CONSTRUCTION of PLANT GENE EXPRESSION VECTORS CARRYING *ath-Mir393a* GENE and ITS TARGET GENE *AFB3*

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CONSTRUCTION of PLANT GENE EXPRESSION VECTORS CARRYING *ath-Mir393a* GENE and ITS TARGET GENE *AFB3*

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Thesis Supervisor: Hikmet Budak

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ABSTRACT

MicroRNAs (miRNA) are a class of non-coding small RNAs that regulate gene expression. Plant miRNAs are involved in a broad range of biological processes including growth, developmental patterning and abiotic stress responses.

In our previous miRNA profiling results in response to glyphosate, ath-Mir393a was found to be up-regulated at the 5 % glyphosate application whereas down-regulated at the 20 % foliar glyphosate application when compared to control samples of *Festuca arundinacea*. Ath-Mir393a is a conserved miRNA species that targets F-box proteins TIR1 and AFB1/2/3 transcripts. F- box motif mediates protein-protein interactions and mostly found in proteins as components of SCF ubiquitin-ligase complexes. Hence they have very important roles in the maintenance of protein stabilities inside the cell. TIR1 and AFBs have shown that they are associated with auxin signaling pathways as auxin receptors.

In order to improve the management of weed invasion on crop lands, the idea of developing genetically engineered cultivars which are glyphosate-resistant has become a necessity. Therefore in this study two plant gene expression vectors carrying *Arabidopsis thaliana ath-Mir393a* gene and *AFB3* gene were constructed by using Gateway technology. *Ath-Mir393a* and *AFB3* genes were first captured in 'entry vectors' and then recombined into pEARLEY Gate100 Gateway compatible 'destination vectors'. These vectors are later used for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. These vectors constructed in this study will be a good starting point for exploring and elucidating the miRNAs associated with regulation of plant responses to glyphosate.

Ath-Mir393a GENİNİ ve HEDEF GENİ OLAN *AFB3`ü* TAŞIYAN BİTKİ GEN İFADE VEKTÖRLERİNİN TASARLANMASI

Mine Bakar

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ÖZET

MicroRNAlar (miRNA) gen ekspresyonunu control eden ve kodlanmayan küçük RNA sınıfındandırlar. Bitki miRNA'ları büyüme, gelişimsel desenlendirme, ve abiyotik stress tepkileri de dahil olmak üzere birçok biyolojik süreçte görev alırlar.

Önceki Glyphosate'e cevaben çıkartılmış miRNA profil sonuçlarımıza göre, *Festuca arundinacea* kontrol örnekleriyle karşılaştırıldığında *ath-Mir393a* % 5 glyphosate uygulamasında yüksek ifade edilmiş, % 20 glyphosate uygulamasında düşük ifade edilmiş bulundu. Ath-Mir393a F-box proteinlerinden olan TIR1 ve AFB1/2/3 transkriptlerini hedefleyen bir miRNA türüdür. F-box motifi protein-protein etkileşimlerine aracılık edip çoğunlukla SCF ubiquitin-ligaz komplekslerinin bileşenleri olarak protein yapısında bulunmaktadır. Bu sebeple bunların hücre içinde protein kararlılığını kontrol etmek gibi çok önemli rolleri vardır. Ayrıca TIR1 ve AFBlerin oksin reseptörü olarak oksin sinyalizasyon yolağı ile ilişkilendirildikleri gösterilmiştir.

Ekili alanları istila eden yabancı otların kontrol edilebilmesi için genetik olarak tasarlanmış glyphosate`e dirençli kültivarların oluşturulması bir gereklilik haline gelmiştir. Bu sebeple bu çalışmada Gateway teknolojisi kullanılarak *ath-Mir393a* genini ve onun hedef geni olan AFB3`ü taşıyan iki bitki gen ifade vektörü tasarlandı. Ath-Mir393a ve AFB3 genleri ilk olarak 'entry vektör' içine alındı ve daha sonra Gateway`e uyumlu pEARLEY Gate100 Gateway 'destinasyon vektörü'ne' birleştirildi. Bu vektörler daha sonra *Arabidopsis thaliana* bitkisinin *Agrobacterium*-aracılı transformasyon yönteminde kullanıldı. Bu çalışmada tasarlanmış bu vektörler, glyphosate'e karşı bitki reaksiyonunun düzenlenmesiyle ilgili mikroRNAların araştırılması ve açıklanması yolunda iyi bir başlangıç noktası olacaktır.

"Dedicated to My Family"

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ABBREVIATIONS

AFB	AUXIN SIGNALING F-BOX
AGO	ARGONAUTE
ath	*Arabidopsis thaliana*
BAC	Bacterial artificial chromosome
bHLH	basic helix-loop-helix
CaMV	*Cauliflower mosaic virus*
CpG	Cytosine-phosphate-Guanine
DCL1	Dicer-like 1
DNA	Deoxyribonucleic acid
dsRBDs	double-stranded RNA Binding Domains
dsRNA	double-stranded RNA
dsRNA	double-stranded RNA
eIF4E	initiation factor 4E
EPSPS	5-enolpyruvylshikimate 3-phosphate synthase
EPSP	5-enolpyruvyl-shikimate-3-phosphate
EST	expressed sequence tag
EXP5	Exportin 5
GSS	genomic survey sequence
GUS	Beta-glucuronidase
HEN1	Hua Enhancer 1
HYL1	HYPONASTIC LEAVES1
LB	Luria-Bertani
miRNA	MicroRNAs
MPSS	Massive Parallel Signature Sequencing
MS	Murashige and Skoog
NC	Negative control
PCR	Polymerase chain reaction
POLR3D	Polymerase (RNA) III (DNA directed) polypeptide D

PPT	Phosphinothricin
Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA
qRT-PCR	quantitative real-time PCR
RISC	RNA-induced silencing complex
RLC	RISC Loading Complex
RNA	Ribonucleic Acid
Rpm	Revolutions per minute
S3P	shikimate-3-phosphate
SCF	Skp, Cullin, F-box containing complex
SE	SERRATE
siRNAs	small RNAs
ssRNA	single-stranded RNA
T-DNA	Transfer DNA
TE	Tris-EDTA
TIR1	TRANSPORT INHIBITOR RESPONSE 1
TRBP	trans-activation responsive RNA-binding protein
UTR	Untranslated Region

1. INTRODUCTION

1.1. Glyphosate

Glyphosate is a wide-spectrum herbicide which has been extensively used to control annual and perennial weed species. In the USA, the herbicide is first registered under the trade name Roundup by Monsanto. In 2000 it was calculated to be the world's leading agrochemical. As compared to the other herbicides against which many species have evolved resistance, glyphosate is an essential tool in modern agriculture due to the rarity of exceedingly resistant species.

Despite glyphosate's extensive usage, there are still naturally occurring glyphosate tolerant species and degree of tolerance between species varies greatly. For that reason, application dosage is determined by depending on the organism. In contrast to annual weeds, higher rates of application are required for the management of perennials, for example, since they have a greater potential for regeneration. Moreover broad-leaved weeds are more resistant than are grasses.

1.1.1. Mode of Action

Glyphosate is a glycine analog with a phosphonomethly group added to its amino group (Figure 1). Glyphosate interacts with the shikimate pathway in plant systems leading to the block in the synthesis of aromatic amino acids tyrosine, tryptophan and

phenylalanine (Figure 2). It inhibits a nuclear encoded and chloroplast localized enzyme, 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) that catalyzes the conversion of shikimate-3-phosphate (S3P) and phosphoenolpyruvate to 5-enolpyruvyl-shikimate-3-phosphate (EPSP) (Franz, Mao, & Sikorski, 1997). Kinetic patterns of EPSPS inhibition by glyphosate indicated that glyphosate is an uncompetitive inhibitor with respect to S3P and competitive inhibitor with respect to PEP (Boocock & Coggins, 1983; Schonbrunn, et al., 2001). This study designated that EPSPS : S3P complex formation is a prerequisite for glyphosate binding. On the other hand, since PEP and glyphosate share the same binding site on the kinetic intermediate (EPSPS: S3P complex), they compete for binding EPSPS: S3P complex. Moreover, in the reverse reaction of EPSPS, glyphosate has been shown to be a non-competitive inhibitor with respect to phosphate and competitive inhibitor with respect to EPSP (Boocock & Coggins, 1983). In addition to interaction with shikimate pathway, it also acts on other physiochemical processes such as reduction in photosynthesis and auxin transport (Baylis, 2000). Although it is a slow-acting herbicide, physiological analysis has disclosed that within 24 hours of application it causes an increase in shikimic acid levels (Stasiak, Hofstra, & Fletcher, 1992).

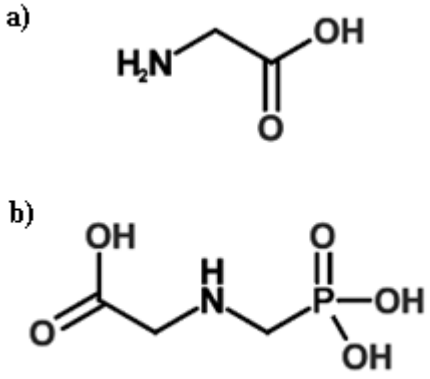

Figure 1: a) Glycine molecule, b) Glyphosate: Glycine (N-phosphonomethyl), (modified from "Glyphosate," 2005)

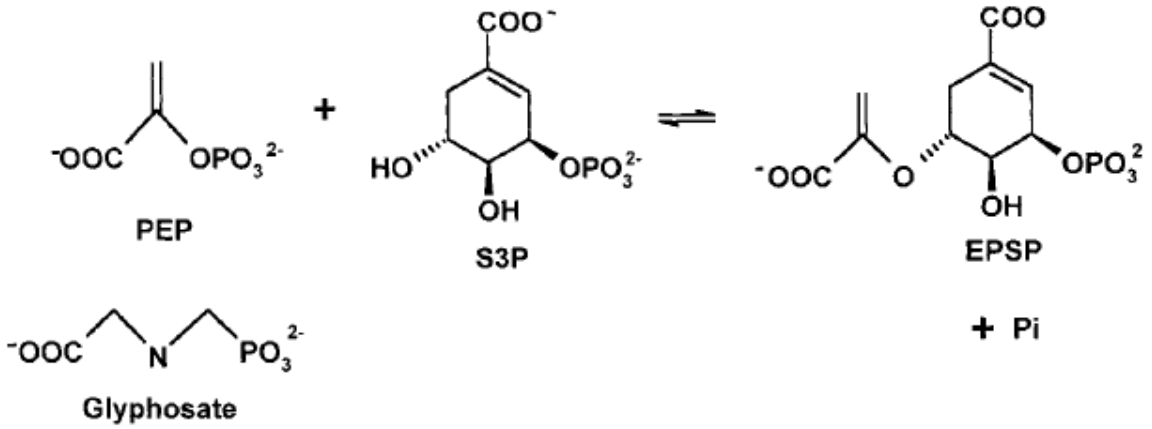

Figure 2: Reaction catalyzed by EPSPS and the structure of inhibitor glyphosate (Brown, et al., 2003)

1.2. Glyphosate-Resistant Crops

In order to improve the management of weed invasion on crop lands, developing genetically engineered cultivars which are glyphosate-resistant has become a necessity. Until now many glyphosate-tolerant crops have been developed such as soybean (*Glycine max*), cotton (*Gossypium hirsutum*), oilseed rape (*Brassica napus*), maize (*Zea mays*), and sugar beet (*Beta vulgaris*) by the heterologous expression of the glyphosate-resistant form of EPSPS obtained from *Agrobacterium* sp strain CP4 (Padgette, et al., 1995). However transgenic soybeans have been shown to produce 6.7 % lower yields than do their non-engineered varieties (Baylis, 2000).

1.3. MicroRNA

MicroRNAs (miRNA) are non-coding small RNA molecules with a limited size of 20-24 nucleotides that regulate gene expression at a post-transcriptional level either by causing mRNA degradation or repressing translation. They carry out their role by binding

to their targets in a sequence specific manner. Most recently it has been discovered that they are also involved in transcription regulation through DNA and/or histone methylation.

They were first discovered in *C.elegans* as regulators of developmental timing. However, the breadth of miRNA-regulatory gene expression was recognized as more miRNAs were discovered in other eukaryotes. Yet it is nearly accepted as a universal mechanism in eukaryotes. Despite differences between animals and plants there are some common themes concerning miRNAs shared by both groups namely (1) they are endogenously expressed (2) processed from precursors and (3) generally evolutionarily conserved (Bartel, 2004).

miRNAs have very distinctive roles inside the cell. They are involved in almost every biological process ranging from cell proliferation to cell death, differentiation, and cellular patterning. Through such extensive covering, 1/3 of mRNAs are predicted to be regulated by miRNAs. The present miRNA database contains 3882 entries for vertabrates, of which 940 belongs to *H.sapiens;* 2520 entries for plants, of 199 belong to *A.thaliana* ("miRBase: the microRNA database," 2004).

