Hungarian University of Agriculture and Life Sciences

Szent István Campus

Agricultural Biotechnology

INVESTIGATING THE FUNCTION OF A NOVEL WHEAT SEED-SPECIFIC miRNA BY OVER-EXPRESSION IN TRANSGENIC WHEAT (*Triticum aestivum L.*) LINES.

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1. Table of Contents

2. ABBREVIATIONS 4

3. INTRODUCTION 5

4. LITERATURE 7

4.1 The importance and genetics of wheat 7

4.2 RNA interference (RNAi) 7

4.2.1 PTGS 8

4.2.2 TGS 11

4.3 Wheat transformation 13

4.4 Modification of microRNAs' expression 16

4.4.1 Strategies based on miRNA for crop improvement. 17

4.4.1.1 STTM (Short Tandem Target Mimic) 17

4.4.1.2 CRISPR (clustered regulatory interspaced short palindromic repeats) 19

5. MATERIALS AND METHODS 20

5.1. Plant growth 20

5.2. Culture media 20

5.2.1 Culture media for bacteria 20

5.2.2. Culture media for wheat 21

5.3. Bacterial Strains 22

5.4 Wheat cultivar 23

5.5 Plasmid Construction 23

5.5.1 Digestion of plasmid and PCR fragments 25

5.5.2 Ligation of PCR fragment into pC61K binary vector 25

5.5.3 Structure of PcubiGRF4:GIF1_NOS vector 26

5.6 Transformation of *E. Coli* 26

5.7. Plasmid miniprep 27

5.8. Transformation of *Agrobacterium* with pC61K_Pre2187A/B/D vector 27
5.9. DNA Extraction 28
5.10. RNA Extraction 28
5.11. PCR amplification 29
5.12. Gel-Electrophoresis 31
5.13. Small RNA northern hybridization 31
5.14. Wheat transformation 32
5.14.1. Preparation of Agrobacterium for transformation 32
5.14.2. Embryo Collection, Sterilization, and Embryo inoculation 33
5.14.3. Collection and sterilization of immature seeds 33
5.14.4. Isolation of immature embryos, inoculation with Agrobacterium, and co-cultivation 33
5.14.5 Resting period, callus induction and selection of transformed material 34
5.14.6. Regeneration, Rooting, and acclimatization 34
6. RESULTS 36
6.1 PCR amplification of precursors 38
6.2 Cloning of precursors into PJET1.2 plasmid 39
6.2 Cloning of precursors into binary vector pC61K 40
6.3 wheat transformation and transgene analysis 43
7. DISCUSSION AND RECOMMENDATIONS 46
8. SUMMARY 48
9. ACKNOWLEDGMENT 50
10. REFERENCES 51

2. ABBREVIATIONS

- miRNA (microRNA)
- RNAi (RNA interference)
- dsRNA (double-stranded RNAs)
- ssRNA (single-stranded RNAs)
- miRISC (miRNA-induced silencing complex)
- pri miRNAs (primary miRNAs)
- RISCs (RNA-induced silencing complexes)
- RdDM (RNA-directed DNA methylation)
- PolII (Polymerase II)
- PTGS (post-transcriptional gene silencing)
- TGS (transcriptional gene silencing)
- AGO (ARGONAUTE)
- CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)
- siRNA (small interfering RNA)
- DCL (Dicer-like proteins)
- mRNA (messenger RNA)
- WIM (wheat inoculation medium)
- WRM (wheat regeneration medium)
- AS (acetosyringone)
- cDNA (complementary DNA)
- Pol (polymerase II)
- RGENs (RNA-guided endonucleases)
- mRNA (messenger RNA)
- RDRs (RNA-dependent RNA polymerases)
- FHB (Fusarium head blight)
- TFs (Transcription factors)
- NRP (nuclear RNA polymerase)
- RNA-seq (RNA sequencing)

3. INTRODUCTION

microRNAs (miRNAs) are small noncoding RNAs crucial for regulating gene expression by targeting specific messenger RNA (mRNA) molecules. In plants, miRNAs are transcribed by RNA Pol II as primary miRNAs (pri-miRNAs). Upon transcription they are processed into mature forms by DICER-LIKE proteins, and these mature miRNAs join with ARGONAUTE (AGO) proteins to form RNA-induced silencing complexes (RISCs), once assembled, they play a crucial role in controlling various aspects of plant development and environmental interactions. Mutations affecting miRNA transcription or processing often result in broad-ranging effects on plant morphology and function, highlighting their importance in coordinating plant development (Dong et al., 2022).

Since posttranscriptional gene silencing (PTGS) and transcriptional gene silencing (TGS), are integral components of RNA interference (RNAi), small RNAs induce gene silencing through various mechanisms. Within the cytoplasm, these small RNAs facilitate PTGS by targeting complementary mRNA molecules, leading to their degradation or translational repression. Conversely, in the nucleus, small RNAs arrange TGS by directing repressive epigenetic modifications, such as DNA cytosine methylation and histone methylation, to homologous genomic regions. In plants, a notable pathway for small RNA-mediated epigenetic modifications is RNA-directed DNA methylation (RdDM). This process involves specialized transcriptional machinery centered around two plant-specific RNA polymerase II (Pol II)-related enzymes: Pol IV and Pol V. While Pol II, Pol IV, and Pol V share several subunits, they also possess specialized subunits designated as nuclear RNA polymerase B (NRPB) for Pol II, NRPD for Pol IV, and NRPE for Pol V. Pol IV transcribes a single-stranded RNA which is copied into a double-stranded RNA by RNA-DEPENDENT RNA POLYMERASE 2 (RDR2). The dsRNA is processed by DICER-LIKE 3 (DCL3) into 24 - nucleotide siRNAs. On the other hand, Pol V transcribes a scaffold RNA that base-pairs with AGO4-bound siRNAs. RNA-DIRECTED DNA METHYLATION 1 (RDM1) links AGO4 and DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), which catalyses de novo methylation of DNA (Matzke & Mosher, 2014).

Identification of microRNAs (miRNAs) serves as the initial step in unraveling their functional roles. Both computational and experimental methods are employed across plant and animal species to achieve this goal. Deep sequencing technology provides insight into the expression levels of each miRNA based on read counts. Given the substantial amount of data

generated by deep sequencing, bioinformatics tools play a critical role in filtering potential miRNAs and predicting their targets based on nucleotide sequence homology (Zhang & Wag, 2015).

With NGS we found a new wheat specific miRNA during small RNA library preparation from grain, based on accession number we call it miRNA2187. PsRNATarget predicted that PolV is a potential target of this miRNA. To prove the target in planta, miRNA2187 overexpression lines were produced.

Objectives

- Design a miRNA overexpression construct to study the function of a novel miRNA to determine its target i.e., Pol V subunit messenger RNA.
- Transform the construct into *Triticum aestivum* L. *cv. Fielder* plants using *Agrobacterium tumefaciens*-mediated transformation method and regenerate transformant plants from callus in the tissue culture.
- Verify overexpression wheat lines with PCR to check the presence of transgenes.
- Check the level of miRNA with small RNA northern hybridization and Pol V subunit messenger RNA with the help of semiquantitative PCR and RTq-PCR.

4. LITERATURE

4.1 The importance and genetics of wheat

The domestication and utilization of wheat have strong connections to humanity's pursuit of food security and control over their food sources. Wheat, including bread wheat (*Triticum aestivum*) and durum wheat (*Triticum durum*), has long been grown in the Western world to provide people with nourishment and energy. Today, China and India are the leading wheat producers globally due to its efficient water usage and its role as a primary ingredient in various processed foods, meeting the demands of modern urban life. Wheat remains a fundamental source of nutrition and sustenance globally (Igrejas & Branlard, 2020). Bread wheat, which is hexaploid and possesses 42 chromosomes derived from its three ancestral genomes and have three sub-genomes A, B and D was initially cultivated in what is now known as the Middle East around 8,000 to 25,000 years ago (Alaux et al., 2018). Due to its hexaploid nature and the presence of numerous repetitive and transposable elements, bread wheat possesses one of the most extensive genomes among crop plants, estimated at around 17 giga base pairs (Gbp). This substantial genome size presents considerable challenges in the fields of genetics, genomics, and breeding (Schilling et al., 2020). Nonetheless, recent progress in sequencing technology has enabled the International Wheat Genome Sequencing Consortium to achieve a top-notch genome assembly and detailed annotation for wheat (Alaux et al., 2018). Extensive RNA sequencing (RNA-seq) studies have offered valuable insights into the expression patterns of homoeologous genes during diverse developmental stages and under various stress conditions. This has resulted in the creation of a valuable resource for conducting more in-depth analyses (Alaux et al., 2018).

4.2 RNA interference (RNAi)

RNA interference (RNAi) is a comprehensive term used to delineate the phenomena of gene silencing instigated by small RNAs. Within plants, RNAi-associated pathways engender various categories of sRNAs, encompassing siRNAs, miRNAs, ta (trans-acting)-siRNAs, and pha (phased)-siRNAs(Rosa et al., 2018). Within the cytoplasm, small RNAs instigate post-transcriptional gene silencing (PTGS) through the targeting of complementary mRNAs for degradation or translational inhibition. Conversely, within the nucleus, small RNAs prompt transcriptional gene silencing (TGS) by guiding repressive epigenetic alterations, such as DNA cytosine methylation and histone methylation, to corresponding segments of the genome. These

epigenetic modifications mediated by Small RNAs are evident in plants, fungi, and metazoans (Matzke & Mosher, 2014). RNA silencing assumes a pivotal role in orchestrating the regulation, endurance, defense, and perpetuation of eukaryotic genomes. It encompasses various mechanisms that consistently rely on fundamental small RNA molecules (sRNAs) to fulfill distinct sequence-specific roles (Vazquez et al., 2010). Ever since its identification by botanical researchers in the latter part of the 1980s, RNA silencing has been acknowledged for its fundamental contributions to developmental processes, stress responses, and safeguarding functions by harmonizing the regulation, defense, durability, and heredity of essentially all eukaryotic genomes. It has been demonstrated to encompass a wide array of mechanisms that execute specialized functions and consistently hinge on small RNAs (sRNAs) measuring 20–40 nucleotides (nt) in length(Vazquez et al., 2010). RNAi can broadly be categorized into two pathways, namely PTGS and TGS, although at times RNAi and post-transcriptional gene silencing are conflated under a singular term(Matzke & Mosher, 2014).

4.2.1 PTGS

In the realm PTGS, both small interfering RNAs (siRNAs) and microRNAs (miRNAs) hold significant prominence and are commonly employed in various translational contexts. The genesis of siRNAs ensues from long double-stranded RNAs (dsRNAs) that stem from the replication of RNA viruses, dsRNAs synthesized via the RNA-dependent RNA polymerase intrinsic to plants, as well as dsRNAs originating from natural sense and antisense transcripts, in addition to single-stranded RNAs that configure hairpin-loop secondary structures. These dsRNA entities are duly identified and cleaved by Dicer-like (DCL) proteins, thereby instigating the production of siRNAs. Notably, a fundamental set of four DCL proteins (DCL1–4) appears to be shared among plants, yielding diverse categories of siRNAs varying in lengths (H. Zhang et al., 2015). Particularly, DCL4 is responsible for generating 21-nucleotide siRNAs, pivotal in the immune response against RNA viruses. Moreover, DCL4 partakes in the biogenesis of trans-acting siRNAs (ta-siRNAs) that modulate gene expression. DCL2, on the other hand, yields 22-nucleotide siRNAs and exhibits synergy with DCL4 in antiviral defense mechanisms; in the absence of DCL4, DCL2 exhibits proficiency in generating copious secondary siRNAs against viral agents. DCL3 contributes to the generation of 24-nucleotide siRNAs, linked to the suppression of transposons and repetitive elements, and purportedly aids in defending against DNA viruses (Xie et al., 2004). Following the

processing of dsRNAs by DCLs into siRNA duplexes, each siRNA duplex is incorporated into the RNA-induced silencing complex (RISC), where the Argonaute (AGO) protein assumes a pivotal role. Within the duplex molecule, one strand is designated as the guide strand, responsible for pinpointing and discerning specific target RNAs via Watson-Crick base pairing, thereby typically instigating cleavage or slicing of the RNA target by RISC. Analogous to DCLs, plants encode a repertoire of AGO proteins(H. Zhang et al., 2015). These AGO proteins are characterized by three principal conserved domains: the PAZ, MID, and PIWI domains. The initial segment of the AGO protein encompasses a variable domain (ND), potentially aiding in disengaging the small RNA–target duplex post-slicing by disrupting the duplex configuration. The PAZ domain, situated proximal to the ND, has been demonstrated to bind the 3' terminus of small RNAs (the guide RNA). The distal domain houses the MID and PIWI domains, with their conjunction forming a binding site that secures the 5' terminus of the guide RNA. The AGO protein loaded with the siRNA-target duplex executes target RNA cleavage via the PIWI domain, housing an RNase-H-like structure and a conserved catalytic site. Consequently, the target RNA undergoes cleavage and degradation(Y. Wang et al., 2008).

Contrary to siRNAs, miRNAs are derived from endogenous noncoding RNAs and serve as crucial regulators of gene expression. In plants, primary miRNAs (pri-miRNAs) exhibit specific secondary structures and undergo initial processing into precursor miRNAs (pre-miRNAs) before being further processed into miRNA duplexes within the nucleus by DCL1. Following this, the miRNA duplexes are loaded onto AGO in the RISC complex, with one strand being chosen as the guide strand, ultimately leading to degradation or translational suppression of targeted mRNAs (Matzke & Mosher, 2014).

