THESIS

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M.Sc. Agricultural Biotechnology

Gödöllő

2023

Hungarian University of Agriculture and Life Sciences

Szent István Campus

Agricultural Biotechnology

Functional Investigation of a novel wheat seed-specific miRNA by short tandem target mimic (STTM)

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Gödöllő

2023

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2. ABBREVIATIONS

- nts (nucleotides)
- bps (billion bases)
- STTM (short tandem target mimic)
- RNAi (RNA interference)
- PTGS (post-transcriptional gene silencing)
- TGS (transcriptional gene silencing)
- miRNA (microRNA)
- cDNA (complementary DNA)
- mRNA (messenger RNA)
- CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)
- BSA (bulked segregant analysis)
- ssRNAs (single-stranded RNAs)
- dsRNAs (double-stranded RNAs)
- RISC (RNA-induced silencing complex)
- shRNA (small hairpin RNAs)
- Transposable element (TE)
- RdDM (RNA-directed DNA methylation)
- Pol IV (polymerase IV)
- AGO (ARGONAUTE)
- NMD (Nonsense-mediated mRNA decay)
- viRNA (viral RNA)
- RDR 6 (RNA-dependent RNA polymerase 6)
- SGS3 (suppressor of gene silencing 3)
- siRNA (small interfering RNA)
- DCL (Dicer-like proteins)
- PepGMV (pepper golden mosaic virus)
- PAR-CLIP (Photoactivatable Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation)
- iCLIP (Individual-nucleotide resolution UV crosslinking and immunoprecipitation)
- IAA (Indole acetic acid)

- pri-miRNAs (primary miRNAs)
- pre-crRNAs (CRISPR RNAs)
- CLIP (crosslinking immunoprecipitation)
- NHEJ (non-homologous end joining)
- WIM (wheat inoculation medium)
- AS (acetosyringone)
- WRM (wheat regeneration medium)

3. INTRODUCTION

Wheat is a crucial cereal crop that plays a significant role in feeding a significant percentage of the world's population. Nevertheless, its production is often hindered by several challenges, such as pest infestation, diseases, and harsh environmental conditions like drought. These challenges significantly impact both the yield and quality of the crop (Baillo et al., 2019). Scientists have been investigating the genetic factors that influence the growth and development of wheat to find ways to overcome the difficulties associated with crop production. RNA interference (RNAi) is one such mechanism that can be utilized to selectively silence genes and control their expression, offering a promising solution to improve crop yield and quality (Kaur et al., 2021). RNAi is a highly effective method for studying gene function and can be accomplished by either post-transcriptional gene silencing (PTGS) or transcriptional gene silencing (TGS) (Mendez et al., 2015; Ashfaq et al., 2020). Among the different RNAi approaches, microRNA (miRNA) knock-down is a popular strategy, that can be implemented using either short tandem target mimic (STTM) or Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) techniques (Teotia et al., 2016).

miRNAs are small RNA molecules that play a crucial role in regulating gene expression after transcriptional modification in plants (Kumar et al., 2018). The identification of several miRNAs in crops, including wheat, has shown their significance in different biological processes such as plant growth, development, response to stress, and disease resistance (Zhou and Luo, 2013; Kumar et al., 2018). Therefore, exploring a novel wheat seed-specific miRNA through the STTM technique presents a promising way of gaining insights into the mechanisms that regulate seed development in wheat.

STTMs have a size of about 100 nucleotides (nt) and consist of two tandemly arranged miRNA binding elements, each of which is designed with a mismatch located at the miRNA cleavage site. The miRNA binding elements are connected by a flexible stem-loop linker that spans between 48-88 nt. STTM technique works by promoting the degradation of most of the target miRNAs, and this is attributed to the action of small nucleases that cleave the miRNA-mimic duplex. The STTM method has been used to silence various miRNA families in *Arabidopsis* and several model and staple crops such as tomato, rice, wheat, tobacco, *Medicago*, soybean, poplar, cotton, common bean, and barley. Additionally, this technique has been employed in animals too. STTMs have been introduced into plant cells through

different methods, including stable transformation, *Agrobacterium*-mediated transient expression, and virus-induced-gene silencing (Peng et al., 2018).

The STTM method's versatility has made it possible to investigate miRNAs' known and undiscovered roles in crops and model plants. Multiple miRNAs are known to regulate specific transcripts in unique plant tissues or in different stress conditions. By manipulating the STTM promoter region, the STTM technique can be customized to achieve expression in specific tissues or response to specific stimuli, minimizing unintended effects. Targeted expression of STTM at specific stages of plant growth can aid in better understanding miRNA function without any negative impact on plant yield or phenotype (Othman et al., 2023).

Objectives

- Design a Short Tandem Target Mimic (STTM) construct in order to block 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 STTM transformed wheat lines with PCR to check the presence of STTM unit.
- Check the level of miRNA 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

Wheat (*Triticum aestivum* L.) is a crop that holds significant economic value and is a crucial source of nutrition for humans and livestock alike (Shewry and Hey., 2015). The latest data from 2020 from the FAO website shows the value of wheat and its products, which was 761,282 Euros/1000 tonnes worldwide. In 2020, in the European Union, the value of wheat and its products for 1000 tonnes was 127,316 Euros (FAO., 2020). The data indicate that wheat production averaged around 680 million tonnes annually between 2008 and 2012, peaking at 700 million tonnes in 2011. Although maize and rice are the two most produced crops globally, wheat ranks third. It can be grown in tropical and subtropical regions and is widely cultivated in countries ranging from Scandinavia to Argentina (Shewry and Hey., 2015).

Wheat is in demand in new markets beyond its climate adaptation zone. During the Industrial Revolution era, the demand for wheat increased due to its use in various food products like bread, pasta, and noodles. Although wheat is primarily considered a source of energy (carbohydrate), it also contains essential nutrients like proteins, and fiber, as well as minor components such as lipids, vitamins, minerals, and phytochemicals. These nutrients contribute to a healthy diet and are crucial for overall well-being (Shewry; 2015).

4.1.1. Wheat grain composition in relation to diet and health

There is a lot of literature available on the composition of wheat grain, that focus on the parts of wheat that are relevant to human health and nutrition, specifically comparing the data for wholegrain and white flour (O. Onipe et al., 2015). It will also consider where the different components are located within the grain tissues and how this affects their recovery during milling (O.Onipe et al., 2015; Giraldo et al., 2019). The composition of wheat grain can be affected by factors such as the wheat variety, environment, and processing method. Figure 1 displays three main components of the wheat grain are the bran, germ, and endosperm. The bran is the outermost layer of the grain and contains essential nutrients such as fiber, vitamins, minerals, and phytochemicals. The germ is found in the innermost part of the grain and is a rich source of vitamins, minerals, antioxidants, and unsaturated fatty acids. The endosperm, which is the largest part of the grain, consists mostly of carbohydrates and some protein (Brouns et al., 2012).

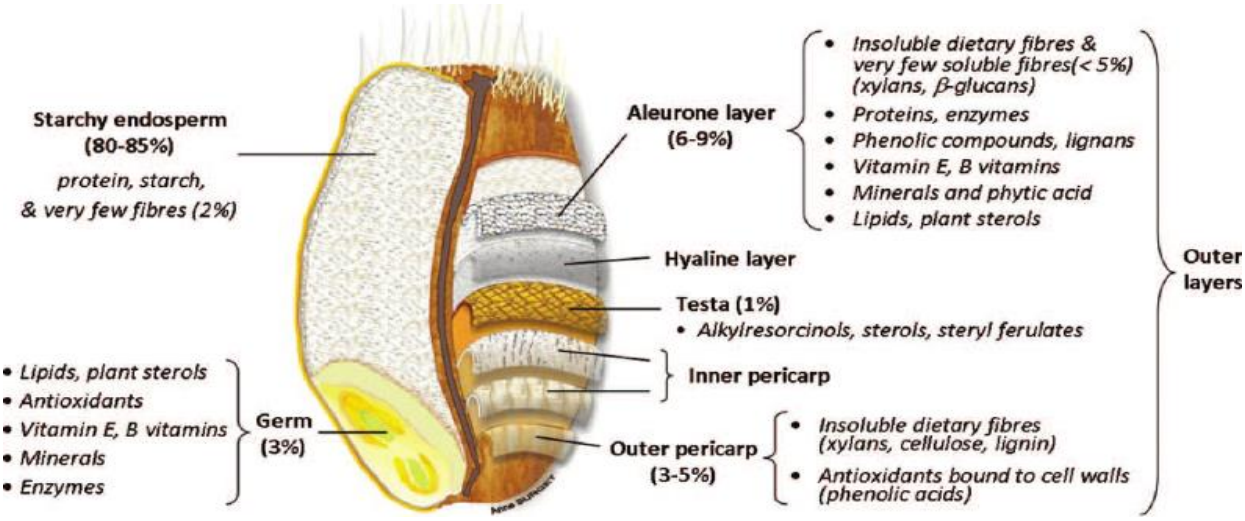

Fig. 1: Histological structure of wheat grain (Brouns et al., 2012)

Consuming whole wheat grains and wheat products is beneficial for health due to their high fiber content, which can aid in digestion, reduce the risk of chronic diseases such as diabetes, heart disease, and cancer, and lower cholesterol levels. Wheat bran and germ also contain essential vitamins and minerals, such as B vitamins, vitamin E, zinc, magnesium, and iron (Liu., 2007; Gill et al., 2011; Sheng et al., 2018). In contrast, refined wheat products like white bread and pasta have reduced nutrient content as they undergo processing, which involves removing the bran and germ. Studies have shown that the consumption of refined wheat grain may increase the risk of obesity, type 2 diabetes, and other chronic diseases (Madina-Remon et al., 2018).

4.1.2. Nutritional Importance of Wheat and Grinding

Wheat is composed of important nutrients such as protein, fat, carbohydrates, and dietary fiber in the form of starch, and iron. Wheat starch is a valuable by-product of wheat and is second in economic value only to wheat gluten (Copper, 2015). Wheat has the ability to self-pollinate, making it easier to select and cultivate distinct domestic varieties for different purposes, including baked goods like bread, cakes, breakfast cereal, pasta, and noodles. It is also used in the production of alcoholic beverages, biofuel, and as a source of food for livestock (Copper, 2015). Wheat-based diets are high in fiber, which is beneficial for intestinal function, glucose response, and cholesterol control. According to The HEALTHGRAIN Program, which is a comprehensive study on the significance of wheat nutrition, the demand for wheat is expected to increase by 60% compared to 2010. To meet

this demand, global annual yield increases must rise from 1% per year between 2001-2010 to 1.6% per year between 2011-2050 (Giraldo et al., 2019).

4.1.3. Genetics of Wheat

Polyploid wheat comprises of tetraploid pasta wheat (*Triticum durum*, 2n=4x=28; AABB genomes) and hexaploid bread wheat (*Triticum aestivum*, 2n=6x=42; AABBDD genomes) that have a common wild emmer ancestor (*Triticum dicoccoides*, 2n=4x=28; AABB genomes). The domestication of wild emmer wheat, which occurred 10,000 years ago, led to the development of tetraploid pasta wheat and subsequent hybridisation with diploid goatgrass (*Aegilops tauschii*, 2n=2x=14; D genome) resulted in the creation of bread wheat. Majority of the genes in tetraploid and hexaploid wheat exist in two to three functional copies, known as homoeologs, with coding sequences having over 97% similarity (Uauy., 2017). Recent improvements in genome reference have facilitated the characterisation of these sequences and allowed for the identification of DNA methylation patterns in wheat promoters that are largely conserved across homoeologs. RNA sequencing analyses have shown no global dominance of a single genome, although some level of genome dominance was observed for all three genomes in a cell-type or stage-specific manner. PacBio complementary DNA (cDNA) reads have led to the identification of complete gene families of agronomic importance, which have been used to align over 400 publicly available RNA-seq samples to develop gene expression atlases in wheat (Uauy., 2017).

Although bulked segregant analysis (BSA) has become a popular method for gene mapping in bread wheat, there are limited user-friendly tools available for researchers without a strong background in bioinformatics (Zhang et al., 2021). The bread wheat genome is known for its complexity, that are divided into three subgenomes. Each subgenome contains seven chromosomes (making n = 21). These subgenomes are further categorized into seven homoeologous groups. Each homoeologous group has three chromosomes that are closely related to one another, one from each of the three subgenomes (Gupta et al., 2008). A large haploid size of over 15 billion bases (bps), and numerous near-identical sequences scattered throughout. Despite many attempts to sequence and assemble the genome (Zimin et al., 2017). It has proven to be a challenging task due to the high proportion of relatively long, near-identical repeats, largely due to transposable elements (TEs). Additionally, as a hexaploid organism, the wheat genome presents an even greater challenge as the three component genomes (A, B, and D) share many regions of high similarity, resulting in intra- and inter-chromosome variation. However, the wheat genome is highly complex due to its composition

of three subgenomes, A, B, and D. It is believed that common wheat resulted from a hybridization event between tetraploid wheat and goat grass, with the A genome donor being *Triticum urartu* and the origin of the B genome remaining unclear (Guan et al., 2020). Therefore, genome assembly programs face a complex task, first due to the genome's repetitive nature and secondly because of the existence of multiple copies of each chromosome with varying degrees of similarity (Zimin et al., 2017).

Bread wheat is widely cultivated and is a crucial source of calories and protein for humans, especially through bread consumption. The hexaploid nature of wheat is advantageous because interactions between subgenomes contribute to its adaptability to different environments. Wheat has been an important model organism for cytogenetic and genetic research, with the Chinese Spring landrace being particularly useful for genetic studies due to its diverse genetic stocks. Therefore, wheat has significantly contributed to our understanding of plant chromosomes and genetic loci underlying important agronomic traits (Guan et al., 2020).

4.2. RNA interference

RNA interference (RNAi) is a biological process where RNA molecules neutralize targeted messenger RNA (mRNA) molecules to inhibit gene expression or translation (Xu et al., 2019). It is one of the RNA silencing pathways that uses small RNAs as guides for sequence-specific silencing. This mechanism has potential for practical applications such as manipulating cellular processes and defenses for therapeutic purposes. RNAi can suppress transcription or activate sequence-specific RNA degradation to limit transcript levels (Svoboda., 2020). The discovery of RNAi has opened up possibilities for RNA-based therapeutics for treating diseases and tissue regeneration (Gupta et al., 2016).

The mechanism of RNAi was initially discovered in the nematode *Caenorhabditis elegans* by Andrew Z. Fire, Craig C. Mello, and their colleagues, who were awarded the Nobel Prize in Physiology or Medicine in 2006 (Fire et al., 1998). RNAi has since been observed in a range of eukaryotic organisms, including fungi, plants, invertebrates, vertebrates, protozoans, and algae, and is thought to have evolved as an antiviral immune response (Zhu and Palli., 2019). Napoli and Jorgensen first observed the phenomenon of transgene-induced „cosuppression" of endogenous genes in hybridized violet petunias, while Romano and Macino reported a similar event in *Neurospora crassa* called „quelling," caused by the introduction of homologous RNA sequences. The discovery of RNAi in animals was reported

by Guo and Kemphues in 1995, where the introduction of sense or antisense RNA led to the degradation of par-1 mRNA (Xu et al., 2019).

