2016).

5.6. *In vivo* editing of cis-regulatory elements with the CRISPR/Cas9 system

Disruption of a gene function is fundamental in plant genetic analysis. Conceptually, this can be achieved effectively by utilizing Cas9/gRNA to induce insertions/deletions (InDels) within the coding region of a gene resulting in frame-shift and a disrupted translation. Notably, the Cas9/gRNA approach has been effectively employed to generate gene knockouts in various plant species such as *Arabidopsis*, rice, tobacco, and sorghum (Feng *et al.*, 2013). The Cas9/gRNA system has emerged as the predominant method for generating knockout mutants in plants. The initial proof-of-concept investigations demonstrated that Cas9/gRNA induces InDels at targeted sites and effectively disrupts target genes with varying degrees of efficiency. Subsequent examinations revealed that the edited genes were reliably transmitted to the subsequent generation in *Arabidopsis* (Feng *et al.*, 2014) and rice (Zhang *et al.*, 2014). Following three years of development, a comprehensive CRISPR/Cas9 toolkit has been established for precise gene knockout in plants. Notably, the expression of multiplex gRNAs has facilitated the advancement of innovative applications that enhance genetic analysis. This toolkit not only enables the knockout of coding genes but also facilitates the investigation of non-coding elements, which have posed challenges in plant genetic analysis (Figure 5).

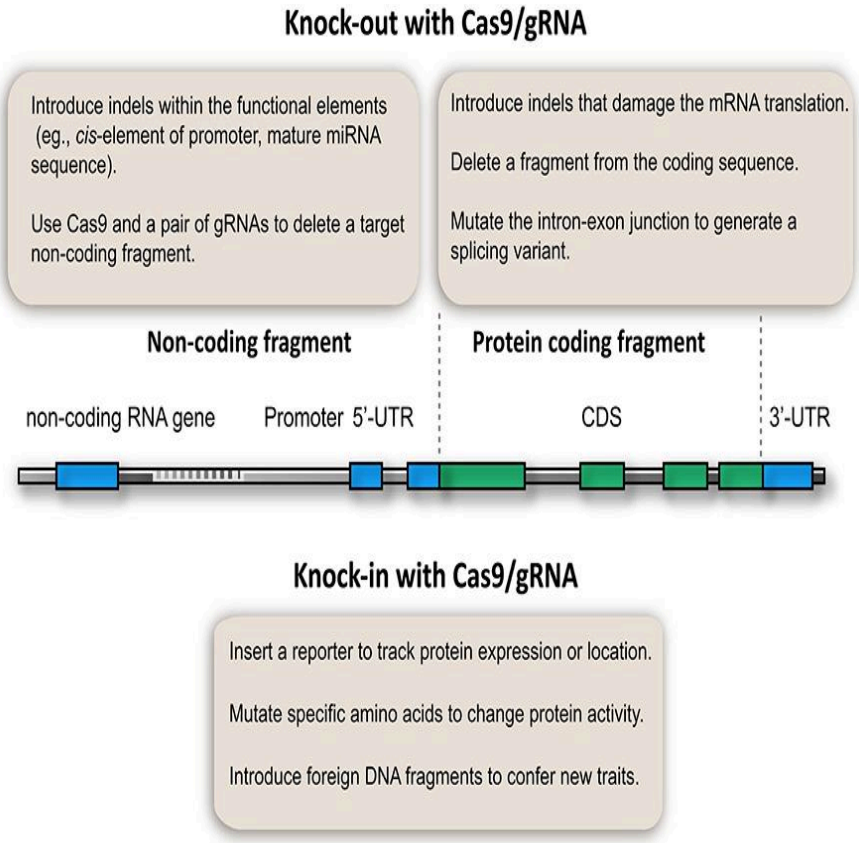

Figure 5: Potential applications of CRISPR/Cas9 genome editing in plant genetic analysis.

For instance, disruption of microRNA genes can be achieved by inducing InDels at the mature sequence if a PAM is present, thereby interfering with the function of the mature microRNA. Alternatively, Cas9 coupled with multiple gRNAs can be employed to delete short microRNA fragments. Consequently, microRNA genes and other non-coding elements (such as cis-elements of promoters, enhancers, and transposons) can be effectively knocked out. This proposed methodology is substantiated by the ability of the Cas9/gRNA system to generate stable plant lines harboring deletions of chromosomal fragments (Ma *et al.*, 2015; Zhou *et al.*, 2015).

Many quantitative trait loci (QTL) and genome-wide association studies (GWAS) conducted in both plant and animal species have unveiled a multitude of genetic alterations responsible for evolutionary shifts, domestication processes, and breeding improvements

predominantly located within cis-regulatory regions (Rodríguez-Leal *et al.*, 2017). In contrast to mutations occurring within coding sequences, which can lead to significant alterations in protein structure, cis-regulatory variants often exhibit reduced pleiotropy and tend to induce subtle changes in phenotype by influencing the timing, pattern, or magnitude of gene expression (Wittkopp and Kalay, 2012). One significant factor contributing to this phenomenon is the intricate nature of transcriptional regulation, characterized by redundancy and modular organization within the numerous cis-regulatory elements (CREs) present in promoters and other regulatory regions. However, the majority of these elements are still not fully understood or characterized (Priest, Filichkin and Mockler, 2009). The intricacy is further enhanced by CRE spacing, interactions between chromosomes, epistasis and the interplay among modules. While these factors offer room for adaptability in processes (Shrawat *et al.*, 2008). They can also pose challenges when anticipating the effects of genetic mutations, within cis regulatory regions (Wittkopp and Kalay, 2012).

Despite being widely utilized in the processes of plant and animal evolution as well as domestication, cis-regulatory variants remain largely untapped and offer potential for increasing allelic diversity in breeding programs. The limited availability of cis-regulatory alleles has also hindered a comprehensive understanding of the impact of regulatory changes on quantitative traits. This includes investigating whether modifications in gene-regulatory landscapes lead to linear or non-linear associations between transcriptional activity and phenotypic variation, and how such responses differ across various genes (Birchler and Veitia, 2012). Therefore, the expansion of cis-regulatory variation not only holds promise for enhancing crop improvement efforts but also for unraveling fundamental principles governing the regulation of quantitative traits. Genome editing provides a potent method for generating new allelic variations (Doudna and Charpentier, 2014; Hsu, Lander and Zhang, 2014). In the realm of plant science, this technology has predominantly been employed to induce mutations within coding sequences, aiming to produce null alleles for subsequent functional investigations (Belhaj *et al.*, 2015).

Building upon prior research, it has been studied that by integrating various components of CRISPR/Cas9 technology, it could be feasible to engineer a wide array of cis-regulatory mutations, each exhibiting distinct types and levels of regulatory alterations (Čermák *et al.*, 2017). Mutations occurring in cis-regulatory regions play a significant role in evolution, not only

due to their subtle phenotypic impacts but also because they tend to have fewer pleiotropic effects compared to mutations in protein-coding regions (Stern, 2000; Galli, Feng and Gallavotti, 2020). Genome editing holds promise for enhancing agricultural traits in numerous crops, as demonstrated in previous research where CRISPR-Cas9 mutagenesis of promoters was employed to engineer quantitative trait variation (Rodríguez-Leal *et al.*, 2017). By targeting conserved non-coding sequences (NCSs), this strategy allows for the simultaneous delineation, allocation, and fine-tuning of the functions of pleiotropic genes. The precise editing of cis-regulatory regions to mitigate pleiotropy could unlock a wealth of new genes and variants with potential applications in agriculture.