The origin of miRNAs is believed to be linked to inverted repeat (IR) genes, specifically MIR genes. Consequently, MIR genes are thought to have evolved from gradual random mutations in initially flawless IRs, resulting in shorter hairpins with increased mismatches and bulges (Vazquez et al., 2010). Plant miRNAs typically consist of 21 nucleotides in length (Cuperus et al., 2010). Members of the DCL family produce small RNAs of varying sizes: 21 nucleotides for DCL1 and DCL4, 22 nucleotides for DCL2, and 24 nucleotides for DCL3(Xie et al., 2004). DCL1 plays a pivotal role in the biogenesis of miRNAs from most of MIR genes in plants (Rogers & Chen, 2013). In plants, pri-miRNA stem-loops are transformed into short double-stranded RNAs (dsRNAs) comprising mature miRNA guide and passenger (miRNA*) strands with 2-nucleotide

overhangs by a DCL RNase III endonuclease (Margis et al., 2006). The structural characteristics of the pri-miRNA stem-loop guide an initial DCL cleavage event close to the stem's base(Bologna et al., 2009). Subsequent cleavage events post excision of the precursor miRNA (pre-miRNA) stem-loop may occur processively(Liu et al., 2012), at intervals of 21 nucleotides along the stem. The accurate identification of the miRNA from the pri-miRNA is reliant on precursor structure-encoded processing signals, including a 14- to 15-bp paired stem proximal to the miRNA/miRNA* duplex, a specific length of paired stem distal to the miRNA/miRNA* duplex, and a terminal loop(Rogers & Chen, 2013).

In animal systems, the terminals of certain intronic pre-miRNA hairpins are produced through intron splicing and debranching (mirtrons), thereby bypassing the necessity for an RNase III cleavage event; analogous mechanisms could potentially exist in plants (Rogers & Chen, 2013).

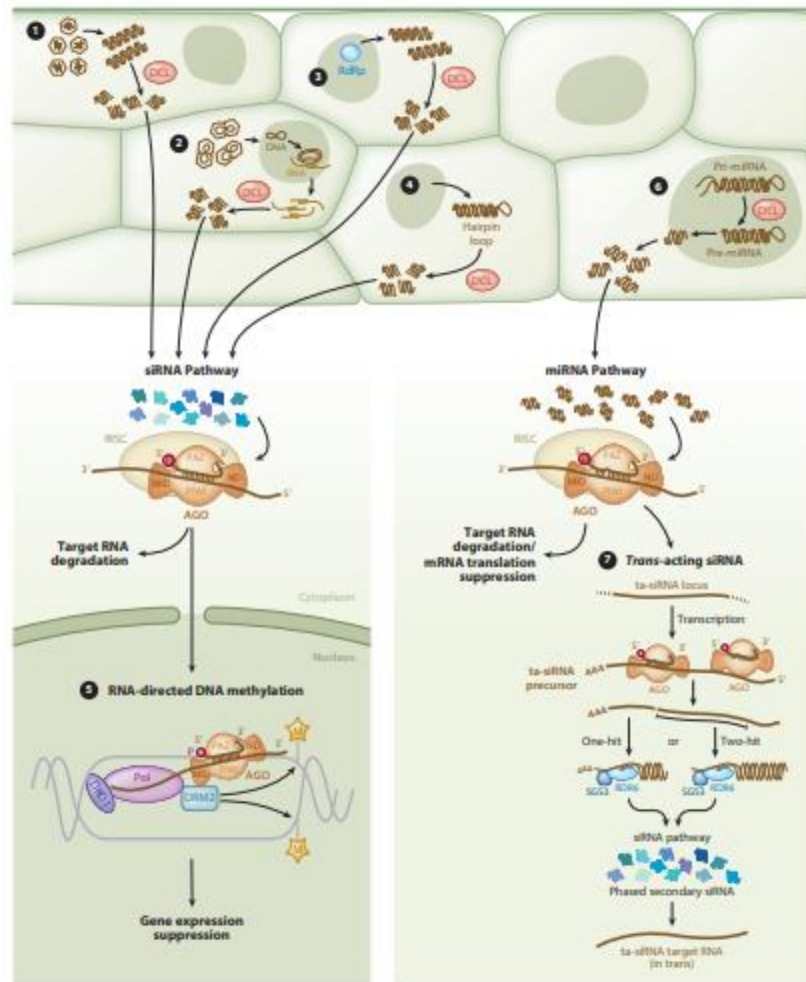

Fig. 1: RNAi pathways in plants. siRNAs can be produced from double-stranded RNAs derived from viral replication and/or gene expression of **1.** RNA and **2.** DNA viruses, **3.** dsRNAs synthesized by plant endogenous RNA-dependent

RNA polymerases, and **4.** single-stranded RNAs that form extensive secondary structures in plant cells. **5.** Specific siRNAs (mostly 24-nt siRNAs) also may enter the nucleus to trigger RNA-directed DNA methylation (RdDM). **6.** miRNAs are derived from endogenous noncoding RNA transcripts [primary miRNA (pri-miRNA)] that fold into specific secondary structures to initiate the miRNA pathway. **7.** Specific miRNAs can direct AGO to target transcripts of the TAS loci (ta-siRNA loci).

4.2.2 TGS

A notable mechanism in plants is RdDM (RNA-directed DNA Methylation), which plays a crucial role in stress response, cell-to-cell communication, and silencing of transposable elements, all vital for maintaining genome stability. This mechanism involves adding DNA methylation to cytosines regardless of specific sequence context, distinguishing it from other plant mechanisms. Fig.2 provides an overview of the diverse biological functions associated with RdDM (Erdmann & Picard, 2020).

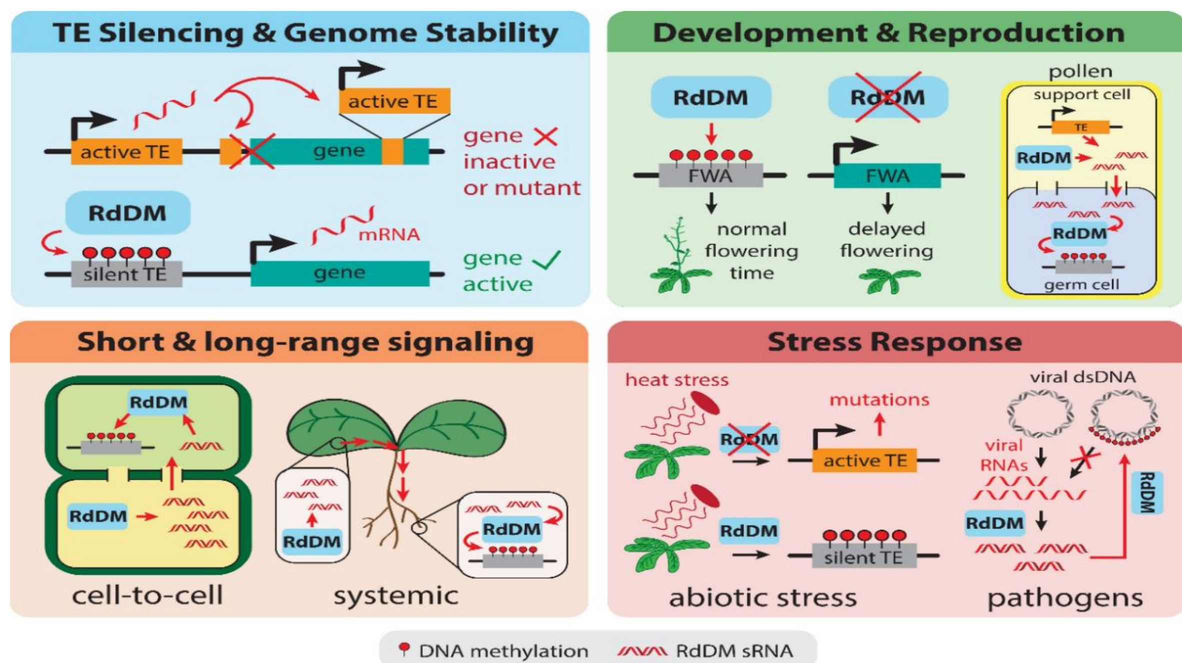

Fig. 2: At a high level, RdDM (RNA-directed DNA Methylation) plays crucial roles in various biological functions within plants. Firstly, it serves as a vital defense mechanism, preventing the activation of transposable elements (TEs) that could otherwise disrupt gene expression. Furthermore, RdDM influences developmental processes, such as regulating flowering time by suppressing genes like FWA. Additionally, during pollen formation, RdDM generates small RNAs (sRNAs) that strengthen the silencing of TEs in germ cells, facilitating their mobility between cells and tissues. Moreover, RdDM contributes to plant responses to abiotic stress, such as heat shock, and acts as a defense mechanism against pathogens by silencing viral DNA through sRNAs derived from viral mRNAs (Erdmann & Picard, 2020).

DNA methylation plays a pivotal role in regulating gene expression and maintaining genome stability in plants. This multifaceted process encompasses three distinct sequence contexts—CG, CHG, and CHH—whose collective actions contribute to the complex landscape of epigenetic modifications (Erdmann & Picard, 2020). At the heart of this regulatory process lies the RNA-directed DNA methylation (RdDM) pathway, led by the enzyme DRM2. DRM2 acts as a crucial component, directing the establishment of DNA methylation patterns across the genome (D. Zhang et al., 2018). The dependence of RdDM on 24-nt small interfering RNAs (siRNAs) produced by Dicer from RNAs transcribed by Polymerase IV (Pol IV) highlights the complex interaction between transcriptional and post-transcriptional mechanisms in epigenetic regulation, as illustrated in Fig.2 (D. Zhang et al., 2018).

The RdDM pathway, depicted in Fig.3, outlines a series of molecular events aimed at meticulously regulating gene expression through the initiation of 24 nt siRNA production. Pol IV plays a crucial role in this process, serving as a catalyst to initiate template synthesis, thereby facilitating the generation of double-stranded RNAs (dsRNAs).(Vazquez et al., 2010). These dsRNAs are then processed into siRNAs by DCL3, an integral step in the pathway's progression (Bond & Baulcombe, 2014). The interaction between the Sawadee Homeodomain Homolog 1 (SHH1) protein and Pol IV at loci characterized by dimethylated histone H3 lysine 9 (H3K9me2) highlights the integration of epigenetic marks and transcriptional machinery in governing DNA methylation dynamics. Furthermore, the participation of the chromatin remodeler Classy 1 (CLSY1) underscores the complexity of chromatin architecture in regulating epigenetic processes. The complete RdDM pathway is depicted in Fig. 3 (Bond & Baulcombe, 2014).

The subsequent binding of siRNAs onto ARGONAUTE (AGO) proteins serves as a pivotal juncture in the RdDM pathway, where these small RNA molecules play a decisive role in determining the specificity of DNA methylation. The capacity of loaded siRNAs to associate with either AGO4 or AGO6 underscores the adaptability of the system in selectively targeting particular genomic loci for methylation(Wierzbicki et al., 2009). The focused methylation process, facilitated by Pol V and DRM2, leads to the suppression of transposable elements and the control of gene expression, ultimately safeguarding genome integrity and facilitating appropriate developmental pathways in plants (Vazquez et al., 2010).

Pol V transcripts, believed to be either triphosphorylated or capped at the 5′ ends and lacking poly(A) tails, are hypothesized to serve as scaffold RNAs that interact with siRNAs and recruit other components involved in gene silencing. Analogous to Pol IV, the precise chromatin characteristics responsible for the recruitment of Pol V to its specific target regions remain not fully elucidated, with no definitive DNA sequence consensus having been established. Through the utilization of chromatin immunoprecipitation experiments coupled with sequencing, it was observed that the majority of Pol V localizes predominantly at transposons and repetitive sequences associated with 24-nucleotide siRNAs and cytosine methylation, thus indicating the crucial role of Pol V in facilitating RdDM at these specific genomic loci (Matzke & Mosher, 2014).

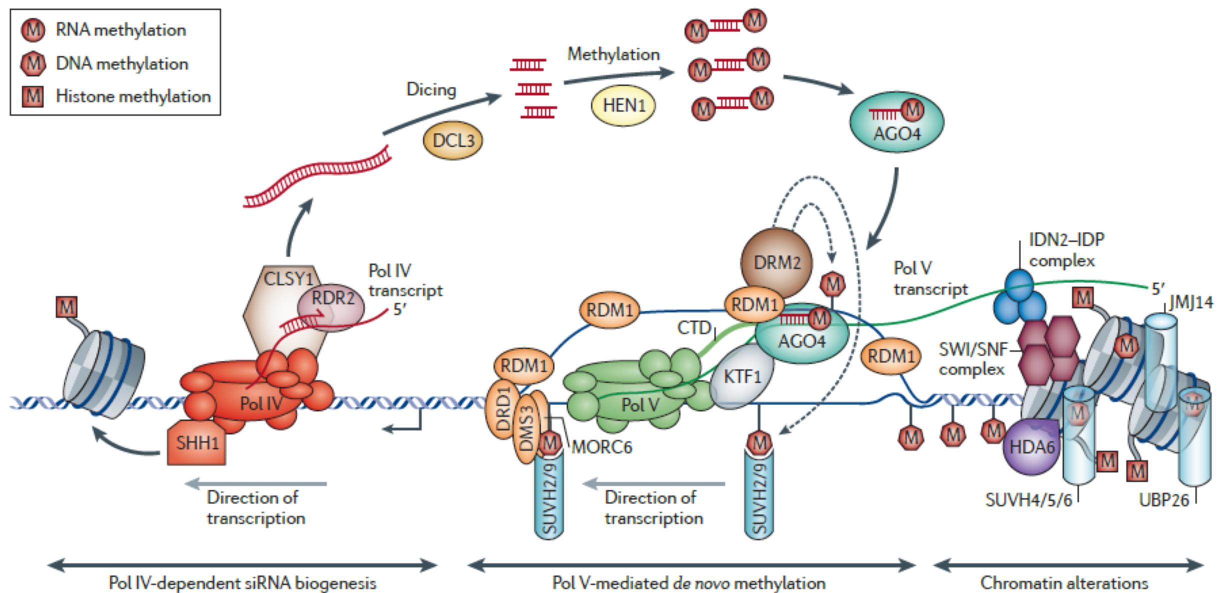

Fig. 3: The RdDM model in plants involves Pol IV transcription of non-coding RNA from specific genomic regions, aided by CLSY1 and SHH1. RDR2 generates complementary RNA copies, processed into 24-nt sRNAs by DCL3. These sRNAs, loaded onto AGO proteins, guide de novo DNA methylation via DRM2 in the nucleus with DDR complex assistance, ensuring stable methylation patterns across (Matzke & Mosher, 2014).