1.3.1. miRNA families

Sequence analysis has revealed that plant genomes have large gene families. Plant miRNAs derived from the same gene family have strong homology not only in their mature structure but also throughout their genes (A. L. Li & Mao, 2007). This suggests they expand via tandem gene duplication or segmental duplication events. 22 of the miRNA gene families in *Arabidopsis*, for example, have found to be conserved between other dicots and monocots (Griffiths-Jones, Grocock, van Dongen, Bateman, & Enright, 2006). Although the members of the same gene family are clustered together in animals, the situation differs in plants, i.e. plants are mostly scattered through the genome. In animals clustered genes are sometimes non-homologous but their target genes are functionally related.

Sequence and structure conservation in miRNAs facilitates the discovery of new miRNAs in both animal and plant systems. Reverse genetics strategies are commonly used to identify new miRNAs. As a reverse genetics strategy, computational approaches are widely used in miRNA identification because of being low cost, high efficiency, fast and comprehensive methodology (Unver, Namuth-Covert, & Budak, 2009). For instance, 26 new *Brachypodium distachyon* miRNAs were identified by comparing the all known plant miRNA sequences with *B. distachyon* expressed sequence tags (ESTs) and genomic survey sequences (GSSs) (Unver & Budak, 2009). In another study, through sequence homology analysis, 28 conserved miRNAs were identified in *Hordeum vulgare*, of which 4 were detected to be dehydration stress-regulated (Kantar, Unver, & Budak). Computationally identified miRNAs are further confirmed by experimental strategies such as cloning and sequencing of small RNA libraries.

1.3.2. miRNA biogenesis

miRNAs are transcribed from MIR genes by RNA polymerase II enzyme. Primary miRNA transcripts (Pri-miRNA) contain a 5'-G-cap and 3'-polyA tail. In contrast to animal miRNAs which are transcribed as polycistronic precursors from a *MIR* gene cluster, plant miRNAs are transcribed from single gene loci that give rise to a single miRNA species. Depending on the differences in terms of sequence, the precursor structure and biogenesis mechanisms of plant miRNAs seem to have evolved separately from those of animal miRNAs (Chapman & Carrington, 2007; Millar & Waterhouse, 2005).

In animals, mature miRNA structure is generated in two endonucleolytic modification steps. A typical pri-miRNA consists of a stem-loop structure that is flanked by single-stranded RNA (ssRNA) regions at both ends. Flanking ssRNA regions are removed by the action of RNase III type protein Drosha in the nucleus. The remaining stem-loop structure is called pre-miRNA. The first cleavage step requires a cofactor DiGeorge syndrome critical region gene 8 (DGCR8) or and a double-stranded RNA (dsRNA) binding protein. Together with DGCR8, Drosha is known as Microprocessor complex. DGRC8

recognizes the ssRNA segment and stem of pri-miRNA and assists Drosha to cleave its substrate ~11bp away from the ssRNA- dsRNA junction. Pre-miRNA is exported to the cytosol by Exportin 5 (EXP5) for a second processing step. Another RNase III type protein Dicer in association with another dsRNA-binding protein, the human immunodeficiency virus trans-activation responsive RNA-binding protein (TRBP) plays a role in the maturation step. Dicer cleaves terminal loop releasing ~22nt miRNA-miRNA* duplexes. Thus while Drosha generates one end of the mature miRNA, Dicer determines the other end.

In plants there is single type of enzyme that orchestrates pri-to-pre-miRNA conversion and mature miRNA formation. Dicer-like 1 (DCL1) together with double-stranded RNA-binding protein HYPONASTIC LEAVES 1 (HYL1) and the C2H2-zinc finger protein SERRATE (SE) completes pri-to-pre-miRNA conversion. Although the pri-to-pre-miRNA or maturation sites are not very clear in plants, subcellular localization studies with fluroscents proteins of DCL1 and HYL1 designate Dicing-bodies (D-bodies) inside the nucleus (Fang & Spector, 2007). On the other hand, both pre-miRNA and miRNA/miRNA* are found to be the cargoes of HASTY, the plant homolog of exportin-5. Studies with *hasty* mutants points out that there is HASTY-independent export pathways because of the decreased accumulation of only some miRNAs in both nuclear and cytoplasmic fractions (Park, Wu, Gonzalez-Sulser, Vaucheret, & Poethig, 2005).

In contrast to animal miRNAs, plant miRNA duplexes are modified from their 3' ends by the S-adenosyl methionine-dependent methyltransferase Hua Enhancer 1 (HEN1) (Aravin, Hannon, & Brennecke, 2007). 2'-*O*-methylated forms of miRNAs are protected against uridylation and degradation (J. J. Li, Yang, Yu, Liu, & Chen, 2005).

1.3.3. RISC formation: Argonaute loading

Following modification, miRNA/miRNA* duplex is loaded onto ARGONAUTE (AGO) protein to form RNA-induced silencing complex (RISC). What determines the

guide strand (miRNA) and degrades the passenger strand (miRNA*) are the relative thermodynamic stabilities of the two ends of the duplex. Studies on miRNA precursors indicate that the strand with the unstable base pairs at 5' end is selected as a guide strand (Khvorova, Reynolds, & Jayasena, 2003). In some cases strands can be selected as a guide strand. Three necessary components for the generation of RISC complex are Dicer, TRBP and AGO proteins which are jointly called RISC Loading Complex (RLC). The mechanism of duplex loading to RISC complex is investigated with synthetic RNA molecules in *D. melanogaster*. R2D2 protein which contains two double-stranded RNA Binding Domains (dsRBDs) binds to more stable end of miRNA/miRNA* duplex together with Dicer-2 enzyme and controls AGO protein orientation (Tomari, Matranga, Haley, Martinez, & Zamore, 2004). AGO protein takes role in the endonucleolytic cleavage of the passenger strand (Matranga, Tomari, Shin, Bartel, & Zamore, 2005).

There are several AGO proteins in plants and animals; however these proteins' diversity varies among different organisms. In the structure of AGO proteins N-terminal domain is the variable domain; the C-terminal domain that consists of PAZ, MID and PIWI domains is the conserved domain. Each part in the conserved domain has unique functions i.e. PAZ domain interacts with the 3' of the small RNAs, MID interacts with the 5' of the small RNAs and PIWI is responsible for the endonuclease activity with its active site Asp-Asp- His (DDH) domain (Hutvagner & Simard, 2008; Rivas, et al., 2005). However, not all the PIWI domains have slicing activity such as does the human Ago3 protein.

There are three types of AGO proteins classified according to their phylogenetic tree and type of small RNA choice. Members of group 1 bind to miRNAs and small RNAs (siRNAs) and are called AGO proteins. In contrast to Group 1 proteins, the second group prefers PIWI- interacting proteins, so called PIWI proteins. The third group has been studied only in *C.elegans* and preferentially bind to secondary siRNAs (Yigit, et al., 2006). Although the detailed mechanism of miRNA mediated gene inactivation is not well known, the mechanism of action is thought be dependent on the type of AGO protein. In animals such as *Droshophila* perfectly matching small RNAs are directed into *Dm*AGO2 whereas, duplexes with central mismatches are directed into *Dm*AGO1. *Arabidopsis* contains ten

different AGO proteins and among them AGO1 responsible for the miRNA mediated gene regulation. The evidence of AGO1 has role in miRNA pathway came from the study with Ago1 mutants which miRNA accumulation is reduced and reciprocally mRNA accumulation is increased (Vaucheret, Vazquez, Crete, & Bartel, 2004).

1.3.4. miRNA mediated gene regulation

Although the complete mechanisms for RNA mediated gene regulation are not completely understood, until now three types of regulation have been disclosed i.e. post-transcriptional regulation, transcriptional regulation and gene activation. All these findings point that it is a highly complex regulation network.

1.3.5. Post-transcriptional gene silencing

There are several proposed mechanisms for the miRNA mediated gene inactivation. There are studies which have shown that miRNAs block translation of its target mRNA either at the initiation step by inhibiting translation initiation factor 4E (eIF4E), or at the elongation step (Humphreys, Westman, Martin, & Preiss, 2005; Lytle, Yario, & Steitz, 2007). Another study has shown that miRNAs cause rapid mRNA decay by inducing deadenylation and decapping (Behm-Ansmant, et al., 2006). They have also been shown to stimulate nascent peptide proteolysis. In some cases AGO proteins have been found to associated with miRNAs and mRNAs in the cytoplasmic P-bodies suggesting that they stimulate target degradation (Rossi, 2005).

miRNAs represent a negative gene regulatory system in both plants and animals. Most of the animal minas exert their function through repressing the translation of their target mRNAs. In plants there are possible mechanisms of gene regulation by miRNAs; they might either cause the degradation of their target mRNAs or like act as those do in animals-- repress mRNA translation (Aukerman & Sakai, 2004; Llave, Xie, Kasschau, & Carrington, 2002). The difference in the mechanism of action is due to miRNA target sites

and complementarity between miRNA and its target mRNA (Millar & Waterhouse, 2005). While most of the animal miRNA target regions lie on the 3' UTR regions of their corresponding mRNAs, the target sites of plant miRNAs are found almost exclusively within the open-reading frame of their targets (Bartel, 2004; Lewis, Shih, Jones-Rhoades, Bartel, & Burge, 2003). However there are some cases which plant miRNAs bind to 3' UTR regions of their targets (Sunkar & Zhu, 2004). Another determining factor for miRNA operation is level of complementarity between miRNA and mRNA. While low-complementarity such as in animals results in translation repression, high-complementarity on the other hand gives mRNA cleavage (Doench, Petersen, & Sharp, 2003; Zeng, Yi, & Cullen, 2003). The third mechanism of operation is found to be related to the number of target sites on mRNA. Most of the animal miRNAs have multiple target regions, whereas plant miRNAs have only one site. If multiple sites are occupied translation is more efficiently attenuated, but if only one site is occupied the efficiency of repression is lowered (Doench, et al., 2003).

1.3.6. Transcriptional gene silencing

Epigenetic studies reveal that miRNAs can also modulate gene expression by targeting promoter regions resulting in transcriptional gene silencing. By methylating CpG residues in the promoter sequences, the transcription of a downstream gene is repressed. In plants transcriptional repression can also be achieved by the modification of the histone proteins surrounding the chromatin. Di-methylation of lysine 9 on histone H3 (H3K9Me2) is an example for small RNA mediated transcriptional regulation via chromatin remodelling (Zilberman, Cao, & Jacobsen, 2003). In contrast to Ago 1 association of miRNAs involved in post-transcriptional regulation, Ago 4 association is observed in the transcriptional regulation components− in the cell. Since DNA methyltransferases' responsibility for DNA methylation suggests that there might be either direct or indirect interactions between DNA methyltransferases and RNA dependent gene regulation components. There are two proposed mechanisms for RNA dependent transcription repression: small RNA in the RISC complex recruited together with DNA methyltransferases binds either to target DNA or to

the nascent transcript. An evidence of a cis-regulatory role of miR320 came from a study which its target sequence is found to be exist with a perfect complementarity in the promoter region of cell cycle gene POLR3D. Transfection of anti-mir320 oligonucleotides into HeLa cells which normally express low levels of POLR3D has been shown to increase relative POLR3D mRNA levels (Kim, Saetrom, Snove, & Rossi, 2008).

1.3.7. Gene activation

Most recently it has been discovered that miRNAs can also induce gene expression by targeting promoter regions. In a study conducted on PC-3 cells, miR373 has been shown to target the promoter region of E-cadherin. Transfection of PC-3 cells with synthetic mir373 precursor causes an induction in E-cadherin expression (Place, Li, Pookot, Noonan, & Dahiya, 2008). It has been suggested that it depends on the promoter structure whether to be activated or suppressed when targeted by small RNAs. If the promoter sequence is low in GC content and high in complexity it might more likely to be activated (Janowski, et al., 2007). Another miRNA mediated positive regulation system has been observed in liver cells. Interestingly, the replication of Hepatatis C virus which is a RNA virus depends on miR122 presence.

1.4. Mir393a

It was previously reported that plant miRNAs are involved in a broad range of biological processes including growth, developmental patterning and abiotic stress responses. According to the evaluated miRNA profiling results of glyphosate treated *Festuca arundinacea* cv. falcon, 34 of the 853 plant miRNAs have found to be differentially expressed in response to treatment (Unver, Bakar, Shearman, & Budak, 2010). MiRNA microarray results have been validated by quantitative real-time PCR (qRT-PCR) analysis of their target transcripts. Among the 34 differentially regulated miRNA species, ath-Mir393a has found to be up-regulated in 5 % glyphosate treated

samples and down-regulated in 20 % treated ones when compared to untreated samples. In addition, its target transcript E3 ubiquitin ligase F-box subunit has been down-regulated at 5 % and up-regulated at 20 %.

