ROLE OF MiR156 and MiR171 IN DROUGHT RESPONSE OF TOBACCO

by

DENİZ ADALI

Submitted to the Graduate School of Engineering and Natural Sciences in partial fulfillment of the requirements for the degree of Master of Science

Sabancı University

June 2015

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APPROVED BY:

Prof. Dr. Hikmet Budak (Thesis supervisor) .....

Prof. Ersin Göğüş .....

Assist. Prof. Bahar Soğutmaz Özdemir .....

DATE OF APPROVAL: 30.06.2015

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Bioengineering and Biological Sciences, MSc. Thesis, 2015

Keywords: microRNA, *Nicotiana*, overexpression, drought

Abstract

microRNAs (miRNAs) are genetic regulator RNA molecules belonging to small RNA family. Functions of miRNAs in plants range in a broad spectrum, one of which is the drought stress that is a major environmental stress factor globally affecting plant growth and development. Overexpression of a gene indicates elevated expression of a certain gene beyond its normal expression level. Studies seeking for function of plant miRNAs in response to overexpression carries significant role for agricultural improvement. Among wide variety of plant miRNAs with diverse functions, miR156 and miR171 have critical roles on plant development and stress tolerance. In the first part of our study, different Turkish tobacco cultivars were subjected to drought stress and RNA from leaf tissue of these plants was utilized for changes of gene expression levels in miR156 and miR171. In the second part of project, leaf explants of grown tobacco cultivars were transformed with *Agrobacterium tumefaciens* containing *MIR156* and *MIR171* genes of tobacco cultivars and successful overexpressed putative transgenic plants were selected. Callus tissue formed from putative transgenic and control leaf explants groups were also subjected to drought stress and drought stress response of miR156 and miR171 expression levels were analyzed by quantitative real time PCR. Scope of this study could be utilized for a better understanding of molecular mechanisms of drought stress and the roles of miRNAs.

ÖZET

MiR156 VE MiR171'İN TÜTÜN KURAKLIK TEPKİSİNDEKİ ROLÜ

Deniz Adalı

Biyolojik Bölümler ve Biyomühendislik Bölümü

Yüksek Lisans Tezi, 2015

Prof. Dr. Hikmet Budak (Tez Danışmanı)

Anahtar Kelimeler: mikroRNA, *Nicotiana*, overekspresyon, kuraklık

MikroRNAlar (miRNA), küçük RNA ailesine ait genetik düzenleyici moleküllerdir.miRNA işlevleri geniş bir yelpazeye sahiptir. Bu işlevlerden biri de bitki büyüme ve gelişmesini global olarak etkileyen ve önemli bir çevresel faktör olan kuraklık stresidir. Bir genin overekspresyonu, genin normal ekspresyon seviyesinden daha fazla ekspres edilmesini ifade eder. Bitki miRNAlarının overekspresyona yanıt olarak taşıdığı işlevleri araştıran çalışmalar, tarımsal gelişimde önemli bir role sahiptir. Çeşitli fonksiyonları olan bir çok miRNA arasından miR156 ve miR171, bitki gelişimi ve stres toleransı açısından kritik bir önem taşımaktadır. Çalışmamızın ilk bölümünde, Türkiye'ye ait farklı tütün kültür bitkileri kuraklık stresine maruz bırakılmış ve tütün yaprak dokusuna ait RNA moleküllerinden yararlanılarak miR156 ve miR171 genlerinin ekspresyon seviyesindeki değişim belirlenmiştir. İkinci kısımda ise, yaprak eksplantlarından elde edilmiş kalluslar *MIR156* and *MIR171* genlerini taşımakta olan *Agrobacterium tumefaciens* ile transforme edilmiş ve başarılı aday transformantlar seçilmiştir. Aday transgenik ve kontrol gruplarına ait kallus dokularına kuraklik stresi uygulanmış, miR156 ve miR171 miRNA'larının kuraklık tepkisi, eş zamanlı Polimeraz Zincir Reaksiyonu'ndan faydalanılarak ekspresyon seviyelerine bakılmak suretiyle ölçülmüştür. Bu çalışma, kuraklık stresinin moleküler mekanizmasının ve miRNAların kuraklıktaki rolünün anlaşılması için kullanılabilir.

To my family and the longest holiday ever

ACKNOWLEDGEMENTS

First of all, I would like to thank my thesis advisor Prof. Hikmet Budak for his guidance, motivation, and patience as well as protective and infinite support since my first day in Sabancı University. His existence is my major driving force to complete this study and helped me to discover my interest in further studies. I will be always grateful to him to let me join his dynamic and innovative team.