4.3 Wheat transformation

Hexaploid wheat (*Triticum aestivum* L.), globally cultivated, has lagged crops such as rice and maize in fully exploiting genetic engineering, primarily due to the obstacle of transformation (Hamada et al., 2017). Despite this, wheat faces numerous challenges, both biotic and abiotic,

impeding its productivity, while modern biotechnology presents promising opportunities to overcome these barriers through plant transformation techniques aimed at enhancing crop traits. Plant transformation serves as a crucial tool in wheat research, enabling the exploration of gene functions and the identification of desirable characteristics. Although various techniques exist for wheat transformation, two main approaches have been utilized: particle bombardment of embryogenic callus and *Agrobacterium*-mediated transformation of immature embryo (Hayta et al., 2019).

Biolistic-mediated transformation

Biolistic or gene gun-mediated transformation offers a method for delivering DNA into plant cells. This technique involves coating DNA onto microprojectiles, such as gold or tungsten particles, which are then propelled into plant tissues using a pneumatic device or gene gun. In contrast to *Agrobacterium*-mediated transformation, biolistic transformation circumvents bacterial infection and tissue culture, streamlining the process. Upon bombardment onto plant tissues, some cells take up the DNA and integrate it into the plant's genome. Verification of successful transformation is carried out through selection based on selectable markers or reporter genes, followed by molecular analysis. The versatility of biolistic transformation lies in its ability to transform a broad spectrum of plant species, including those resistant to *Agrobacterium infection*. Nonetheless, challenges like the random integration of transgenes, varying expression levels, and possible gene silencing persist. Moreover, the high costs associated with equipment and consumables for biolistic transformation present financial barriers. Potential physical harm to plant tissues during bombardment might further reduce regeneration efficiency. Addressing these challenges necessitates ongoing research efforts to optimize protocols, improve integration strategies, and lower costs, ultimately broadening the utilization of biolistic transformation in wheat genetic engineering pursuits(Hayta et al., 2021).

Efforts have been made to enhance the efficiency of transformation in model cultivars (Hamada et al., 2017). While debates continue regarding the advantages and disadvantages of both techniques, biolistic transformation has garnered attention due to its flexibility in incorporating emerging technologies like genome editing tools and its reliability in generating genome-edited plants devoid of DNA and markers (Tian et al., 2019). Nevertheless, these methods have limitations, as they may not be universally applicable to all cultivars and can be time-consuming,

potentially resulting in somatic variations; many premium commercial cultivars lack the essential traits for efficient culture and regeneration, posing hindrances to successful transformation endeavors (Hamada et al., 2017).

***Agrobacterium*-mediated transformation**

Agrobacterium-mediated transformation is considered a fundamental technique in the realm of plant genetic manipulation, providing a reliable and effective approach for introducing exogenous DNA into plant genomes. Within dicotyledonous plant species, the preferred gene transfer mechanism has long been the utilization of *Agrobacterium* tumefaciens, a soil bacterium capable of mediating the transformation process. This bacterium has the ability to deliver its DNA, known as T-DNA or transfer DNA, to plant cells, inducing the formation of tumor-like structures called crown galls(Ream, 1989). By the 1980s, strategies had been devised to create transgenic plants in species like tobacco and petunia without inducing tumorigenesis(Fraley et al., 1986). Initially, it was widely accepted that *A. tumefaciens* could not genetically modify monocotyledonous plants due to their natural resistance to crown gall disease(*Scopus - Document Details - The Host Range of Crown Gall*, n.d.). The unique capability of *A. tumefaciens* to transfer limited copies of relatively large DNA fragments with precise boundaries to plant chromosomes, resulting in minimal genetic alterations, made it an attractive option for cereal crops. While early attempts to apply similar transformation methods as those used in dicotyledonous plants to cereals were unsuccessful, breakthroughs were eventually achieved. Transgenic plants were successfully generated from callus tissues derived from mature rice embryos and immature maize embryos following co-cultivation with *A. tumefaciens*(Ishida et al., 2007).The key to these successes lay in utilizing cells that were undifferentiated, actively dividing, and competent for plant regeneration, attributes found in mature and immature embryos. These accomplishments paved the way for further investigations in monocotyledonous plants, expanding the scope of genetic engineering in this plant group. The sluggish progress in wheat transformation was primarily attributed to the complex nature of its genome and the challenges posed by tissue culture. Both *Triticum aestivum* and Triticum durum are polyploid species, with the former possessing a genome size significantly larger than that of rice and maize(Ishida et al., 2019).

Over time, significant advancements have been made in overcoming the obstacles associated with tissue culture. Subsequently, following the successful establishment of efficient

Agrobacterium-mediated transformation protocols in rice and maize, the wheat cultivar Bobwhite was also genetically modified using *A. tumefaciens*. As seen in other cereal crops, the starting material for transformation was immature embryos. Although progress in wheat transformation has been slower compared to other crops, the reported transformation efficiency has generally been below 5% of the initial tissue inoculum(Hayta et al., 2019). Through optimization efforts, (Ishida et al., 2015) managed to achieve a transformation frequency of up to 90% in the Fielder cultivar, highlighting the critical factors influencing wheat transformation efficiency, such as genotype selection, quality and developmental stage of embryos, culture medium composition, *A. tumefaciens* strain, embryo pretreatment, and tissue handling procedures, which are more restricted compared to rice and maize (Richardson et al., 2014).

4.4 Modification of microRNAs' expression

Past research utilizing microarray and high-throughput sequencing methodologies has furnished extensive insights into plant miRNAs and their regulatory targets. These investigations underscore the pivotal roles miRNAs play in various facets of plant biology, encompassing development, morphology, stress responses, and hormonal signaling. Given the pivotal roles of miRNAs in plant biology, they have emerged as a promising avenue for genetic engineering in plants. Different strategies, such as miRNA gene overexpression, knockdown, RNA interference, short tandem target mimicry, and artificial miRNA, are deployed to develop miRNA-based genetically modified crops (Richardson et al., 2014).

Wang et al. (2020) Investigated the role of miR156 in enhancing anthocyanin production in poplar trees by overexpressing this microRNA. It was found that miR156 overexpression altered the expression of 228 microRNAs, particularly miR160h and miR858, which are linked to key regulatory genes affecting anthocyanin synthesis. The metabolomics analysis revealed increased levels of anthocyanins and other flavonoids, along with reduced lignin content in the transgenic poplars compared to wild types. These findings highlight miR156's potential to modify anthocyanin biosynthesis through a complex regulatory network involving microRNAs and transcription factors (Richardson et al., 2014).

Another study demonstrated the effectiveness of Potato virus X (PVX)-based expression systems for miRNA silencing in *Nicotiana benthamiana*. By applying short tandem target mimic (STTM) technology against miR165/166 and miR159, the research induced specific phenotypic

changes in all treated plants, showcasing a powerful tool for studying miRNA function in plants (Zhao et al., 2016).

4.4.1 Strategies based on miRNA for crop improvement.

Addressing constraints such as limited arable land, environmental stress, and the imperative of food security has increased the demand for high-yielding, stress-resistant crops. Research indicates that microRNAs (miRNAs) serve as key regulators of gene expression across various biological processes. Consequently, harnessing miRNA-based genetic modifications represents a promising avenue for cultivating superior crop varieties. Plant scientists have devised numerous strategies to achieve this. miRNA biogenesis and the various methods to manipulate miRNA activity and its influence on gene expression as shown in below Fig. 4.Initially, the miRNA gene produces primary miRNA in the nucleus, which is processed into mature miRNA in the cytoplasm, and then pairs with the RISC to target mRNA, regulating its expression. Technologies depicted include RNA interference to prevent miRNA maturation, target mimicry to block miRNA activity, and the use of CRISPR/Cas9 to edit miRNA or its target genes. Additionally, methods to overexpress miRNA-resistant genes or the miRNAs themselves are shown, offering multiple strategies to adjust gene expression for research or therapeutic purposes (Mandal et al., 2021).

4.4.1.1 STTM (Short Tandem Target Mimic)

The Short Tandem Target Mimic (STTM) technology is an innovative tool designed to silence specific microRNAs (miRNAs) in plants. With its artificial, non-coding RNA structure, STTM effectively decoys miRNAs, preventing them from exerting their regulatory functions. One notable feature of STTM is its incorporation of dual binding sites for miRNAs, enhancing its efficiency in decoying different miRNAs compared to other mimic tools. In plant development studies, STTM has demonstrated its versatility by successfully modulating fruit shape and size through the manipulation of miRNA expression. Researchers utilizing STTM technology have gained valuable insights into miRNA functions, particularly regarding their quantitative effects on organ development. This approach offers a powerful means of uncovering the intricate roles of miRNAs in regulating plant architecture across various developmental stages (Othman et al., 2023).

Due to its versatility, the STTM method is commonly used in plant miRNA research. It involves a structure with two miRNA-binding sites, which can effectively silence some highly

expressed miRNAs and result in visible phenotypic changes. This technique can also be used to investigate interactions between two miRNAs, silence multiple distinct miRNAs, and complement CRISPR/Cas9 in certain scenarios Fig. 5 (Teotia et al., 2016). In a recent study, STTM166 plants showed a significant decrease in the size and number of metaxylem vessels in their stems and leaf veins. This was linked to the down-regulated expression of genes related to cell and intercellular components, with two cellulose synthesis genes showing opposite expression patterns. The inactivation of miR166, which regulates IAA levels, could provide an alternative pathway for vascular development and may contribute to the abiotic stress resistance and brittle leaf basal seen in STTM166 plant (Li et al., 2020).

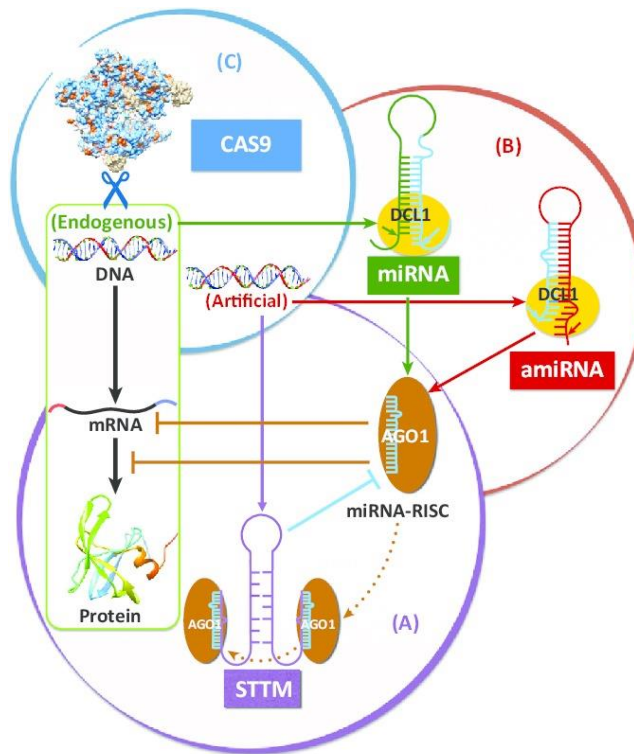

Fig. 5: There are three RNA-based technologies used to study gene function: STTM for noncoding miRNA genes, amiRNA for coding genes, and CRISPR/Cas9 for gene editing. (A) STTM creates short RNA molecules that bind to the miRNA gene of interest to prevent its function. (B) amiRNA creates an artificial miRNA molecule that targets and degrades mRNA produced by the coding gene of interest, leading to reduced expression. (C) CRISPR/Cas9 uses an enzyme to cut DNA at a specific location, allowing the introduction of RNA to guide the enzyme to desired locations to alter the genetic code and investigate the effects on cellular processes(Teotia et al., 2016).

4.4.1.2 CRISPR (clustered regulatory interspaced short palindromic repeats)

CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats. It's a revolutionary gene-editing technology inspired by the natural defense mechanisms found in bacteria. Essentially, CRISPR allows scientists to make precise changes to the DNA of organisms, including plants, animals, and even humans. Using CRISPR, researchers can edit, delete, or replace specific sections of DNA with remarkable accuracy. This technology has enormous potential for various applications, from curing genetic diseases to developing drought-resistant crops. With the help of the efficient CRISPR/Cas9 tool, scientists can now dive deeper into understanding how tiny molecules called microRNAs (miRNAs) work in plants. This tool lets them tweak the spots where miRNAs attach to genes, creating what they call "in-frame mutants." These mutants still do their jobs, but they mess up the way miRNAs usually control them. For example, there's this miRNA called miR396 that usually targets important genes like OsGRF4 and OsGRF8, which affect how big grains grow in rice and how plants defend against pests like the brown planthopper. By using CRISPR/Cas9 to make changes in these genes, scientists made mutants with bigger grains and stronger pest resistance. This exciting use of CRISPR/Cas9 shows how we can improve specific traits in plants, helping farmers grow better crops and making agriculture even more innovative (Lin et al., 2021).