5.7. Promoter activity reporters utilizing fluorescent proteins

In order to fulfill their roles effectively, both endogenous and foreign proteins need to be directed to specific locations within or outside the cell. One approach to track a protein of interest in living organisms involves fusing it with a fluorescent protein (FP) that has the ability to absorb and emit light at distinct wavelengths. Fluorescent reporter genes serve as valuable tools for biological imaging purposes. Among the commonly utilized fluorescent reporter genes are green fluorescent protein (GFP) and red fluorescent protein (RFP) (Li *et al.*, 2018).

The initial discovery of a green fluorescent protein (AvGFP) stemmed from research on the *Aequorea victoria* jellyfish, whereas the first red fluorescent protein (DsRed) was identified through genetic analysis of a coral species known as Discosoma (Shimomura, Johnson and Saiga, 1962; Prasher *et al.*, 1992; Matz *et al.*, 1999). Certain characteristics of wild-type fluorescent proteins (FPs) pose challenges to their effectiveness as markers in transgenic organisms. For instance, their absorption spectra are intricate and wide-ranging, they operate as multimers, and their ideal maturation temperature is considerably lower than that of mammalian cells (Baird, Zacharias and Tsien, 2000). Visualizing plant tissues presents distinctive difficulties due to the presence of autofluorescent substances (such as chlorophyll, flavonoids, alkaloids, and tannins) and structures (including chloroplasts, cell walls, and cuticles) (García-Plazaola *et al.*, 2015). Visualizing fluorescent proteins (FPs) within the apoplast, encompassing cell walls and intercellular spaces, presents notable hurdles. Aside from autofluorescence, the pH of the

apoplast is frequently acidic (e.g., around 5.4 in meristematic cells of Arabidopsis roots) and exhibits substantial variability (ranging from pH 3.5 to 8.3), depending on factors such as tissue type, species, growth conditions, or responses to biotic or abiotic stressors (Barbez *et al.*, 2017).

The fluorescence emitted by Green Fluorescent Protein (GFP) in tomatoes has been extensively investigated for diverse applications (Wieczorek *et al.*, 2020). Notably, studies have demonstrated the successful creation of genetically stable infectious clones of tomato torrado virus, incorporating sGFP, which enables the visualization of virus infection in vivo. Additionally, fluorescence imaging has been employed to track tomato freshness during storage, revealing distinct fluorescence patterns across different parts of the tomato, facilitating the classification of freshness (Nurulhuda *et al.*, 2021). Moreover, the induction of chlorophyll fluorescence has been proposed as a method for evaluating tomato maturity, with fluorescence parameters decreasing as fruit maturation progresses (Abdelhamid *et al.*, 2020). Furthermore, GFP fluorescence intensity has been utilized as a dependable indicator for screening transgenic potato plants, exhibiting a positive correlation with the content of target mRNA (Yuorieva *et al.*, 2018). In essence, the analysis of GFP fluorescence in tomatoes serves as a valuable tool for investigating virus infections, monitoring freshness, assessing maturity, and screening transgenic plants.

The emission spectra of GFP variants such as YFP and CFP are closely similar, posing a challenge in visually distinguishing between them using readily accessible imaging systems (Feng *et al.*, 2000). Moreover, the establishment of reporter strains often necessitates a double reporter system, prompting the need for easily distinguishable, spectrally unique colors, such as red. In recent years, several red fluorescent proteins (RFPs) emitting orange, red, and far-red fluorescence have been identified from anthozoans (corals), offering versatile options for various biological applications (Chudakov *et al.*, 2010; Stepanenko *et al.*, 2011). The first red fluorescent protein (RFP) discovered from *Discocoma* sp. was named DsRed1 (Baird, Zacharias and Tsien, 2000). Due to its slow maturation times and inadequate solubility, enhancements were introduced to DsRed1, resulting in the creation of the mutant DsRed S197Y (Verkhusha *et al.*, 2001). An enhanced version of DsRed, termed DsRed.T3, was developed *via* random mutagenesis techniques (Bevis and Glick, 2002). Following the observation that DsRed.T3 can aggregate, a series of monomeric red fluorescent proteins (RFPs) were subsequently developed (Campbell *et*

al., 2002). Campbell et al. (2002) pioneered the creation of the first true monomeric RFP, known as monomeric RFP 1 (mRFP1). This variant was later employed to investigate the expression of native mRFP1 in ES cells and its transmission through the germline (Long, Lackan and Hadjantonakis, 2005). Their findings revealed that mRFP1 expression across various tissues is compatible with normal development and fertility in transgenic mice expressing mRFP1. Presently, numerous monomeric RFPs derived from DsRed or other fluorescent proteins are available and widely utilized in biological research and transgenic reporter strains (Chudakov *et al.*, 2010; Stepanenko *et al.*, 2011). Among these, mCherry and tandem dimer Tomato (tdTomato) are notable examples. mCherry, which is brighter, matures more rapidly, and exhibits higher photostability compared to mRFP1, has already been utilized to generate ubiquitous mCherry transgenic reporter lines (Armstrong *et al.*, 2010).

5.8. *Agrobacterium*-mediated genetic transformation of tomato

The genetic transformation of plant cells by *Agrobacterium* involves the transfer from the bacterium and integration into the plant nuclear genome of a segment originating from a large tumor-inducing (Ti) or rhizogenic (Ri) plasmid present in Agrobacterium. Ti plasmids are on the order of 200 to 800 kbp in size (De Vos *et al.*, 1981). *Agrobacterium*-mediated genetic transformation of plants presents a compelling alternative to direct DNA delivery techniques. The method offers several advantages, including the insertion of a distinct DNA segment into the recipient genome at a low copy number. This stands in contrast to the intricate rearrangements and multiple integration events often associated with biolistic DNA delivery methods (Peng *et al.*, 1995). Advancements in tissue culture techniques and the development of more efficient T-DNA vectors have expanded the applicability of *Agrobacterium*-mediated DNA delivery to certain cereal species. Rice and maize have been successfully transformed using this method, as evidenced by various studies. More recently, barley and wheat have also been reported to undergo stable transformation through *Agrobacterium*-mediated T-DNA delivery, indicating the widening scope of this approach in cereal crop genetic engineering (Cheng *et al.*, 1997; Tingay *et al.*, 1997). *A. tumefaciens*-mediated transformation offers notable benefits compared to naked DNA delivery, such as the insertion of a limited number of gene copies into the plant genome, efficient co-expression of introduced genes, and straightforward manipulation *in vitro*.

Agrobacterium tumefaciens-mediated transformation is commonly used in tomato research for gene overexpression, silencing, and genome editing, utilizing selectable marker genes for resistance to various compounds (Van Eck, Keen and Tjahjadi, 2019). The tomato plant has been employed as a model organism for *Agrobacterium*-mediated transformation due to its unique characteristics, such as its relatively small and compact genome size (Carvalho *et al.*, 2011). Therefore, genetic transformation is an essential process for enhancing plants, and significant efforts have been dedicated to advancing the genetics of the tomato plant. Nearly three decades ago, the first transgenic tomato plant was generated using the *A. tumefaciens*-mediated transformation technique (Horsch *et al.*, 1985). Genetic modification of tomato plants has been utilized for a range of objectives, such as enhancing tolerance to various biotic and abiotic stresses (Jia *et al.*, 2002), as well as for identification, characterization, and synthesis of foreign proteins (Goel *et al.*, 2010; Khare *et al.*, 2010). Nonetheless, the absence of an effective transformation system to many tomato varieties present a significant hurdle to the advancement of genetically modified tomatoes (Velcheva *et al.*, 2005). The reported transformation efficiency in tomatoes ranged from 1.4% to 34% (Chyi and Phillips, 1987; Pozueta-Romero *et al.*, 2001). Various factors have been identified as significant contributors to the efficiency of tomato transformation, including *Agrobacterium* cell density, conditions for regeneration and co-cultivation (Frary and Earle, 1996; Costa *et al.*, 2000), acetosyringone presence, cell competence following injury (Davis, Miller and Lineberger, 1991), and the nature of gene constructs (Krasnyanski *et al.*, 2001). Optimization of kanamycin concentration during different stages of shoot initiation, elongation, and rooting of tomato regenerants has been reported to enhance transformation efficiency (Hu and Phillips, 2001). Pozueta-Romero et al. achieved successful regeneration of tomatoes post-Agrobacterium transformation on kanamycin selection media; however, only 1.4% of these regenerated plants were confirmed as transgenic (Pozueta-Romero *et al.*, 2001). Increasing the contact area between the explant surface and the selective media was found to increase selection efficiency, resulting in 48% of transformed meristems.