RNAi plays a natural role in safeguarding the genome against mobile genetic elements, including viruses and transposons, and in regulating the developmental programs of eukaryotic organisms. Different aspects of RNAi have been covered in separate reviews (Agrawal et al., 2003). RNAi is a complex mechanism that regulates various biological processes such as defense against viruses and TEs, maintenance of chromosome structure and stability, and regulation of developmental timing and differentiation. Organisms have genes for miRNAs that function as innate developmental small interfering RNA (siRNAs). Unlike siRNAs, miRNAs are derived from single-stranded RNAs that fold back on themselves to create small „stem-loops" of RNA. When incorporated into the RNA-induced silencing complex (RISC), miRNAs inhibit mRNAs that are not fully complementary to them, allowing a single miRNA to regulate the expression of hundreds of genes (Pandita., 2018).

Humans generate around 500 miRNAs, and deficiencies in specific miRNAs have been linked to various diseases (Pandita., 2018). Two types of small RNA, siRNA and miRNA, are derived from longer double-stranded RNA (dsRNA) precursors. dsRNA can come from repetitive DNA elements or RNA molecules that can form dsRNA segments. Dicer, a RNase III class ribonuclease, cleaves dsRNA to produce siRNAs or processes it into miRNAs. The miRNA biogenesis pathway differs from siRNA production because miRNAs are generated from introns of endogenous coding genes or noncoding transcripts (Martienssen and Moazed., 2015). RNAi is generally viewed as a negative switch that regulates gene expression through siRNA sequence specificity. Different types of RNAs, including synthetic siRNAs, small hairpin RNAs (shRNAs), long dsRNAs, endoribonuclease-prepared short interfering RNAs, and pro-siRNAs, can be introduced to induce RNAi. The first two only have a single sequence, while the others contain multiple sequences (Xu et al., 2019).

4.2.1. Transcriptional gene silencing (TGS)

RdDM (RNA-directed DNA methylation) is a mechanism in plants that plays a crucial role in various biological processes such as stress responses, cell-to-cell communication, and maintenance of genome stability through TE silencing. This mechanism is responsible for adding DNA methylation to cytosines, irrespective of their sequence context, which is unique to RdDM among all plant mechanisms. An outline of RdDM's various biological functions is depicted in Figure 2. (Erdmann and Picard., 2020).

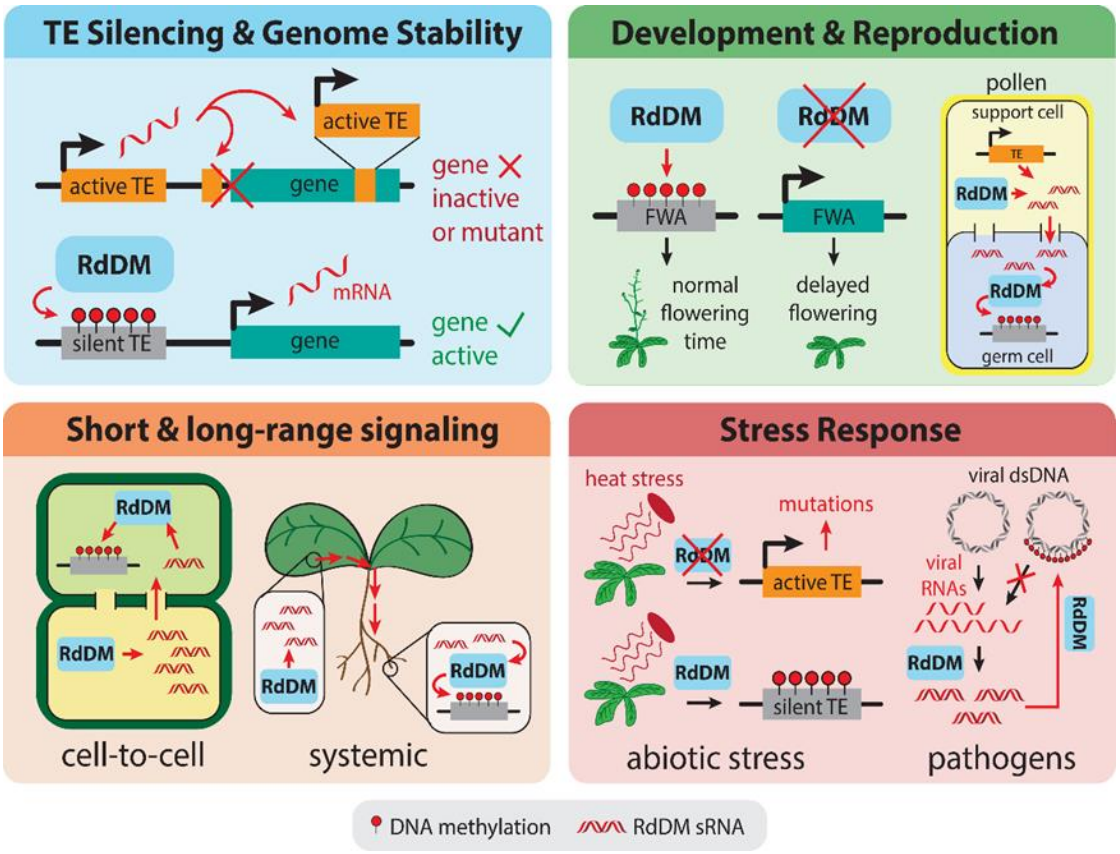

Fig. 2: High-level overview of several of the biological functions of RdDM. RdDM is a crucial mechanism that prevents the activation and transposition of transposable elements (TEs), which can otherwise disrupt gene expression or result in a mutant protein. RdDM also plays a vital role in development, as it represses FWA, thereby affecting flowering time. During pollen formation, TEs are activated, and sRNAs for RdDM are produced, which move to the germ cell and reinforce TE silencing. RdDM-mediated silencing is mobile, and the sRNAs can move between cells through plasmodesmata or systemically via the vasculature, allowing the silencing to spread to distal tissues. In addition, RdDM is involved in several abiotic stress responses, including the heat shock response, and it can silence TEs that would otherwise become active and transpose under heat stress. Moreover, RdDM is involved in pathogen defense by using sRNAs derived from viral mRNAs to silence viral DNA, either as a viral minichromosome or an integrated provirus (Erdmann and Picard., 2020).

In plants, DNA methylation is categorized into three types, CG, CHG, and CHH, based on the sequence context of the methylated cytosine. These pathways are responsible for maintaining pre-existing DNA methylation patterns. The RdDM pathway is involved in establishing DNA methylation in all sequence contexts (Erdmann and Picard., 2020). In plants, primarily through the enzyme DRM2. The canonical RdDM pathway uses 24-nt siRNAs generated by polymerase IV (Pol IV), while non-canonical RdDM pathways use sRNAs from various sources to direct RdDM. Pol IV is an essential component of the RdDM

pathway that produces short transcripts converted into double-stranded RNA by RNA-DEPENDENT RNA POLYMERASE 2 (RDRP 2). An outline of RdDM's mechanism in plants is depicted in Figure 3. In Arabidopsis, it is thought that the RNA polymerase IV (Pol IV) is responsible for initiating the production of 24 nt siRNA, which serves as a template for RNA-dependent RNA polymerase 2 to generate double-stranded RNAs (dsRNA) and dsRNA cleavage done by DCL3 into siRNA incorporated into ARGONAUTE (AGO) proteins (Zhang et al., 2018). The Sawadee Homeodomain Homolog 1 (SHH 1) protein helps to recruit Pol IV to the targeted loci, which have dimethylated histone H3 lysine 9 (H3K9me2). The Pol IV-dependent siRNA production also requires the interaction of a chromatin remodeler, Classy 1 (CLSY1), with Pol IV. The sRNAs incorporated into AGO4 and/or AGO6 can trigger Pol V- and DRM2-dependent methylation of complementary DNA sequences, which may lead to transcriptional gene silencing and silencing of transcriptionally active TEs (Gallego-Bartolom, 2020).

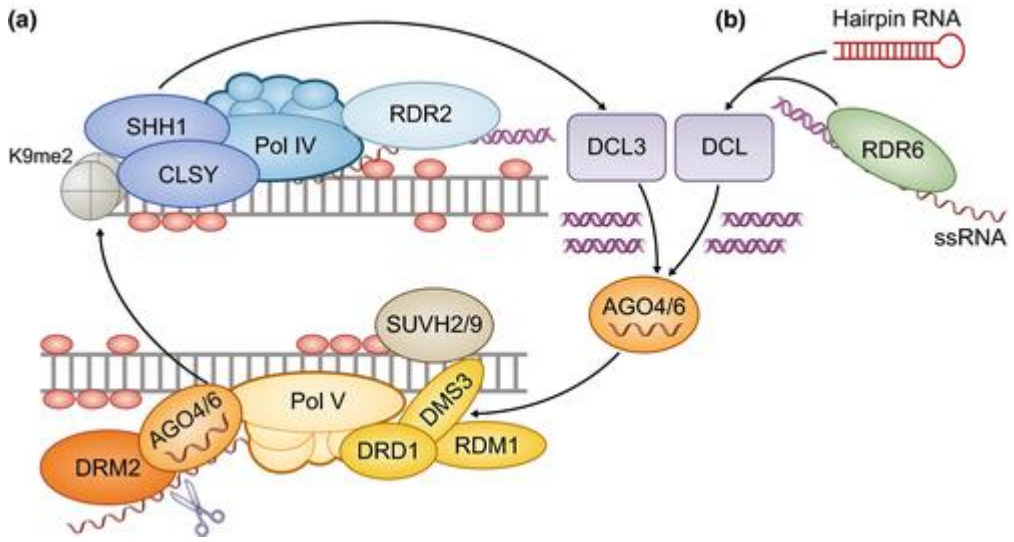

Fig. 3: RNA-directed DNA methylation in plants. (a) The RNA-directed DNA methylation (RdDM) pathway in plants involves RNA polymerase IV (Pol IV) transcripts being converted into double-stranded RNAs (dsRNAs) by RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) and then cut into 24-nt short-interfering RNAs (siRNAs) by DICER-LIKE 3 (DCL3). These siRNAs are incorporated into AGO4 and AGO6, which interact with Pol V and trigger the recruitment of the DNA methyltransferase DRM2. The histone reader SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1) and chromatin remodelers CLSY are required for Pol IV association with chromatin, while SUVH2 and SUVH9, together with the DDR complex (DMS3, DRD1, and RDM1), are required for Pol V recruitment to chromatin. (b) In addition to the canonical pathway, non-canonical RdDM can occur through RNAs from different origins, such as viruses and Pol II-dependent transcripts, which can serve as entry points for the generation of small RNAs to feed into RdDM. These RNAs are converted into dsRNAs by RDR6 and cut by different DCL proteins into 21-24-nt small RNAs, which can be loaded into the AGO4/AGO6 family (Zhang et al., 2018; Gallego-Bartolom, 2020).

Small RNA-directed transcriptional gene silencing (TGS) was identified as a phenomenon in which RNA-dependent DNA methylation and Argonaute protein family members are required for TGS in Arabidopsis. Post-TGS (PTGS) was later discovered as an RNAi mechanism that targets messenger RNAs (mRNAs) in plants and *Caenorhabditis elegans* (Weinberg and Morris., 2016). Co-suppression, a related phenomenon, results in the suppression of transgene expression and endogenous gene expression. Co-suppression can occur through TGS, which involves changes such as DNA methylation and chromatin remodeling that lead to transcription shutdown in the nucleus. TGS is heritable and does not cause systemic silencing (Ashfaq et al., 2020). TGS is a well-studied mechanism of gene regulation that is observed in various species, including *Arabidopsis thaliana, Caenorhabditis elegans*, and *Schizosaccharomyceae pombe* (Mendez., 2015). Unlike PTGS, TGS leads to long-lasting changes in gene expression that can be inherited by daughter cells during cellular

division. Studies have shown that siRNA-directed TGS operates through a distinct epigenetic mechanism in the nucleus, which is different from the cytoplasmic RNAi-mediated PTGS mechanism (Weinberg and Morris., 2016). In plants, TGS plays a crucial role in defending against DNA viruses by inducing DNA methylation and generating vi-siRNAs that suppress the transcription of viral genomes. Additionally, TGS has been linked to biological functions such as symptom remission in plant-virus interactions, such as in the case of pepper and Pepper golden mosaic virus (Sanan-Mishra et al., 2017).

4.2.2. Post-transcriptional gene silencing (PTGS)

Post-transcriptional gene silencing (PTGS) is a process that occurs in the cytoplasm and is used to target and degrade specific mRNA transcripts of genes. Various methods of PTGS exist, including RNA interference (RNAi), CRISPR/Cas9, and NMD, and studies have been conducted to enhance plant resistance to pathogens, improve drought tolerance, and engineer the lignocellulose pathway using these methods (El-Sappah et al., 2021). In plants, transgene PTGS, also called cosuppression, was first observed in transgenic plants, which showed disrupted expression of both transgenes and their cognate endogenous genes. Earlier discoveries in RNA-based viral resistance in plants, quelling effect in fungi, and RNA interference in animals shared similar mechanisms with PTGS. While highly expressed transgenes and invading viral genes frequently undergo PTGS, most endogenous genes do not trigger PTGS, except for a few endogenous genes involved in siRNA production (Zhang et al., 2016).

Post-transcriptional gene silencing (PTGS) is a mechanism that is similar to natural processes like cross-protection and RNA-mediated viral resistance in plants. PTGS acts as a protective measure against viral infections in plants, and the mechanism has been adopted to induce desirable traits in crop plants by targeting specific gene transcripts (Ashfaq et al., 2020). The process of PTGS involves targeting mRNA and viral RNA, and double-stranded RNA (dsRNA) is the key inducer of gene silencing. This dsRNA can be formed by various means, including the transcription of inverted repeats, bidirectional transcription of a locus, or the action of RNA-dependent RNA polymerases (RDRs) that convert single-stranded RNA to dsRNA (Taochy et al., 2017). In plants, PTGS pathways use suppressor of gene silencing 3 (SGS3) and RNA-dependent RNA polymerase 6 (RDR6) to transform single-stranded RNA into double-stranded RNA, which is subsequently processed into 21 nt small interfering (si)RNAs by the DICER-like enzyme 4 (De Alba et al., 2015). The process of PTGS involves four well-defined steps, including detection of the dsRNAs, generation and amplification of

small interfering RNAs, silencing of viral target genes, and spreading of the signal between plant cells and within the host through the vasculature. The cytoplasmic dsRNAs serve as strong signaling molecules recognized by nucleases, known as Dicer-like proteins (DCL), that cleave dsRNA to short fragments (Wieczorek and Obrępalska-Stęplowska., 2015).