5. MATERIALS AND METHODS

5.1. Plant growth

Seeds of the spring wheat (*Triticum aestivum* L.) *cv.* 'Fielder' are first sown in a Jiffy. After germination plants are transferred into 13 cm diameter round pots containing peat and sand mix (5:1) for continued development. Plants were risen in growth chambers (Sanyo) under 16h/8h light/dark period, 70% humidity with light levels of 50 µE $m^{-2}s^{-1}$ provided by fluorescent tubes and tungsten lighting. Plants are not sprayed with fungicides or insecticides at any stage of growth.

5.2. Culture media

5.2.1 Culture media for bacteria

We used LB and YEB media for the growth of *Escherichia coli* and *Agrobacterium* respectively.

Table-1: Component used for LB media.

Reagent Measure for	1 L
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar*	15 g

pH was adjusted to 7 and autoclaved for 25 minutes.

Antibiotics were added (If needed): Kanamycin (25mg/L)

*For solid media

Table-2: Component of YEB media.

Reagent Measure	**for 1 L**
Beef extract	5g
Bacto yeast extract	1g
Bacto peptone	1g
Sucrose	5g

pH was adjusted to 5.8 and autoclaved for 25 minutes.

Antibiotics were added (If needed): rifampicin (10mg/L), Kanamycin 25mg/L

5.2.2. Culture media for wheat

Table-3: Component of wheat transformation media

WIM-liq		**1 L**
	MS Inc. Vit. **M0222**	0,44 g
	glucose	10 g
	MES	500 mg
	(make before use!!)	
WIM-inf	**WIM-liq+**	**1 L**
(before use!)	Silwet L-77	250 µl
	acetosyringone 1M	100 µl
WIM-AS	**WIM liq +**	**1 L**
	AgNO3 (0,85mg/ml)	1ml
	CuSO4*5H2O (1,25mg/L)	1 ml
	acetosyringone 1M	100 µl
	agarose (P5575)	**8 g**
WCI		**1 L**
	MS Basal Salt, **M0221**	4,4 g
	Myo-inositol	350 mg
	Proline	690 mg
	Thiamin HCL (1mg/ml)	1 ml
	Casein	1 g
	maltose	30 g
	2,4-D (2,5mg/mL)	200 µl
	Picloram (2mg/ml)	1 ml
	CuSO4*5H2O (1,25mg/L)	1 ml
	agarose	**5 g**
WCI-Res	**WCI+**	
	cefatoxime	300 mg
WCI-H20	**WCI+**	
	cefatoxime	300 mg
	hygromycin (500mg/mL)	40 µl
WCI-H40	**WCI+**	**1 L**
	cefatoxime	300 mg
	hygromycin	80 µl
WRM		**1 L**
	MS Inc. Vit. **M0222**	4,4 g
	sucrose	20 g
	MES	500 mg
	Zeatin (0,5mg/ml)	1 ml

	CuSO4*5H2O (1,25mg/L)	2 ml
	cefatoxime	300 mg
	Gelzan	**3 g**
WRM-H20	**WRM+**	
	hygromycin (500mg/mL)	40 µl
LSF-H20		**1 L**
	MS Basal Salt, **M0221**	4,4 g
	Myo-inositol	350 mg
	Proline	690 mg
	Thiamin HCL (1mg/ml)	1 ml
	Casein	1 g
	maltose	30 g
	Gelzan	**3 g**
	cefatoxime	300 mg
	hygromycin (500mg/mL)	40 µl

Make up all media using water from an ultra-pure water. All media should be adjusted to pH 5.8 and all reagents should be added slowly under sterile conditions in a laminar flow hood. Prepare all medium components for solid media except the gelling agent as double concentrates and filter sterilize. Make up the gelling agent separately as a double concentrate (in H2O) and autoclave. After autoclaving, maintain the 2× gelling agents at 60°C and warm the filter-sterilized 2× medium components to 60°C prior to mixing the two together and pouring the plates. Add the phytohormones and antibiotics as filter-sterilized stocks just before pouring. All media in petri plates and tubes should be stored at 10°C. Use of media >2 weeks old is not recommended.

5.3. Bacterial Strains

We used *Escherichia coli* for cloning and *Agrobacterium tumefaciens* for plant transformation (Table-4).

Table-4: Bacterial strains

Bacteria	**Strains**
Escherichia coli	DH5α
Agrobacterium tumefaciens	AGL1

5.4 Wheat cultivar

We used 'Fielder' a hexaploid common wheat cultivar, an American, soft, white, pastry-type wheat released in 1974 and known for its amenability to *Agrobacterium tumefaciens*-mediated transformation.

5.5 Plasmid Construction

Precursor amplification and cloning in pJET1.2 blunt cloning vector

First of all, precursors were amplified with the help of PCR using universal primers for all three A, B and D sub-genome precursors. PCR fragment was ligated into PJET1.2 blunt cloning commercial vector using manufacturer instructions. Transformed colonies were selected on solid LB-ampicillin media. Colony PCR amplicons were digested with specific enzymes to sort out all three precursors based on unique restriction enzyme site. Unique restriction sites present only on precursor B or D are listed in the table below. Non-digested PCR fragments with either of the enzymes were considered as A precursor. Final vector was called as PJET1.2_Pre2187A, PJET1.2_Pre2187B, PJET1.2_Pre2187D.

Table-5: Unique restriction sites in B precursor only

Restriction enzyme	A Precursor	B Precursor	D Precursor
*BIp*I	0	1	0
*Bsu*36I	0	1	0
*Mae*I	0	1	0
*Nhe*I	0	1	0
*Xmn*I	0	1	0

Table-6: Unique restriction sites in D precursor only

Restriction enzyme	A Precursor	B Precursor	D Precursor
*Apa*I	0	0	1
*Ban*II	0	0	1
*Bse*SI	0	0	1

*Bsp*120I	0	0	1
*Sfi*I	0	0	1

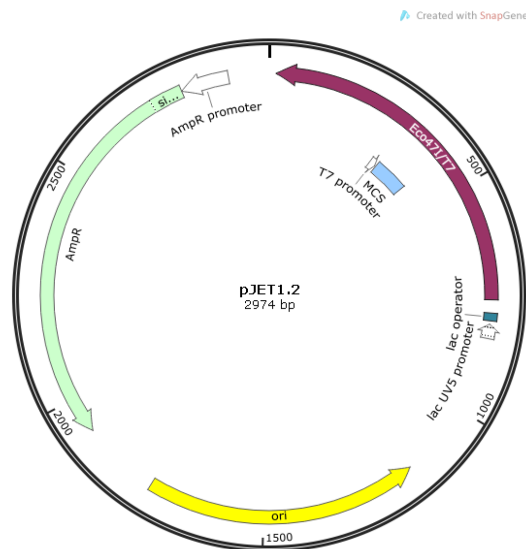

Fig. 6: Map of commercial pJET1.2 plasmid.

Finally, after separation of all three precursors, it was integrated into final vector pC61K with the help of restriction digestion and ligation cloning. Primers were redesigned by integration of *Kpn*I site in forward primer and *Bam*HI site in reverse primer. PCR was carried out using PJET1.2_Pre2187A, PJET1.2_Pre2187B, PJET1.2_Pre2187D plasmids as a template. Amplified PCR products were digested with *Bam*HI and *Kpn*I and ligated into pC61K vector which was already opened with *Bam*HI and *Kpn*I. Finally, the final vector was called as pC61K_Pre2187A, pC61K_Pre2187B, pC61K_Pre2187D. All three precursors were first tested transiently in *Nicotiana benthamiana* and based on expression level pC61K_Pre2187B vector was chosen to be transformed into Wheat cultivar 'Fielder'.

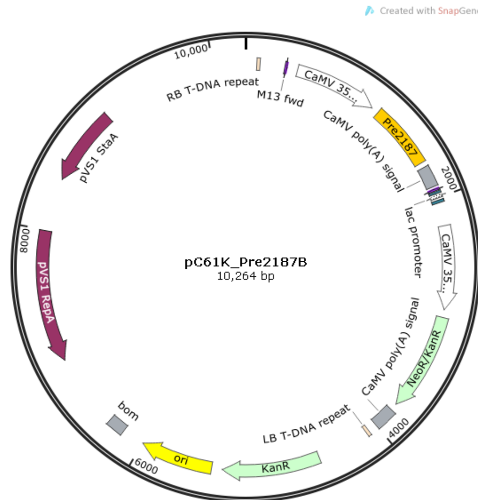

Fig. 7: Map of pC61K_Pre2187B

5.5.1 Digestion of plasmid and PCR fragments

PCR and pC61K_Pre2187A/B/D plasmid were digested with *Bam*HI and *Kpn*I

Table-7: Protocol for plasmid digestion

Reagents	Amount
pC61K_Pre2187A/B/D PCR fragments	3 µL
Tango Buffer (1x)	3 µL
*Bam*HI, *Kpn*I	2-2 µL
Nuclease Free Water	20 µL
Total Volume	30 µL

Mix gently and spin down for few seconds and incubate it for 1h and 30 minutes at 37 °C.

5.5.2 Ligation of PCR fragment into pC61K binary vector

We used pC61K binary vector designed by (Kis et al., 2016). Precursors were amplified with the help of PCR and digested with *Bam*HI and *Kpn*I enzymes and ligated into pC61K binary vector.

Table-8: Protocol for ligation of PCR fragments into digested pC61K binary vector

Reagent	Measure
Digested vector (pC61K)	1 µL

Digested PCR fragment	3 µL
Reaction buffer	1 µL
T4 ligase	1 µL
Nuclease free water	4 µL

Incubate at room temperature for 1hour.

5.5.3 Structure of PcubiGRF4:GIF1_NOS vector

To increase the regeneration efficiency of wheat transformation a vector PcubiGRF4:GIF1_NOS was adopted and modified (Juan M et al., 2020) This vector has a fusion protein combining wheat GROWTH-REGULATING FACTOR 4 (GRF4) and its cofactor GRF-INTERACTING FACTOR 1 (GIF1) under the Ubiquitin promotor and NOS terminator which can, substantially increases the efficiency and speed of regeneration in wheat. This vector also contains hygromycin and kanamycin resitant genes (Fig 8).

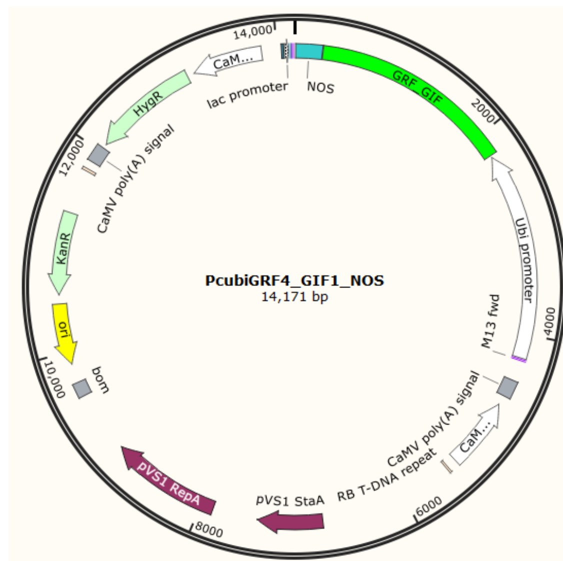

Fig. 8: Map of PcubiGRF4:GIF1_NOS vector. GRF:GIF chimera gene (in green) can be seen under the Ubi promotor and NOS terminator.

5.6 Transformation of *E. Coli*

The transformation of *E. coli* followed a standard protocol. Competent *E. coli* cells were thawed on ice for 30 minutes. Then, 100 µL of these competent cells were transferred to sterile Eppendorf tubes. To this, 2-3 µL of the ligated plasmid mixture was added. After incubating the mixture on

ice for 10 minutes, a brief heat shock was given at 42 °C for 30 seconds, followed by immediate return to ice. Subsequently, 500 µL of SOC media was added to the tubes, and they were gently flicked to mix. The tubes were then incubated at 37 °C for 40 minutes. After this incubation period, the transformed cells were smeared onto LB plates containing the appropriate antibiotic. The plates were then incubated overnight at 37 °C to allow for colony growth.

5.7. Plasmid miniprep

E. coli colonies that contain transformed plasmid were grown in 5 mL LB medium containing Kanamycin overnight. Plasmids were extracted from *E. Coli* with the help of manufactures (Fisher Scientific UK) instructions. Plasmid concentration was measured by NanoDrop spectrophotometer 1000.

Table. 9: Plasmid concentration on Nanodrop spectrophotometer 1000.

Sample name	Amount (ng/µL)	A260/280	A260/230
pC61K_Pre2187A/B/D	686 ng/µL	2.11	1.89

5.8. Transformation of *Agrobacterium* with pC61K_Pre2187A/B/D vector

Freeze-thaw method.

Once a desired molecule is constructed in *E. Coli*, the molecule can be transferred into *Agrobacterium* by the freeze-thaw method. Although the transformation frequency by these methods is low (approximately 103 transformants per µg DNA) compared to the triparental mating method, the technique is reliable and very rapid. This transformation procedure also eliminates much of the plasmid rearrangement that often occurs during triparental mating.