6. MATERIALS AND METHODS

6.1. Plant growth conditions

In order to perform the agrobacterium mediated transformation in tomatoes we need to grow tomato plants under specific plant growth conditions necessary for the procedure. Eighty seeds of *Solanum lycopersicum* cv. Moneymaker were counted on a clean paper and kept in a 50 ml greiner. Each seed went through the sterilization process under the laminar air flow. Seeds were washed with 70% Ethanol for 5 minutes then rinsed thoroughly with sterile deionised water (milliQ water). Then seeds we shaked in a 50% sodium hypochlorite solution at 100 rpm on the shaker for 20 minutes. All the seeds were washed more than 5 times with sterile deionised water until there is no foam observed. Then we put all the seeds on sterilized paper and they were sown (16 seeds per jar) in 5 jars with MS media (500 ml prepared earlier with 3g plant agar, 2.2g of MS vitamins, 5g of saccharose), each jar containing 100 ml of MS Media. All jars were sealed with breathable tape and kept in a dark and cold room (~ 5 °C) for at least 3 weeks for synchronized or even seed germination. Then after 3 weeks jars were shifted to the light room where seeds were grown under long day conditions (16 h light/8h dark) at 21 °C temperature and 80 ± 5% of relative humidity. After one week in the light room tomato seedlings were observed around 13 cm in height with a lot of fresh green cotyledons, which were used for agrobacterium mediated transformation.

6.2. Plant Genomic DNA Extraction

Protocol according to (Li *et al.*, 2013) was followed to obtain high quality plant genomic DNA. To prepare genomic DNA suitable for PCR, a 1.2 mL extraction buffer was formulated for 4 samples (two from each Wild type and mutant A120 plant leaves) by using Extraction Buffer (constituted of 100 mM Tris-HCI pH 8.0, 1 M NaCl, 50 mM EDTA, 1.25% SDS, 1% β-mercaptoethanol 10 mL: 4.65 mL water, 1 mL 1 M Tris-HCI pH 8.0, 2 mL 5 M NaCl, 1 mL 0.5 M EDTA, 1.25 mL 10%), SDS, 100 µL β-mercaptoethanol Sol III (5 M K-acetate pH 5.2), Isopropanol, 70% ethanol, 0.1× TE buffer pH 8.0 (1 mM Tris-HCI pH 8.0, 0.1 mM EDTA)

Approximately 0.5 cm² of leaf tissue was transferred into a 1.5 mL Eppendorf tube and added 300 µL of extraction buffer (EB), then homogenized the mixture using a pestle. Add 150 µL of Sol III to the homogenized sample and mix thoroughly, followed by centrifugation at maximum speed for 5 minutes to pellet the cellular debris. Transfer approximately 300 µL of the clear supernatant to a new tube, then add 200 µL of isopropanol and mix well to precipitate the DNA. Centrifuge the tubes again at maximum speed for 5 minutes to pellet the DNA, and carefully pour off the supernatant before placing the tubes upside down on a paper towel to remove excess liquid. Add 1 mL of 70% ethanol to the pellet and vortex thoroughly until detachment, then centrifuge the tubes again at maximum speed for 5 minutes to pellet the DNA. Pour off the supernatant once more, place the tubes upside down on a paper towel to remove excess liquid, and dry the DNA pellets in a speedvac for 10 minutes. Finally, resuspend the DNA pellets in 150 µL of 0.1× TE buffer for downstream applications, using 6 µL of the resuspended DNA in a 50 µL PCR reaction as required.

6.3. Modular cloning of Promoter gene constructs

To create modular clones of promoter reporter gene constructs we followed the protocol as described by Rodríguez-Leal et al. (Rodríguez-Leal *et al.*, 2017) by using modular cloning and the principle of golden gate assembly. We started from amplification of the promoter and 3'-UTRs from the WT (D2-Pro) and A120 mutant (DNA was extracted previously by V3 protocol from leaves of Wild type and A120 mutant plants were used as templates of DNA in this amplification process. They were checked using gel imaging as well). For amplification we setup the PCR based on 6 samples of reporter modules with these combinations as: Forward primer, Reverse primer, Promoter:

I. D2_pro_BsaI_F(TGGTCTCTGGAGATAAAAAGTACTATAAATTTCAATTTTTTACTTATC), D2_5utr_BsaI_R(TGGTCTCTCCATTCTGCAGTTCTGCAAACTGG), D2-promoter (4009 bp)
II. D2_pro_BsaI_F(TGGTCTCTGGAGATAAAAAGTACTATAAATTTCAATTTTTTACTTATC), D2_5utr_BsaI_R(TGGTCTCTCCATTCTGCAGTTCTGCAAACTGG) , A120-promoter (1905 bp)

III. D2_3utr_BsaI_F(TGGTCTCTGTAAACATAGCTACCCAAGTACTC), D2_3utr_BsaI_R(TGGTCTCTAGCGTATGTGTAGCAAAGAACTTATAAT), D2 3'-UTR (443 bp)

IV. GFP_BsaI_F2(TGGTCTCTATGGGAAGTAAAGGAGAAGAACTTTTCAC), GFP_BsaIdom_R(TGGTCTCaTCTCTCTTTTCGTTGGGATCTTTCG), GFP plasmid (666 bp)

V. GFP_BsaIdom_F(TGGTCTCTGAGAtCACATGGTCCTTCTTGAGTTTG), GFP_BsaI_R2(TGGTCTCTTTACTATTTGTATAGTTCATCCATGCCAT), GFP (93 bp)

VI. SlDML2_M9_F_BsaI(TGGTCTCCATGGTGAGCAAGGGCGAG), SlDML2_M9_R_BsaI(TGGTCTCTTTACTTGTACAGCTCGTCCA), mCherry (727 bp)

Reaction compositions for PCR are as follows: Final volume was 50 µL (25 µL 2x Phusion master mix, 16 µL H2O, 3 µL MgCl2, 2.5 µL Forward primer, 2.5 µL Reverse primer, 1 µL DNA).

The temperature program of PCR for module III, IV, V to amplify the promoter and 3'-UTRs from the WT (D2-Pro) and GFP is described in Table 1.

Table 1. The PCR temperature program for amplifying the promoter and 3'-UTRs from D2-Pro and A120 (Module III, IV, V)(09h53m04s)

Step	Temperature	Time	Number of cycles
Initial Denaturation	98 °C	30 s	1
Denaturation	98 °C	10 s	35
Primer Annealing	60 °C	30 s	
Extension	72 °C	2.5 min	
Final Extension	72 °C	10 min	1
Hold	12 °C	∞	1

The temperature program of PCR for module I, II to amplify the promoter and 5'-UTRs from the WT (D2-Pro) and A120 mutant is described in Table 2.