There are two Mir393 precursors, *ath-Mir393a* and *ath-Mir393b*, residing on chromosome 2 of *Arabidopsis* which are encoded by AT2G39885.1 and AT3G55734.1 genes respectively. Mir393a is a conserved miRNA species that targets F-box proteins TIR1 and AFB1/2/3 and also bHLH transcription factor (Jones-Rhoades & Bartel, 2004; Sunkar & Zhu, 2004). It has been experimentally cloned (Sunkar & Zhu, 2004) and sequenced using MPSS and 454 parallel sequencing technologies together with the method of miRNA population enrichment in *rdr2* mutants (Lu, et al., 2006).

The *AFB3* is an Auxin Signaling F-Box protein that belongs to a subfamily of seven related F-box proteins including TIR1, and AFB1/2 (Dharmasiri, et al., 2005). The F- box motif mediates protein-protein interactions and is mostly found in proteins as components of SCF ubiquitin-ligase complexes. Hence they have very important roles in the maintenance of protein stabilities inside the cell. TIR1 and AFBs have also been associated with auxin signaling pathways as auxin receptors (Cecchetti, Altamura, Falasca, Costantino, & Cardarelli, 2008). The histological analysis of GUS stained AFB3: GUS *Arabidopsis* anthers showed that it is highly expressed during late stamen development at different stages mostly in the tissues surrounding the theca, in microspores.

Previously it has been shown that Mir393 contributes to antibacterial resistance by repressing auxin signalling (Navarro, et al., 2006). Analysis performed on transgenic *Arabidopsis* expressing the P1-Hc-Pro, P19, and P15 viral proteins that suppress miRNA-guided functions indicate that bacterial flagellin, f22, stimulates Mir393 activation causing reduction in TIR1 and AFB3 expression. It is further claimed that down-regulation of auxin signaling is required for disease resistance.

Another study, indicates that Mir393 takes role in cadmium stress mediation in *M. truncatula, B. napus* and rice (Xie, et al., 2007). RT-PCR analysis of cadmium treated *Brassica napus* and *M. truncatula*, revealed a down-regulation in Mir393 expression.

1.5.Aim of the study

The objective of the present study was to construct two plant gene expression vectors carrying *ath-Mir393a* gene and its target gene *AFB3* for compatible use in *Arabidopsis* transformation. Therefore these constructs can readily be used for genetically engineer grass species in response to glyphosate.

2. MATERIALS and METHODS

2.1. Materials

2.1.1. Plant Material

Arabidopsis thaliana Col-5 seeds were purchased from The Arabidopsis Information Resource Center (The Arabidopsis Information Resource stock number: CS1644) (Holub, 2002).

2.1.2. Chemicals

All chemicals, growth media, antibiotics and enzymes used in this research are listed in Appendix A.

2.1.3. Growth media, buffers and solution

The growth media, buffers and solutions used in this study were prepared according to the protocols by Sambrook et al. (2001) unless otherwise stated.

2.1.4. Cells, plasmids and primers

2.1.4.1. Cells

E.coli DH5α cells were used during entry clone and destination clone selection studies. *E.coli* TOP10 BAC clone carrying cDNA of *Arabidopsis thaliana* AT1G12820 locus was purchased from The Arabidopsis Information Resource Center (The Arabidopsis Information Resource stock number: U24298) (Ecker, 2003). *Agrobacterium* AGL-1 strain was used during plant transformation studies.

2.1.4.2. Plasmids

pDONR/Zeo (Invitrogen Cat. No. 12535-035), pEXP7-tet (Invitrogen- Cat. No. 11789-020) vectors were used during entry clone construction studies. pEARLEY Gate 100 used during destination clone construction studies was purchased from The Arabidopsis Information Resource Center (The Arabidopsis Information Resource stock number: CD3-724) (Pikaard, 2005). All the maps of plasmids used in this research are listed in Appendix B.

2.1.4.3. Primers

All primers were commercially synthesized in Integrated DNA Technologies (IDT, USA).

2.1.4.4. Equipments

All equipments used in this study were listed in Appendix D.

2.2. Methods

2.2.1. Construction of Plant Gene Expression Vector Carrying *ath-Mir393a* Gene

2.2.1.1. Plant material and growth conditions

Dry deeds were sprinkled onto wet filter paper and placed to petri dish and left for cold treatment at 4 °C for 5 days. After cold treatment seeds were incubated at room temperature (25 °C) for 8 days until cotyledons fully open and rosette leaves were greater than 1mm in length. Then seedlings were placed gently with forceps onto soil. Pots were covered with nylon. Seedlings were incubated in plant growth chamber at 23 °C at extremely high humidity (70 %) for long day conditions (16h light, 8h dark).

2.2.1.2. DNA isolation from plant leaf tissue

40 mg of 6 weeks-old plant leaves were grinded in tissue lyser (Qiagen Retsch, USA) and plant genomic DNA was isolated using Wizard Genomic DNA Purification Kit (Promega- Cat. No A1125) according to the manufacturer's protocol. 600 µl Nuclei Lysis solution was added and mixture was incubated at 65 °C for 15 minutes. 3 µl of RNAse solution was added and incubated at 37 °C for 15 minutes and cooled to room temperature for 5 minutes. 200 µl Protein Precipitation Solution was added and vortexed. Sample was centrifuged at 13000 g for 3 minutes. Supernatant was transferred to clean tube containing 600 µl room temperature isopropanol. It was mixed by inversion and centrifuged at 13000 g for 1 minute. Then after addition of 600 µl 70 % (v/v) ethanol, it was centrifuged at 13000 g for 1 minute. The ethanol part was aspirated and the pellet was air dried. 100 µl DNA Rehydration Solution was added and the tube was incubated at 65 °C for 1 hour.

2.2.1.3. Concentration determination of the isolated DNA samples

1 µl of DNA sample was used for concentration determination on NanoDrop ND-1000 spectrophotometer. Concentration of DNA sample was determined to be 180 ng/µl with absorbance ratios 0.62 (A_{260} / A_{230}) and 1.43 (A_{260} / A_{280}).

2.2.1.4. *Ath-Mir393a* gene amplification via PCR

Ath-Mir393a gene was cloned as full-length pri-miRNA sequence from plant (Col5) genomic DNA. The amplification was performed by using *Ath-Mir393a* specific forward (MIR393F) and reverse (MIR393R) primers flanked by 5' terminal attB sequences. Annealing temperature for PCR amplification of those primers was optimized to 63.1 °C. 50 µl of final volume and following components were mixed; 5 µl Col5 (180 ng/µl) genomic DNA, 5 µl Taq Buffer (10X) (100 mM Tris-HCl with pH 8.8 at 25 °C, 500 mM KCl), 2 µl dNTP (10 mM from each), 4 µl MgCl2 (25 mM), 1µl *Taq* DNA polymerase (5 U/µl), 2 µl forward primer (10µM) and 2 µl reverse primer (10µM) and sterile distilled water up to 50 µl. PCR cycling conditions were 95 °C for 3 minutes as an initial denaturation step, 35 cycles of three steps of denaturation at 95 °C for 1 minute, annealing at 63.1 °C for 1 minute and extension at 72 °C for 1 minute. Amplified PCR products were loaded on 2% (m/v) agarose gel and analyzed.

Ath-Mir393 gene specific primers

MIR393F: 5'- **GGGGACAAGTTTGTACAAAAAAGCAGGCTGT**gagagagatagagagttgaaca-3' MIR393R: 5'- **GGGGACCACTTTGTACAAGAAAGCTGGGTC**catacgatcgaagaggaag-3'

attB sequences are written in bold characters.

2.2.1.5. Cloning *att*B-Ath-Mir393a PCR Products via the BP Reaction

Amplified PCR products were gel extracted with QIquick Gel Extraction Kit (QIAGEN- Cat. No. 28756) according to the manufacturer's protocol and then used for BP reaction. Reaction mix was added to 1.5 ml ependorf tube as in the Table 1. BP CLONASE enzyme mix (Invitrogen- Cat. No. 11789-020) was added lastly. All the steps were performed on ice. Reaction tubes were vortexed and incubated at 25 °C for 6 hours. BP reaction was stopped by adding 1 µl proteinase K and incubating the reaction tubes at 37 °C for 10 minutes. BP reaction was followed by transformation.

Table 1: BP reaction components

Component	**Tube 1 Negative Control**	**Tube 2 Positive Control**	**Tube 3 Sample Clone**
*att*B-PCR product (50fmol)	1.3 µl	-	1.3 µl
pEXP7-tet, Positive Control, 50 ng/µl	-	2 µl	-
pDONR/Zeo, Donor Vector, 150 ng/µl	1 µl	1 µl	1 µl
TE Buffer pH8.0	To 10 µl	5 µl	To 8 µl
BP CLONASE Enzyme Mix	-	2 µl	2 µl
Total Volume	10 µl	10 µl	10 µl

2.2.1.6. Transformation of BP Reaction products

Prior to transformation of plasmid into the cells, *E.coli* DH5α cells were made competent. A single colony of *E.coli* cells was inoculated into 2 mL LB medium without any antibiotic. Cells were incubated in a shaking incubator at 37 °C, 250 rpm overnight. 1 mL of overnight grown culture was inoculated into 100 mL of LB medium in a sterile 2-liter flask and incubated in a shaking incubator at 37 °C, 250 rpm, to an absorbance (A590 nm) of 0.375. Culture was aliquated into two 50-mL tubes and put in ice for 10 minutes. Cells were centrifuged for 10 minutes at 4000 rpm. Supernatant was discarded and pellet was resuspended in 5 mL ice-cold sterilized $CaCl_2$ (4 mM) solution. Cells were centrifuged for 5 minutesute at 2500 rpm and supernatant was discarded. Pellet was resuspended again in 2 mL of ice-cold $CaCl_2$ (4 mM) solution. The centrifugation was repeated once. Pellet was resuspended in 2 mL of ice-cold CaCl2 (4mM) solution and dispensed into sterile prechilled eppendorf tubes. *E.coli* DH5α competent cells were transformed with BP reaction products.

1 µl of BP reaction was transformed into 100 µl of DH5α Competent cells. Cells were incubated on ice for 30 minutes and heat-shocked at 42 °C for 45 seconds. Tubes were placed on ice for 1-2 minutes. 1 ml of pre-warmed LB broth added and incubated at 37 °C for exactly 1 hour. After incubation cells were spinned down. 750 µl of supernatant was discarded and cells were suspended in the remaining part and negative control and sample clone were plated onto low salt LB agar plates containing zeocin (50 µg/ ml). Positive control clone was plated on LB agar plate containing tetracycline (20 µg /ml).

2.2.1.7. Selection of ath-Mir393a entry Clones

Several colonies were picked up from sample clone plates and over-night cultured in shaking incubator at 37 °C, 225 rpm. The following day plasmid isolation was performed by using High Pure Plasmid Isolation kit (ROCHE- Cat. No. 11754777001) according to the manufacturer's protocol. 2.5 ml of overnight culture was pelleted and supernatant was

discarded. 250 µl Suspension buffer (+RNase) was added and cells were resuspended. 250 µl Lysis Buffer was added and the solution was mixed gently and incubated at room temperature for 5 minutes. After addition of 350 µl chilled Binding Buffer the tube was mixed gently and incubated on ice for 5 minutes and centrifuged at 13000 g for 10 minutes. Supernatant was transferred to High Pure Filter Tube which Collection tube was inserted. The mixture was centrifuged at 13000 g for 1 minute. Flow-through liquid was discarded and to wash cells 700 µl Wash Buffer II was added to the upper reservoir of Filter Tube. After discarding the flow-through liquid, the tube was centrifuged at 13000 g for 1 minute and collection tube was discarded. Filter tube was placed on a clean 1.5 ml eppendorf tube and 100 µl Elution Buffer was added to the upper reservoir of Filter Tube. After 1 hour incubation at room temperature the tube was centrifuged at 13000 g for 1 minute. Plasmid DNA was stored at 4 °C.

For the confirmation of *ath-Mir393a* insertion into entry vector clones were sent for sequencing. Clones were sequenced with M13 reverse primer. Sequencing results of construct is given in Appendix C.

2.2.1.8. Transferring *ath-Mir393a* gene from entry clone into destination vector via the LR reaction

Isolated *ath-Mir393a* gene entry clones were used for LR reaction. Reaction mix was added to 1.5 ml ependorf tube as in the Table 2. LR CLONASE enzyme mix (Invitrogen-Cat. No. 11791-020) was added lastly. All the steps were performed on ice. Reaction tubes were vortexed and incubated at 25 °C for 2 hours. LR reaction was stopped by adding 1 µl proteinase K and incubating the reaction tubes at 37 °C for 10 minutes. LR reaction was followed by transformation.