To begin the transformation process, an *Agrobacterium* strain with a suitable helper Ti plasmid was cultured overnight in 5 ml of YEB medium at 28°C. Subsequently, 2 ml of this overnight culture was transferred to a 250-ml flask containing 50 ml of YEB medium. The flask was then vigorously shaken at 28°C until the culture reached an OD600 of 0.5 to 1.0. After achieving the desired cell density, the culture was chilled on ice and then centrifuged at 3000 g for 5 minutes at 4°C. The supernatant was carefully removed, and the cell pellet was resuspended in 1 ml of ice-cold 20 mM CaCl2 solution. 0.1-ml aliquots of the resuspended cells were dispensed into prechilled Eppendorf test tubes. Next, approximately 1 µg of plasmid DNA was added to each

tube containing the cells. The cells were then frozen in liquid nitrogen and subsequently thawed by incubating the test tubes in a 37°C incubator for 10 minutes. Following thawing, 1 ml of YEB medium was added to tube, and the tube was incubated at 28°C for 2-4 hours with gentle shaking. This incubation period allowed the bacteria to express the antibiotic resistance genes carried on the plasmid. After incubation, the tubes were centrifuged for 30 seconds, and the supernatant was discarded. The cells were then resuspended in 0.1 ml of YEP medium. Finally, the transformed cells were spread onto YEB plates containing the appropriate antibiotic at 28°C. Transformed colonies were expected to appear within 2-3 days.

Note: It was noted that the cells could be frozen in liquid nitrogen and stored at -80°C for future transformation experiments.

5.9. DNA Extraction

Total DNA was obtained from young leaf samples with a direct DNA extraction method. Leaf pieces of approximately 5×5 mm was placed into a 1.5 mL Eppendorf tube containing 100 µL of Extraction solution (E7526- 24ML, Sigma-Aldrich, St. Louis, MO, USA) together with a stainless-steel bead (3 mm diameter, Qiagen Sciences, Germantown, MD, USA). The samples were homogenised in a mixer mill (Bullet Blender Storm Pro, Next Advance, Troy, NY, USA) at speed grade 8 for 30 s. The mixture was incubated at 95 °C for 15 min in a dry heat block followed by cooling on ice for 1 min. Finally, 100 µL of Dilution solution (D5688-12ML, SigmaAldrich) was added, and after vortexing, the samples were spun for 1 min at 18,000 × g. The supernatant (100 µL) was transferred into a new 1.5 mL Eppendorf tube and the DNA was stored at -20 °C until required.

5.10. RNA Extraction

The process begins by rubbing the seed or leaf in liquid nitrogen. Then the powder was transferred in 650 ul of extraction buffer (EB) in a 2ml tube, followed by vigorous vortexing. Next, 650 ul of phenol is added, and the mixture is vortexed. After centrifugation for 5 minutes at 13,000 g at room temperature, the supernatant is carefully transferred to a new 2ml Eppendorf tube containing 650 phenol again, this step is to increase the purity of RNA samples, followed by another round of vortexing and centrifugation at 13,000 g at room temperature. Subsequently, supernatant was transferred in 650 phenol-chloroform in a 2ml Eppendorf tube, vortexed, and centrifuged again for

5 minutes at 13,000 g at room temperature. Finally supernatant was transferred in 600 phenol chloroform isomyl-alcohol in a new 2ml Eppendorf tube, vortexed and centrifuged once more at same RPM and temperature. Following this, 600ul of the supernatant is combined with 1.2 ml of 100% ethanol and 60ul of 3M sodium acetate in a new tube, and the mixture is allowed to precipitate for 1 hour at -70 ° C. After centrifugation for 25 minutes at 13,000 g at 4 ° C, the pellet is washed with 1ml of 70% ethanol and centrifuged for 10 minutes at 13,000g at 4 ° C. The pellet is then dried in a speedvac for 10 minutes before being eluted in 50ul of either TE or MilliQ water.

5.11. PCR amplification

Direct DNA from Fielder wheat and 1 µg plasmid DNA were used in PCR reaction with Phusion Green Hot Start II High-Fidelity DNA Polymerase (F537S). To setup parallel reactions and to minimize the possibility of pipetting errors, prepare a PCR master mix by mixing water, buffer, dNTPs, specific primers, and DNA polymerase. Prepare sufficient master mix for the number of reactions plus one extra. Gently vortex and briefly centrifuge all solutions after thawing (Table-8,9,10).

Table-10: Sequence of primers and LNA probe used.

Name of the primer	Sequence of the primer	Tm°C
Pre2187_clone_KpnI_Fw	ATGGGTACCCAGAATTAGACTTGGGCCCAT	64.1
Pre2187_clone_BamHI_Rv	ATGGGATCCGAGTTATCGGTGGCGGAAGC	66.5
Pre2187_uni_Fw	CAGAATTAGACTTGGGCCCAT	54.8
Pre2187_uni_Rv	GAGTTATCGGTGGCGGAAGC	58.3
Hyg_Det_Fw	GCAAACTGTGATGGACGACA	55.6
Hyg_Det_Rv	CTCCATACAAGCCAACCACG	56.1
GRF4:GIF1_Det_Fw	AACGACTACATTCCCGCTCT	55.9
GRF4:GIF1_Det_Rv	ATGAGATCAGTGGTGGCAGT	56.1
tae2187 LNA probe	[Btn]G[+C]AC[+C]AG[+T]CG[+A]CG[+G]AG[+C]CG[+C]G	

Table-11: Phusion PCR mix.

HF buffer	4 µL
Forward primer (0.5 µM)	1 µL
Reverse primer (0.5 µM)	1 µL
Template DNA or plasmid	1 µL
dNTP (0.2 µM each)	0.4 µL
Phusion HS II enzyme	0.2 µL
Nuclease free water	12.4 µL
Total volume	20 µL

Gently vortex the samples and spin down.

PCR Programs

For Precursor amplification: 98 °C 30 sec (98 °C 10 sec, 59.8 °C 15 sec, 72 °C 3 minutes) x34 cycles, 72 °C 10 minutes.

For Hygromycin and GRF:GIF detection: 98 °C 30 sec (98 °C 10 sec, 63 °C 15 sec, 72 °C 12 sec) x34 cycles, 72 °C 10 minutes.

Table-12: Colony PCR mix.

Template DNA	1 µL
HF buffer	4 µL
dNTP (0.2 µM)	0.4 µL
Phusion HS II enzyme	0.2 µL
Forward primer (0.5 µM)	1 µL
Reverse rimer (0.5 µM)	1 µL
Nuclease free water	12.4 µL
Total volume	20 µL

Note: Colonies were diluted in 10 µL of water and heated on 95 °C for 10 minutes.

Gently vortex the samples and spin down.

PCR Program: 98 °C 3 mint, (98 °C 10 sec, 65 °C 30sec, 72 °C 10sec) x32cycles, 72 °C 10 minutes.

5.12. Gel-Electrophoresis

PCR amplification was confirmed by gel electrophoresis on 1.2 % (w/v) agarose in 1 × TBE (45 mM Tris-borate, 1 mM EDTA) buffer for 25 minutes at 130 Volts. PCR reactions were carried out on a Mastercycler® Nexus gradient (Eppendorf, Germany). All gel images were captured after staining with ethidium bromide (0.5 μg/ml gel) using the ChemiDoc™ MP Imaging System (BIORAD, USA).

10x TBE buffer.

1) 10g Tris (Mw7=121.1g)
2) 55 g boric acid.
3) 40 ml 0.5 M EDTA (pH=8.0)
4) H_20 to 1L.

5.13. Small RNA northern hybridization

Prepare a 12% denaturing polyacrylamide gel containing 20 gm Urea, 4 ml 4X TBE, 12 ml 40% acrylamide and add 5 ml distilled water. Heat the solution until urea is dissolved followed by cooling on ice. Assemble the gel casting frame and casting stand. Add 240 µL of 10% (w/v) ammonium persulfate and the add 16 µL of TEMED. Mix the solution quickly, and then pour the gel into the mold of a gel apparatus with a 20 well comb. Allow the gel to polymerize for 40 min at room temperature.

Mix 5 µg of total RNA sample with an equal volume of formamide loading die. Heat the mixture for 10 min at 95˚C, and then place the tubes on ice. Wash the wells of the gel to remove urea. This is essential to produce uniform, straight bands. Then, carefully load RNA sample into each well of the gel. Run the gel in 1× TBE buffer at 450 V until the bromophenol blue reaches the bottom of the gel (approx. 1.5 h).

Now cut a piece of Hybond NX nylon membrane and 6 pieces of blotting paper to the dimensions of the gel and soak them in 1× TBE buffer. Place three pieces of pre-soaked, paper on the surface of the semi dry transfer equipment. Remove air bubbles by rolling the paper with a clean roller. Place the presoaked membrane on top of the blotting papers and roll out the air bubbles again. Carefully lay the gel on top of the membrane and place the other three pieces of pre-soaked, blotting paper on top of the gel, and roll out the air bubbles again. Connect the cathode to the sandwich stack, and then place the safety cover on the unit. Transfer at constant voltage of 10 V for 1 h.

After transfer, place the membrane on a piece of filter paper, and cross-link the RNA to the membrane with 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Pour the EDC solution on the top of the filter paper and then place the membrane on it. Wrap in plastic foil and keep it at 60 ℃ in an incubator for 1 h. Followed by washing with sterile distilled water for 30 seconds.
Now put the membrane into a tube having 25 ml 2X SSC solution in the hybridization oven for 15 min at 50 ˚C with rotation. Throughout solution and fill it with 12 ml ultrahyb prehybridization solution for 30 min for prehybridization. Pour back the solution into the falcon tube and reuse it. Now add 12 ml ultrahyb solution containing 1 µL probe at 50 ˚C with rotation for overnight.
Next day, wash the membrane two times for 10 minutes each with 50 ml 2XSSC + 0.1 % solution. Warm the blocking buffer, substrate equilibration buffer, and wash buffer. Place the membrane in aluminium tray having 16 ml blocking buffer and agitate for 30 minutes. Which is followed by addition of 50 µL of streptavidin in the same blocking buffer and agitate again for 30 minutes. Wash the membrane for 3 times with 1X washing buffer. Cover the membrane with SEB buffer for 5 minutes by continuous agitation.
Finally prepare the detection solution by mixing 950 µL Luminol and same amount of 950 µL Hydrogen peroxide. Put the membrane on an x-ray film and pour the working solution onto the membrane and incubate for 1 mint.
Now detect the signal under the chemidoc machine using the function "Chemi High Resolution, and at the last take a colorimetric image as well to find the exact position on the membrane.

5.14. Wheat transformation

5.14.1. Preparation of Agrobacterium for transformation

From the inoculated plate single colony of *Agrobacterium* strain AGL1 containing the desired vector, were inoculated into 5 ml of liquid YEB medium supplemented with the appropriate antibiotics. The cultures were then incubated at 30 °C in a rotary shaker, shaken at 200 rpm for approximately 16 hours. The inoculated medium was then incubated at 30 °C in a rotary shaker, shaken at 200 rpm overnight, for approximately 16 hours, to allow for optimal growth and preparation for the transformation process.
To prepare standard inoculums for transformation, a mixture of glycerol and culture was employed. 850 µL *Agrobacterium* culture and 250 µL of 87% sterile glycerol were combined, and

1000 µl aliquot was dispensed into 1.5-ml microcentrifuge tube. This aliquot was subsequently frozen at -80°C for long-term storage and for subsequent transformation events.

5.14.2. Embryo Collection, Sterilization, and Embryo inoculation

On the day of transformation, the bacteria were pelleted by centrifugation in a 50-ml Falcon tube at 3100 rpm for 10 minutes at 24°C. Following centrifugation, the supernatant was discarded, and the cells were gently resuspended in 10 ml of wheat inoculation medium (WIM) to achieve an optical density of 0.5 OD (600 nm). Subsequently, 100 mM acetosyringone (AS) was added to a final concentration of 100 µM. The culture was then incubated for 1-2 hours at room temperature. This period allowed the activation vir genes before the transformation process.

5.14.3. Collection and sterilization of immature seeds

To begin the wheat transformation process, wheat spikes were collected approximately 14 days post-anthesis, when the immature embryos reached a diameter of 1-1.5 mm and were at the early milk stage. Kernels from florets 1 on the central spikelet were specifically chosen for transformation. Prior to processing, the awns were removed from the ears, leaving approximately 3-5 mm from the grain. The immature grains were then separated from the ear and placed in a sterile 150-ml jar. Under aseptic conditions, such as within a laminar flow cabinet, the grains were sterilized using 70% (v/v) ethanol for 1 minute. Following ethanol treatment, the grains were rinsed once with sterile distilled water. Subsequently, they were submerged in 10 ml of 10% (v/v) sodium hypochlorite solution and allowed to shake for 7 minutes. After the hypochlorite treatment, the grains were washed three times with sterile distilled water to remove any residual disinfectant.

5.14.4. Isolation of immature embryos, inoculation with Agrobacterium, and co-cultivation

To isolate the embryos from the immature grains, fine forceps and knives were used. Subsequently, the embryos were transferred to 1.5-ml microcentrifuge tubes containing 1 ml of wheat inoculation medium (WIM) supplemented with 0.05% Silwet L-77, with approximately 100 embryos placed in each tube. After isolating all the embryos, the WIM was removed, and fresh WIM was added to the microcentrifuge tube(s). The isolated embryos were then centrifuged for 10 minutes at 14,000 rpm, 4°C. Following centrifugation, the WIM was removed using a pipette, and 1 ml of

Agrobacterium solution was added to each tube. The tubes were inverted for 30 seconds, and then incubated at room temperature in the dark for at least 20 minutes. After the incubation period, the *Agrobacterium* suspension with the embryos was poured into an empty sterile petri plate, and the suspension was removed with a pipette. Subsequently, 25 embryos, with the scutellum side facing up, were transferred to fresh petri plates of wheat co-cultivation medium. The petri plates were sealed with Micropore tape and incubated at 24±1°C in the dark for 3 days for co-cultivation.