Mechanisms of post-transcriptional gene silencing

Research groups studying gene silencing across different organisms have observed common features in the mechanism of PTGS. The trigger for PTGS can be either homologous genes, dsRNA, or siRNAs, suggesting a conserved mechanism across kingdoms. However, there are slight variations in the mechanisms among different organisms, with organism-specific genes fine-tuning the PTGS mechanism (Ashfaq et al., 2020). Plants have established RNA quality control systems to prevent inappropriate RNA silencing of important protein-encoding genes. These systems monitor mRNA quality and direct aberrant mRNAs towards degradation through processes such as shortening the poly(A) tail and degradation by XRN and SKI complexes. The core protein of the exosome complex, RRP45B/CER7, is involved in the 3'-to-5' degradation of mRNAs (Li et al., 2019).

4.2.3. RNAi in plant biotechnology

The investigation of the possible uses of RNAi in crop protection has been expanding, and it is apparent that RNAi-based strategies could play a significant part in achieving sustainable agriculture and integrated pest management (Mezzetti et al., 2020). Breeders are provided with additional opportunities for varietal improvement in Agricultural plant biotechnology through the unique features of RNAi compared to genome editing technologies such as CRISPR/Cas or TALENs. One unique feature that a gene knockdown effect can be achieved through the use of RNAi in plant biotechnology, depending on the dsRNA (length and sequence) utilized, rather than a complete knockout (Wagner et al., 2011). The effectiveness of this method within the plant depends on the selection of a suitable transformation methodology (Jothi Kanmani et al., 2023). By introducing RNAi constructs to suppress specific genes in plants, transgenic plants that utilize RNAi have been developed to improve crops, plant growth, and other desirable traits. The efficacy of RNAi in crop improvement has been demonstrated in various ways, including the development of seedless fruits, regulation of plant biomass, enhancement of flower color and scent, prolongation of shelf-life, regulation of secondary metabolites, and improvement of abiotic stress tolerance (Guo et al., 2026).

Xiong et al. (2005) applied RNAi technology to create genetically modified tomato plants. They introduced a double-stranded RNA unit that targeted the 1-Aminocyclopropane-1-carboxylate (ACC) oxidase gene, which is responsible for the production of ethylene gas that causes fruit to ripen and spoil. The modified plants produced only minimal amounts of ethylene, resulting in their fruit staying fresh for more than 120 days. In another experiment, researchers used RNAi to target two other genes involved in fruit ripening, α-mannosidase (α-Man) and β-D-N-acetylhexosaminidase (β-Hex), which reduced the softness of the tomatoes and extended their shelf life by nearly 30 days (Meli et al., 2010). Researchers used RNAi technology to alter the original blue color of *Torenia hybrida* flowers to white or pale colors by targeting the chalcone synthase (*CHS*) gene, as reported by Fukusaki et al. (2004). The *CHS* gene was also targeted in another study by Schijlen et al. (2007) to produce seedless tomatoes, also known as parthenocarpic tomatoes, by reducing the production of flavonoids. Additionally, the biosynthesis or signaling of plant hormones such as auxin and gibberellins were manipulated in the study (De jong et al., 2009).

Saurabh et al. (2014) and Guo et al. (2016) reported that RNAi technology has been utilized to regulate plant metabolite profiling for improving nutrition, biofortification, and eliminating allergens or toxins. In another study, Davuluri et al. (2005) used RNAi technology to increase the production of carotenoids and flavonoids in tomato plants. Furthermore, Zhang et al. (2009) employed RNAi with hpRNA to target *squalene synthase* (SQS) and generate transgenic *Artemisia annua* plants. RNA interference (RNAi) technology has been found to have potential benefits in various aspects of plant growth, development, ripening, nutritional content, and physiology, as shown in the reports discussed (Das and Sheif, 2020).

4.3. Wheat transformation

Commonly grown worldwide, hexaploid wheat (*Triticum aestivum* L.)) has been slower to benefit from genetic engineering than other crops such as rice and maize, due in part to difficulties in transformation (Hamada H et al., 2017). However, as with other crops, wheat faces various biotic and abiotic challenges that limit its productivity, and modern biotechnology offers potential solutions through plant transformation for crop improvement. Plant transformation is a valuable tool for studying gene function and discovering traits, and is essential for both applied and fundamental research in wheat. Wheat transformation has been achieved through two primary methods: particle bombardment of embryogenic callus and *Agrobacterium*-mediated transformation of immature, embryo-derived, re-generable callus (Hamada et al., 2017; Tian et al., 2019).

Model cultivars have been modified using these methods to improve transformation efficiency (Hamada et al., 2017). While the advantages and disadvantages of the two methods are still being debated, the biolistic transformation has become a commonly used approach due to its ability to integrate new technologies such as genome editing tools and provide a robust platform for the development and recovery of DNA- and marker-free genome-edited plants (Tian B et al., 2019). However, these methods have limitations as they are not suitable for all cultivars and can be time-consuming, leading to somatic variations. Furthermore, many elite commercial cultivars lack the necessary characteristics for culture and regeneration, making them difficult to transform (Hamada et al., 2017).

Transformation is a crucial tool for both applied and fundamental research in wheat. The development of efficient transformation protocols mediated by *Agrobacterium tumefaciens (A. tumefacien)*, which can transfer defined DNA segments with low copy numbers to plant chromosomes with minimal rearrangements, was first established in rice and maize. Subsequently, *A. tumefaciens*-mediated transformation was also applied to wheat, but progress was slow compared to other cereals, which showed a significant improvement in the efficiency of gene transfer and the range of transformable genotypes. In the early 2000s, embryos and cultured cells of several wheat genotypes were tested, and various factors were examined, but the transformation frequency was mostly less than 5% of the inoculated tissue pieces. (Ishida et al., 2015).

Genetic engineering involves introducing genes of interest into living organisms, bypassing natural barriers to achieve desired results. Despite worldwide research efforts, genetic engineering in wheat lags behind that of other important crops like rice and maize, likely due to wheat's complex and highly redundant genome, which is much larger than those of other crops. In addition, most wheat varieties are difficult to culture and regenerate in vitro, further hindering progress in wheat genetic engineering (Borisjuk et al., 2019).

Plant transformation is a crucial tool for crop improvement and functional genomic studies. The *A. tumefaciens* mediated process can be divided into two phases: T-DNA transfer and incorporation into the genome, and selection and regeneration of transformed cells into viable plants. Factors influencing T-DNA transfer include the binary vector, *Agrobacterium* strain, pre-treatments of embryos, *Agrobacterium* inoculation, and co-cultivation. Factors influencing regeneration in vitro include cultivar, donor material quality, stage of immature embryos, handling of material, and media composition. Wheat transformation efficiency has been low, but advancements in gene delivery tools, genetic manipulation, and tissue culture

techniques have led to higher efficiency, throughput, and cost-effectiveness. DNA cloning approaches are integral to gene function studies (Hayta et al., 2019).

4.3.1. Growth-Regulating Factor 4 (GRF4) and its cofactor GRF-Interacting Factor 1 (GIF 1)

The conserved GRF transcription factor genes across angiosperms, gymnosperms, and mosses encode proteins that interact with DNA and other proteins through their QLQ and WRC domains (Omidbakhshfard et al., 2015). MiR396, a microRNA that diminishes the function of GRFs in fully developed tissues, targets many GRF genes in angiosperms and gymnosperms (Debernardi et al., 2012). In vivo, the GRF proteins create complexes with GIF cofactors that interact with chromatin remodeling complexes. The efficiency of functional GRF-GIF complex assembly is regulated at multiple levels (Vercruyssen et al., 2014; Debernardi et al., 2014). The reduced organ size observed in plants with loss-of-function mutations in GIF genes mimics the effect of GRF loss-of-function mutants or plants that overexpress miR396 (Rodriguez et al., 2010; Li et al., 2016). Conversely, the overexpression of GIF genes promotes organ growth and can increase the activity of GRFs (Shimano et al., 2018; Zhang et al., 2018). The development of larger leaf sizes is observed when there is a simultaneous increase in the expression of Arabidopsis GRF3 and GIF1, which interact to form a protein complex. This finding indicates that increasing the expression of both genes together produces a stronger effect than increasing the expression of either gene individually (Debernardi et al., 2014).

In 2020, Debernardi et al. demonstrated that the efficiency and speed of regeneration in wheat, triticale, and rice can be substantially increased by expressing a fusion protein that combines wheat GROWTH-REGULATING FACTOR 4 (GRF4) and its cofactor GRF-INTERACTING FACTOR 1 (GIF1). The expression of GRF4-GIF1 also boosts the number of transformable wheat genotypes. The transgenic plants produced from the expression of GRF4-GIF1 were fertile and showed no observable developmental defects. Furthermore, GRF4-GIF1 prompted efficient wheat regeneration even in the absence of exogenous cytokinins, which facilitates the selection of transgenic plants without selectable markers. In addition, the researchers combined GRF4-GIF1 using CRISPR-Cas9 genome editing and generated 30 edited wheat plants with disruptions in the gene Q (AP2L-A5). Finally, the study indicates that a chimera of dicot GRF-GIF enhances the regeneration efficiency in citrus, suggesting that this method can be applied to dicot crops (Debernardi et al., 2020).

4.4. MicroRNA Knock-Down

miRNAs are short, non-coding RNA molecules that are typically 20-24 nucleotides in length. They can regulate gene expression at the post-transcriptional or translational level by binding to the 3′ untranslated region of target genes. It's interesting to note that one miRNA can potentially regulate hundreds or even thousands of target genes, while one gene can be influenced by multiple miRNAs. Scientists commonly use gain- and loss-of-function approaches to study the effects of miRNAs on target genes. Synthetic mimics or expression vectors are often employed to increase the abundance of miRNAs in cells for both basic and translational research purposes (Chang et al., 2016). MiRNAs play crucial roles in animal development and are involved in various biological processes. Abnormal miRNA expression is linked to many human diseases. Additionally, miRNAs can be secreted into extracellular fluids and have been suggested as potential biomarkers for various diseases. They also serve as signaling molecules that mediate cell-to-cell communication (O'Brien et al., 2018). MiRNA-biology emerged through the evolution of a mechanism that enabled the processing of long endogenous transcripts into short RNA duplexes. In animals, this process involves the Microprocessor complex, which comprises the Rnase III endonuclease Drosha and its co-factor Pasha. Initially, it was believed that miRNAs evolved separately in animals and land plants. However, it is also possible that a common miRNA-like pathway for post-transcriptional regulation was already in use by the last common ancestor of these lineages (Dexheimer and Cochella., 2020).

The machinery responsible for miRNA maturation and assembly is also involved in the RNA interference (RNAi) pathway, which was first discovered in worms and plants. While the miRNA maturation process is well understood, new surprises continue to be uncovered, such as the discovery that miRNAs can be processed from other types of non-coding RNAs (Virciglio et al., 2021). The primary miRNAs (pri-miRNAs) that give rise to miRNAs are typically composed of stem-loop-like structures and can be classified into three types based on their genomic location. Intergenic miRNAs have their own promoters, while intronic and exonic miRNAs are regulated by the promoters of their host genes (Luo et al., 2015). Concatenated miRNA genes, which consist of several hairpins controlled by a single promoter, offer a powerful tool for biotechnology, as multiple genes can be targeted simultaneously to increase knockdown efficiency. Several concatenation strategies have been developed, including the use of natural polycistronic miRNA backbones, synthetic miRNA hairpins, or DROSHA-independent intronic miRNAs (Rousset et al., 2019). Many researchers opt to use shRNAs due to their potential for greater knockdown, but there is a growing interest

in enhancing the knockdown potency of miRNAs. Researchers are exploring the targeted optimization of miRNA scaffolds using insights gained from a better understanding of miRNA function. Recent studies have revealed conserved sequence elements of miRNA backbones that are associated with increased miRNA biogenesis and enhanced knockdown efficiency (Fowler et al., 2015). Additionally, certain miRNAs have evolved to be packaged into exosomes to carry out their biological functions, and their loading into exosomes is dependent on 3′-end uridylated isoforms and on the levels of miRNA targets in producer cells (Alexander et al., 2015). Recent advancements in crosslinking immunoprecipitation (CLIP) based methods, such as CLIP-seq, PAR-CLIP, and iCLIP, have provided insights into how miRNAs interact with their target sites through the identification of Argonaute binding sites. However, CLIP-based approaches do not specifically identify the miRNA responsible for the identified interaction, which can be problematic for families of miRNAs that share the same seed sequence or for sites with no obvious pairing to known miRNAs (Broughton and Pasquinelli., 2016).

4.4.1. STTM (Short Tandem Target Mimic)

STTM is an artificial noncoding RNA that is about 100 nucleotides long and can be produced through stable plant transformation or transient expression using a virus-based system. It contains two miRNA binding sites separated by a 48-88 nucleotide spacer and has three nucleotide mismatches at the miRNA cleavage site, which creates a bulge that prevents cleavage by target miRNAs. Target mimics, molecular sponges, and short tandem target mimics (STTMs) can be used to knock down the expression of all members of a miRNA family (Teotia et al., 2017). STTMs consists of 21 nucleotide sequences that are complementary to mature miRNAs, with a loop sequence inserted around the 10th and 11th positions of the binding sites that makes them resistant to cleavage by miRNA complexes (Othman et al., 2023). STTMs have been successfully used to silence miRNA families in several plants and animals, including Arabidopsis, tomato, wheat, tobacco, soybean, and common bean, using stable transformation, *Agrobacterium*-mediated transient expression, and virus-induced-gene silencing. STTMs induce the degradation of most, if not all, cognate miRNAs through the action of small degrading nucleases (Peng et al., 2018).

STTM Techniques in Plant

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 (Figure 3) (Chen et al., 2021). 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 plants (Li et al., 2020).