Table 2: LR reaction components

Component	Tube 1 Negative Control	Tube 2 Positive Control	Tube 3 Sample Clone
Ath-Mir393a Entry Clone	-	-	0.8 µl (150ng)
Destination Vector (pEARLEY Gate 100)	1.2 µl	1.2 µl	1.2 µl
pENTR GUS 150 ng/µl	-	2 µl	-
TE Buffer pH8.0	To 8 µl	To 8 µl	To 8 µl
LR CLONASE Enzyme Mix	2 µl	2 µl	2 µl
Total Volume	10 µl	10 µl	10 µl

2.2.1.9. Transformation of LR reaction products

1 µl of LR reaction was transformed into 100 µl of DH5α Competent cells. Competent cell preparation was as described in part 2.2.1.6. Cells were incubated on ice for 30 minutes and heat-shocked at 42 °C for 45 seconds. Tubes were placed on ice for 1-2 minutes. 1 ml of pre-warmed LB broth added and incubated at 37 °C for exactly 1 hour. After incubation cells were spin-downed. 750 µl of supernatant was discarded and cells were suspended in the remaining part and plated onto kanamycin plates (50 µg/ ml).

2.2.1.10. Selection of ath-Mir393a destination clones

Several colonies were picked up from sample clone plates and over-night cultured in shaking incubator at 37 °C, 225 rpm. The following day plasmid isolation was performed by using High Pure Plasmid Isolation kit (ROCHE- Cat. No. 11754777001) according to the manufacturer's protocol as described in part 2.2.1.7. Plasmid DNA was stored at 4 °C.

For the confirmation of *ath-Mir393a* transfer into destination vector clones were sent for sequencing. Clones were sequenced with *ath-Mir393a* specific forward (MIR393F) or reverse primer (MIR393R).

2.2.1.11. Transformation of *ath-Mir393a* gene destination clones into *Agrobacterium tumafaciens*

Prior to transformation, competent *Agrobacterium* cells were prepared. *Agrobacterium* strain AGL1 was cultured in 5 mL of LB (Kanamycin 100 µg/ ml, Carbenicillin 200 µg/ ml) at 28 °C, 250 rpm for 40 hours. 2 ml of culture was added to 50 ml LB in 250 ml flask and shaked vigorously at 28 °C, 250 rpm until culture reached OD600 of 0.6. Culture was added to 50 ml falcon tube, chilled on ice, and centrifuged for 20 minutes at 4 °C in swing bucket centrifuge (4000 rpm). Supernatant was discarded and cells were resuspened in 1 ml of 20 mM $CaCl_2$ (ice cold). 100 µl aliquots were dispensed into prechilled 1.5 ml eppendorf tubes and freezed in liquid nitrogen and stored at -80 °C.

1 ug of ath-Mir393a destination vector was used for the transformation of *Agrobacterium*. 1 ug of ath-Mir393a destination vector added to a frozen tube of competent cells. Cells were thawed at 37 °C for 5 minutes. 1 ml LB (no antibiotic) was added to the tube and incubated for 3 hours at 28 °C 140 rpm. 20 µl, 50 µl and 100 µl of cells were spread onto agar plates containing Kanamycin (100 µg/ ml) and Carbenicillin (200 µg/ ml). Plates were incubated at 28 °C.

Colony PCR was performed for selection. Colony was picked up by toothpick and streaked onto kanamycin, carbenicillin plate and incubated at 28 °C. The following day colony PCR was performed by mixing a bunch of colony taken with toothpick to the following components: 2.5 µl Taq Buffer (10X) (100 mM Tris-HCl with pH 8.8 at 25 °C, 500mM KCl), 1 µl dNTP (10 mM from each), 2 µl MgCl2 (25 mM), 0.5 µl *Taq* DNA polymerase (5 U/µl), 1 µl MIR393F (10µM) and 1 µl MIR393R (10µM) and sterile distilled water up to 25 µl. PCR cycling conditions were 95 °C for 3 minutes as an initial denaturation step, 35 cycles of three steps of denaturation at 95 °C for 1 minute, annealing at 63.1 °C for 1 minute and extension at 72 °C for 1 minute. Amplified PCR products were loaded on 2 % (m/v) agarose gel and analyzed.

2.2.1.12. *Arabidopsis* floral dip transformation

Agrobacterium transformed with ath-Mir393a destination vector was cultured in 5 mL of LB (Kanamycin 100 µg/ ml, Carbenicillin 200 µg/ ml) at 28 °C, 250 rpm for 40 hours. 5 ml of culture was added to 500 ml LB in 2.5 lt flask and shaked vigorously at 28 °C, 250 rpm until culture reached OD600 of 0.6. Cells were collected by centrifugation for 15 minutes in swing bucket.

Supernatant was discarded and cells were resuspended in 300 ml of 5 sucrose, 0.1 % (v/v) Silwet solution (per 300 ml: 15 g sucrose, 30 ul Silwet). 6 weeks-old *Arabidopsis* (flowers were starting to open, but before siliques were formed) was soaked into resuspended cells for 45 seconds. Plants were laid on side for 10 minutes and placed in plastic box with plastic dome over top. And the box was placed to growth chamber. Plants were let for growing at 23 °C at extremely high humidity (70 %) for long day conditions (16h light, 8h dark). The dome was partially removed after 2 days. 1 week later an apparatus was put over plants to collect seeds inside and plants were covered with a plastic with micropores on it. They were started growing at 24 hours day light conditions.

2.2.1.13. Surface sterilization of *Arabidopsis* seeds

The collected *Arabidopsis* seeds were taken into eppendorf tube and surface sterilized with 70% (v/v) ethanol for 5 minutes and 10 % (v/v) sodium hypochloride for 20 minutes and rinsed 5 times with sterile distilled water. The seeds were kept in sufficient amount of distilled water and placed on culture medium with the help of micropipette.

2.2.1.14. Selection of transgenic seeds

Surface sterilized seeds were placed into MS media containing 20 µg/mL of BASTA. Magenta was firstly placed into cold room for 2 days and then transferred to plant tissue culture room. The daylight conditions in the culture room were 24 hours of constant light. After 1 week seedlings were sprayed with 120 µg/mL of BASTA.

2.2.2. AFB3 gene over expression in *Arabidopsis thaliana*

2.2.2.1. Plasmid Isolation from AFB3 BAC clones

E. coli BAC clone carrying AT1G12820 locus (The Arabidopsis Information Resource stock number: U24298; Ecker, 2003) was streaked onto agar plates with kanamycin (50 µg/ ml) and over-night incubated at 37 ˚C. The next day a single colony was picked up from the plate and over-night cultured in a shaking incubator at 37 °C, 225 rpm (Kanamycin, 50 µg/ ml). The following day plasmid isolation was performed by using High Pure Plasmid Isolation kit (ROCHE- Cat. No. 11754777001) according to the manufacturer's protocol as described in part 2.2.1.7. Plasmid DNA was stored at 4 °C.

2.2.2.2. *AFB3* gene amplification via PCR

AFB3 gene was cloned as full-length cDNA from AFB3 plasmid. The amplification was performed by using *AFB3* specific forward (AFB3F1) and reverse (AFB3R1) primers. Annealing temperature for PCR amplification of those primers was optimized to 53 °C. 50 µl of final volume and following components were mixed; 2.5 µl AFB3 plasmid (0.5 ng/µl), 5 µl Taq Buffer (10X) (100 mM Tris-HCl with pH 8.8 at 25 °C, 500mM KCl), 1 µl dNTP (10 mM from each), 2 µl MgCl2 (25 mM), 1µl *Taq* DNA polymerase (5 U/µl), 1.5 µl forward primer (10µM) and 1.5 µl reverse primer (10µM) and sterile distilled water up to 50 µl. PCR cycling conditions were 95 °C for 5 minutes as an initial denaturation step, 30 cycles of three steps of denaturation at 95 °C for 1 minute, annealing at 53 °C for 1 minute and extension at 72 °C for 2 minute. Amplified PCR products were loaded on 1% (m/v) agarose gel and analyzed.

AFB3 gene specific primers

AFB3F1: 5'- ATGAATTATTTCCCAGACGAGGTTATAGAGC-3'
AFB3R1: 5'- CCCTTTCCAAGT TTGATA AAAGGCAAAAAC-3'

2.2.2.3. Transferring *AFB3* gene from entry clone into destination vector via the LR Reaction

U24298 AFB3 BAC clone was an entry clone of *AFB3* gene. Isolated *AFB3* gene entry clones were used for LR reaction. Reaction mix was added to 1.5 ml ependorf tube as in the Table 3. LR CLONASE enzyme mix was added lastly. All the steps were performed on ice. Reaction tubes were vortexed and incubated at 25 °C for 2 hours. LR reaction was stopped by adding 1 µl proteinase K and incubating the reaction tubes at 37 °C for 10 minutes. LR reaction was followed by transformation.

Table 3: LR reaction components

Component	Tube 1 Negative Control	Tube 2 Positive Control	Tube 3 Sample Clone
AFB3 Entry Clone	-	-	0.8 µl (150ng)
Destination Vector (pEARLEY Gate 100)	1.2 µl	1.2 µl	1.2 µl
pENTR GUS 150 ng/µl	-	2 µl	-
TE Buffer pH8.0	To 8 µl	To 8 µl	To 8 µl
LR CLONASE Enzyme Mix	2 µl	2 µl	2 µl
Total Volume	10 µl	10 µl	10 l

2.2.2.4. Transformation of LR Reaction Products

1 µl of LR reaction was transformed into 100 µl of DH5α Competent cells. Competent cell preparation was as described in part 2.2.1.6. Cells were incubated on ice for 30 minutes and heat-shocked at 42 °C for 45 seconds. Tubes were placed on ice for 1-2 minutes. 1 ml of pre-warmed LB broth added and incubated at 37 °C for exactly 1 hour. After incubation cells were spin downed. 750 µl of supernatant was discarded and cells were suspended in the remaining part and plated onto kanamycin plates (50 µg/ ml).

2.2.2.5. Selection of *AFB3* Destination Clones

Several colonies were picked up from sample clone plates and over-night cultured in shaking incubator at 37 °C, 225 rpm. The following day plasmid isolation was performed by using High Pure Plasmid Isolation kit (ROCHE- Cat. No. 11754777001) according to the manufacturer's protocol as described in part 2.2.1.7. Plasmid DNA was stored at 4 °C.

2.2.2.6. Transformation of *AFB3* gene Destination Clones into *Agrobacterium tumafaciens*

1 ug of AFB3 destination vector was used for the transformation of *Agrobacterium*. 1 ug of AFB3 destination vector added to a frozen tube of competent cells. Cells were thawed at 37 °C for 5 minutes. 1 ml LB (no antibiotic) was added to the tube and incubated for 3 hours at 28 °C 140 rpm. 20 µl, 50 µl and 100 µl of cells were spread onto agar plates containing Kanamycin (100 µg/ ml) and Carbenicillin (200 µg/ ml). Plates were incubated at 28 °C.

Colony PCR was performed for selection. Colony was picked up by toothpick and streaked onto kanamycin, carbenicillin plate and incubated at 28 °C. The following day colony PCR was performed by mixing a bunch of colony taken with toothpick to the following components: 2.5 µl Taq Buffer (10X) (100 mM Tris-HCl with pH 8.8 at 25°C, 500mM KCl), 0.5 µl dNTP (10 mM from each), 1 µl MgCl2 (25 mM), 0.5 µl *Taq* DNA polymerase (5 U/µl), 1 µl AFB3F1 (10µM) and 1 µl AFB3R1 (10µM) and sterile distilled water up to 25 µl. PCR cycling conditions were 95 °C for 10 minutes as an initial denaturation step, 30 cycles of three steps of denaturation at 95 °C for 1 minutes, annealing at 53 °C for 1 minutes and extension at 72 °C for 2 minutes. Amplified PCR products were loaded on 1% (m/v) agarose gel and analyzed.

2.2.2.7. *Arabidopsis* floral dip transformation

Agrobacterium transformed with *AFB3* Destination vector was cultured in 5 mL of LB (Kanamycin 100 µg/ ml, Carbenicillin 200 µg/ ml) at 28 °C, 250 rpm for 40 hours. Remaining part of the protocol was the same as described in part 2.2.1.12.

2.2.2.8. Surface sterilization of *Arabidopsis* seeds

The collected *Arabidopsis* seeds were taken into eppendorf tube and surface sterilized with 70% (v/v) ethanol for 5 minutes and 10% (v/v) sodium hypochloride for 20 minutes and rinsed 3 times with sterile distilled water. The seeds were kept in sufficient amount of distilled water and placed on culture medium with the help of micropipette.

2.2.2.9. Selection of transgenic seeds

Surface sterilized seeds were placed into MS media containing 20 µg/mL of BASTA. Magenta was firstly placed into cold room for 2 days and then transferred to plant tissue culture room. The daylight conditions in the culture room were 24 hours of constant light. Seedlings were sprayed with 120 µg/mL of BASTA after 1 week.