5.14.5 Resting period, callus induction and selection of transformed material

After a 3-day co-cultivation period, the embryogenic axes were excised from the embryos using knives. These embryos were then transferred to fresh callus induction plates (WCI), containing a medium consisting of 2 mg L−1 Picloram, 0.5 mg L−1 2,4-dichlorophenoxyacetic acid (2,4-D), 160 mg L−1 cefotaxime, and 5 mg L−1 agarose. The plates were subsequently incubated at 24 ± 1 °C in the dark for 5 days. During this incubation, cefotaxime was included in the media to control *Agrobacterium* during the resting period. Following this period, the embryos were transferred, with the scutellum side facing up, to fresh WCI plates supplemented with 20 mg mL−1 Hygromycin. They were then incubated at 24 ± 1 °C in the dark for 2 weeks. At the next transfer, the calli were split into two clumps of approximately 4 mm−2, and callus pieces derived from each single embryo were carefully labelled to keep track of their origin. These calli were then transferred to fresh selection plates (WCI) containing 40 mg L−1 Hygromycin (Selection 2) and incubated at 24 ± 1 °C in the dark for another 2 weeks. At Selection 2, the number of explants per plate was reduced by approximately half. After 2 weeks, the calli were transferred to a lit culture room under fluorescent lights (100 µmol m−2 s−1) at 24 ± 1 °C with a 16-hour photoperiod and covered with a single layer of paper towel for an additional week. During this period, putative transformed lines should begin to green and produce small shoots, indicating successful transformation and growth.

5.14.6. Regeneration, Rooting, and acclimatization

Regeneration of transgenic plants

For the regeneration of transgenic plants, the calli were subjected to a final transfer to wheat regeneration medium (WRM) after three weeks on selection 2 medium. This transfer was conducted in deep boxes. All regenerating callus derived from a single embryo was meticulously labelled to facilitate tracking of its origin. Upon transfer to WRM, the paper covering was removed

from the dishes, and the calli were cultured under fluorescent lights (100 μmol m^{-2} s^{-1}) at 24 ±1 °C with a 16-hour photoperiod. This environment provided the necessary conditions for the regeneration process to proceed efficiently.

Rooting

Regenerated shoots, measuring 1–2 cm in length and displaying visible roots, were carefully transferred to petri dish contained WCI medium without growth regulators, solidified with 3 g L−1 Gelzan, and supplemented with 300 mg L−1 Cefatoxim and 20 mg L−1 Hygromycin. This medium composition supported further growth and development of the shoots in a controlled environment. Subsequently, putative transformed plants exhibited a robust root system characterized by the development of root hairs. This observation indicated successful transformation and demonstrated the health and vigor of the transgenic plants.

Acclimatization

After approximately 10 days, the regenerated plantlets with strong root systems were gently removed from the plates using forceps. The roots were then washed with cool running water to eliminate any residual culture medium. Subsequently, the plants were planted in Jiffy. To ensure successful establishment, the plants remained covered with the lid for approximately 1 week, maintaining high humidity around them. This allowed the plants to acclimate to the soil environment. Following this period, the plants were transferred in 13mm round pots containing peat and sand mix (5:1) and grown under the same conditions as the donor plants within a controlled environment room. Once the plants were well established in the soil, leaf samples could be collected for further analysis to confirm the presence of the introduced genes.

6. RESULTS

Preliminary findings

A novel wheat seed specific miRNA was found during the library preparation from 10-, 20- and 30-day old seeds of wheat *Triticum aestivum* L. cv. Bankuti (B35 and B52 line) and *Triticum spelta* in two biological replicates. Small RNA libraries were prepared from either total RNA or the isolated small RNA fraction to compare the sequencing data generated from small RNA library (Nagy et al., 2016). During data analysis, in addition to known miRNAs, a cluster of previously unidentified 21-nucleotide-long miRNAs (tae-2187) was detected, as shown in the second bar of the graph (see Fig. 9).

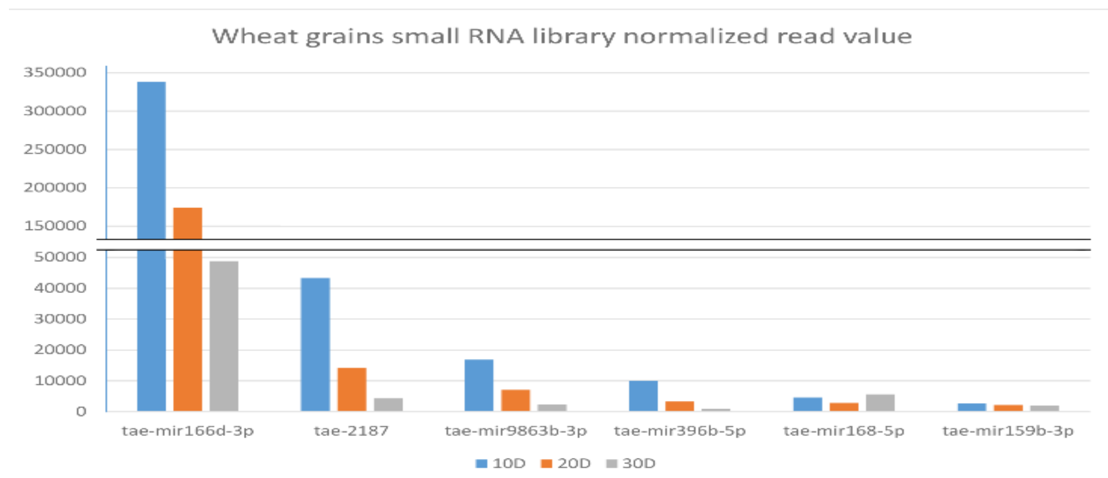

Fig. 9: This bar graph showing wheat grains small RNA library normalized read in *Triticuam aestivum* cv. Bánkúti. A high number of reads for an unknown miRNA can be seen in the second left bar (tae2187).

Small RNA northern hybridization

Initially, the biological presence of this recently found miRNA was confirmed through small RNA northern hybridization. RNA samples were extracted from seeds of Bankuti BK35, Bankuti BK52, and *Triticum spelta* at 10, 20, and 30 days post-anthesis. Following hybridization with a DNA probe specific to the miRNA and subsequent detection using chemiluminescence, a band of the anticipated size was detected on the membrane (See fig. 9). Notably, this novel miRNA was exclusively detected in seed tissue and was absent in libraries generated from other tissues such as leaves (not shown).

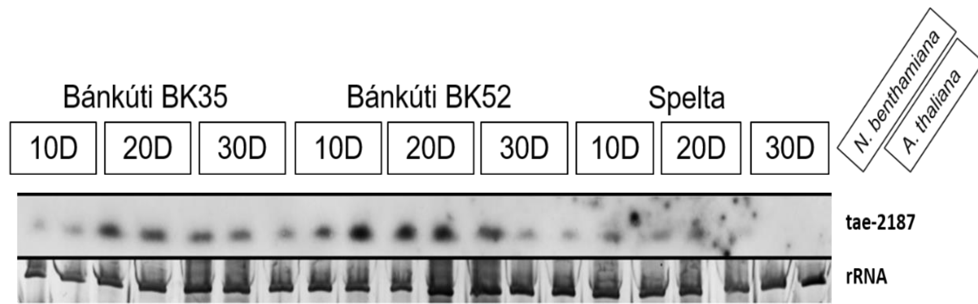

Fig. 10: Small RNA northern blotting analysis showing the presence of a new miRNA (tae2187). The miRNA tae2187 is present in the *Triticum aestivum* L. cv. Bankuti BK35, Bankuti BK52 and *Triticum spelta*.

Following the confirmation of its presence, a sequence of bioinformatic analyses was conducted. The precursor of this miRNA was identified across the wheat sub-genomes A, B, and D. The secondary structure of the precursor was forecasted using the RNAfold web Server software (accessible at http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). The resulting secondary structure can be seen in Fig 11.

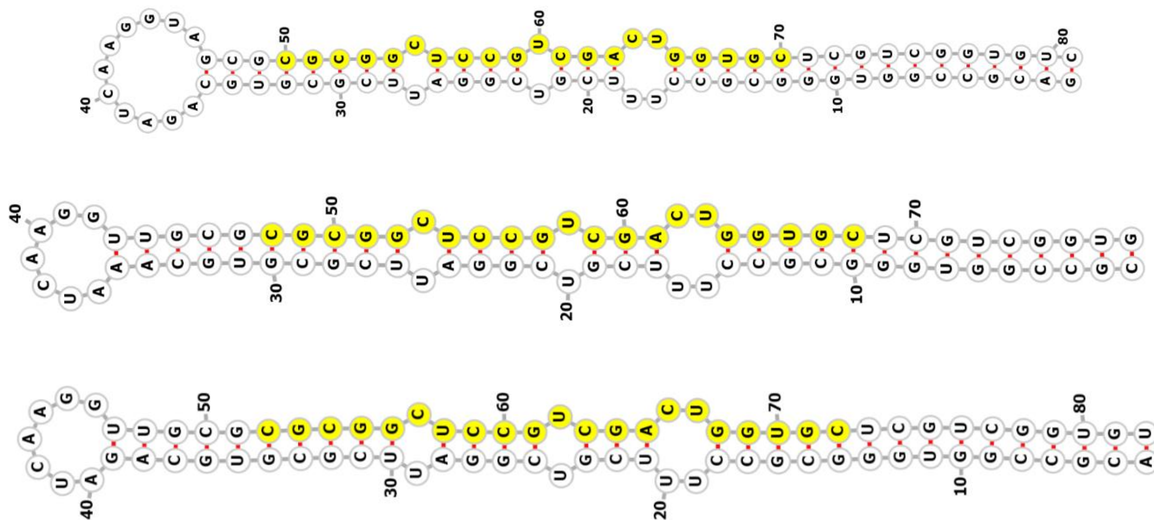

Fig. 11: Secondary structure of the precursor of miRNA tae2187. The top image is of A precursor, Middle is showing B precursor and bottom image is depicting D precursor. The miRNA sequence in all three precursor is highlighted in yellow colour.

In further examination, the target of this miRNA was identified using psRNATarget, revealing that a messenger RNA encoding the DNA-directed RNA Polymerase V subunit is a potential target of this novel miRNA. Consequently, this messenger RNA will be referred to as NRPE1. This nomenclature is based on the homology of proteins shared between *Arabidopsis thaliana* and *Triticum aestivum*. Given the similarity in protein sequences and the presence of identical essential protein domains in both genes, the designation NRPE1 was assigned. It's important to note that while there is a lack of experimental evidence to definitively confirm identical functionality, based on bioinformatic discoveries and protein similarities, it is termed NRPE1 (as illustrated in Fig. 12).

Alignment	Target Description	Inhibition	Multiplicity

`miRNA 21 CGUGGUCAGCUGCCUCGGCGC 1`
`::: :.:::::::::::::::`
`Target 5887 GCAGCGGUCGACGGAGCCGCG 5907`
	cdna chromosome:IWGSC:6B:155759318:155777793:-1 gene:TraesCS6B02G151900 gene_biotype:protein_coding transcript_biotype:protein_coding description:DNA-directed RNA polymerase subunit [Source:UniProtKB/TrEMBL;Acc:A0A341XB43]	Cleavage	1

`miRNA 21 CGUGGUCAGCUGCCUCGGCGC 1`
`::: :.:::::::::::::::`
`Target 5683 GCAGCGGUCGACGGAGCCGCG 5703`
	cdna chromosome:IWGSC:6B:155759318:155777793:-1 gene:TraesCS6B02G151900 gene_biotype:protein_coding transcript_biotype:protein_coding description:DNA-directed RNA polymerase subunit [Source:UniProtKB/TrEMBL;Acc:A0A341XB43]	Cleavage	1

`miRNA 21 CGUGGUCAGCUGCCUCGGCGC 1`
` :: :.:::::::::::::::`
`Target 5729 CCAACGGUCGACGGAGCCGCG 5749`
	cdna chromosome:IWGSC:6D:81230475:81247577:-1 gene:TraesCS6D02G113900 gene_biotype:protein_coding transcript_biotype:protein_coding description:DNA-directed RNA polymerase subunit [Source:UniProtKB/TrEMBL;Acc:A0A341XKG8]	Cleavage	1

`miRNA 21 CGUGGUCAGCUGCCUCGGCGC 1`
` :: :.:::::::::: ::::`
`Target 5786 CCAACGGUCGACGGAGGCGCG 5806`
	cdna chromosome:IWGSC:6A:97808607:97829562:-1 gene:TraesCS6A02G123800 gene_biotype:protein_coding transcript_biotype:protein_coding description:DNA-directed RNA polymerase subunit [Source:UniProtKB/TrEMBL;Acc:A0A341WDG7]	Cleavage	1

Fig. 12: psRNATarget score of this miRNA on target Pol V. Pol V messenger RNA on genome 6B and 6D has a better score than on genome 6A because it has a mismatch.

6.1 PCR amplification of precursors

For the overexpression of miRNA tae2187, Precursor sequences were amplified with the help of PCR using universal primers for all the sub-genomes A, B and D using the DNA from the leaf of wheat cultivar Fielder. Expected mixed PCR amplicons were observed on the gel (Fig. 13).