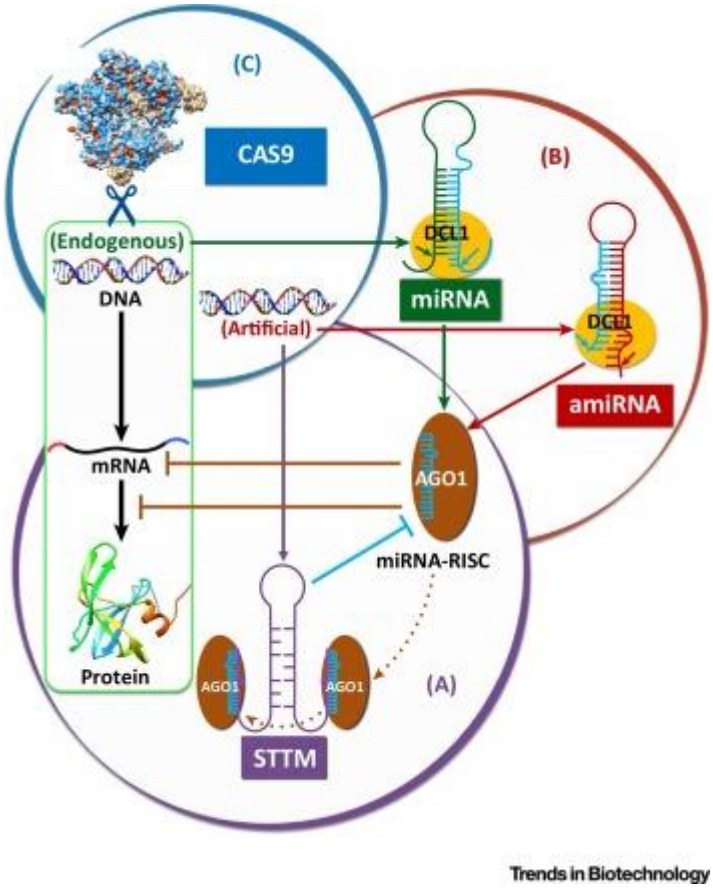

Fig. 4: There are three RNA-based technologies used to study gene function: STTM for non-coding miRNA genes, amiRNA for coding genes, and CRISPR/Cas9 for gene editing. (A) STTM creates short RNA molecules that bind to the miRNA gene of interest to prevent its function. (B) AmiRNA creates an artificial 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).

4.4.2. CRISPR (clustered regulatory interspaced short palindromic repeats)

The CRISPR-Cas9 system is a powerful tool for targeted genetic editing, allowing for the generation of DNA double-strand breaks with high accuracy and efficiency. It has revolutionized the development of improved plant varieties, bypassing the limitations of traditional breeding methods (Mujtaba et al., 2021). CRISPR, which was first identified in *Escherichia coli* K12 in 1987, is a set of palindromic repeats interspersed with spacers containing exogenous nucleotides from invading viruses or plasmids. These loci are often flanked by associated endonucleases, such as as. When transcribed into precursor CRISPR RNAs (pre-crRNAs) and processed into crRNAs, they can form a complex with Cas protein to cleave target DNA sequences (Chang et al., 2016). This complex cuts a specific genomic site 3-4 nucleotides upstream of a PAM (NGG) sequence, leading to variable sizes of insertions or deletions when repaired by the nonhomologous end joining (NHEJ) system. CRISPR/Cas9 was originally discovered as a genome editing tool and has since become a dominant tool for gene editing in cell and animal models in biomedical research (Wang et al., 2013; Matano et al., 2015).

Applications in Plants

The CRISPR-Cas9 system has proven effective in developing plants resistant to geminiviruses and managing citrus pathogens. Its applications have also led to the creation of tomato varieties resistant to bacterial speck disease. Different delivery systems have been employed to incorporate the Cas9 protein into plant cells, including plant virus vectors, *Agrobacterium*-mediated gene transfer, and nanoparticle platforms such as chitosan (Mujtaba et al., 2021). The approach has also been successful in knocking out miRNA genes in soybean and rice, resulting in improved plant traits related to architecture. However, there is still room for improvement in using CRISPR-Cas9 technology for miRNA-based crop improvement (Chaudhary et al., 2021).

5. METHODS AND MATERIALS

5.1. Plant growth

Seeds of the spring wheat (*Triticum aestivum* L.) genotype 'Fielder' are sown at weekly intervals in a peat and sand mix (5:1). Fielder was risen in growth chambers (Conviron, Winnipeg, Canada) under 16h/8h light/dark period.

They are initially sown in 5 cm diameter pots and after approximately 30 days the germinated plants are transferred into 13 cm diameter round pots containing peat and sand mix (5:1) for continued development. 70% humidity with light levels of 800 $\mu mol.m^{-2}s^{-1}$ 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-1,2)

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 5.7 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

Wheat medium 1L (Hayta et al. 2019 & Ishida et al., 2015)			
WIM-liq		**1 L**	**0,25 L**
	MS Inc. Vit. **M0222**	0,44 g	0,11 g
	glucose	10 g	2,5 g
	MES	500 mg	125 mg
	(make before use!!)		
	(filter sterilized)		
WIM-inf	**WIM-liq+**	**1 L**	**0,2 L**
(before use!)	Silwet L-77	250 µl	50 µl
	acetosyringone 1M	100 µl	20 µl
WIM-AS	**WIM liq +**	**1 L**	**0,5 L**
	AgNO3 (0,85mg/ml)	1ml	0,5ml
	CuSO4*5H2O (1,25mg/L)	1 ml	0,5 ml
	acetosyringone 1M	100 µl	50 µl
	agarose (Sigma)	**8 g**	**4 g**
WCI		**1 L**	**0,5 L**
	MS Basal Salt, **M0221**	4,4 g	2,15 g
	Myo-inositol	350 mg	175 mg
	Proline	690 mg	345 mg
	Thiamin HCL (1mg/ml)	1 ml	0,5 ml
	Casein	1 g	500 mg
	maltose	30 g	15 g
	2,4-D (2,5mg/mL)	200 µl	100 µl
	Picloram (2mg/ml)	1 ml	0,5 ml
	CuSO4*5H2O (1,25mg/L)	1 ml	0,5 ml
	agarose	**5 g**	**2,5 g**
WCI-Res	**WCI+**		
	cefatoxime	300 mg	150 mg
WCI-H20	**WCI+**		
	cefatoxime	300 mg	150 mg
	hygromycin (500mg/mL)	40 µl	20 µl
WCI-H40	**WCI+**	**1 L**	**0,5 L**
	cefatoxime	300 mg	150 mg
	hygromycin	80 µl	40 µl
WRM		**1 L**	**0,5 L**
	MS Inc. Vit. **M0222**	4,4 g	2,2 g
	sucrose	20 g	10 g
	MES	500 mg	250 mg

	Zeatin (0,5mg/ml)	1 ml	500 µl
	CuSO4*5H2O (1,25mg/L)	2 ml	1 ml
	cefatoxime	300 mg	150 mg
	Gelzan	**3 g**	**1,5 g**
WRM-H20	**WRM+**		
	hygromycin (500mg/mL)	40 µl	20 µl
LSF-H20		**1 L**	**0,5 L**
	MS Basal Salt, **M0221**	4,4 g	2,2 g
	Myo-inositol	350 mg	175 mg
	Proline	690 mg	345 mg
	Thiamin HCL (1mg/ml)	1 ml	500 µl
	Casein	1 g	500 mg
	maltose	30 g	15 g
	Gelzan	**3 g**	**1,5 g**
	cefatoxime	300 mg	150 mg
	hygromycin (500mg/mL)	40 µl	20 µl

Note: Make the liquid medium in half volume (500 or 250 ml) and filter sterilized in a double volume flask. 2. Make the gelling agent in half volume (500 or 250 ml) and autoclaving. 3. Warmed the two components to 60 °C and mix both before pouring. Put the antibiotics before pouring. pH was adjusted to 5.8 by adding KOH or HCL.

5.3. Bacterial Strains

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

Table-4: Bacterial strains

Bacteria	Strains
Escherichia coli	DH5α
Agrobacterium tumefaciens	AGL1

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 and genome editing.

5.4 Plasmid Construction

5.4.1 pBlueScript II SK (+)

Commercial vector pBluescript II SK (+) was used to first integrate STTM in it with the help of PCR. 2187STTM unit contains a miRNA on both ends with a 3-nucleotide bulge at the nucleotide position 10 of the real miRNA sequence. An 80 nt long linker sequence linked both 2187STTM miRNA (Shown in figure 5). pBlueScript II SK (+) vectors is powerful cloning vectors for a range of research applications. Featuring several unique restriction enzyme recognition sites, which makes this vector a suitable for a range of DNA sequencing and cloning processes.

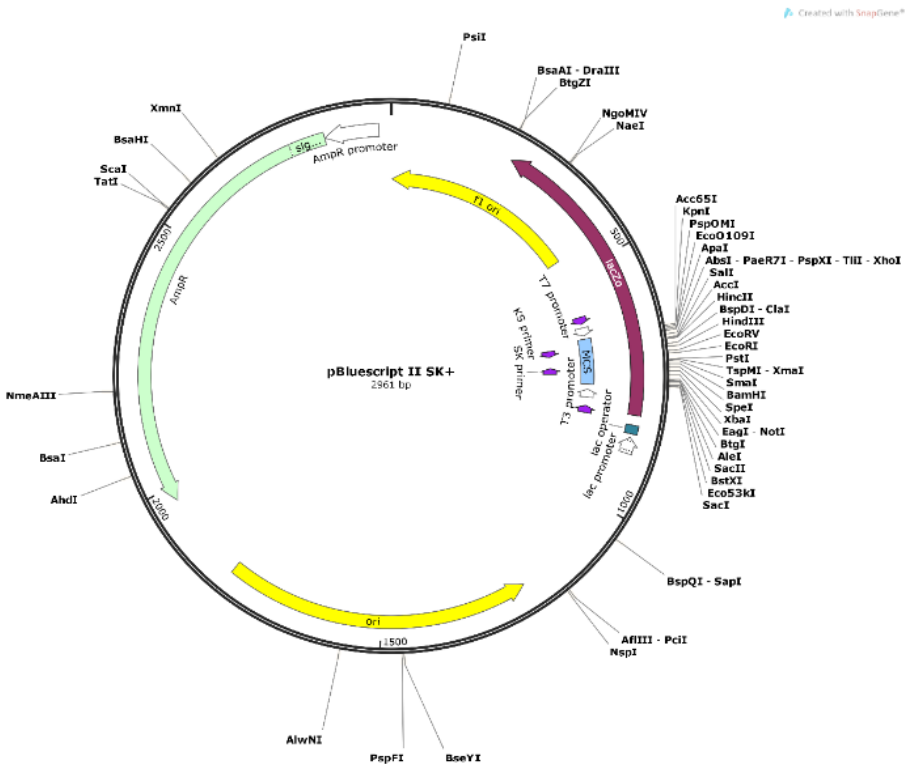

Figure. 5: Map of commercial pBluescript SK II (+) plasmid.

5.4.2 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 contain hygromycin and kanamycin resitant genes (Figure 6).

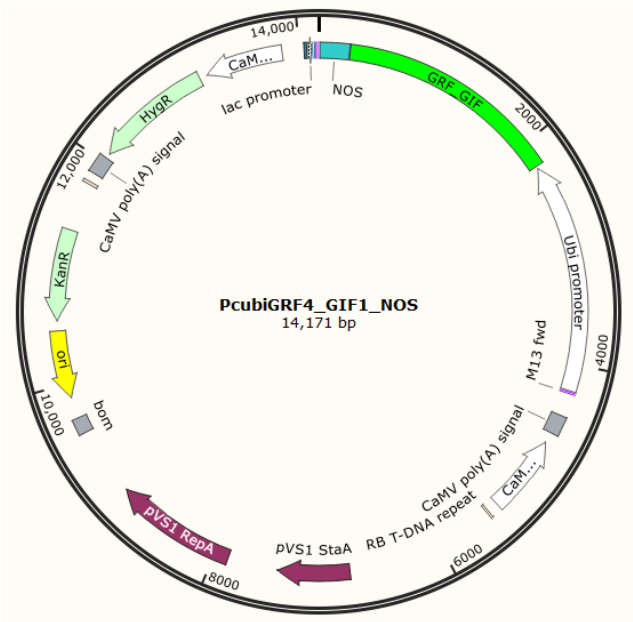

Figure. 6: Map of PcubiGRF4:GIF1_NOS vector. GRF:GIF chimera gene (in green) can be seen under the Ubi promotor and NOS terminator.

5.4.2 Digestion of plasmid

pBlueScript_2187STTM and Pcubi_Tae2187_STTM were digested with *BamHI* and *KpnI*

Table-5: Protocol for plasmid pBlueScript digestion

Reagents	Amount
pBlueScript_2187STTM (10µg) or Pcubi_Tae2187_STTM (10µg)	2 µL
Tango Buffer (1x)	3 µL
BamHI, KpnI	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.

5.4.3. Purification of digested fragment and plasmid

Digested fragment from vector pBlueScript_2187STTM using Gel Purification Kit (K199925) and digested Pcubi_Tae2187_STTM was purified using GeneJET PCR Purification Kit (K0702) according to manufacturer instructions.

5.4.4. Cloning of 2187STTM unit into pCubiNOS vector

We used pCubiNOS plasmid, a modified pCAMBIA2300 designed by (Kis et al., 2016). STTM unit was cut out using BamHI and KpnI enzymes from pBluescript_STTM and ligated into pCubiNOS vector. pCubiNOS vector was already digested and opened with the same enzymes. Finally, the final vector was called as Pcubi_Tae2187_STTM.

Table-6: Protocol for ligation of digested 2187STTM unit and digested plasmid

Reagent	Measure
Digested vector (pCubiNOS)	1 µL
2187STTM unit	3 µL
Reaction buffer	2 µL
T4 ligase	1 µL
Nuclease free water	3 µL

Incubate at 18°C for overnight.

5.5 Transformation of E. Coli

For the transformation of *E. Coli* following protocol was used.

- Defrost competent *E. Coli* for 30 minutes on ice.
- Take 100 µL competent cell and pipetted them into sterile Eppendorf tubes.
- Added 2- 3 µL ligated product to the 100 µL competent cell.
- Kept them on ice for 10 minutes.
- Heat shock was given at 42°C for 30 seconds.
- Immediately put back on ice.
- Now add 500 µL SOC media and flick the tube few times.
- Incubate at 37°C for 60 minutes.
- Now smear them on LB plate containing antibiotics (kanamycin).

- Incubate the plate at 37 °C for overnight.

5.6. Miniprep

E. coli colonies that contain our 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. 7: Plasmid concentration on Nanodrop spectrophotometer 1000.

Sample name	Amount (ng/µL)	A260/280	A260/230
pBluescript_Tae2187_STTM	1192 ng/ µL	1.68	1.89
pCubi_Tae2187_STTM	889 ng/ µL	2.08	2.44

5.7 Transformation of Agrobacterium with pCubi_Tae2187_STTM 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.

Steps in the procedure

1. Grow an Agrobacterium strain containing an appropriate helper Ti plasmid in 5 ml of YEP medium overnight at 28 °C.
2. Add 2 ml of the overnight culture to 50 ml YEP medium in a 250-ml flask and shake vigorously (250 rpm) at 28°C until the culture grows to an OD600 of 0.5 to 1.0.
3. Chill the culture on ice. Centrifuge the cell suspension at 3000 g for 5 min at 4°C.
4. Discard the supernatant solution. Resuspend the cells in 1 ml of 20 mM CaCl2 solution (ice-cold). Dispense 0.1-ml aliquots into prechilled Eppendorf test tubes.
5. Add about 1 µg of plasmid DNA to the cells.
6. Freeze the cells in liquid nitrogen.