3. RESULTS and DISCUSSION

3.1. Ath-Mir393a transformation studies

3.1.1. Ath-Mir393a entry clone construction

Ath-Mir393a pri-miRNA sequence was amplified successfully from six week-old *Arabidopsis thaliana* leaves (Figure 3). The annealing temperature of MIR393F and MIR393R primers was optimized to be 63.1 °C. These primers were designed to amplify the *ath-Mir393* gene from the *Arabidopsis thaliana* genome with flanking attB sequences on each site which were necessary for entry vector construction via Gateway cloning technology. Hence 257 bp amplified full-length pri-miRNA fragment was flanked by the attB1 sequence on its 5' terminal site and the attB2 sequence on its 3' terminal. The Gateway system uses bacteriophage lambda site-specific recombination pathway for transferring DNA segments between vectors. Once the entry vector is generated, it can be transferred into numerous types of destination vectors including expression vectors.

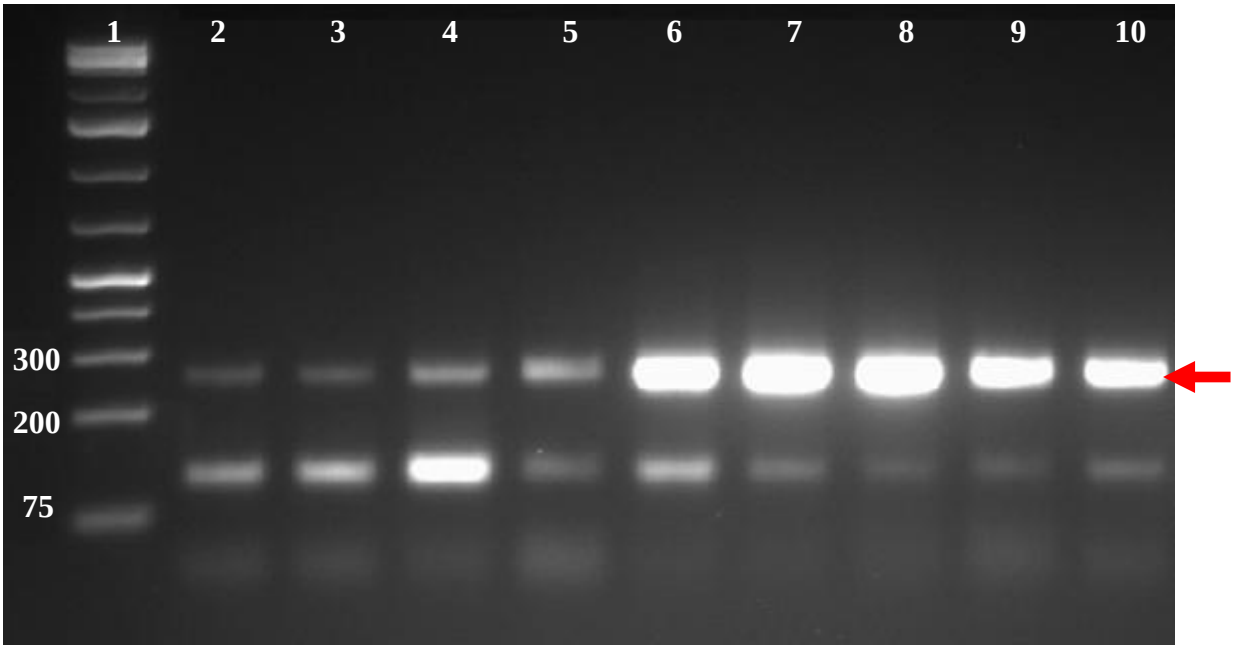

Figure 3: Ath-Mir393a amplification by gradient PCR (257bp region is indicated with an arrow)

2% agarose gel picture. 1st well Fermentas SM1331 1kb plus DNA ladder, 2nd-10th wells 257bp ath-Mir393a PCR fragment. Annealing temperature of PCR reaction is between 61.5 and 63.1°C in 0.2 °C

To construct an entry clone of *Ath-Mir393a*, BP reaction was performed. In this abbreviation, B stands for bacterial's 'B' and P stands for phage's 'P'. During BP reaction pDONR/Zeo Gateway (INVITROGEN) was used as a target vector which bears attP sites (Appendix B). In the pDONR/Zeo Gateway vector there is a ccdB gene sequence between the P1 and P2 sites which is required for negative selection. If this vector is propagated in cddB survival strain such, as DH5α, the bacteria transformed with this vector is not going to live because of the toxic effects of *ccdB* gene product. After a successful BP reaction the region between the P1 and P2 sites is replaced by the region between the attB sites of the attB PCR product. Hence all the colonies in the sample clone plate will contain the desired gene insertion. Zeocin antibiotic in the negative control plate and sample plate was used as a positive selection marker. As a positive control, pEXP7-tet vector was used which contains the tetracycline positive selection gene between the attB sites. After a successful

BP reaction the *ccdB* gene in pDONR/Zeo Gateway vector was replaced by tetracycline gene in the pEXP7-tet vector. The clones transformed with this reaction product were selected on LB agar plate containing the tetracycline antibiotic.

In the *Ath-Mir393a* entry clone construction step, colonies were observed in the positive control plate and in sample clone plate but not in negative control plate as expected (Figure 4). These results indicate that *Ath-Mir393a* gene was successfully replaced by the *cddB* gene in the entry vector. *Ath-Mir393a* insertion into entry vector was also confirmed by sequencing with M13 reverse primer.

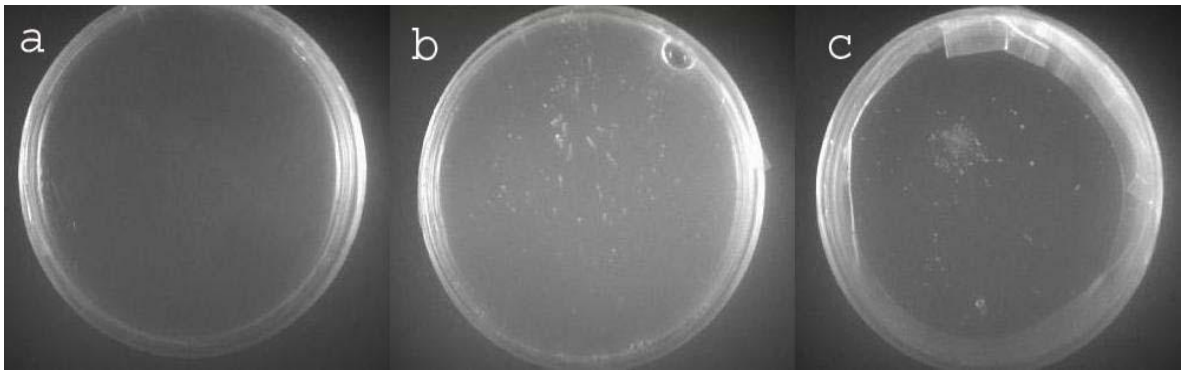

Figure 4: BP reaction plates
a: Negative control (zeocin plate), b: Positive control (tetracycline plate), c: Sample clone (zeocin plate)

3.1.2. Ath-Mir393a destination clone construction

To transfer *ath-Mir393a* from entry vector into the destination vector, LR reaction was performed. In this abbreviation, L stands for Left's 'L' and R stands for right's 'R'. The resulting vector after the BP reaction contains the reorganized sequence structure of attB and attP sites, creating a attL1 site. During the LR reaction pEARLEY Gate100 destination vector was used which bears attR sites (Appendix B). In the pEARLEY Gate100 destination vector there is a *ccdB* gene sequence between the R1 and R2 sites which is required for negative selection. If this vector is propagated in cddB survival strain

such, as DH5α, the bacteria transformed with this vector is not going to live because of the toxic effects of *ccdB* gene product. After a successful LR reaction the region between the R1 and R2 sites is replaced by the region between the attL sites of the entry vector. Hence all the colonies in the sample clone plate will contain the desired gene insertion. Kanamycin resistance gene in pEARLEY Gate100 vector provides a positive selection marker when the cells plated onto LB agar plates containing the kanamycin antibiotic.

In the ath-Mir393a destination clone construction step, colonies were observed both in positive control and sample clone plate, but not in negative control plate (Figure 5). These results indicate that, *cddB* gene in pEARLEY Gate100 destination vector was replaced by *ath-Mir393a* gene in ath-Mir393a entry vector, thus creating an expression clone. *Ath-Mir393a* insertion into destination vector to the downstream of CaMV 35S strong promoter region was also confirmed by sequencing with *ath-Mir393a* gene specific primer.

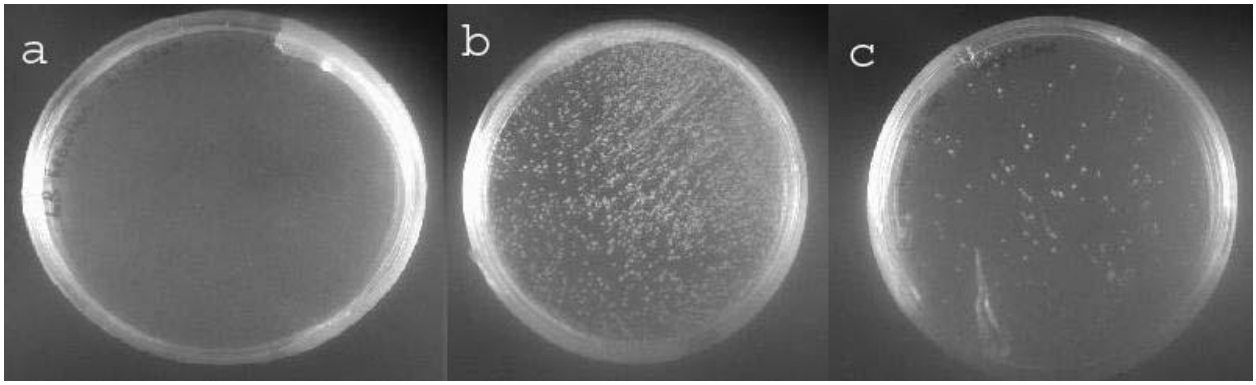

Figure 5: LR reaction plates

a: Negative control (kanamycin plate), b: Positive control (kanamycin plate),

c: Sample clone (kanamycin plate)

3.1.3. Transformation of *Agrobacterium* cells

The previously constructed *ath-Mir393a* destination vector was transformed into competent *Agrobacterium* (Figure 6a). Since both *Agrobacterium* strain (AGL1), and pEARLEY Gate-100 destination vector contain kanamycin selection marker, colony PCR was performed to select the desired clone that contains the destination vector. Hence several colonies were picked up from the plate and streaked onto new plates (Figure 6b). Colony PCR was performed for these colonies to confirm the presence of the gene and the products were analyzed on agarose gel (Figure 7). The clones which *ath-Mir393a* could be amplified were chosen to be used in *in planta* transformation.

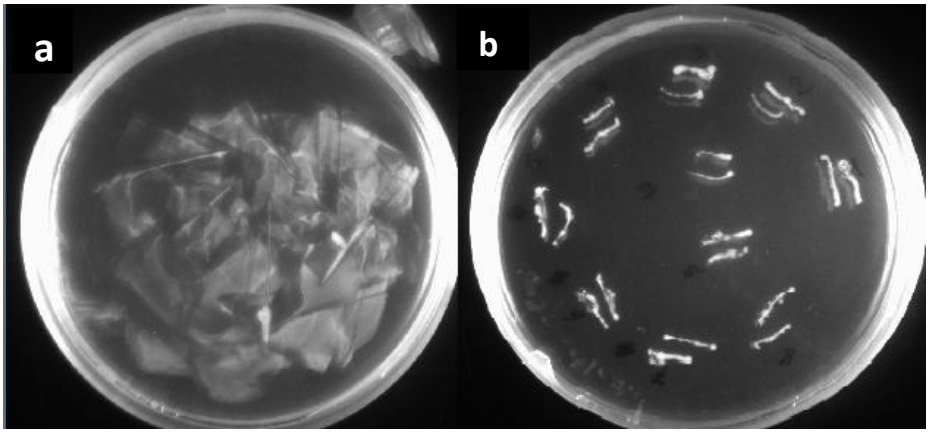

Figure 6: Ath-Mir393a *Agrobacterium* Clones

a: *Agrobacterium* clones transformed with Ath-Mir393a destination vector (kanamycin, carbenicillin plate) b: *Agrobacterium* clones selected from plate a (kanamycin, carbenicillin plate)