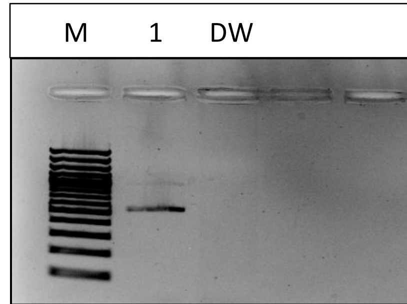

Fig. 13: PCR amplification of precursor sequences using genomic DNA as a template extracted from the leaf of Fielder wheat (expected size ~548 bp), M: Molecular marker, Thermo Scientific TM GeneRuler 100bp+ DNA Ladder.

6.2 Cloning of precursors into PJET1.2 plasmid

Amplified PCR fragments were then ligated into the plasmid PJET1.2 by following the manufacturer instructions. Finally ligated plasmids were transformed into *E coli* and transformed *E. coli* colonies were selected on the LB medium supplemented with ampicillin antibiotic. Colony PCR was performed to check integration of precursors into PJET1.2 plasmid. Expected PCR amplicon size was seen on the gel (Fig. 14).

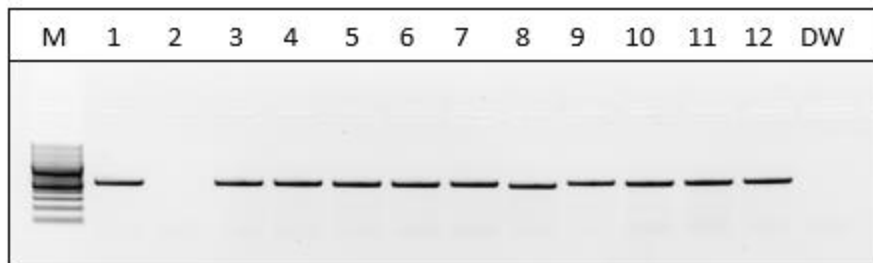

Fig. 14: Colony PCR amplification of precursor sequences using transformed *E. coli* cells with PJET1.2 plasmid ligated with either precursor A, B or D (expected size ~548 bp), M: Molecular marker, Thermo Scientific TM GeneRuler 100bp + DNA Ladder.

Based on restriction digestion all three possible precursors were separated. First, 3 µl of colony PCR amplicons were digested with *Nhe*I restriction enzyme for B precursor. Out of 11 amplicons it was observed that PCR amplicons coming from colonies 4, 5, 9 and 10 were digested with *Nhe*I that means these colonies contain PJET1.2 plasmid ligated with precursor B (Fig. 15).

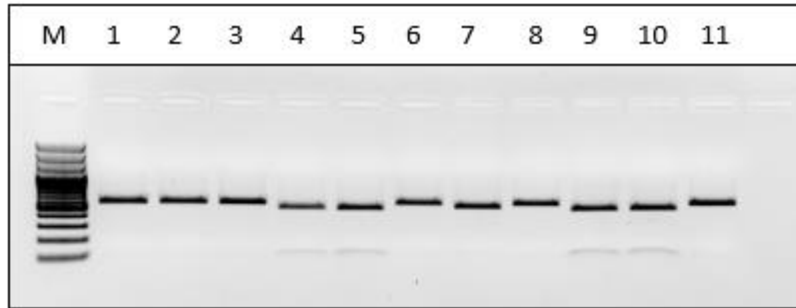

Fig. 15: Colony PCR amplicon digestion with *Nhe*I restriction enzyme for precursor B, PCR amplicon digestions from colonies 4, 5, 9 and 10 were digested. M: Molecular marker, Thermo Scientific TM GeneRuler 100bp + DNA Ladder.

For D precursor, again 3 µl of PCR amplicons were digested with *Sfi*I restriction enzyme (Fig 16.). It was found that out of 11 PCR amplicons, only PCR coming from colony 7 was digested that means it contained a precursor D.

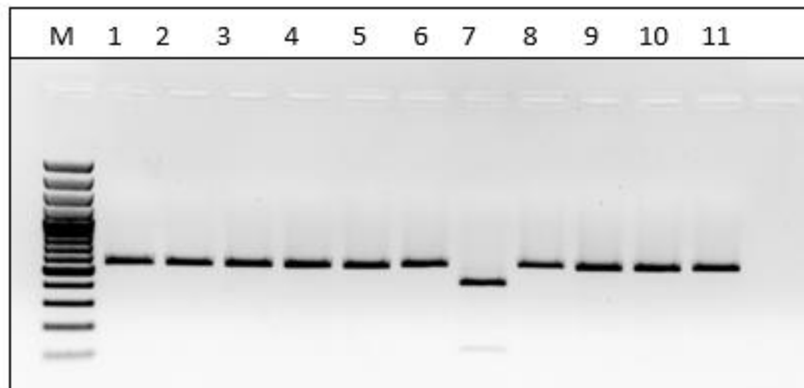

Fig. 16: PCR amplicon digestion with *Sfi*I restriction enzyme for precursor D, only PCR amplicon from colony 7 was digested. M: Molecular marker, Thermo Scientific TM GeneRuler 100bp+ DNA Ladder.

PCR amplicons neither digested with *Nhe*I nor *Sfi*I restriction enzymes were considered as precursor A, for example amplicons 1, 2, and 3 because these two enzymatic sites were absent on precursor A.

6.2 Cloning of precursors into binary vector pC61K

In the next step, colonies having plasmid ligated with A, B and D precursors were grown in liquid LB-amp medium. Plasmids were extracted from *E. coli* colonies having PJET1.2_Pre2187A,

PJET1.2_Pre2187B, and PJET1.2_Pre2187D. Before amplification and ligation of precursors into the binary vector pC61K. Once more the integration of precursors into the plasmid PJET1.2 was checked with restriction digestion. Plasmids were digested with *Xba*I and *Xho*I restriction enzymes. An expected size of cleaved fragment was observed on the gel, which can be seen in (Fig. 17).

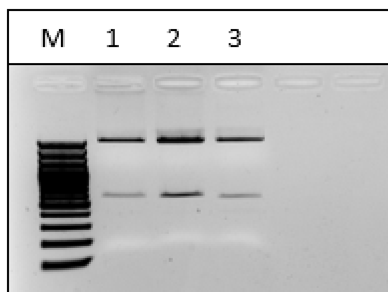

Fig. 17: Plasmid digested with *Xba*I and *Xho*I restriction enzymes, 1: PJET1.2_Pre2187A, 2: PJET1.2_Pre2187B, 3: PJET1.2_Pre2187D, M: Molecular marker, Thermo Scientific TM GeneRuler 100bp + DNA Ladder.

Finally, precursor A, B and D were amplified with PCR to clone in binary vector pC61K using PJET1.2_Pre2187A, PJET1.2_Pre2187B and PJET1.2_Pre2187D plasmids as a template. The same primer pairs which were used to amplify precursors from Fielder wheat were used again to amplify precursors from plasmids but with slight modifications. The forward primer was flanked with *Kpn*I restriction site and reverse primer was flanked with *Bam*HI restriction site which helps to ligate amplified precursor fragments into pC61K binary plasmid. On the gel expected PCR amplicons of precursors were observed (Fig. 18)

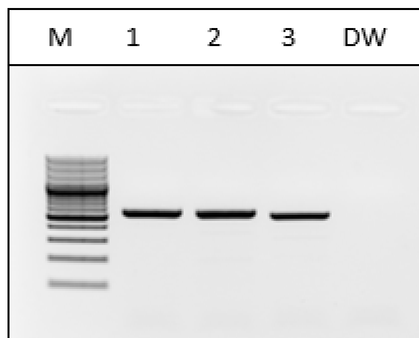

Fig. 18: PCR amplification of precursors A, B and D using plasmid PJET1.2_Pre2187A, PJET1.2_Pre2187B and PJET1.2_Pre2187D as template (from left to right), M: Molecular marker, Thermo Scientific TM GeneRuler 100bp+ DNA Ladder.

PCR amplicons were digested with *Bam*HI and *Kpn*I restriction enzymes and were purified. Binary vector pC61K was also digested with same *Bam*HI and *Kpn*I restriction enzymes and was purified. 10- and 20-times dilution series were made of PCR amplicons and plasmid. Which were then loaded on the gel to find appropriate amount for ligation (Fig. 19).

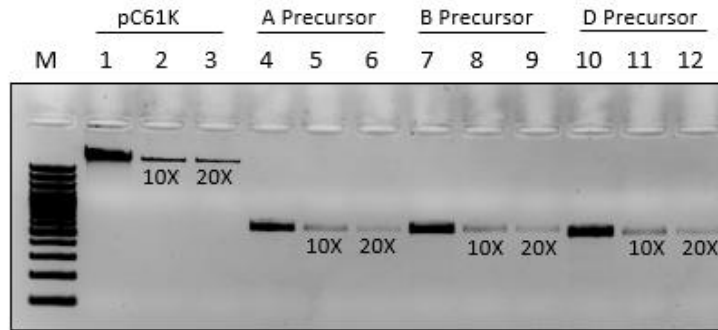

Fig.19: Gel photo representing dilution of plasmid pC61K, and of precursor A, Precursor B, and precursor D. M: Molecular marker, Thermo Scientific TM GeneRuler 100bp + DNA Ladder.

Ultimately, after deciding the appropriate amount of precursors and plasmid, precursors were ligated into pC61K binary vector. Following the ligation, the mixture was transformed into competent *E. coli* cells. Transformed colonies with pC61K_Pre2187A, pC61K_Pre2187B and pC61K_Pre2187D plasmids were selected on LB-Kanamycin plate. Colony PCR was carried out to check if the transformation was successful with desired plasmid and they contain the precursors. The expected size of PCR amplicons can be seen in the gel photo (Fig. 19). Plasmids were extracted from the first clone of each precursor construct and were sequenced to confirm that the integrated precursor sequence is good without any point mutation which may occur during PCR.

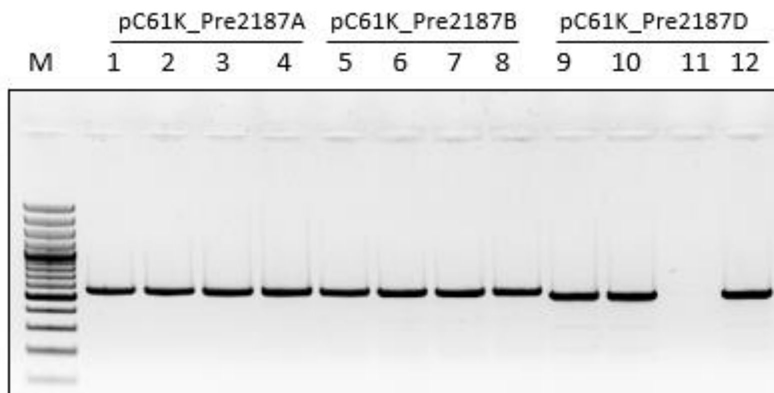

Fig.20: Colony PCR amplification of precursors using diluted colonies as template (expected size ~548 bp), M: Molecular marker, Thermo Scientific TM GeneRuler 100bp + DNA Ladder.

6.3 wheat transformation and transgene analysis

Wheat plants of the Fielder cultivar, cultivated under specified conditions, underwent transformation according to the methodology outlined by Hayta et al. (2019) with some modifications. The transformation process involved the co-transformation of the pC61K_Pre2187B and pCubi_GRF4:GIF1_NOS binary vectors. A total of 3 lines were successfully regenerated from the transformation event. Approximately 150 embryos were utilized for the co-transformation. Notably, transformation efficiency of around 10% was achieved. To confirm successful transformation, DNA was extracted using the direct DNA extraction method, followed by first PCR analysis targeting hygromycin and precursor B with specific primers to the genes, naming Hyg_det_Fw and Rv primer, and Pre2187B_uni_Fw and Rv primer respectively. For the positive sample, a mixture of plasmid pC61K_Pre2187B and a plasmid having hygromycin was used, +VE and DW stands for positive sample and distilled water respectively and are indicated in same manner in all below gel pictures (Fig. 2).

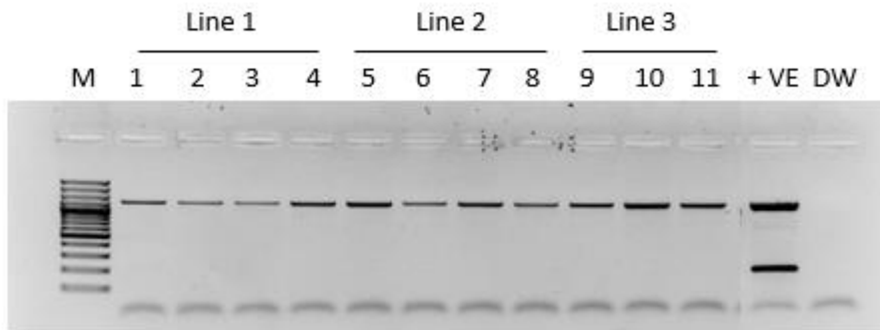

Fig. 21 PCR amplification of hygromycin resistance gene (*hptII*), a plasmid having hygromycin gene mixed with pC61K_Pre2187B plasmid used as positive control. M: Molecular marker, Thermo Scientific ™ GeneRuler 100bp+ DNA Ladder.

In a second round of PCR, GRF4:GIF1 chimera gene with primer GRF:GIF_det_Fw and Rv primer, which helps in regeneration, was amplified with the help of PCR. All plants of every three lines were also positive for GRF4:GIF1 chimera gene. Plasmid pCubi_GRF4:GIF1_NOS was used for positive control in PCR reaction (Fig. 22).