7. Thaw the cells by incubating the test tubes in a 37°C water bath for 5 min.
8. Add 1 ml of YEP medium to the tube and incubate at 28°C for 2-4 h with gentle shaking. This period allows the bacteria to express the antibiotic resistance genes.
9. Centrifuge the tubes for 30 s in an Eppendorf centrifuge. Discard the supernatant solution. Resuspend the cells in 0.1 ml YEP medium.
10. Spread the cells on a YEP agar plate containing appropriate antibiotic selection. Incubate the plate at 28°C. Transformed colonies should appear in 2-3 days.

Notes:

4. The cells can be frozen in liquid nitrogen and stored at ‹80°C. The frozen cells can be used for future transformation experiments. Add about 1 µg of DNA to the frozen cells and follow the steps 7-10.

5.8. DNA Extraction

The direct DNA extraction method was used to extract genomic DNA from a young leaf for PCR.

- Approximately 5×5 mm leaf section was taken.
- It was homogenized in a 1.5 ml Eppendorf tube with a stainless-steel bead (3 mm diameter, Qiagen, USA).
- The Eppendorf already contains 100 µl of Extraction solution (E7526-24ML, Sigma Aldrich, USA).
- Homogenization was done using a mixer mill (Bullet blender storm 24, Next Advance, Averill Park NY, USA) at speed point 8 for 30 seconds.
- The mixture was then incubated at 95 ℃ for 15 minutes and cooled on ice for 1 minute.
- 100 µl Dilution solution (D5688-12ML, Sigma Aldrich, USA) was added.
- After vortexing the samples were centrifuged at 18000 × g for 1 minute at room temperature.
- The resulting supernatant (100 µl) was transferred to a new Eppendorf tube and stored at -20 ℃ until needed.

5.9. 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-8: Sequence of primers used.

Name of the primer	Sequence of the primer	Tm
STTM_Clone_ KpnI_Fw	NNNATTTAAATATGGTCTAAAGAAG AAGAATATGGTCTAAAGAAGAA GAATGCACCAGTCGTGTACGGAGCCG CGggtacccaattcgccctatagtgagtcgtat	
STTM_Clone_ BamHI_Rv	NNNATTTAAATTAGACCATAACAA CAACAACTAGACCATAACAACAACA ACCGCGGCTCCGTACACGACTGGT GCggatccactagttctagagcggccgccaccg	
Hyg_Det_Fw	GCAAACTGTGATGGACGACA	
Hyg_Det_Rv	CTCCATACAAGCCAACCACG	
STTM_Det_Fw	ACGACTGGTGCATTCTTCTTC	
STTM_Det_Rv	TGGTCTAGTTGTTGTTGTTATGGT	
GRF4:GIF1_Det_Fw	AACGACTACATTCCCGCTCT	
GRF4:GIF1_Det_Rv	ATGAGATCAGTGGTGGCAGT	

Table-9: 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 2187STTM unit detection: 98°C 30 perc, (98°C 10 sec, 61°C 15 sec, 72°C 3 minutes) x34 cycles, 72°C 10 minutes.

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

Table-10: 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, 61°C 30sec, 72°C 10sec) x32cycles, 72°C 10 minutes.

5.10. 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.

5.11. Wheat transformation

5.11.1. Preparation of Agrobacterium for transformation

- From the inoculated plate, select single colonies of Agrobacterium AGL1, containing the desired vector were picked and inoculated in 10 ml liquid LB medium containing the appropriate antibiotics.
- Incubate at 28°C in a rotary shaker, shaken at 200 rpm for ~65 hr.
- Prepare Agrobacterium standard inoculums for transformation by mixing glycerol and culture.
- Mix equal quantities of the Agrobacterium culture and 30% sterile glycerol. Make 400-µl aliquots in 1.5-ml microcentrifuge and freeze at −80°C.
- The standard inoculum aliquots are stored at −80°C until required. They can be stored almost indefinitely.
- The day before wheat transformation, use a single 400-µl standard Agrobacterium inoculum to inoculate 10 ml of YEB liquid medium without antibiotics and incubate at 28°C in a rotary shaker, shaken at 200 rpm overnight (~16 hr).

5.11.2. Embryo Collection, Sterilization, and Embryo inoculation

- On the day of transformation, pellet the bacteria by centrifugation in a 50-ml Falcon tube at 3100 rpm for 10 min at 24°C.
- Discard the supernatant and resuspend the cells gently in 10 ml wheat inoculation medium (WIM) to an optical density of 0.5 OD (600 nm).
- Then, add 100 mM acetosyringone (AS) to 100 µM final concentration.
- Incubate the culture for 4-6 hr at room temperature with gentle agitation (80 rpm) in the dark (wrap Falcon tube with aluminum foil) before proceeding to Basic Protocol

5.11.3. Collection and sterilization of immature seeds

- Collect the wheat spikes ~14 days post anthesis when the immature embryos are 1-1.5 mm in diameter and at the early milk stage.
- Use kernels from florets 1 on central spikelet for transformation. Cut off the awns from the ears ~3-5 mm from the grain.
- Separate the immature grains from the ear and place in a 150-ml Sterile in jar.

- Sterilize the grains under aseptic conditions (e.g., laminar flow cabinet) using 70%(v/v) ethanol for 1 min.
- Rinse once with sterile distilled water and then place in 10 ml 10% (v/v) sodium hypochlorite and let stand 7 min. Then wash the grains three times with sterile distilled water.

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

- Isolate the embryos from the immature grains using fine forceps.
- Transfer embryos to 1.7-ml microcentrifuge tubes containing 1 ml WIM with 0.05%Silwet L-77, placing ~100 embryos in each tube.
- After isolating all the embryos, remove the WIM and add fresh WIM to the microcentrifuge tube(s). Centrifuge the isolated embryos 10 min at 14,000 rpm, 4°C.
- Remove WIM with a pipette, add1ml Agrobacterium solution and invert the tubes for 30s and finally incubate at room temperature in the dark for at least 20 minutes.
- After the incubation period pour the Agrobacterium suspension with the embryos into an empty sterile50-mm diameter petri plate and then remove the suspension with a pipet.
- Transfer 25 embryos, scutellum side up, to a fresh plate(s)of wheat co-cultivation medium in 90-mm-diameter single-vent petri plates.
- Seal the petri plates with Micropore tape and incubated at 24±1°C in the dark for 3 days of co-cultivation.

5.11.5 Resting period, callus induction and selection of transformed material

- After 3 days' co-cultivation, the embryogenic axes were excised from the embryos using forceps.
- The embryos were transferred to the fresh callus induction plates (WCI) based on the media containing 2 mg L−1 Picloram (Sigma-P5575), 0.5 mg L−1 2,4-dichlorophenoxyacetic acid (2,4-D), 160 mg L−1 cefotaxime and 5 mg L−1 agarose and incubated at 24 ± 1 °C in the dark for 5 days.
- Cefotaxime was added to control Agrobacterium during the resting period. The embryos were transferred, scutellum side up, to fresh WCI plates as above with 15 mg mL−1 Hygromycin and incubated at 24 ± 1 °C in the dark for 2 weeks.

- The calli were split at the next transfer into clumps of approximately 4 mm−2, callus pieces derived from each single embryo were labelled to keep track of their origin.
- The calli were transferred to fresh selection plates (WCI) as above, but with 30 mg L−1 Hygromycin (Selection 2) and incubated at 24 ± 1 °C in the dark for 2 weeks.
- The number of explants per plate were reduced by approximately half at Selection 2. 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-h photoperiod and covered with a single layer of paper towel for a further week.
- During this period putative transformed lines should start to green and produce small shoots

5.12. Regeneration, Rooting, and acclimatization

Regeneration of transgenic plants

- The following procedure was used for regeneration of transgenic plants.After the three weeks on selection 2 medium, the calli are transfer one final time to wheat regeneration medium (WRM).
- In deep Petri dishes (tissue culture dish, 90 mm diameter x 20 mm, Falcon 353003). All regenerating callus derived from a single embryo is labelled to track its origin. The paper covering is removed and the calli are cultured under fluorescent lights (100 µmol m2 s-1) at 24 ±1 °C with a 16-hour photoperiod.

Rooting

- Regenerated shoots which were 1–2 cm in length with visible roots (Fig. 1j) were transferred to “De Wit” culture tubes (Duchefa, W1607) containing 8 mL of WCI without growth regulators, solidified with 3 g L−1 Gelzan and supplemented with 160 mg L−1 Timentin and 15 mg L−1 Hygromycin.
- A strong root system with root hairs developed on putative transformed plants.

Acclimatization

- After ∼10 days, gently remove regenerated plantlets with strong root systems from the tubes using long forceps.
- Wash the roots with cool running water to remove any remaining tissue culture medium. Plant the plants in gify.

- The plants should remain covered with the propagator lid for ~1 week to maintain high humidity around them while they become established in soil.
- The plants are grown under the same conditions as donor plants within a controlled environment room. Once the plants are well established in the soil, leaf samples can be collected for further analysis to confirm the presence of the introduced genes.

6. RESULTS

Preliminary results

A novel wheat seed specific miRNA was found in the library prepared from 10-, 20- and 30-day old seeds of developing wheat *Triticum aestivum* L. cv. Bankuti (B35 and B52 line) and Spelta in two biological replicates. Small RNA library was prepared from total RNA or the isolated sRNA fraction for comparison of data obtained by sequencing small RNA libraries (Nagy et al., 2016). In the data analysis, together with known miRNAs a pool of previously unknown 21nt long miRNA (second highest read) was found shown in the second bar of the graph (Figure 7).

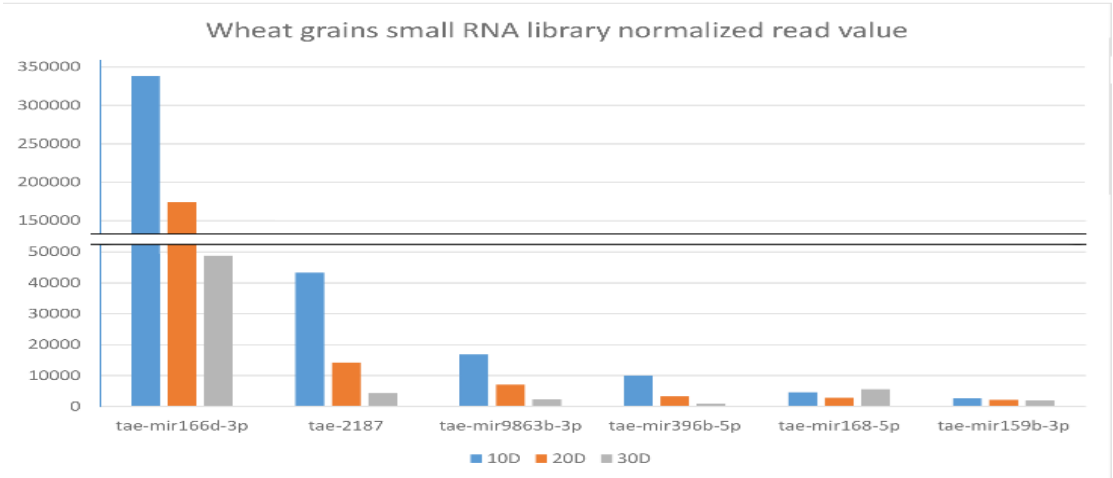

Figure. 7: This bar graph is depicting wheat grains small RNA library normalized read in *Triticuam aestivum* cv. Bánkúti. A high number of reads can be seen in the second left bar (tae2187).

Small RNA northern hybridization

First of all, the presence of this new miRNA was validated by small RNA northern hybridization. RNA was isolated from 10-, 20- and 30-day post anthesis from the seed of Bankuti BK35, Bankuti BK52 and Spelta. After hybridization with DNA specific probe and chemiluminescence-based detection a band on expected size was observed on the membrane. It is worth to mention that this novel miRNA was only observed in the seed and was not present in the libraries made from other tissue such as leaf (Shown in figure 8).

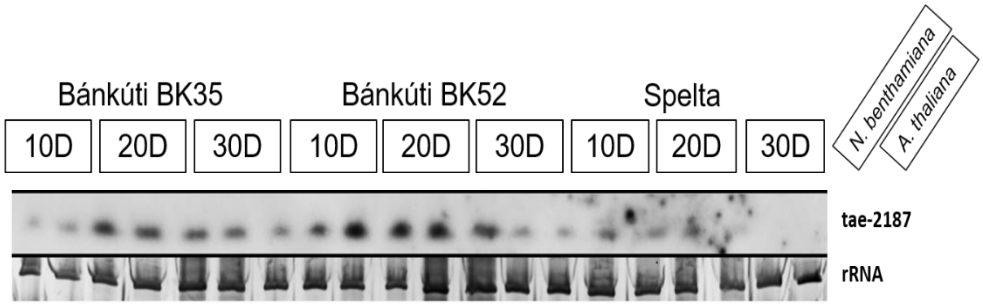

Figure. 8: Northern blot is showing the presence of a new miRNA (tae2187 based on reading) which was validated by small RNA northern hybridization. The miRNA is present in the Bankuti BK35, Bankuti BK52 and Spelta. Tae-mir9678 was used as a control.

After the presence validation a series of bioinformatic analysis was performed. The precursor of this miRNA was found on the wheat sub-genome A, B and D. The precursor secondary structure was predicted using the software RNAfold web Server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). Secondary structure can be seen in the (Figure 9).

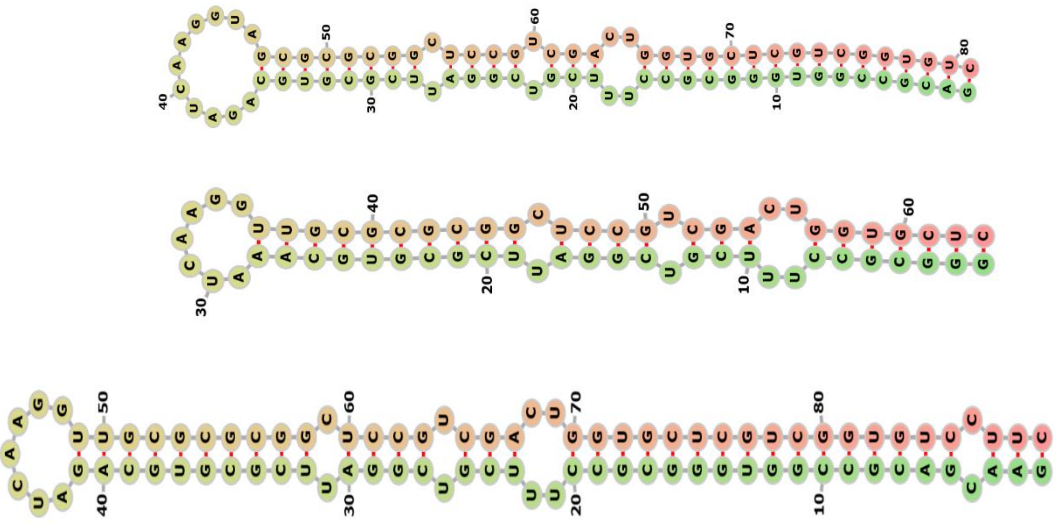

Figure. 9: Secondary structure of the precursor of miRNA2187. The top image is of A precursor, Middle is showing B precursor and bottom image is depicting D precursor.