Table 2. The temperature program of PCR for module I and II.

Step	Temperature	Time	Number of cycles
Initial Denaturation	98 °C	30 s	1
Denaturation	98 °C	10 s	10
Primer Annealing	65 °C -0.5 °C/cycle	30 s	
Extension	65 °C	5 min	
Denaturation	98 °C	10 s	30
Primer Annealing	60 °C	30 s	
Extension	65 °C	5 min	
Final Extension	65 °C	20 min	1
Hold	12 °C	∞	1

The PCR temperature program of amplification for module VI (mCherry) is described in Table 3.

Table 3. The temperature program of PCR for module VI (mCherry).

Step	Temperature	Time	Number of cycles
Initial Denaturation	98 °C	30 s	1
Denaturation	98 °C	10 s	35
Primer Annealing	60 °C	10 s	
Extension	72 °C	10 s	
Final Extension	72 °C	30 s	1
Hold	12 °C	∞	1

PCR cleanup/Gel extraction/DNA purification:

To remove polymerases, primers, dNTPs from our DNA we performed DNA purification. We added 9 µL of DNA dye buffer (green buffer) to all samples (6 samples). 1 % agarose gel (0.3 g Agarose gel, 0.5 µL EtBr, 30 ml TBE) was prepared and all samples were loaded in the wells. After 20 minutes of gel electrophoresis we cut the DNA fragments on a UV box at 365 nm and put every fragment into separate columns. We weighed empty columns prior fragment cutting and after adding DNA fragments to calculate the amount of DNA in the column. Centrifuge for 10 minutes, remove the tubes and check the volume. To make it 100 µL we added 40 µL of water to the first four tubes and 80 µL of water to the last two tubes. Add sodium acetate (pH 5.2) of 20 µL each, 80 µL of Isopropanol and mix thoroughly. Then we centrifuge for 10 minutes, throw the fluid and wash with 70 % ethanol (1 ml each). Centrifuge again for 5 minutes then throw the fluid and dry out the tubes in the vacuum for 10 minutes. Atlast add 150 µL of 0.1x TBE to resuspend the pellets.

After obtaining PCR products of 6 reporter modules we started cloning of PCR products into pJET vector using the golden gate assembly method where it employs Type IIS restriction enzymes, which cleave DNA outside of their recognition sites. Because these recognition sites are non-palindromic, the precise cut site depends on the orientation of the recognition sequence. These enzymes have the ability to cut any sequence, enabling seamless fusion of DNA fragments. The cutting and joining of fragments occur simultaneously in the T4 DNA ligase buffer. When the recognition sites are positioned correctly, after cleavage and ligation to another fragment with compatible ends, the original recognition sites are no longer present. Concentration of the pJET vector (pICH86966::AtU6p::sgRNA_PDS) used was 99.5 ng/µL or 23.3 nM. Before starting the ligation reaction we need to dilute our reporter modules and composition for dilution is described in Table 4.

Table 4. Composition for dilution of reporter modules.

Reporter module Samples	Molar concentration (nM)	Plasmid (µL)	Water (µL)
D2-Pro_WT_1.1	331.2	3.0	7.0
D2-Pro_WT_1.2	530.6	1.9	8.1
D2-Pro_A120_2.1	380.4	2.6	7.4
D2-Pro_A120_2.2	246.4	4.1	5.9
D2-3'UTR_3.1	493.3	2.0	8.0
D2-3'UTR_3.2	412.4	2.4	7.6
GFP-dom_R_4	579.8	1.7	8.3
GFP-dom_F_5	563.4	1.8	8.2
mCherry_6.1	777	1.3	8.7
mCherry_6.2	612.5	1.6	8.4

DNA Ligation:

For cloning we performed DNA ligation where we joined pJET vector and DNA fragments and later on will proceed with agrobacterium transformation. Reaction recipe for DNA ligation is as follows: Total Volume was 20 µL containing 10 µL of TG DNA ligase buffer, 1 µL of pJET vector, 8 µL of DNA fragments (6 different DNA fragments prepared before), 1 µL of TG DNA ligase. Reaction was prepared in separate 6 eppendorf tubes and kept at room temperature for 30 minutes for successful ligation.

Transformation:

LB Media and plates were prepared for the bacterial transformation process. 1L LB (Luria Broth) containing 10 g of triptone, 5g of YEB (Yeast Extract Broth) which will serve as a medium for preparing competent cells for bacterial transformation, 10 g of NaCl, 900 ml of

water and pH will be set to 7.0 with 1N NaOH. 500 ml of LB media was used containing 7.5 g of Agar and cooked for 15 minutes in cooker and 500 ml of Ampicillin was added after cooling down the media. Then 18 plates were filled with this media containing ampicillin and was stored in a cold room. To grow colonies we will use our ligated products on ampicillin LB plates and keep them overnight at 37 °C and next colonies will be collected and inoculated in LB liquid media and keep it shaker overnight at 37 °C. Next day we used inoculated samples and performed plasmid miniprep that allows us to isolate plasmid DNA from bacterial cultures.

Plasmid miniprep:

First, transfer 2 mL of the 5 mL overnight (ON) culture into a 2 mL tube and centrifuge at 8000 rpm for 5 min. After centrifugation, pour off the liquid and repeat the centrifugation step. Then, pour off the remaining liquid and place the tube upside down on a paper towel to drain any residual liquid. Next, resuspend the pelleted cells in 200 µL Sol I by gently grating using a rack. Following that, add 400 µL Sol II and mix thoroughly until the suspension becomes transparent with a slimy bottom. Briefly spin the tubes to collect the slimy material and add 300 µL Sol III, mixing gently by shaking. Incubate the tubes on ice for 15 min before centrifuging at maximum speed for 5 min. Transfer approximately 900 µL of the clear supernatant into a new 1.5 mL tube and add 600 µL isopropanol, mixing well by inversion and incubating at room temperature for 5 min. Centrifuge again at maximum speed for 5 min to pellet the DNA, then carefully pour off the supernatant and drain any residual liquid. Add 1 mL 70% cold ethanol to the pellet and vortex until detached. Briefly spin down the tubes and carefully pour off the supernatant, repeating the washing step once more. Remove any remaining liquid and dry the pellets in a Speedvac for 10 min. Finally, resuspend the pellet in 50 µL 0.1 TE pH 7.5 or 8.0, or Milli-Q water. Prepare the RNase solution by combining 49 µL TE pH 7.5 and 1 µL RNase A per sample, add 50 µL RNase solution to the resuspended plasmids, and incubate at 37 °C for 1 h. Afterward, add 5 µL 20% SDS to the samples, vortex, then add 50 µL Sol III, vortex, and incubate on ice. Centrifuge at maximum speed for 5 min and transfer approximately 150 µL of the clear supernatant into a new 1.5 mL tube. Add 100 µL isopropanol, mix well, incubate at room temperature for 5 min, and centrifuge at maximum speed for 5 min. Pour off the supernatant, drain the remaining liquid on a paper towel, and repeat the washing step. Spin down the tubes briefly, carefully pour off the

supernatant, remove any remaining liquid, and dry the pellets in a Speedvac for 10 min. Resuspend the pellet in 50 µL 0.1 TE pH 7.5 or 8.0.

We performed test digestion using the BsaI restriction enzyme. Total volume was 10 µL containing 8 µL of water, 1µL of buffer, 0.5 µL of BsaI and 0.5 µL of DNA. Samples were loaded on 1% agarose gel and gel electrophoresis was performed. All the plasmid constructs were analyzed with the presence of insert and plasmid size.