I would also like to thank Prof. Ersin Göğüş and Assist.Prof. Bahar Soğutmaz Özdemir to give me the honor of being a part of my thesis defense jury. Their ideas, guidance and comments for this study are more than invaluable for me.

I would also like to express all my gratitude to my lab members: Dr. Stuart James Lucas for his infinite guidance, patience and kindness, Dr. Anı Akpınar and Dr. Melda Kantar for their everlasting support and guidance since the first day of my internship till the last day of my master education in Sabancı University. I would like to thank my other lab members Reyyan Bulut and Burcu Alptekin for their knowledge, disciplined approach, kind friendship and all other lab members for their existence.

I also owe lots of thanks to Dr. Dilek Tekdal, Dr. Ines Karmous, Dr. Yasemin Yozgat, Ersoy Çolak and Aslı Yenenler for being there whenever I need any material, technical equipment, any idea, and most importantly warmth of a close friend. When it comes to friends I can never forget infinite love, support and positive energy of Sinem Müderrisoğlu, Begüm Erol, Ece Demir, İpek Özdemir, Burçin Üstbaş, Canhan Şen, Onur Özensoy, Hazal Yılmaz and members of Office G022.

Last but not the least, I would like to thank my beloved family Cansel, Nail, Çınar Adalı and Serkan Sırlı for their unconditional love, infinite patience and ever-lasting faith in me. I owe you till the end of my life for all your efforts to raise me.

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ABBREVIATIONS

°C	Degree Celcius
µ	Micro
ABA	Abscisic acid
AGO	Argonaute
BLAST	Basic Local Alignment Search Tool
bp	Basepair
cal	Calorie
cDNA	Complementary DNA
DCL1	Dicer-Like 1 Protein
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide
g	Gram
HEN1	HUA ENHANCER1
HST	HASTY
HYL1	HYPONASTIC LEAVES 1
k	Kilo
l	Liter
m	Mili
M	Molar
MES	2-(*N*-morpholino) ethanesulfonic acid
MS	Murashige and Skoog
$MgCl_2$	Magnesium Chlorides
min	Minute
MIR	microRNA Encoding Gene
miRNA	microRNA
miRNA*	microRNA Passenger Strand
mol	Mole

mRNA	Messenger RNA
n	Nano
NCBI	National Center for Biotechnology Information
ncRNA	Non coding RNA
nt	Nucleotide
PCR	Polymerase Chain Reaction
PIWI	P-element induced wimpy testis
pre-miRNA	Preliminary microRNA
pri-miRNA	Primary microRNA
qRT-PCR	Quantitative-Real Time Polymerase Chain Reaction
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
RNase	Ribonuclease
RT	Reverse Transcription
RT-PCR	Reverse Transcription PCR
s	Second
SE	SERRATE
Taq	*Thermus Aquaticus*
U	Unit

1. INTRODUCTION

1.1microRNA

microRNAs (miRNAs) are genetic regulator RNA molecules belonging to small RNA family. These single stranded, small, 19-24nt long, noncoding RNA molecules have vital roles to suppress unwanted genetic materials and transcripts, which is known as posttranscriptional silencing (Ghildiyal and Zamore 2009, Ishizu, Siomi et al. 2012). miRNA genes are comprised of one of the most abundant gene families among animals, plants, protists and viruses (Griffiths-Jones, Saini et al. 2008). First discovered miRNA was belonging to *Caenorhabditis elegans*, named lin-4, responsible for regulation of developmental timing via translational repression (Wightman, Ha et al. 1993). Since discovery of first plant miRNA in Arabidopsis over a decade ago, numerous miRNAs are identified and studied in a variety of plants (Llave, Xie et al. 2002).

MicroRNAs are highly conserved among plant species and different miRNAs in the same species could show cross talk in miRNA pathways in response to biotic and abiotic stresses (Liu, Tian et al. 2008, Lu, Sun et al. 2008, Jagadeeswaran, Saini et al. 2009, Jia, Wang et al. 2009, Xin, Wang et al. 2010). Functions of miRNAs in plants range in a broad spectrum involving growth, development, metabolism, response to mechanical stress (Lu, Sun et al. 2005), response to biotic stress such as fungal, bacterial and viral attack as well as response to abiotic stress such as salt, drought, heavy metal, oxygen and temperature (Zhao, Jiang et al. 2012). Action mechanisms of microRNAs could be listed as translational inhibition, mRNA degradation and cleavage.