In the further analysis the target of this miRNA was revealed using psRNATarget and it was found out that a messenger RNA of DNA directed RNA Polymerase V subunit is a potential target of this novel miRNA. Hence after this messenger RNA will be called NRPE1. The name was given based on protein homology shared in between *Arabidopsis thaliana* and

Triticum aestivum. Because they shared a similar protein sequence, and both the genes have the same essential protein domains. We cannot be 100% sure if they have same function because there is no experiment to determine this statement, but based on bioinformatics findings, protein similarities we call it NRPE1 (Shown in figure 10).

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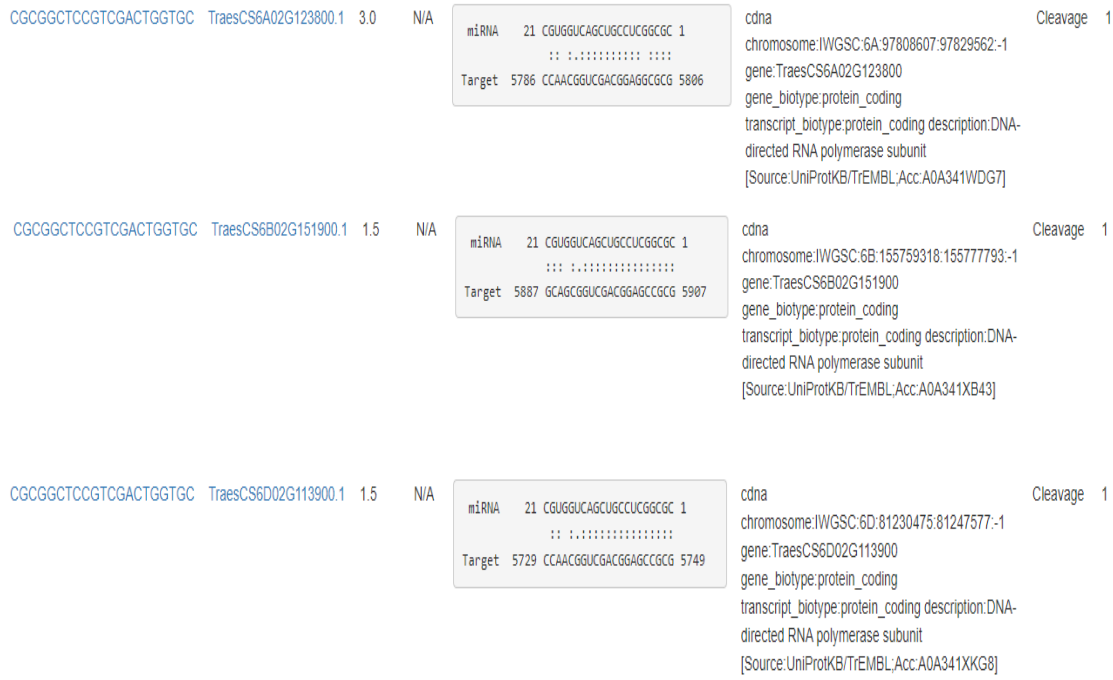


Figure. 10: Showing psRNATarget score of this miRNA on target. NRPE1 gene messenger RNA on genome 6B and 6D has better score than on genome 6A because it a mismatch.

6.1. Blocking function of a miRNA with Short Tandem Target Mimic (STTM)

Designing of 2187STTM-unit

Plasmid pBluescript II SK (+) was used a template to integrate the PCR 2187STTM-unit into the plasmid. The primers were designed in such a manner that they were 20 nt complementary to pBlueScript II SK (+) plasmid and were flanking with 2187STTM miRNA and half of the linker sequence with SwaI (Shown in Figure 11). SwaI site was integrated to circulate the plasmid after the PCR because PCR amplicon can be digested and ligated. Both primers have SwaI restriction site at the 5' ends (Figure 12).

Fig. 11: Showing STTM unit with miRNA with a bulge at both ends in green color and the linker sequence is highlighted in red colour.

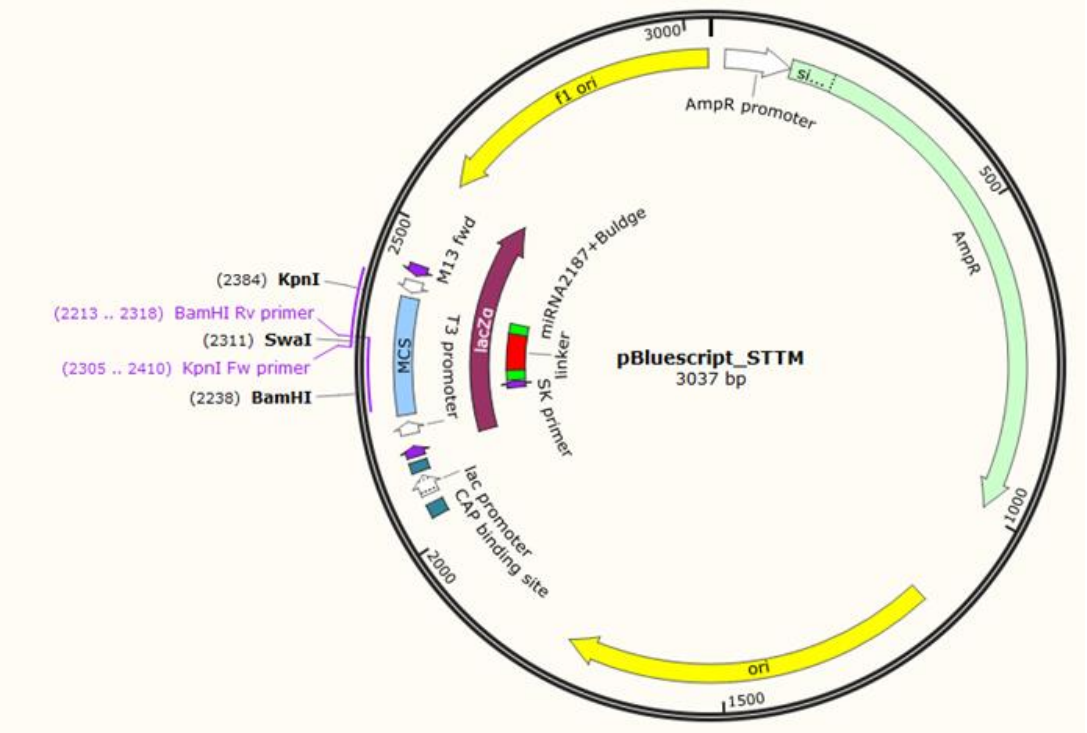


Figure. 12: Showing vector pBluescript SK II (+) containing STTM unit

Expected PCR amplicon size was observed on the gel. PCR was amplicon was digested with SwaI and was self-ligated. Finally ligated plasmids were transformed into *E.coli* (Figure 13). While designing primers it was also kept in mind to integrate KpnI restriction site in the forward primer and BamHI restriction site in the reverse primer. These sites can be used to cut off STTM unit and finally ligated into our final vector.

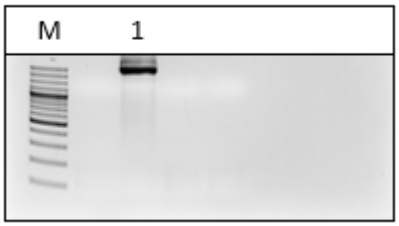


Figure. 13: PCR amplification of the plasmid pBluescript II SK (+) for integration of 2187STTM-unit (expected size=3094 bp), M: Molecular marker, Thermo Scientific ™ GeneRuler 100bp + DNA Ladder.

Plasmid was extracted from the transformed *E.coli* colonies and were put for the digestion using SwaI restriction enzyme to check if plasmids contain 2187STTM-unit. The gel photo (Figure 14) below clearly shows some opened plasmid with SwaI restriction enzyme.

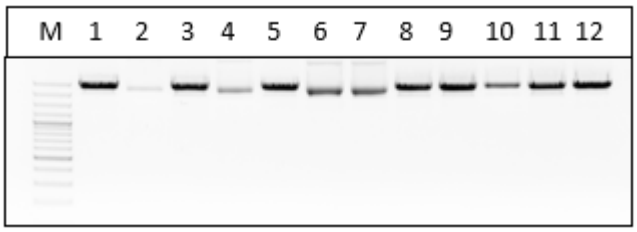


Figure. 14: Plasmid digestion with SwaI restriction enzyme to check the integration of 2187STTM-unit, M: Molecular marker, Thermo Scientific ™ GeneRuler 100bp + DNA Ladder.

6.2. Cloning of 2187STTM unit into pCubiNOS

Plasmid pBlueScript II SK (+) having 2187STTM unit were put for digestion with KpnI and BamHI to cut out 2187STTM unit. Vector pCAMBIA2300 designed by Kis et al. (2016) was already digested with KpnI and BamHI to make it compatible with the fragment. We used pCubiNOS plasmid, a modified vector of pCAMBIA1300. Finally, the final vector was called as Pcubi_Tae2187_STTM (shown in figure 15).

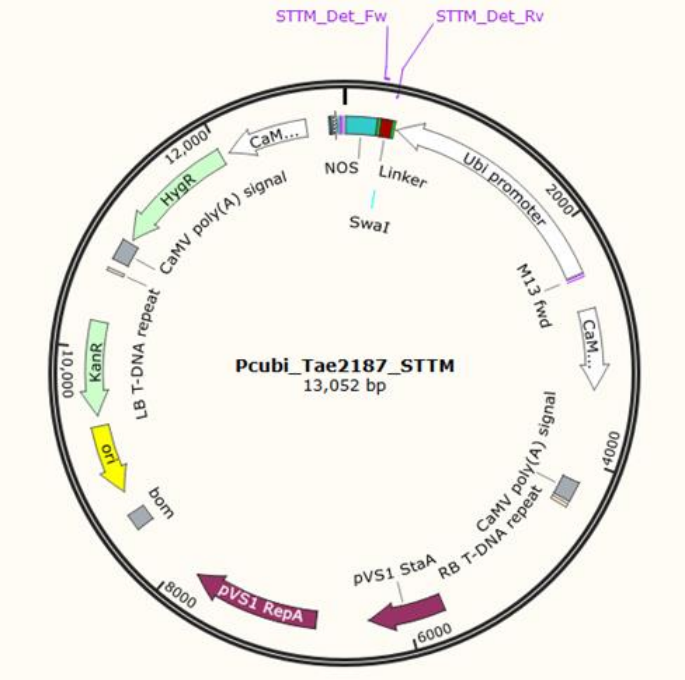


Figure. 15: Showing final vector Pcubi_Tae2187_STTM

Gel image shows the expected size fragment which is around 136 bp cut out product from plasmid. Fragmented product was cleaned up using the Macherey-Nagel™ NucleoSpin™ Gel and PCR Clean-up kit and finally was ligated into the destination vector i.e., pCubiNOS. (Figure 16).

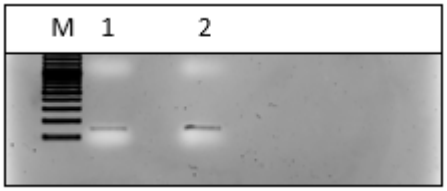


Figure. 16: Digested and purified STTM fragment with KpnI and BamHI restriction enzymes (expected size = 136bp), M: Molecular marker, Thermo Scientific ™ GeneRuler 100bp + DNA Ladder.

Finally, ligated pCubi_2187STTM_NOS plasmid was transformed into *E. coli* and plasmid was extracted from transformed *E.coli* colonies. Extracted plasmid was put for digestion with KpnI and BamHI to check the 2187STTM unit integration into the pCubiNOS. Expected fragment size can be seen in the gel photo below (Figure 17).

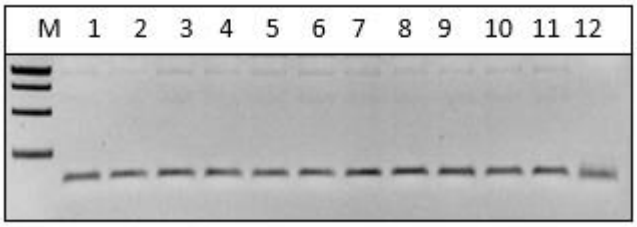


Figure. 17: pCubi_2187STTM_NOS plasmid digestion with KpnI and BamHI restriction enzymes to check the 2187STTM-unit fragment integration (expected size = 136bp), M: Molecular marker, Thermo Scientific ™ GeneRuler 1kb DNA Ladder.

6.3. Wheat transformation

Fielder wheat grown in the conditions mentioned above were transformed using the protocol Hayta. et al., (2019). Embryos were transformed with only vector pCubi_2187_STTM or co-transformation was done with vector pCubi_2187_STTM and pCubi_GRF4:GIF1_NOS. Successfully 12 lines were regenerated. About 150 embryos were used for each single vector transformation and co-transformation and almost 40% efficiency was achieved in the case of co-transformation and about 24% efficiency was achieved in single vector transformation. For the transformation confirmation DNA was extracted using direct DNA extraction method. DNA was subjected to PCR against hygromycin, GRF4:GIF1 and 2187STTM-unit (Figure 18).