Final constructs (D2pro-GFP, A120pro-GFP) were prepared using Level 1 modules by the Golden gate assembly method. PCR reaction is follows; Total volume of the reaction was 20 µL containing 11.6 µL of water, 2 µL of 10X Ligase buffer, 1 µL of pICH86966 vector, 1 µL of promoter module, 1 µL GFP or mCherry module, 1 µL of 3'UTR module, 1.2 µL of BsaI HF and 1.2 µL of T4-DNA Ligase. Temperature program of PCR for final reporter promoter modules is described in Table 5.

Table 5. Temperature program of Final reporter modules

Step	Temperature	Time	Number of cycles
Digestion and ligation	37 °C	1 h	1
Digestion	55 °C	5 min	1
Denaturation	80 °C	5 min	1
Hold	12 °C	∞	1

6.4. Agrobacterium transformation

The gene constructs were transformed into *Agrobacterium tumefaciens* strain LBA4404 competent cells with the freeze-thaw method (Chen, Nelson and Sherwood, 1994). Agrobacteria were cultivated in YEB medium supplemented with rifampicin (50 µg/mL) and streptomycin (25 µg/mL) overnight at 30 °C with agitation. Subsequently, 2 mL of this initial culture was transferred into 50 mL of YEB medium (without antibiotics) and allowed to grow for four hours

until reaching an OD_{600} of approximately 0.5. The culture was then transferred to 50 mL Falcon tubes, cooled on ice for ten minutes, and centrifuged at 3000 rpm for ten minutes in a refrigerated centrifuge. The resulting cell pellets were resuspended in 1 mL of ice-cold 20 mM $CaCl_2$, and aliquots of 100 µL were dispensed into cold 1.5 mL Eppendorf tubes for the transformation process. For transformation, 1 µg of plasmid DNA was combined with 100 µL of competent cells, flash-frozen in liquid nitrogen for five minutes, thawed at 37 °C for five minutes, and supplemented with 1 mL of YEB medium (without antibiotics). The mixture was then incubated for two hours at 30 °C with agitation at 200 rpm. Subsequently, the cells were harvested by centrifugation at 3000 rpm for ten minutes, 1 mL of the supernatant was discarded, and the cells were resuspended in the remaining solution before being plated onto YEB agar plates supplemented with rifampicin (50 µg/mL), streptomycin (25 µg/mL), and kanamycin (50 µg/mL).

6.5. Tomato transformation

Tomato transformation was conducted according to (Fernandez *et al.*, 2009). Briefly, small segments of cotyledons from *Solanum lycopersicum* cv. Moneymaker were excised and placed onto KCMS plates supplemented with vitamins, hormones, and acetosyringone, and incubated overnight at 25 °C under subdued light. Concurrently, overnight cultures of *Agrobacterium* were prepared in YEB solution containing kanamycin (50 µg/mL), rifampicin (50 µg/mL), and streptomycin (25 µg/mL). These cultures were grown until reaching an OD_{600} of approximately 1, then pelleted, and resuspended in KCMS solution to achieve an OD_{600} of 0.05. The cotyledon pieces were immersed in the *Agrobacterium* suspension and gently agitated for 30 minutes at 25 °C. Subsequently, the explants were extracted, dried on paper filters, and transferred to KCMS plates with the abaxial side facing upwards, with 30–40 explants per plate. Following a two-day dark incubation period at 25 °C, the explants were moved to 2Z plates, a medium containing zeatin riboside (2 µg/mL) and timentin (250 µg/mL), for shoot regeneration. They were grown under long-day conditions (16 hours light at 25 °C, 8 hours dark at 18 °C) for two weeks, with regular transfer to fresh 2Z plates every two weeks. Healthy shoots originating from callus tissues were then isolated and transferred to glass jars containing a root regeneration medium. These plants were cultivated for six weeks under the same light conditions until robust roots developed. Following root formation, the plants were carefully transplanted into soil-filled

pots and maintained in a controlled light environment until genotyping was completed. Finally, the mutants were transferred to the greenhouse for further growth and analysis.

7. RESULTS AND DISCUSSION

7.1. Characterization of the A120 mutant

7.1.1. In vivo mutagenesis of the SlDML2 promoter and isolation of the A120 mutant

A120 which is a functional null mutant was created using the CRISPR/Cas9 genome editing system from Wild type tomato in which 2104 bp were deleted. Eight guide RNAs were designed using the CRISPOR server. These guide RNAs can be spotted at hundreds of base pairs from each other. Lot of mutants were created by our group earlier by creating deletions either in the proximal (those were grouped as A such as, A120) or distal part (grouped as B) of the coding sequence. Figure 6 shows the portion deleted in mutant A120.

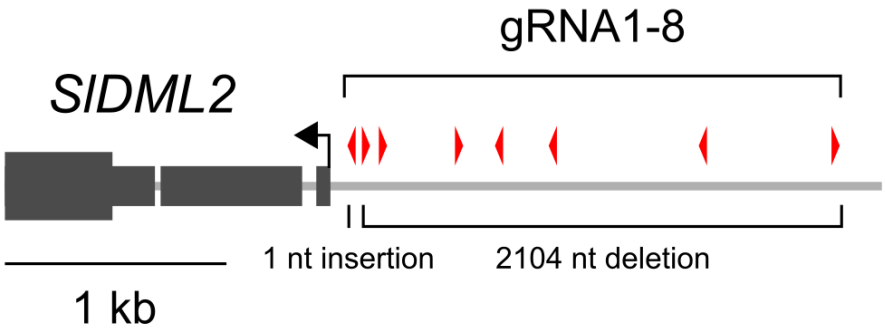

Figure 6. Portion of deletion in mutant A120

This is the illustration of A120 mutant where we can observe gRNAs targeting the SlDML2 promoter. At gRNA 1 there is 1 bp insertion and in between other 7 guide RNAs there is a major deletion of 2104 base pairs.

7.1.2. Phenotype of the A120 mutant

During the initial stages of our project, we cultivated both Wild type and A120 mutant plants under controlled growth conditions to examine their genetic and phenotypic characteristics. We noticed distinct differences between the two. The young leaves of the mutants appeared broader and thicker, with an abundance of white hairs on both surfaces, whereas those of the wild type were narrower, thinner, and had fewer white hairs. The mutants exhibited a

bushy appearance with altered apical dominance, while the wild type plants maintained a regular shape. Additionally, the wild type plants flowered earlier than the mutants. Leaf deformation was evident in the mutants, whereas the leaves of the wild type plants were typically compound in shape. Furthermore, the fruits of the mutants displayed parthenocarpy, resulting in seedlessness and elongated shape, whereas the wild type tomato fruits were round and seeded. Phenotypic comparison between wild type and A120 plants is shown in Figure 7.