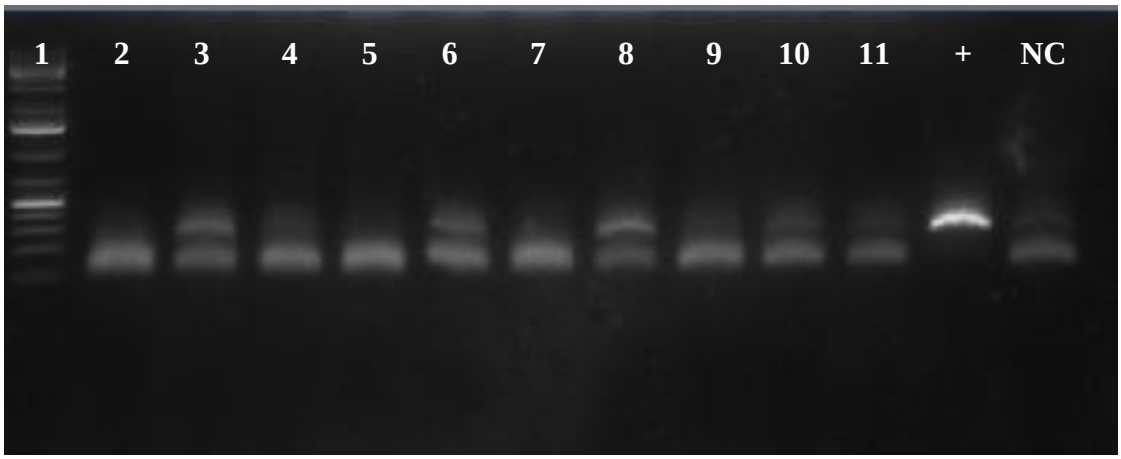

Figure 7: Colony PCR of Ath-Mir393a *Agrobacterium* Clones

2% agarose gel picture. 1st well Fermentas SM1331 1kb plus DNA ladder, 2nd-11th wells *Agrobacterium* clones, + positive control (257bp Ath-Mir393a fragment), NC negative control

3.1.4. *Arabidopsis* transformation

To generate transgenic *Arabidopsis thaliana*, *Agrobacterium* floral dip transformation protocol was used. The best stage for an efficient transformation via floral dip transformation method is when flowers are starting to open, but before siliques are formed. This stage of *Arabidopsis* is almost after 6 weeks of seedling in our growth chamber conditions. But it can be delayed 1-2 weeks by clipping the tips of plant. One week before *in planta* transformation inflorescences were clipped to obtain more floral buds. Afterwards, a dipping procedure was performed. The solution that *Agrobacterium* cells were resuspended after pelleting contains glucose to provide density and Silwet as a surfactant agent. For *Agrobacterium* cells to efficiently infect and insert their T-DNA into plant genome humidity is very important. Hence after dipping plants were kept in a box covered with nylon for about a week (Figure 8a). While doing this process, it was important to take care for the humidity of the soil as well. Watering the plant as usual provided this

condition. After a week plant was covered with an apparatus that would help collect the seeds without any loss (Figure 8b).

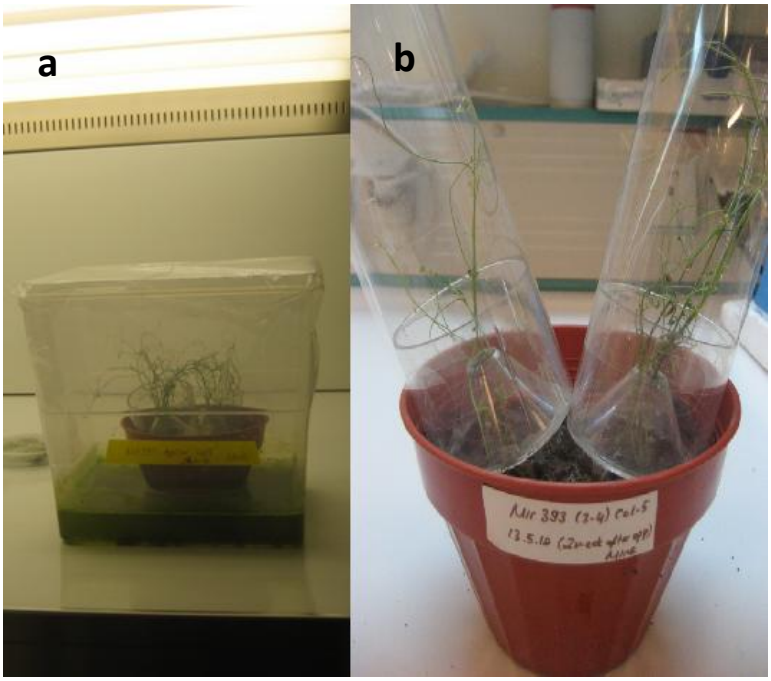

Figure 8: *Arabidopsis thaliana* after *Agrobacterium* Floral dipping

3.1.5. Selection of T_0 transgenic plants

Seeds collected from transformation applied *Arabidopsis* were selected in BASTA containing culture medium (Figure 9). The Bar gene in the T-DNA region of destination vector confers resistance against phosphinothricin (PPT). Selection of trangenic seeds is in progress.

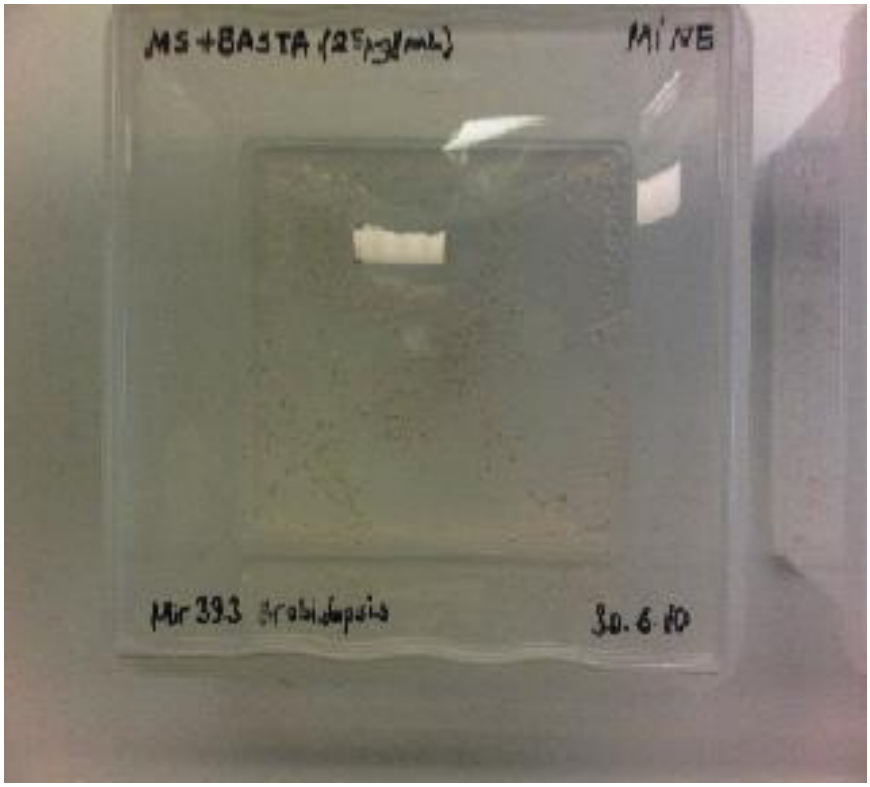

Figure 9: Ath-Mir393a overexpressing T_0 transgenic plant selection

3.2. AFB3 transformation studies

3.2.1. AFB3 destination clone construction

AFB3 gene was amplifed successfully from BAC clone purchased from TAIR (Figure 10). It was pENTR/SD-DTOPO vector containing *AFB3* gene and a Gateway Entry vector itself (Appendix B). 1801 bp amplified AFB3 full-length cDNA fragment also contained the attL sites on its 5' and 3' terminal sequences, which were necessary for destination vector construction via Gateway cloning technology.

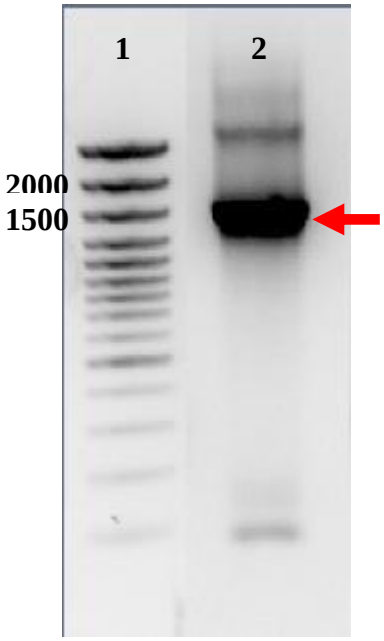

Figure 10: AFB3 Amplification indicated with an arrow

1% agarose gel picture. 1st well Fermentas SM0323 100bp plus DNA ladder, 2nd well 1801bp AFB3 cDNA

To transfer *AFB3* gene form entry vector into destination vector, the LR reaction was performed. During the LR reaction pEARLEY Gate100 destination vector was used which bears attR sites (Appendix B). In the pEARLEY Gate100 destination vector there is a *ccdB* gene sequence between the R1 and R2 sites which is required for negative selection. If this vector is propagated in cddB survival strain such, as DH5α, the bacteria transformed with this vector is not going to live because of the toxic effects of *ccdB* gene product. After a successful LR reaction the region between the R1 and R2 sites are replaced by the region between the attL sites of the entry vector. Hence all the colonies in the sample clone plate will contain the desired gene insertion. Kanamycin resistance gene in pEARLEY Gate100 vector provides a positive selection marker when the cells plated onto LB agar plates containing the kanamycin antibiotic.

In the AFB3 destination clone construction step, colonies were observed both in positive control and the sample clone plate, but not in negative control plate (Figure 11). These results indicate that, *cddB* gene in pEARLEY Gate100 destination vector was replaced by *AFB3* gene in AFB3 entry vector creating an expression clone. *AFB3* insertion into destination vector to the downstream of CaMV 35S strong promoter region was also confirmed by sequencing with *AFB3* gene specific primers.

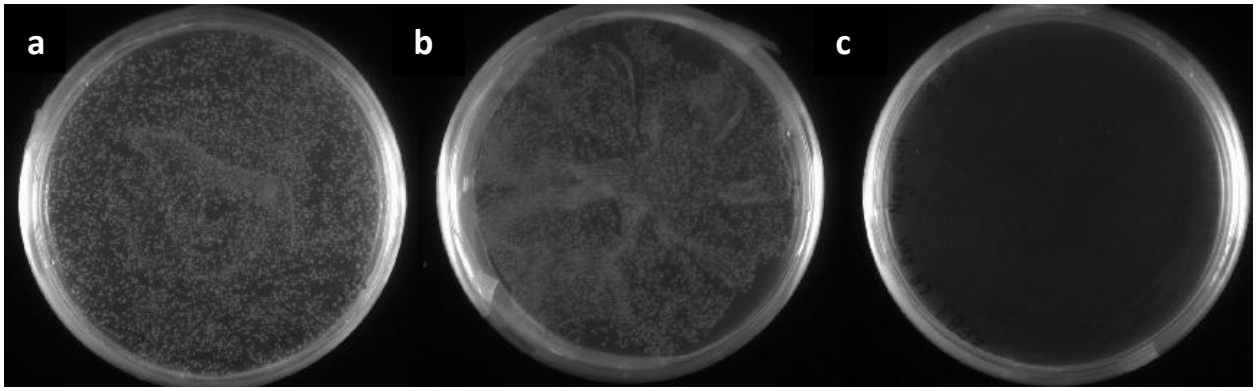

Figure 11: LR reaction plates

a: Positive control (kanamycin plate), b: Sample clone (kanamycin plate), c: Negative control (kanamycin plate)

3.2.2. Transformation of *Agrobacterium* cells

AFB3 destination vector was transformed into competent *Agrobacterium* (Figure 12a). Since both *Agrobacterium* strain (AGL1), and pEARLEY Gate-100 destination vector contains kanamycin selection marker, colony PCR was performed to select the desired clone that contains the destination vector. For this reason several colonies were picked up from the plate and streaked onto new plates (Figure 12 b1/ b2). Colony PCR was performed for these colonies to confirm the presence of the gene and the products were analyzed on agarose gel (Figure 13). The clones which *AFB3* could be amplified were chosen to be used in *in planta* transformation.