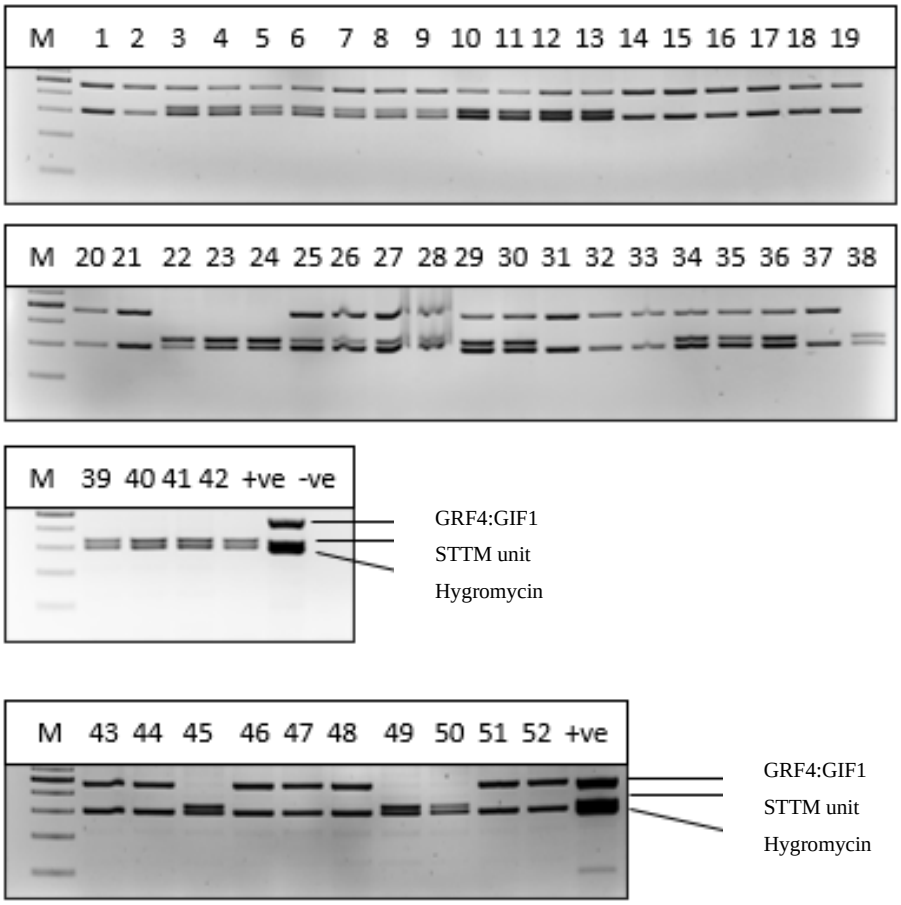


Figure. 18: MPCR amplification of genes Hygromycin, GRF4:GIF1 and 2187STTM unit in sample 1 to 52 (expected size=299bp, 330bp and 485bp respectively) from STTM transformed wheat plants, M: Molecular marker, Thermo Scientific ™ GeneRuler 100bp + DNA Ladder.

Transformed wheat plants either with only 2187STTM unit positive or plants having both genes GRF4:GIF1 and 2187STTM unit were chosen for growth and for further analysis. Three plants without 2187STTM unit were also grown as a negative control.

6.4. Expression analysis of 2187STTM unit in transformed wheat plants

RNA was extracted from the leaf of first plant of every line using phenol-chloroform method. RNA quantity was measured on the NanoDrop® ND-1000 UV-Vis Spectrophotometer and also it was run on the 1.2% gel. 3 µg of RNA was taken to make cDNA using RevertAid RT Reverse Transcription Kit (K1691) by the instructions of manufacturer. After cDNA synthesis and reverse transcriptase deactivation the quantity was measured again on The NanoDrop® ND-1000 UV-Vis Spectrophotometer to normalize the quantity. cDNA was used as a template in the PCR for the 2187STTM unit detection. Gel electrophoresis analysis revealed that

sample 2 and 8 have the strongest expression followed by in the lines 9 and 11. Line 6 has the moderate expression of 2187STTM unit. Lines 4, 5 and 10 are poorly expressing. Line 12 which was positive in PCR against STTM unit did not show any expression of the STTM unit. It could be due the fact this STTM unit was supressed by any small RNA via RNA interference mechanism. As expected, control lines (1, 3, 7) did not show any amplification (shown in figure 19).

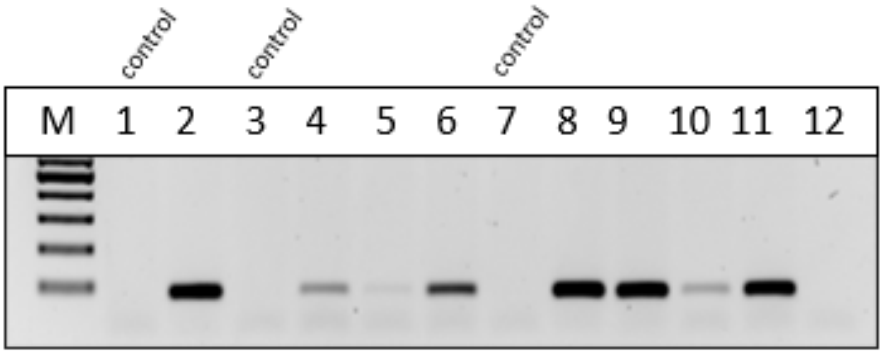


Figure. 19: PCR amplification of 2187STTM unit using cDNA as a template in sample 1 to 12 (expected size=90bp), M: Molecular marker, Thermo Scientific ™ GeneRuler 100bp + DNA Ladder.

6.5. Analysis of NRPE1 messenger RNA level

Based on Expression Atlas analysis (Home < Expression Atlas < EMBL-EBI) we decided to check the NRPE1 mRNA level in the seed at 8 dpa. RNA was extracted from few transformed STTM lines. Finally, 3 µg of RNA was taken to make cDNA using RevertAid RT Reverse Transcription Kit (K1691) as described by instructions of manufacturer. After cDNA synthesis and reverse transcriptase deactivation the quantity was measured again on The NanoDrop® ND-1000 UV-Vis Spectrophotometer to normalize the quantity (Shown in figure 20).

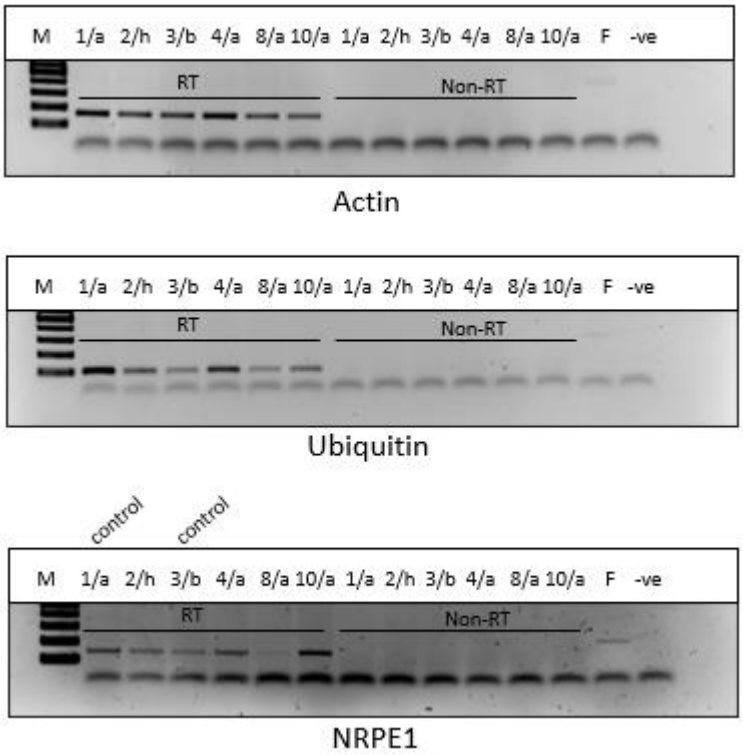


Figure. 20: Semi-quantitative PCR amplification of NRPE1 mRNA using cDNA as a template, M: Molecular marker, Thermo Scientific ™ GeneRuler 100bp + DNA Ladder.

Semi-quantitative PCR was performed in order to check the NRPE1 mRNA level. Primer pair was designed on two different exons to avoid the amplification from DNA. Two control and 4 transformed lines were included in the first analysis. Gel analysis revealed that STTM transformed lines seems to have higher expression of NRPE1 as compared to control lines. Transformed line 10/a seems to have relatively highest expression of NRPE1. Actin and Ubiquitin were used as housekeeping control genes. RT and non-RT were used in the semi-quantitative PCR to check the DNA contamination.

Analysis was extended further and this time seeds were collected at 8 dpa as well as 12 dpa. RNA was extracted and cDNA was synthesized as mentioned above. As Actin seems working more uniformly as compared to Ubiquitin so this time only Actin was included into the experiment.

Semi-quantitative PCR was performed to check the NRPE1 mRNA level (Figure 21). 4 transformed lines at 8 dpa and 2 transformed lines at 12 dpa were analysed. Line 1/a and 3/b were used as control for 8 and 12 dpa respectively. Gel analysis revealed that in the case

of 8 dpa seeds, line 2/b and 4/c have higher expression of NRPE1 mRNA as in the case of control line 3/b. Line 5/a showed almost equal level of expression as in the control 3/b and line 11/b seems not very high expressing but looks having a very slight elevation of NRPE1 mRNA level than control line. In the case of 12 dpa seed result, we cannot see any obvious increasement in the transformed line as compared to control l/a line. But this also could be due to technical error because we can see that Actin expression is also not very uniform. This time only Actin was used as housekeeping control gene. RT and non-RT were used in the semi-quantitative PCR to check the DNA contamination.

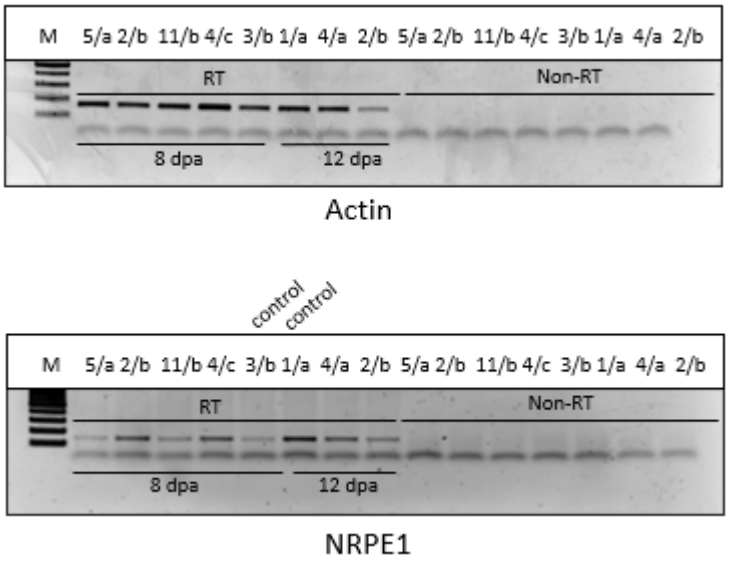
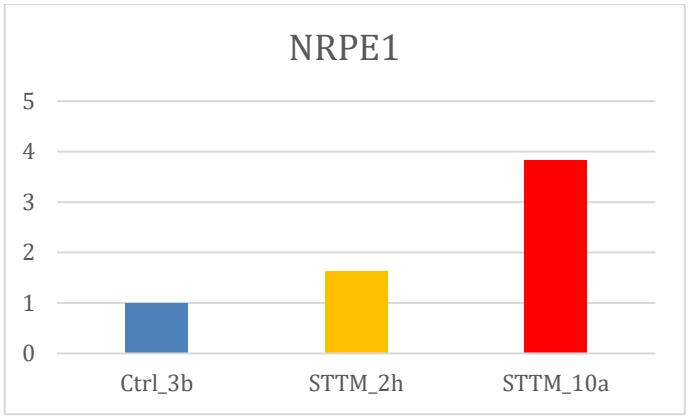


Figure. 21: Semi-quantitative PCR amplification of NRPE1 mRNA using cDNA as a template, M: Molecular marker, Thermo Scientific ™ GeneRuler 100bp + DNA Ladder.

6.6. Real time PCR analysis of NRPE1 mRNA from 8 dpa seeds

Based on semi-quantitative PCR analysis two promising lines were chosen for Real Time PCR analysis. Line 3/b was control and Line 2/h and 10/a were taken as experimental lines. Together with NRPE1 level, STTM-unit level was also measured. As it can be seen the bar graph (Figure 22) that NRPE1 level is higher in the experimental lines than control one. Which is justifying the results of semi-quantitative PCR. But interesting to mention that there is no co-relation between the level of 2187STTM-unit and NRPE1 level. Although line 10/b which has relatively low 2187STTM-unit expression has more NRPE1 mRNA level.



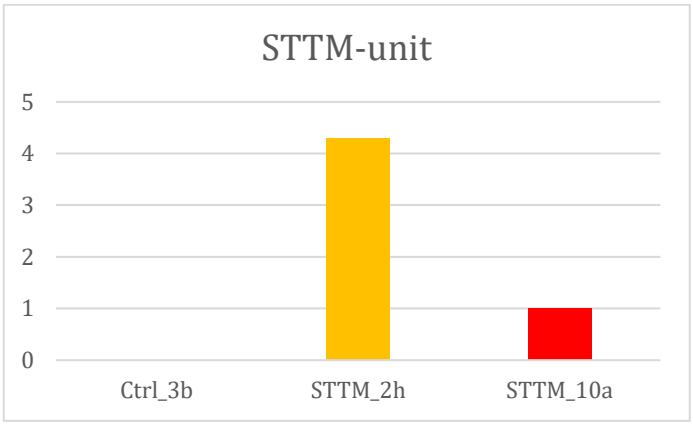


Figure. 22: Bar graph depicting the expression level of NRPE1 gene and 2187STTM unit. Control line 3/b has less expression level of NRPE1 as compared to experimental lines 2/h and 10/a (above bar graph). Expression level of 2187STTM unit can be also seen (below bar graph).

6.7. NRPE1 expression level at different days in control lines

To extend our analysis further we decided to find a time point where the level of NRPE1 expression is very low. So, at this time point we can check the NRPE1 mRNA level in the STTM transformed wheat lines. From the control line 1/a total 9 samples were collected at different days post anthesis (dpa). From line 3/b total 4 samples were collected and from line 3/d total 3 samples were collected at different dpa.

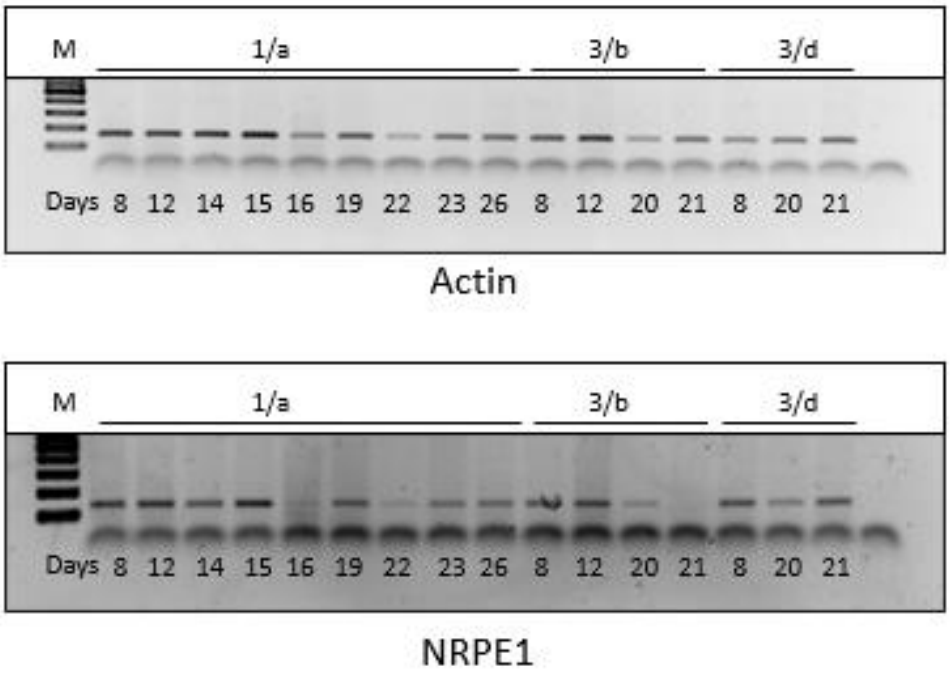


Figure. 23: Semi-quantitative PCR amplification of NRPE1 gene using cDNA as a template at different time point, M: Molecular marker, Thermo Scientific ™ GeneRuler 100bp + DNA Ladder.