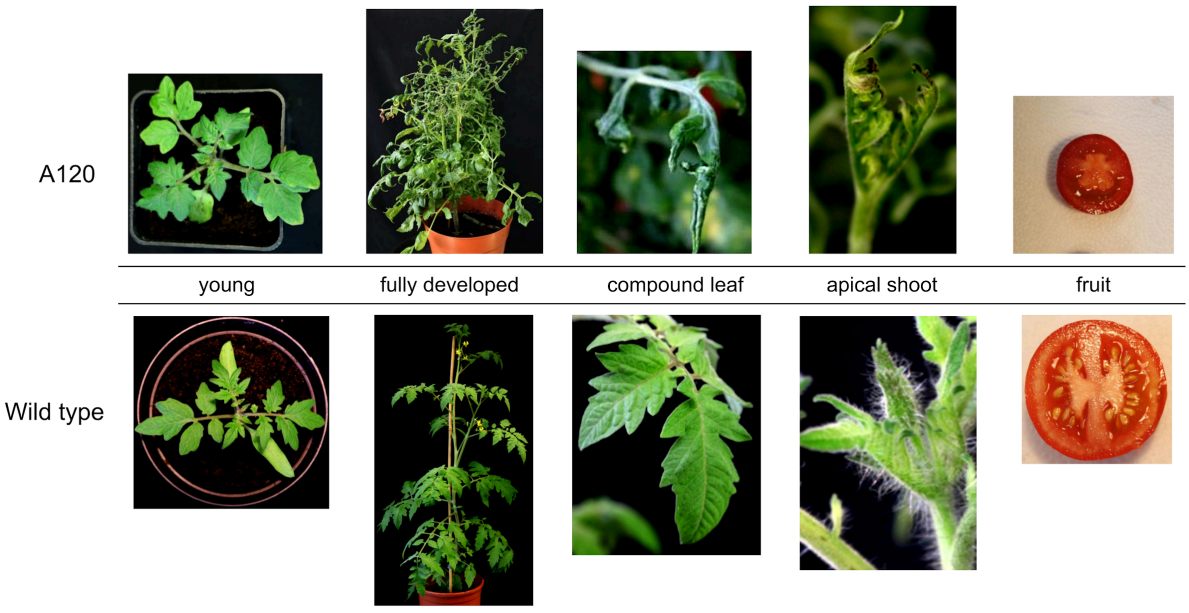

Figure 7. Phenotype of the A120 mutant in comparison with the wild-type plant

7.1.3. Expression of the SlDML2 gene in the A120 mutant

Data on the expression of tomato pericarp across various stages of fruit development and tissues, including samples obtained through laser-capture microdissection, were collected as shown in Figure 8. The analysis focused on tracking the expression levels of SlDML2, a specific gene of interest, in different fruit tissues such as the pericarp, placenta, and seeds, over various time intervals. By examining the expression dynamics of SlDML2 across these specific fruit parts at different developmental stages, researchers aimed to elucidate its potential role in fruit development and ripening processes.

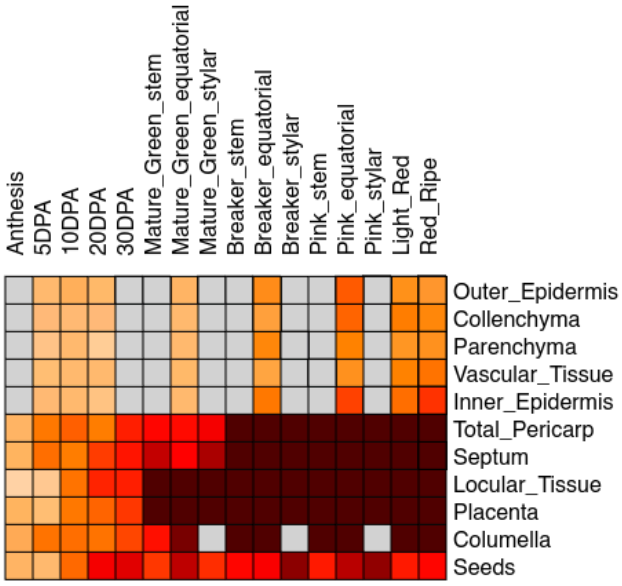

Figure 8. Expression of SlDML2 in different parts of the fruit at different time points. Data were obtained from the Tomato Expression Atlas (https://tea.solgenomics.net/expression_viewer/input)

We measured the expression of SlDML2 in the shoots with RT-qPCR and found that the expression level was lower in the mutant (A120) as shown in Figure 9 . However, this method is not suitable to investigate the spatial and temporal expression in high detail.

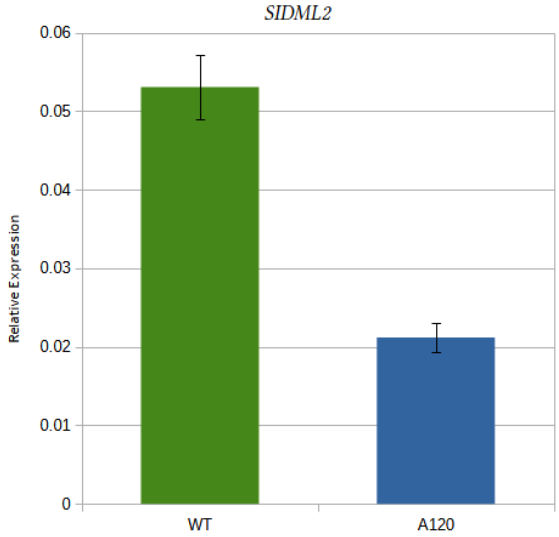

Figure 9. Relative expression calculated from qPCR results in Wild Type (WT) and Mutant (A120) shoots.

7.2. Creating promoter reporter gene constructs

We created two types of promoter reporter gene constructs , a mutant (A120) and a wild-type SlDML2 promoter driven GFP gene construct to investigate the expressional difference of SlDML2 in tomato in a non-invasive way. The constructs contained a 3000 bp (WT) and a 897 bp (mutant) fragment of the promoter upstream of the transcription start site of the SlDML2 gene, a 985 bp 5'-utr of SlDML2, the GFP coding sequence, and a 419 bp 3'-utr of SlDML2. The modules were assembled using the Golden Gate assembly method. This technique employs Type IIS restriction enzymes, which target asymmetric DNA sequences and cleave outside of their recognition sites, typically within 1 to 20 nucleotides. This unique property enables the seamless joining of DNA fragments in a specific orientation. Because the enzyme cleaves outside its recognition sequence, it generates sticky ends based on the orientation of the asymmetric recognition site. If the recognition sites are oriented towards the fragment, they will be lost after cutting and subsequent ligation (refer to Figure 10).

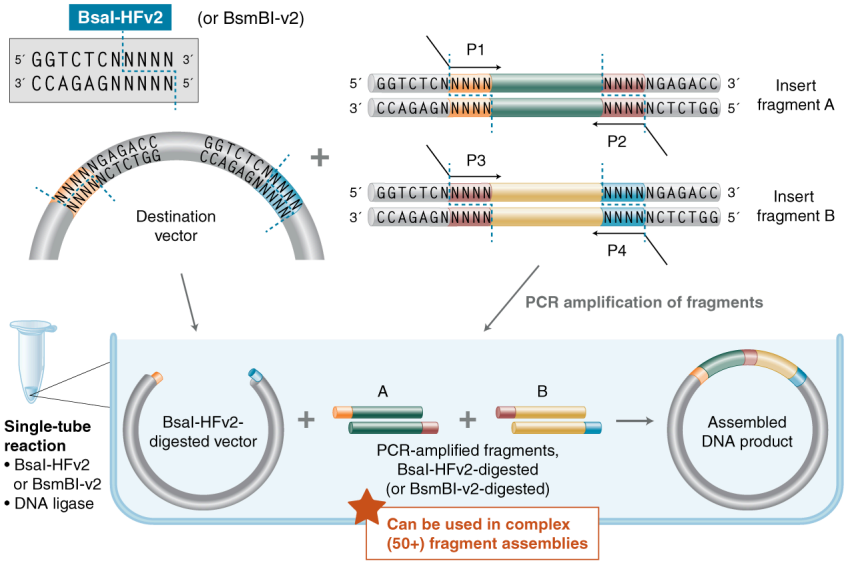

Figure 10. Golden Gate Assembly Workflow (NEBridge Golden Gate Assembly Kit (BsaI-HF v2) | NEB)

Golden Gate Assembly necessitates the incorporation of a Type IIS recognition site, such as BsaI-HFv2 (GGTCTC) or BsmBI-v2 (CGTCTC), at both ends of a double-stranded DNA fragment. Following digestion, these sites remain intact, leaving each fragment with specific 4-base overhangs that guide the assembly process.