1.1.1 miRNA Biogenesis

In plants, mature miRNAs are generated from double stranded primary microRNAs (pri-miRNAs), which are transcribed by RNA polymerase II and their length is highly

heterogenous. pri-miRNAs have such structure that folding back on themselves into imperfectly paired hairpin loop structures, which is thought to be stabilized by DDL (DAWDLE, a DCL1 interacting protein) until further processing of pri-miRNA (Yu, Bi et al. 2008, Kantar, Akpinar et al. 2012) Processing of pri-miRNA into pre-miRNA is conducted by DICER –LIKE1 nuclease (DCL1), which is a member of RNase III endonuclease family and main component of miRNA processing (Kantar, Akpinar et al. 2012). This protein is accompanied by zinc-finger protein SERRATE (SE), nuclear cap binding complex (CBC) and double stranded RNA binding protein HYPONASTIC LEAVES1 (HYL1) in order to improve cleavege mechanism of DCL1 (Vazquez, Gasciolli et al. 2004, Lobbes, Rallapalli et al. 2006, Dong, Han et al. 2008, Laubinger, Sachsenberg et al. 2008). Cleavage of pri-miRNA performed by DCL1 in nucleus results in precursor miRNA (pre-miRNA), which is miRNA/miRNA* duplex bounded to each other from the stem of the hairpin(Kim 2005). Then, 3' ends of both miRNA and miRNA* is methylated by the nuclear protein HEN1 in order to protect the duplex from degradation before export from nucleus to cytoplasm (Yu, Yang et al. 2005). Exportation to cytoplasm is carried out by HASTY, exportin-5 homologue (Park, Wu et al. 2005). In cytoplasm, mature miRNA is attached to Argonaute family protein (AGO) and become part of RNA-induced silencing complex (RISC) while miRNA* is degraded(Bologna, Schapire et al. 2013).

Compared with plants, miRNA biogenesis in animals differs in several points. First, cleavage function of primary miRNA is conducted by Drosha instead of DCL1 in animals (Ha and Kim 2014). Second, unlike plants, animal miRNA processing is not completed in nucleus but continues in cytoplasm instead (Ha and Kim 2014). After 1st cleavage by DROSHA and DGCR8 in nucleus, pre-miRNA is exported to cytoplasm and second cleavage by DICER takes place accompanied other double stranded DNA binding proteins like TRBP or PACT(Bologna, Schapire et al. 2013). Third, miRNA/miRNA* duplex of animals is not methylated unlike plant miRNAs (Bologna, Schapire et al. 2013). Comparison of plant and animal miRNA biogenesis is summarized in Figure 1.