As it can be seen the gel image above that NRPE1 expression is very low at around 21 to 22 dpa. Almost Similar level can be observed in all the three control lines. Actin was included as a house keeping control. Based on this finding we decided to check NRPE1 level in STTM plants at 21-22 dpa (Shown in figure 23).

6.8. Analysis of NRPE1 messenger RNA level at 21 days post anthesis

Based on above finding that at 21 to 22 days post anthesis the level of NRPE1 is really low in control lines. We collected seeds at 21 dpa from transformed STTM wheat plants. cDNA was made according the same procedures as mentioned above. First, NRPE1 mRNA level was analysed with semi-quantitative PCR. 4 transformed lines and 1 control line was considered for this analysis. Actin was used a house keeping control gene. Gel analysis showed that NRPE1 level is really high in all STTM transformed lines except 8/a as compared to control line. Only line 8/a seems having equal or only slightly higher level of NRPE1 (Shown in figure 24).

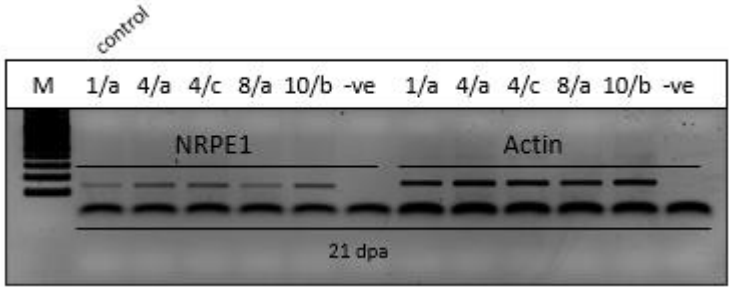


Figure. 24: Semi-quantitative PCR amplification of NRPE1 gene using cDNA as a template at 21 days post anthesis, M: Molecular marker, Thermo Scientific ™ GeneRuler 100bp + DNA Ladder.

6.9. Real time PCR analysis of NRPE1 mRNA from 21 dpa seeds

Another round of RT-qPCR was performed. This time 6 lines at 21 dpa and 2 lines at 22 dpa were included based on the finding of semi-quantitative PCR analysis. Mix was prepared as described by the provider. RT-qPCR analysis revealed that All STTM transformed lines have significantly higher copy of NRPE1 mRNA as compared to control 7/a line in the case of 21 days post anthesis. The result from 22 dpa seeds the amount of NRPE1 doesn't seems increasing in STTM transformed line as compared to control (Shown in figure 25). It could be due to some technical error. There should be another round of RT-qPCR at 22 dpa with more consideration and care. It is still can be seen that there is no corelation between the expression of 2187STTM-unit and NRPE1 mRNA level. The reason of it unknown.

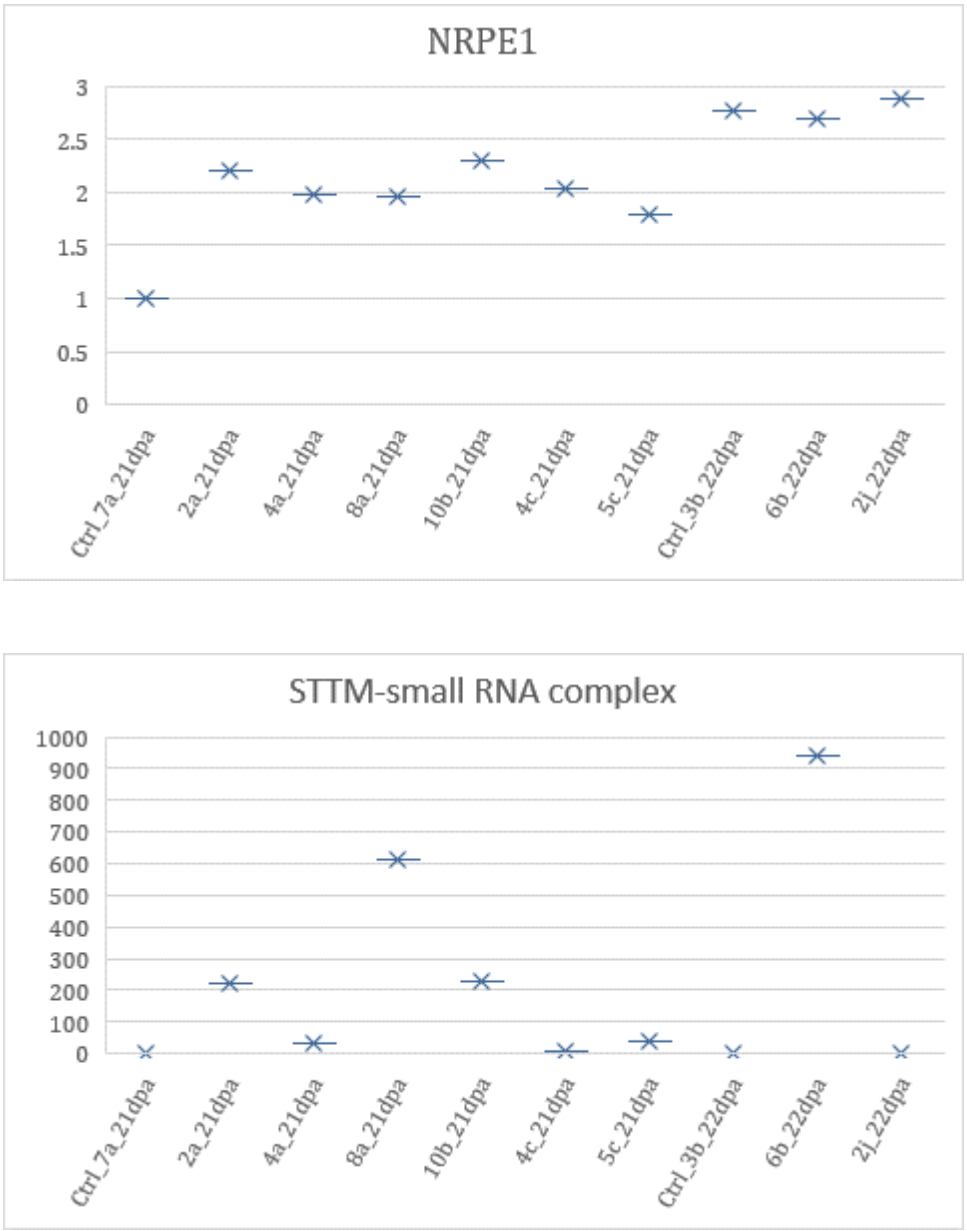


Figure. 25: Box and whisker graph depicting the expression level of NRPE1 gene and 2187STTM-unit at 21- and 22-days post anthesis. Control line 7/a has less expression level of NRPE1 as compared to experimental lines (above graph). Expression level of 2187STTM-unit can be also seen (below graph).

7. DISCUSSION AND RECOMMENDATIONS

The main aim of this experiment was to express 2187STTM-unit in order to study the most potential target of a newly found miRNA in wheat seed specific libraries. The main target of this novel miRNA was a mRNA of Polymerase V. Eukaryotes have three multisubunit, nuclear DNA-dependent RNA polymerases: RNA polymerase I (Pol I) transcribes large ribosomal RNAs, Pol II transcribes mRNA precursors, and Pol III transcribes tRNA and 5S rRNA. Plants have two additional RNA polymerases known as Pol IV and Pol V, both of which evolved from Pol II and are specialized for RNA-directed DNA methylation (RdDM). We named this miRNA as miRNA2187 based on the reading in the libraries. To implement Short Tandem Target Mimic (STTM) technology, we need stable transformation of wheat hence wheat cultivar Fielder was transformed using Agrobacterium method. Wheat embryos were used as an explant for transformation. It is important to choose a perfect stage embryo for transformation. We used protocol described in the protocol described by (Hayeta et. al., 2019). They achieve almost 25% of transformation efficiency, which is almost similar to our finding. We also got almost 24% efficiency in the case of single transformation but in the case of co-transformation with GRF4:GIF1 chimera gene vector, we achieved 40% efficiency which is significantly high. This is because of increasing the efficiency and speed of regeneration in wheat.

The expression of 2187STTM-unit is different in all plants it can be due the copy number or STTM unit is targeted by interference in some lines in some extent. In the transformed line 5 the plants were positive for the transformation based on the PCR analysis but showed no expression of 2187STTM-unit, the most possible reason was this would be a very strong RNA interference where this STTM unit is degraded by siRNA. NRPE1 target analysis with semi-quantitative PCR in all the experiment the PCR amplicons doesn't seems very uniform the reason of this could be the error in the taken amount of RNA during cDNA synthesis or may be concentration difference after DNAase treatment. Because these changes can also be seen in the case of Actin and Ubiquitin control as well not only in the case of NRPE1 target. Other possibility of these differences also can be due to the amount difference of cDNA as a template during the PCR reaction preparation.

During the RT-qPCR analysis the results are significant against control line 7/a, but results are not promising if we compare with control line 1/a. The variations in the control lines can be because of the difference in the time collection. It seems the level of NRPE1 mRNA level shows a drastic decline over time. It also seems that the level of NRPE1 mRNA

shows a rise again after 20-23 days post anthesis which is very unusual finding. The reason of this can be explored further. The whole RT-qPCR can be performed again with more carefulness specially the consideration should be paid in the timing of sample collection. There can be seen that there is no corelation between the expression level of 2187STTM-unit and in the increment of pol V mRNA level. That means expression of 2187STTM-unit start to show a decline after few days.

This experiment can be continued further by knocking out the miRNA gene with CRISPR/Cas9 technology. It is worth to mention that this miRNA is also studied in the Zoltan Havelda group by sensor technology which showed very promising results and give another round of target confirmation of this novel miRNA. It would be also interesting to reveal the relationship of this miRNA with pol V subunit messenger RNA by 5' Rapid Amplification of cDNA Ends (5'RACE) technology.

8. SUMMARY

Thesis Title: Functional Investigation of a novel wheat seed-specific miRNA by short tandem target mimic (STTM)

Written by: Abdul Razzak

Course: Master of science in Agricultural Biotechnology

Institute: Institute of Genetics and Biotechnology

Primary thesis advisor: Dr. Kis Andras, PhD, Research Fellow

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Wheat is a crucial cereal crop that plays a significant role in feeding a significant percentage of the world's population. Scientists have been investigating the genetic factors that influence the growth and development of wheat to find ways to overcome the difficulties associated with crop production. RNAi is a highly effective method for studying gene function and can be accomplished by either post-transcriptional gene silencing (PTGS) or transcriptional gene silencing (TGS). Among the different RNAi approaches, microRNA (miRNA) knock-down is a popular strategy, that can be implemented using short tandem target mimic (STTM). Therefore, exploring a novel wheat seed-specific miRNA through the STTM technique presents a promising way of gaining insights into the mechanisms that regulate seed development in wheat.

STTMs have a size of about 100 nucleotides (nt) and consist of two tandemly arranged miRNA binding elements, each of which is designed with a mismatch located at the miRNA cleavage site. The miRNA binding elements are connected by a flexible stem-loop linker that spans between 48-88 nt. The STTM method has been used to silence various miRNA families in *Arabidopsis* and several model and staple crops such as tomato, rice, wheat, tobacco, *Medicago,* soybean, poplar, cotton, common bean, and barley. Our main aim was to make a STTM construct to block the function of a novel wheat seed specific miRNA2187 to prove its potential target that is Pol V subunit messenger RNA, predicted bioinformatically. STTM construct was introduced into plant cells through *Agrobacterium*-mediated wheat transformation.

Seeds of the spring wheat (*Triticum aestivum* L.) genotype ‘Fielder’ were sown at weekly intervals in a peat and sand mix (5:1). Fielder was risen in growth chambers (Conviron, Winnipeg, Canada) under 16h/8h light/dark period. 70% humidity with light levels of 800 μmol.m-2s-1 provided by fluorescent tubes and tungsten lighting. Plants are not sprayed with fungicides or insecticides at any stage of growth. The STTM construct having

two flanking miRNA sequence with 3 nt bulge at the 10th nucleotide, linked by an 88 nt linker sequence. The construct was made and first introduce 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. Single vector transformation and co-transformation with 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 giffy followed by transfer into the soil.

Transformed plants were put for PCR analysis, simultaneously hygromycin, GRF4:GIF1 chimera gene and STTM unit were targeted. Plants either positive with only STTM unit or STTM unit and GRF4:GIF1 were taken for further analysis. Total RNA was extracted from transformed and control lines seeds at 8 and 12dpa. First several transformed lines were analysed with semiquantitative PCR including controls for comparison. Actin and Ubiquitin were used as housekeeping control genes. Promising lines in semiquantitative PCR were subjected for RTq-PCR analysis. Clearly it was found that PolV messenger (NRPE1) RNA level was elevated in STTM transformed lines as compared to control line.

9. ACKNOWLEDGMENT

The path toward this thesis has been circuitous. This thesis becomes a reality with the kind support and help of many individuals. I would like to extend my sincere thanks to all of them first and foremost, I want to offer this endeavour to Almighty Allah for the wisdom He bestowed upon me, the strength, peace of mind, and good health (ALHAMDULILLAH) in order to finish this thesis. I would like to express my gratitude towards my family as they constantly supported me in every situation of my life. Without their constant support it wouldn't have been possible.

I owe a deep sense of gratitude to my supervisor Dr. Kis Andras. His dedication and keen interest above all his overwhelming attitude to help his students had been solely and mainly responsible for completing my work. Above all Dr. Kis Andras is a joyful soul who stood by me encouraging this whole time. His timely advice, meticulous scrutiny, scholarly advice and scientific approach have helped me to a great extent. The knowledge I gain from you surely will not go in vain.

Also I thank Mohammad Ali for his help in the laboratory during my practical work and my class mate Mohammad Umar.

Thank you!

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