Beginning with amplification of the promoter and 3'-UTRs from both wild-type (D2-Pro) and A120 mutant genomic DNA, we utilized the V3 protocol for DNA extraction from young and green leaves of the respective plant types. Gel imaging confirmed the quality of the extracted DNA . DNA extraction image is shown in Figure 11 and Amplification of modules in Figure 12.

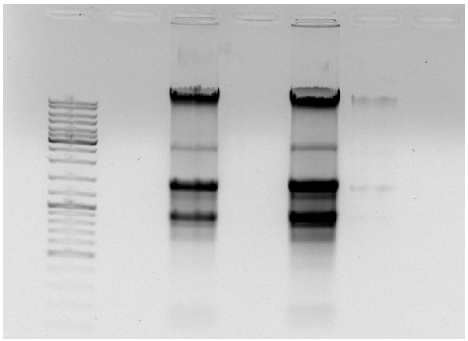

Figure 11. Gel image showing Genomic DNA extracted from WT and A120

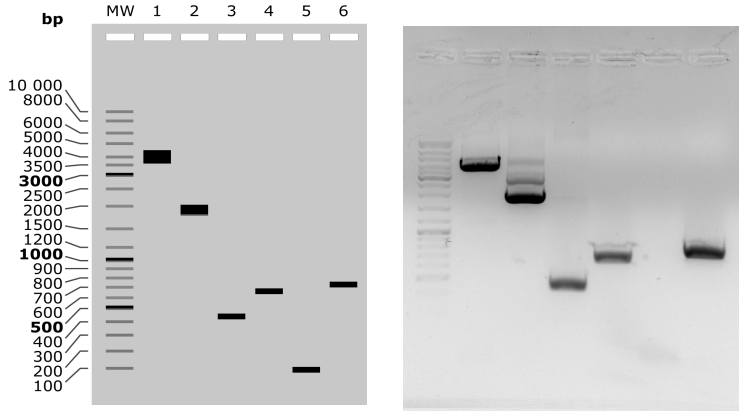

Figure 12. Amplification of 6 reporter modules

A. (left image) Simulated agarose gel image. MW: GeneRuler ladder, 1: D2pro_5utr (4009 bp), 2: A120pro_5utr (1905 bp), 3: D2_3utr (443 bp), 4: GFPdom_PCR1 (666 bp), 5: GFPdom_PCR2 (93 bp), 6: mCherry (727 bp) **B.** (right image) Actual agarose gel image. The sample order is the same as in panel A.

Following PCR amplification, we performed DNA purification, using 1% agarose gel electrophoresis to separate and extract the DNA fragments, which were then subjected to

purification steps involving centrifugation, washing with ethanol, and drying in a vacuum. The resulting purified DNA fragments served as templates for subsequent cloning steps.

Following successful ligation, we diluted the reporter modules and proceeded with DNA ligation, combining the pJET vector with the DNA fragments in separate reactions. After incubation at room temperature, the ligated products were ready for bacterial transformation. For transformation, LB media and plates containing ampicillin were prepared, and colonies were grown overnight at 37°C. The resulting colonies were collected, inoculated in LB liquid media, and subjected to plasmid miniprep to isolate the plasmid DNA from bacterial cultures. The plasmids were digested with EcoRI and one correct plasmid was subjected to Sanger sequencing. According to the sequence analysis, the clone was correct, therefore, it was transformed into Agrobacterium tumefaciens strain LBA4404. Finally, the final constructs were prepared using Level 1 modules in the Golden Gate assembly method, involving a PCR reaction with specific components including the pICH86966 vector, the promoter with 5'-UTR, the GFP/mCherry fragment, and the 3'-UTR modules cloned in the pJET vector as shown in Figure 13. Subsequently, we verified the assembled constructs by testing clones with EcoRI digestion to ensure proper assembly and insert orientation.

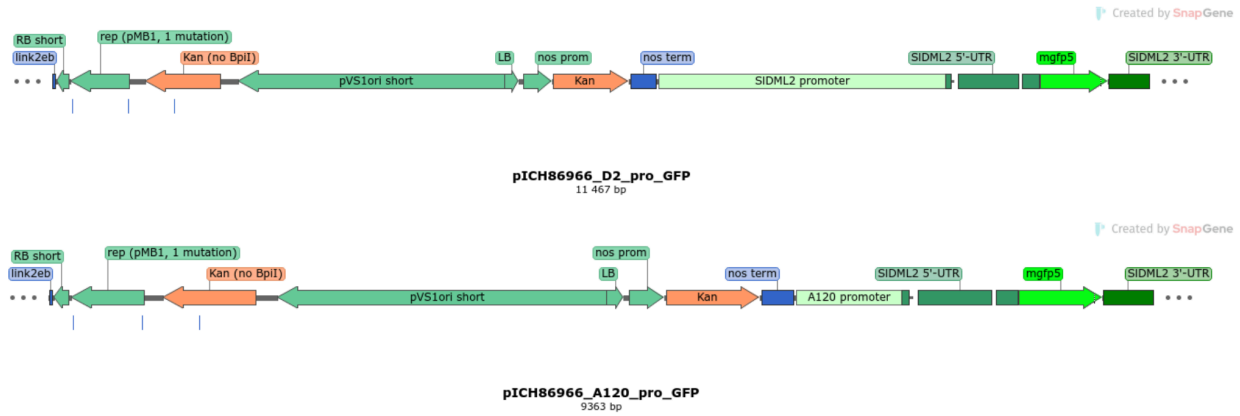

Figure 13. Schematic of Promoter reporter constructs created using SnapGene (https://www.snapgene.com/)

Gel images of the test digestion (simulated and real) of the Level1 modules (D2 and A120 promoters, 3'-UTR, GFP and mCherry in pJET vector) can be seen in Figure 14.

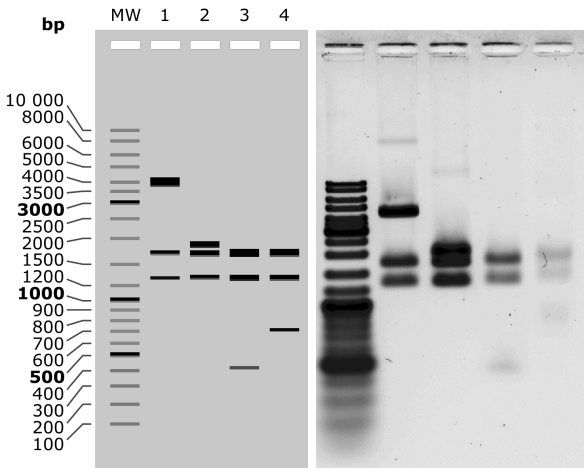

Figure 14. Test digestion of the Level1 modules with *Bsa*I.

A. (left image) Simulated agarose gel image. MW: GeneRuler ladder, 1: pJET D2pro_5utr (3989, 1697, 1297 bp), 2: pJET A120pro-5utr (1885, 1697, 1297 bp), 3: pJET D2_3utr (1697, 1297, 423 bp), 4: pJET GFPdom (1697, 1297, 719 bp). **B.** (right image) Actual agarose gel image.

Final constructs (D2pro-GFP, A120pro-GFP) were verified using test digestion with the restriction enzyme, EcoRI as shown in Figure 15.