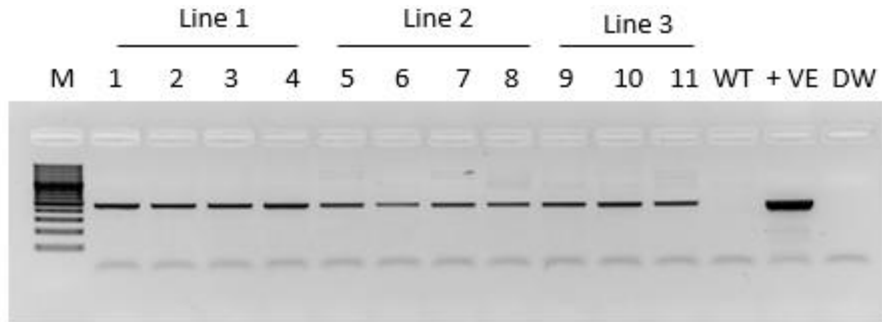

Fig. 22: PCR amplification of GRF4:GIF1 chimera gene, pCubi_GRF4:GIF1_NOS plasmid used as positive control. M: Molecular marker, Thermo Scientific TM GeneRuler 100bp+ DNA Ladder.

Finally, a PCR reaction was carried out against precursor B sequence using specific primers, naming Pre2187_uni_Fw and Rv. But with PCR it failed to amplify. (Fig. 23).

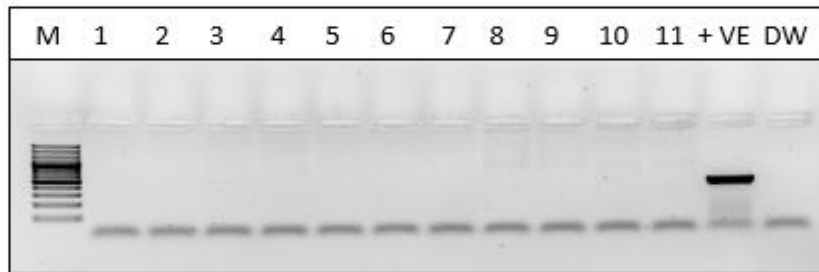

Fig. 23PCR amplification of precursor B sequence, sample from 1-11 represents transgenic lines, pC61K_Pre2187B plasmid used as positive control. M: Molecular marker, Thermo Scientific TM GeneRuler 100bp+ DNA Ladder.

In parallel, miRNA tae2187 was detected with small RNA northern hybridization. For this RNA was extracted from all the 11 transgenic plants of each 3 lines RNA was also extracted from young WT leaf and flag WT leaf (Fig. 24).

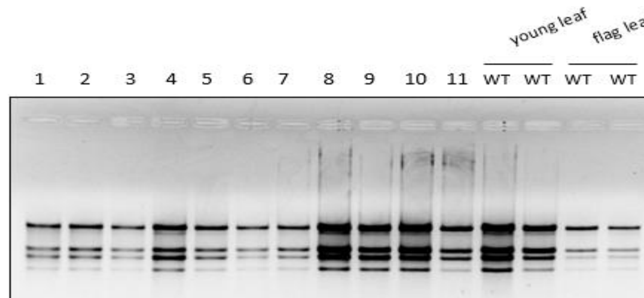

Fig. 24: RNA extraction from transgenic lines, from young leaf of WT Fielder and from flag leaf of WT Fielder.

Small RNA northern hybridization was performed using LNA probe against the miRNA tae2187. miRNA was detected in GRF:GIF transgenic plant young leaves but not in the WT plant young leaf. Intrestingly this miRNA was also detected in flag leaf of WT plant. RNA from WT embryo and endosperm were used as a positive control during small RNA northern hybridization. WT embryo has the highest expression of the miRNA tae2187 which is followed by endosperm (Fig. 25).

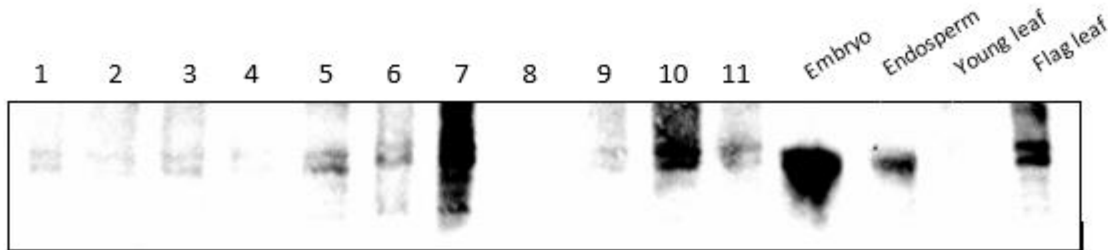

Fig. 25: Small RNA northern hybridization using LNA probe against miRNA tae2187. Embryo and endosperm are used as positive control. WT young leaf and flag leaf as a negative control.

Because transgenic lines gave a very poor signal for the expression of miRNA tae2187, A further PCR reaction was carried out against the Kanamycin resistance gene. But by PCR no any amplicons were detected on the gel. Only PCR amplicon was present in positive control using pC61_Pre2187B plasmid as a control (Fig. 26).

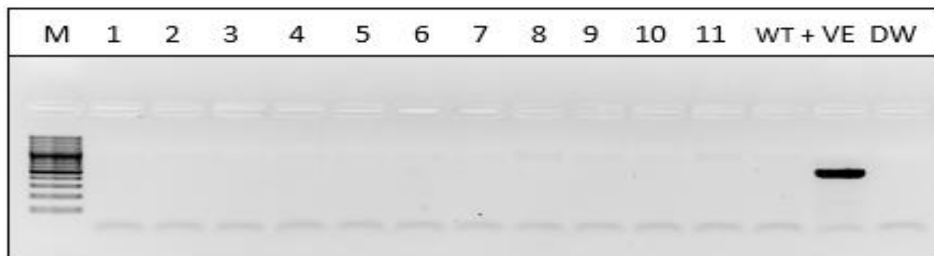

Fig. 2: PCR amplification of Kanamycin resistance gene, sample from 1-11 represents transgenic lines, pC61K_Pre2187B plasmid used as positive control. M: Molecular marker, Thermo Scientific ™ GeneRuler 100b + DNA Ladder.

7. DISCUSSION AND RECOMMENDATIONS

The main aim of this project was to overexpress a newly found wheat seed specific miRNA tae2187 to study the most potential target of this miRNA based on psRNATarget. This new miRNA was found during small RNA library preparation from different tissues of wheat *Triticum aestivum* L. *cv*. 'Bankúti' and *Triticum spelta* for another project (Nagy et al., 2018). The main target of this novel miRNA is the messenger RNA of Polymerase V subunit. In plants, a prominent pathway for small RNA-mediated epigenetic modifications is RNA-directed DNA methylation (RdDM). RdDM is distinctive in plants because it relies on specialized transcriptional machinery centered around two plant-specific RNA polymerase II (Pol II)-related enzymes known as Pol IV and Pol V.

To study and prove the target of this miRNA, overexpression lines of wheat cultivar Fielder was produced using *Agrobacterium* mediated transformation method. Wheat embryos (14DPA) were used as an explant for transformation. It is important to choose a perfect stage embryo for transformation. In our experience we failed the transformation once because of inappropriate stage of embryos as it is mentioned in the literature that wheat has a narrower window of inappropriateness than rice and maize (Ishida et al., 2019). A protocol outlined in (Hayeta et. al., 2019) was utilized for the Fielder wheat transformation. They achieve almost 25% of transformation efficiency, but we got only almost 10% efficiency which is comparatively low. The reason behind this could be, embryo size, plant health, growing conditions etc. Although co-transformation of binary vectors, pC61K_Pre2187B and pCubi_GRF4:GIF1_NOS was tried but unfortunately only vector pCubi_GRF4:GIF1_NOS T-DNA containing hygromycin selection gene was integrated. Interestingly, plants being not transformed with pC61K_Pre2187B overexpression vector, gives miRNA tae2187 expression based on small RNA northern blot, although very faint signals were seen, in the young leaf by small RNA northern hybridization and the signal was absent in young leaf of WT plants, but present in wild type flag leaf too. The most suited hypothesis of this can be the induction of this miRNA gene which may occur during the callus induction and plant regeneration from tissue culture. Because alteration in DNA methylation pattern is very frequent in the plant genome during the tissue culturing (Ghosh et al., 2021). It is also interesting to highlight that this miRNA is present in WT flag leaf as well. And the reason behind this could be that flag leaves play a crucial role in wheat reproductive development, contributing around 45% to 58% of the total photosynthesis activity and approximately 41% to

43% of the carbohydrates required for grain filling. Due to their unique significance, flag leaves exhibit a more intricate regulatory network of miRNAs compared to other tissues (Han et al., 2014). We also observed that the miRNA in the seed was 21 nt in size, while the miRNA in the young leaves of tissue-cultured plants and flag leaf was present in two sizes. This is presumably due to differences in the Dicer proteins involved in the maturation of the miRNA precursor.

This experiment can be extended further by doing the transformation again with pC61K_Pre2187B binary vector to produce miRNA tae2187 overexpression lines. Another experiment should be performed by completely knocking out the miRNA gene with CRISPR/Cas9 technology with a hope that the knockout will not be lethal. It would be also interesting to reveal the relationship of this miRNA with pol V subunit messenger RNA by Rapid Amplification of cDNA 5'Ends (5'RACE) technology in the leaf of overexpression lines. The miRNA is already being studied with STTM technology which also give a positive result about this miRNA target i.e. Pol V.

8. SUMMARY

Thesis Title: Investigating the function of a novel wheat seed-specific miRNA by over expression in transgenic wheat (*triticum aestivum* L.) lines.

Written by: Mohammad Danish

Course: Master of science in Agricultural Biotechnology

Institute: Institute of Genetics and Biotechnology

Primary thesis advisor: Dr. Kis András, PhD, Research Fellow, Institute of Genetics and Biotechnology

Secondary thesis advisor: Mohammad Ali, PhD candidate, Institute of Genetics and Biotechnology

microRNAs (miRNAs) are small noncoding RNAs crucial for regulating gene expression by targeting specific messenger RNA (mRNA) molecules. Within the cytoplasm, small RNAs facilitate PTGS by targeting complementary mRNA molecules, leading to their degradation or translational repression. Conversely, in the nucleus, small RNAs arrange TGS by directing repressive epigenetic modifications, such as DNA cytosine methylation and histone methylation, to homologous genomic regions. In plants, a notable pathway for small RNA-mediated epigenetic modifications is RNA-directed DNA methylation (RdDM). This process involves specialized transcriptional machinery centered around two plant-specific RNA polymerase II (Pol II)-related enzymes: Pol IV and Pol V. Pol IV transcribes a single-stranded RNA which is copied into a double-stranded RNA by (RDR2). The dsRNA is processed by DCL3 into 24 - nucleotide siRNAs. On the other hand, Pol V transcribes a scaffold RNA that base-pairs with AGO4-bound siRNAs. RDM1 links AGO4 and DRM2, which catalyses de novo methylation of DNA.

Identification of microRNAs (miRNAs) serves as the initial step in unraveling their functional roles. Deep sequencing technology provides insight into the expression levels of each miRNA based on read counts. With NGS we found a new wheat specific miRNA during small RNA library preparation from grain, and it was called miRNA tae2187. PsRNATarget predicted that Pol V is a potential target of this miRNA. To prove it in planta, miRNA overexpression lines were produced. Seeds of the spring wheat (*Triticum aestivum* L.) *cv.* 'Fielder' were grown under controlled environment in growth chambers (Sanyo) under 16h/8h light/dark period, 70% humidity with light

levels of 50 μE $m^{-2}s^{-1}$ provided by fluorescent tubes and tungsten lighting. Plants were not sprayed with fungicides or insecticides at any stage of growth.

The overexpression construct having precursor B sequence was made with the help of restriction digestion and ligation assay. The construct was made and first introduced into *E. coli* (strain DH5α) and was finally introduced into *Agrobacterium tumefaciens* strain AGL1. Wheat embryos were extracted at 14 days post anthesis and were put for cocultivation with *Agrobacterium* for transformation. Co-transformation of pC61K_Pre2187B with a vector having GRF4:GIF1 chimera gene was taken into consideration. Callus was induced and went through several selection media. Successfully regenerated plants were put into the LSF medium for root growth. Plants with strong roots were transferred into Jiffy followed by transfer into the soil.

DNA was extracted from regenerated lines and were put for PCR analysis against hygromycin, Kanamycin, GRF4:GIF1 chimera gene and precursor B sequence. Plants positive with hygromycin resistance gene (*hptll*) were taken for further analysis. Total RNA was extracted from the leaf of transformed and WT control lines. First, miRNA expression was checked in leaf with the help of small RNA northern hybridization. Although miRNA expression was seen but later it was found that plants were only transformed with pCubiGRF4:GIF1_NOS vector not with over expression pC61K_Pre2187B vector. The most suited hypothesis of this can be the induction of this miRNA gene which may occur during the callus induction and plant regeneration from tissue culture.

9. ACKNOWLEDGMENT

Yet journey leading to the completion of this thesis has been marked by twists and turns, yet it culminates with gratitude towards those who have contributed to its realization. I am indebted to the following individuals, Firstly, I extend my deepest gratitude to the Almighty Allah for granting me the wisdom, resilience, and good health to see this endeavor through. His guidance has been paramount in navigating the challenges along the way. To my beloved family ,especially my father and my elder brother Adv. Mohd Jahangeer and my younger brother Mohd Asif. I offer my heartfelt thanks for their unwavering support and encouragement. Their constant presence and belief in my abilities have been my pillars of strength throughout this journey. I am profoundly grateful for their love and understanding.

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