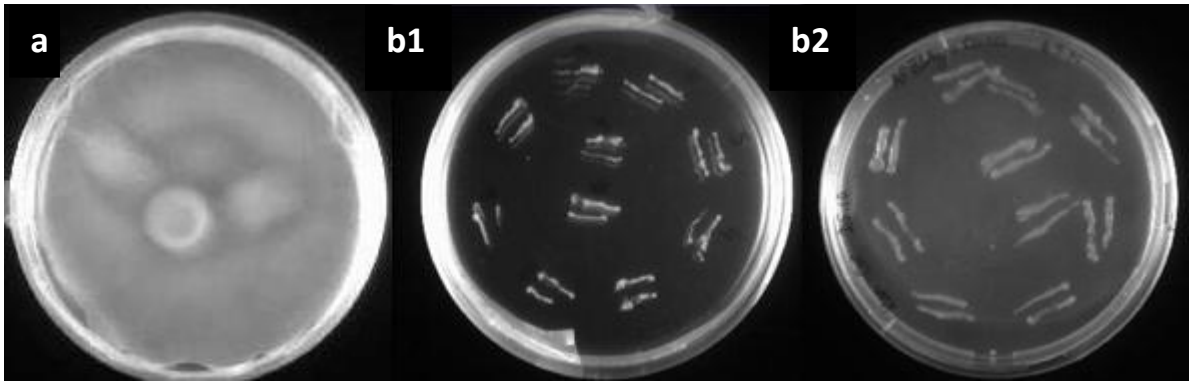

Figure 12: AFB3 *Agrobacterium* Clones

a: *Agrobacterium* clones transformed with AFB3 destination vector (kanamycin, carbenicillin plate) b: *Agrobacterium* clones selected from plate a (kanamycin, carbenicillin plate)

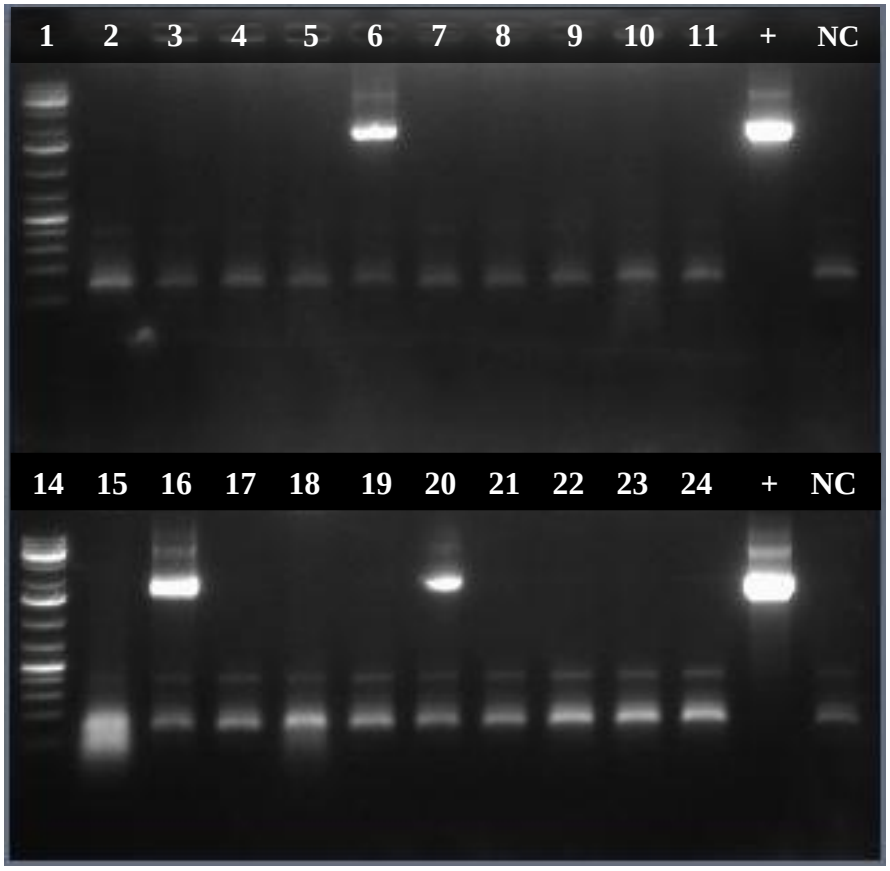

Figure 13: Colony PCR of AFB3 *Agrobacterium* Clones

1% agarose gel picture. 1st, 14th well Fermentas SM1334 1kb plus DNA ladder, 2nd-11th and 15th- 24th wells *Agrobacterium* clones, + positive control (1801bp AFB3 fragment), NC negative control

3.2.3. *Arabidopsis* transformation

To generate transgenic *Arabidopsis thaliana*, *Agrobacterium* floral dip transformation protocol was used. Dipping was performed when flowers were starting to open, but before siliques were formed. 1 week before *in planta* transformation the inflorescences were clipped to obtain more floral buds. Afterwards, a dipping procedure was performed. After dipping, plants were kept in a box covered with nylon for about a week. One week later nylon was removed and the plant was covered with an apparatus that would help to collect the seeds without any loss (Figure 14).

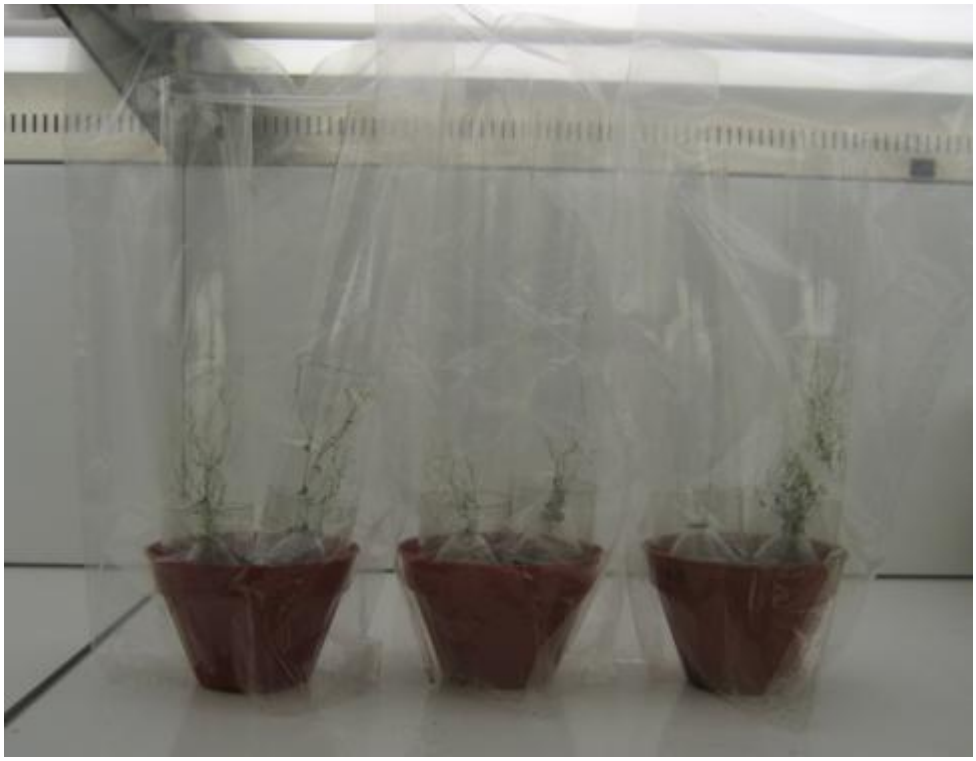

Figure 14: *Arabidopsis thaliana* after *Agrobacterium* Floral dipping

3.2.4. Selection of T_0 transgenic plants

Seeds collected from transformation applied *Arabidopsis* were selected in BASTA containing culture medium (Figure 15). The Bar gene in the T-DNA region of destination vector confers resistance against phosphinothricin (PPT). Selection of trangenic seeds is in progress.

Figure 15: AFB3 overexpressing T_0 transgenic plant selection

4. CONCLUSION

To evaluate the molecular functions of mir393a and AFB3 in *Arabidopsis thaliana*, expression vectors carrying these genes were constructed, as a first step, in this study. The results of vector constructions are summarized below.

Vectors carrying *ath-Mir393a* and *AFB3* gene were constructed by using gateway technology which makes use of site-specific recombination system of bacteriophage lambda. AttB-PCR product of Ath-Mir393a was amplified and then captured in entry clone via BP reaction.

Entry clones of ath-Mir393 and AFB3 were recombined into destination vectors via LR reaction. These vectors were later used for *Agrobacterium*-mediated *Arabidopsis* transformation. Selection of T_0 transgenic plants is still in progress.

According to the miRNA microarray results of our previous study ath-Mir393a was found to be up-regulated in 5% and down-regulated in 20% foliar glyphosate applied *Festuca arundinacea* samples. RT-PCR results of its target gene *AFB3* were correlated with miRNA microarray results. This finding suggests that Mir393a and/or AFB3 might play an important role in the glyphosate resistance mechanism. To study molecular function of Mir393a and AFB3 in glyphosate resistance, future work would include: Real-time RT-PCR analysis of transgenic lines, nothern blot analysis of transgenic plants, as well as morphological and physiological characterization of transgenic plants after glyphosate application.

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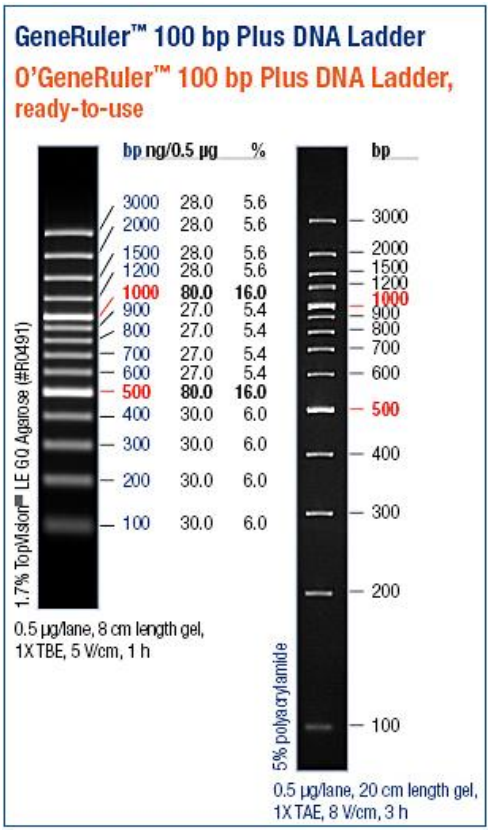
APPENDIX A

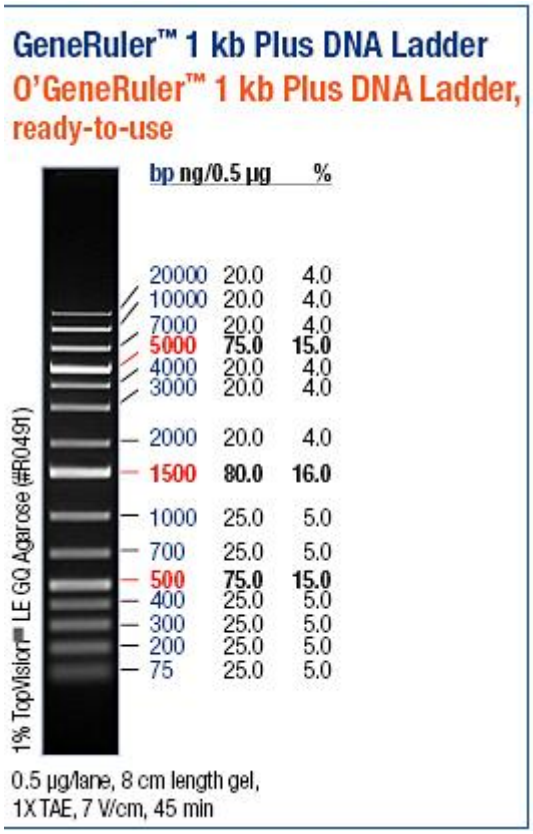
All chemicals, growth media, antibiotics and enzymes used in this research are listed below.