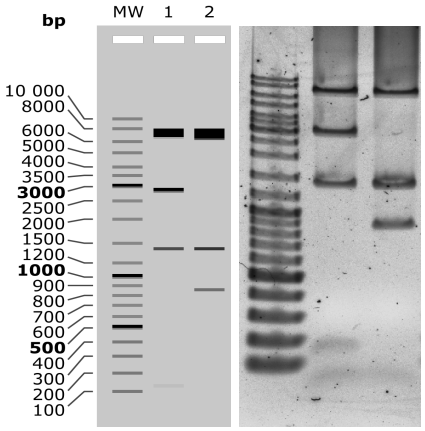

Figure 15. Test digestion of the final constructs with *Eco*RI.

A. (left image) Simulated agarose gel image. MW: GeneRuler ladder, 1: pICH86966 D2pro-GFP (7088, 2835, 1413, 131 bp), 2: pICH86966 A120pro-GFP (7088, 1413, 862 bp). **B**. (right image) Actual agarose gel image.

7.3. Transformation of tomato plants with the promoter reporter gene constructs

Transformation of tomato with the final constructs of promoter reporter modules was the major and prolonged part of this research with the help of which we could deliver gene constructs in a non-invasive way to investigate spatial and temporal expression of DNA DEMETHYLASE 2 or SIDML2 gene in tomato. Our main goal was to deliver this gene through natural agrobacterium mediated transformation and then raise plants from the obtained callus from the successful transformants. Later acclimatizing those plants and investigating through GFP expression analysis using fluorescence microscopy or bioluminescence imaging.

For our transformation we followed the protocol created by (Fernandez *et al.*, 2009) as described in the Materials and methods (Chapter 6.5). Small segments of cotyledons from *Solanum lycopersicum* cv. Moneymaker were obtained from the plantlets (70) grown under certain growth conditions as described in the Materials and methods (Chapter 6.1). Unfortunately our transformation was unsuccessful a couple of times due to the fungal infections which we later on figured out and started our third transformation which will hopefully succeed. We are planning to grow plants from our transformants. Currently, the extent to which transformants can be preserved until callus formation remains uncertain. Please refer to Figure 16.

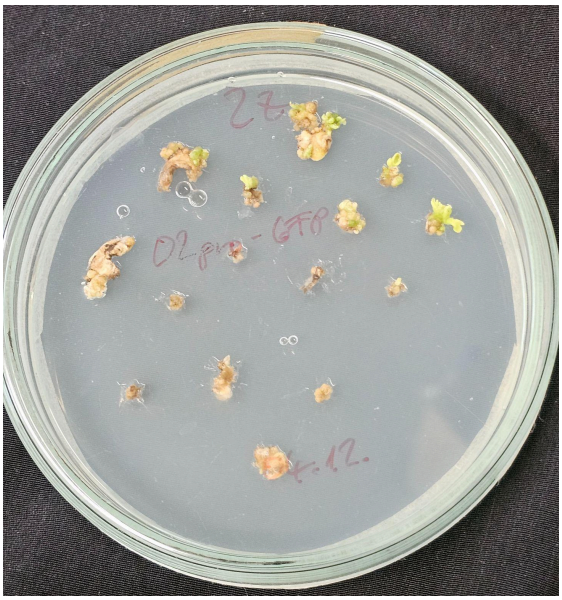

Figure 16. Callus formation in the transformants preserved (D2-proGFP)

8. CONCLUSIONS AND FUTURE PLANS

Our work was divided into four main goals. One of the major goals was to raise wild type and CRISPR mutant (A120) tomato plants and characterize them with their phenotype and genotype. Second major portion of our work was to create promoter reporter gene constructs including GFP and mCherry reporter genes which were successfully assembled using the golden gate assembly method. Third major and prolonged part of our research was to deliver these gene constructs through Agrobacterium mediated transformation in wild tomatoes which was delayed due to some difficulties in the way but now it is under process and we are hoping to complete our transformation in some period of time. After successful tomato transformation we will be able to perform expression analysis in vivo of mutant and wild-type promoter driven GFP and mCherry fluorescence through fluorescence microscopy in different tissues at different developmental stages which will eventually complete our last and fourth major goal of research.

The successful transformation of these constructs opens avenues for in-depth analysis of gene expression patterns, particularly driven by mutant and wild-type promoters, through the visualization of GFP fluorescence in vivo. This approach promises to offer valuable insights into the spatiotemporal dynamics of gene expression and regulatory networks in tomato plants, shedding light on key molecular processes governing plant development, stress responses, and physiological functions.

Furthermore, leveraging the reporter data obtained from the mutant and wild-type promoter-driven GFP fluorescence, comparative analyses of transcriptomes and DNA methylomes are planned. By integrating these datasets, we aim to unravel the intricate interplay between transcriptional regulation and epigenetic modifications in tomato plants. This holistic approach holds immense potential for uncovering novel regulatory pathways, identifying key genes involved in specific biological processes, and unraveling the functional significance of epigenetic modifications in shaping plant phenotypes and responses to environmental stimuli. Ultimately, these findings will contribute to a deeper understanding of plant biology and may inform the development of strategies for crop improvement and agricultural sustainability.

This research was additionally showcased at an international conference through a poster presentation at the VBC symposium 2023: "A Mixtape of Science," hosted at the Vienna

BioCenter (VBC) in Vienna on the 9th and 10th of November 2023. The presentation focused on the comparative analysis of expression patterns between a mutant and wild-type tomato DNA DEMETHYLASE 2 promoters, utilizing reporter gene constructs. This scientific contribution underscored the significance of investigating promoter-driven gene expression dynamics in elucidating the regulatory mechanisms underlying plant development and responses to environmental cues.

9. SUMMARY

Epigenetics refers to heritable changes in gene expression that occur without modification of the underlying DNA sequence. DNA methylation and demethylation are two of the many mechanisms by which epigenetic changes can occur. These changes can affect gene expression and contribute to plant development. DNA demethylation can occur either passively or actively. Active DNA demethylation is regulated by DNA demethylases. There are four DNA demethylases in tomato (SlDML1-4). SIDML2 controls fruit ripening. In line with this, its expression is induced during the breaker stage of ripening. Altering the cis-regulatory elements (promoters) of genes *in vivo* could result in phenotypes different from that of a null mutant. To map the regulatory elements that govern SlDML2 expression, a deletion series was created in the SlDML2 promoter by multiplex CRISPR/Cas9 genome editing.

This research is structured around four major goals. Firstly, wild-type and CRISPR mutant (A120) tomato plants were raised and characterized based on phenotype and genotype. A120 mutants contained a 2104 bp deletion and a 1 bp insertion in the proximal part of the SlDML2 promoter which was shown to be differentially methylated during ripening. The mutant had severe developmental defects that could be a result of the deletion in its promoter. We measured the expression of SlDML2 in the tomato shoots with RT-qPCR and found that the expression level was lower in the mutant. However, this method is not suitable to investigate the spatial and temporal expression in high detail. Therefore, we successfully created two, a mutant and a wild-type SlDML2 promoter driven GFP gene constructs to investigate the expressional difference of SlDML2 in plants in a non-invasive way. These constructs (pICH86966 D2pro-GFP and pICH86966 A120pro-GFP) were assembled using the Golden Gate assembly method which completed our second major goal. Thirdly, despite some delays, efforts are underway to deliver these gene constructs through Agrobacterium-mediated transformation in wild tomatoes.

Finally, upon successful transformation, expression analysis will be conducted in vivo to compare mutant and wild-type promoter-driven GFP and mCherry fluorescence in different tissues and developmental stages. The successful completion of these goals will provide valuable insights into the regulatory mechanisms governing gene expression and epigenetic modifications in tomato plants.

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