Absolute ethanol	Riedel de Haen	32221
Agarose	PRONA	8016
Calcium chloride	Sigma	C5080
Carbenicillin	Phytotechlab	C346
Cloroform	Merck	102.445
dNTP mix	Fermentas	R0193
Ethidium bromide	Applichem	A1151
Ethylenediaminetetraaceticacid (EDTA)	Calbiochem	324503
Glycerol	Sigma	J8773
Hydrochloric acid	Merck	100314
Isopropanol	Merck	1.09634
Kanamycin	Phytotechlab	K378
LB Agar	Sigma	L2897
LB Broth	Sigma	
Murashige and Skoog (MS) medium	Duchefa	M0222
Plant agar	Duchefa	P1001

PPT (DL- Phosphinothricin)	Duchefa	P0159
VAC-IC-STUFF (Silwet-77)	LEHLE SEEDS	VIS-01
Sucrose	Duchefa	S0809
Taq DNA polymerase	Fermentas	EP0401
Trisbase	Sigma	T1503



Fermentas SM0323 100bp plus DNA ladder

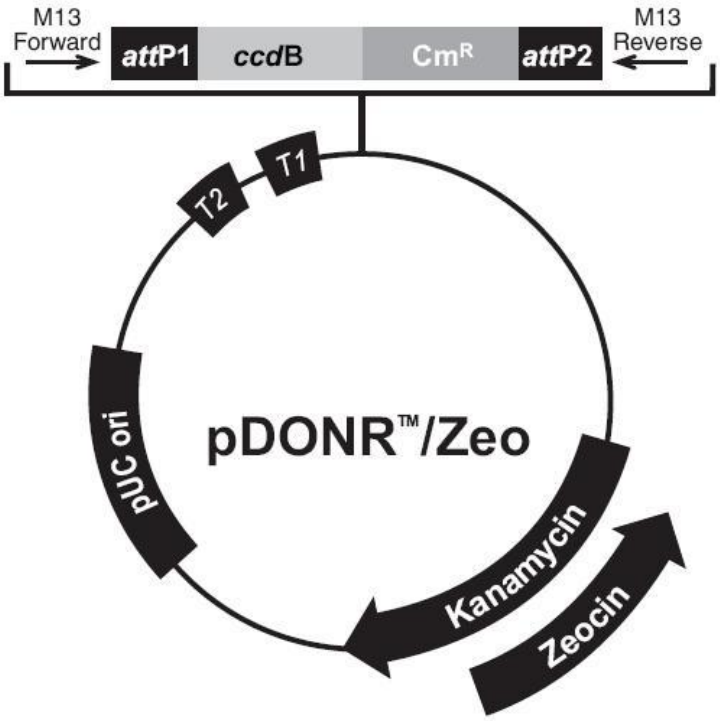


Fermentas SM1331 1kb plus DNA ladder

APPENDIX B

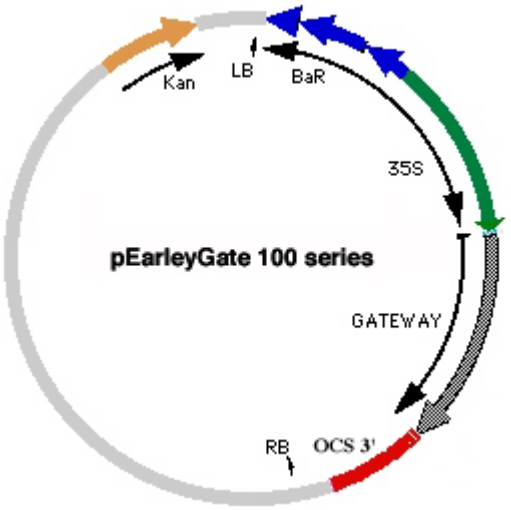
Maps and sequences of primers used in this research are as given below.

pDONR/Zeo Vector Map

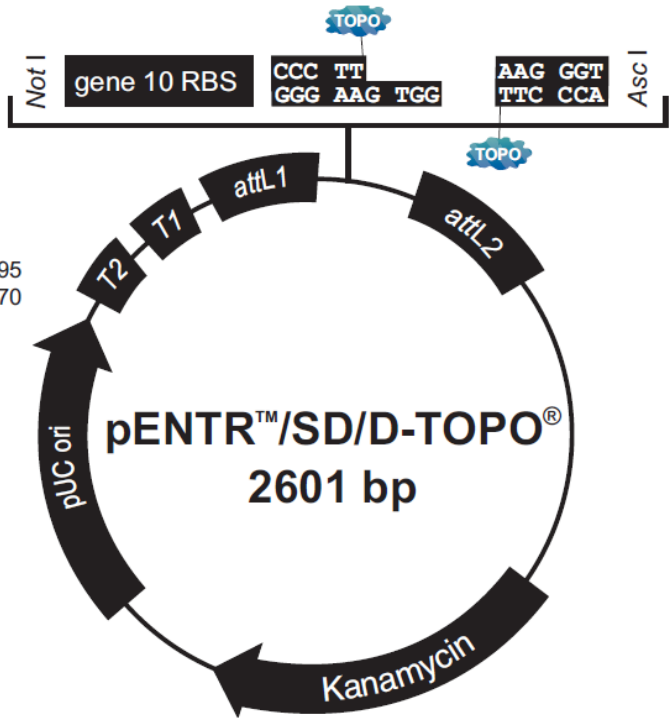


Comments for:	**pDONR™/Zeo** **4291 nucleotides**
*rrn*B T2 transcription termination sequence (c):	268-295
rrnB T1 transcription termination sequence (c):	427-470
M13 Forward (-20) priming site:	537-552
*att*P1:	570-801
*ccd*B gene (c):	1197-1502
Chloramphenicol resistance gene (c):	1847-2506
*att*P2 (c):	2754-2985
T7 Promoter/priming site (c):	3003-3022
M13 Reverse priming site:	3027-3043
Kanamycin resistance gene:	---
EM7 promoter (c):	3486-3552
Zeocin resistance gene (c):	3111-3485
pUC origin:	3615-4288

pEARLEY Gate100 Vector Map



pENTR/SD/D-TOPO Vector Map



Comments for pENTR™/SD/D-TOPO®
2601 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
*att*L1: bases 569-668 (c)
T7 gene 10 translational enhancer: bases 684-692
Ribosome binding site: bases 694-700
TOPO® recognition site 1: bases 701-705
Overhang: bases 706-709
TOPO® recognition site 2: bases 710-714
*att*L2: bases 726-825
T7 Promoter/priming site: bases 842-861 (c)
M13 reverse priming site: bases 866-882
Kanamycin resistance gene: bases 995-1804
pUC origin: bases 1925-2598

APPENDIX C

Sequencing Results

C1- Sequencing results of *ath-Mir393a* entry clone constructs. Sequence results were aligned to ath-Mir393a pri-miRNA sequence.

```
>lcl|27143
Length=748

 Score =  246 bits (133),  Expect = 5e-70
 Identities = 133/133 (100%), Gaps = 0/133 (0%)
 Strand=Plus/Plus

Query  1    AGAGGAAGGATCCAAAGGGATCGCATTGATCCTAATTAAGGTGAATTCTCCCCATATTTT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19   AGAGGAAGGATCCAAAGGGATCGCATTGATCCTAATTAAGGTGAATTCTCCCCATATTTT  78

Query  61   CTTTATAATTGGCAAATAAATCACAAAAATTTGCTTGGTTTTGGATCATGCTATCTCTTT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79   CTTTATAATTGGCAAATAAATCACAAAAATTTGCTTGGTTTTGGATCATGCTATCTCTTT  138

Query  121  GGATTCATCCTTC  133
            |||||||||||||
Sbjct  139  GGATTCATCCTTC  151
```

C2- *AFB3* cDNA sequence on U24298 BAC clone (Mir393 target site is indicated in bold characters) (Retrieved from: http://www.arabidopsis.org/servlets/TairObject?id=1001084359&type=clone)

1 ATGAATTATT TCCCAGACGA GGTTATAGAG CACGTGTTTG ACTTCGTAGC
51 TTCTCACAAA GACAGGAACT CGATATCTCT GGTCTGCAAA TCATGGCACA
101 AGATCGAGAG GTTTAGTAGG AAGGAAGTGT TCATCGGAAA CTGCTACGCG
151 ATTAACCCGG AGAGGTTGAT CAGGAGGTTT CCATGTCTCA AATCCTTAAC
201 TTTAAAAGGG AAGCCTCATT TTGCAGACTT CAACTTGGTT CCTCATGAAT
251 GGGGAGGTTT CGTGCATCCT TGGATTGAAG CTTTGGCTAG AAGCCGTGTG
301 GGACTTGAGG AGCTGAGGTT GAAGCGGATG GTTGTAACAG ATGAAAGCTT
351 GGACCTTCTT TCACGTTCTT TTGCAAATTT CAAGTCTTTG GTTCTTGTTA
401 GCTGTGAAGG GTTTACCACT GATGGCTTAG CTTCCATTGC CGCTAATTGC
451 AGGCATCTTC GTGAGCTGGA CTTGCAAGAG AATGAGATTG ATGATCATAG
501 AGGTCAATGG CTGAACTGTT TTCCAGATAG CTGCACTACT CTTATGTCGT
551 TGAATTTCGC TTGCCTTAAA GGAGAGACCA ATGTTGCTGC TTTAGAAAGG
601 CTTGTTGCTA GGTCACCAAA CCTGAAGAGC TTGAAGTTAA ACCGTGCAGT
651 ACCGCTTGAC GCACTCGCAA GGTTAATGAG TTGTGCGCCG CAGCTAGTGG

701 ACTTAGGAGT AGGGTCTTAT GAGAATGAGC CAGATCCTGA ATCTTTTGCA
751 AAACTCATGA CTGCCATTAA GAAATACACA TCGTTAAGGA GCTTGTCTGG
801 CTTTTTAGAG GTTGCTCCAC TCTGCCTCCC AGCGTTCTAC CCAATTTGCC
851 AAAACCTTAT CTCTTTGAAC CTCAGCTATG CAGCTGAAAT CCAAGGCAAC
901 CACCTCATTA AGCTTATTCA GCTTTGCAAG AGACTTCAAC GATTATGGAT
951 ATTGGATAGT ATTGGTGACA AAGGACTTGC GGTTGTCGCT GCCACATGTA
1001 AAGAGTTACA AGAGCTTAGA GTTTTTCCCT CTGATGTACA TGGTGAAGAA
1051 GATAACAACG CATCTGTGAC TGAGGTTGGA CTAGTCGCCA TTTCCGCAGG
1101 TTGCCCTAAA CTTCATTCGA TTCTGTACTT CTGCAAACAG ATGACAAACG
1151 CAGCGCTCAT AGCCGTGGCC AAAAACTGTC CAAACTTCAT CCGGTTCAGG
1201 CTATGCATTC TCGAGCCACA CAAACCTGAC CACATTACAT TTCAATCACT
1251 GGACGAGGGC TTTGGTGCAA TCGTACAAGC TTGCAAGGGT CTAAGACGGC
1301 TCTCTGTCTC CGGTCTCTTA ACCGATCAAG TCTTTCTCTA CATCGGTATG
1351 TACGCGGAAC AGCTCGAGAT GCTTTCGATA GCTTTTGCGG GGGACACTGA
1401 CAAAGGAATG CTCTATGTGT TGAATGGATG CAAAAAAATG AGGAAGCTGG
1451 AGATAAGGGA CAGTCCTTTT GGGAACGCTG CGCTTCTTGC TGACGTGGGT
1501 AGGTAC**GAAACAATGCGATCCCTTTGGA**TG TCGTCTTGTG AAGTAACACT
1551 CGGTGGCTGC AAGAGGCTCG CGCAGAATTC GCCACGGCTT AACGTAGAGA
1601 TCATCAACGA GAATGAGAAT AATGGGATGG AACAGAATGA AGAAGATGAA
1651 AGAGAGAAGG TTGATAAACT TTACCTCTAC CGAACAGTGG TTGGGACTAG
1701 AAAAGATGCA CCACCATATG TTAGGATTCT TTAGTCTCTT TGCACCTTCA
1751 TGTGTTTTCA AACTTTTCTT TGTACTAGTT TTTGCCTTTT ATCAAACTTG
1801 G

APPENDIX D

Equipment list

Autoclave:	Hirayama, Hiclave HV-110, JAPAN
	Nüve, OT 032, TURKEY
Balance:	Sartorius, BP 221 S, GERMANY
	Schimadzu, Libror EB-3200 HU, JAPAN
Centrifuge:	Beckman Coultier ™ Microfuge® 18 Centrifuge, USA
	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
Deep-freeze:	-80ºC, Thermo Electron Corporation, USA
	-20ºC, Bosch, TURKEY
Deionized water:	Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Biogen Inc., USA
	Biorad Inc., USA
	SCIE-PLAS, TURKEY
Gel documentatiton:	UVITEC, UVIdoc Gel Documentation System,UK
	BIO-RAD, UV-Transilluminator 2000, USA
Heating block:	Bioblock Scientific, FRANCE
	Bio TDB-100 Dry Block Heating Thermostat, HVD Life Sciences, AUSTRIA
Ice machine:	Scotsman Inc., AF20, USA
Incubator:	Memmert, Modell 300, GERMANY
	Memmert, Modell 600, GERMANY
	Nüve EN 120, TURKEY

Laminar flow:	Kendro Lab. Prod., Heraeus, Herasafe HS12, GERMANY
Magnetic stirrer:	VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY
	VELP Scientifica, Microstirrer, ITALY
Micropipette:	Gilson, Pipetman, FRANCE
	Eppendorf, GERMANY
Microwave Oven:	Bosch, TURKEY
pH meter:	WTW, pH540 GLP Multical®, GERMANY
	HANNA, pH213 microprocessor pH meter, GERMANY
Power Supply:	Wealtec, Elite 300, USA
	Biogen, AELEX, USA
Refrigerator:	+4º, Bosch, TURKEY
Shaker:	Excella E24 Shaker Series, New Brunswick Sci., USA
	GFL, Shaker 3011, USA
	Innova™ 4330, New Brunswick Sci., USA
Spectrophotometer:	BIO-RAD, SmartSpec™ 3000, USA
	VARIAN, Cary 300 Bio Uvi-visible spec., AUSTRALIA
Speed vacuum:	Savant, Refrigerated Vapor Trap RVT 400, USA
Thermocycler:	PE Applied biosystems, GeneAmp PCR System 9700, USA
Tissue lyser	Qiagen Retsch, USA
	MJ Research, PTC-100, USA
	TECHNE, TC 512, UK
Water bath:	TECHNE, Refrigerated Bath RB-5A, UK
	JULABO, TW 20, USA