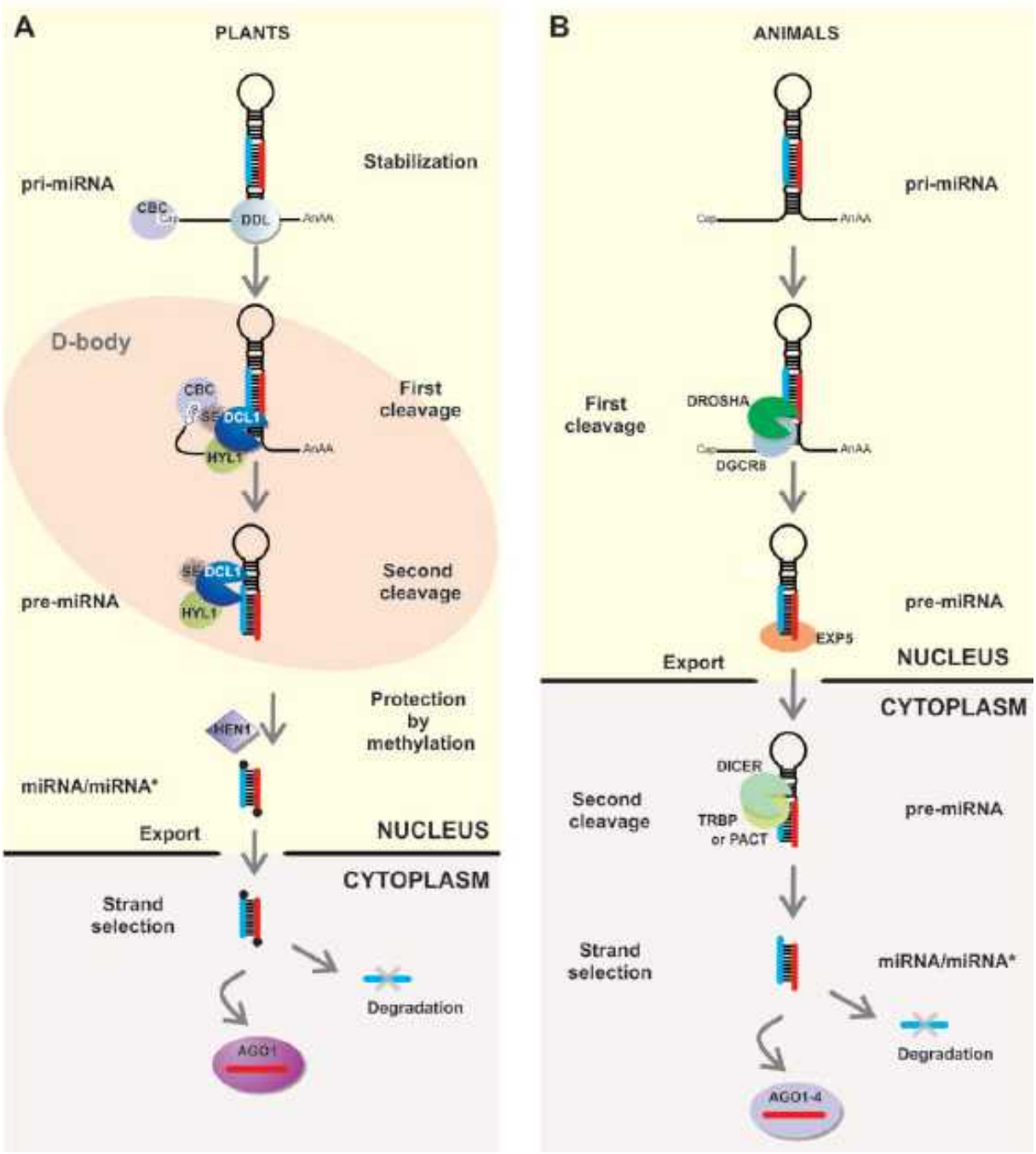


Figure 1 miRNA processing pathways in plants and animals (Bologna, Schapire et al. 2013)

1.1.2 miRNA Mediated Gene Regulation

Until recent studies, miRNAs are believed to be involved in regulation of genes in only repressive manner; however, stimulative regulation of gene expression is considered very likely according to recent updates (Vasudevan 2012). In other words, miRNAs are capable of posttranscriptional up-regulation as well as mRNA cleavage, mRNA deadenylation, translational repression and transcriptional repression; namely, DNA methylation and histone modification.

1.1.2.1 mRNA Cleavage

In plants, mRNA cleavage is considered as the most common regulation mechanism. Plant miRNAs reported to show complementary to its target mRNA in high degrees, which is a requirement for efficient target slicing (Mallory and Vaucheret 2010, Ma, Cao et al. 2013).Upon mature miRNA is attached to AGO protein at the end of miRNA biogenesis process, RNaseH-like is formed by PIWI domain of AGO protein fold with a slicer endonuclease activity to cut target mRNA as shown in Figure 2.

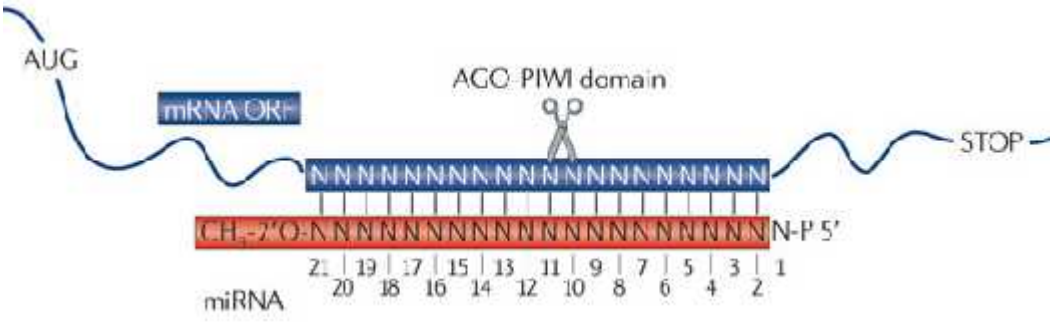


Figure 2 miRNA mediated gene regulation via mRNA cleavage in plants(Huntzinger and Izaurralde 2011)

1.1.2.2 Translational Inhibition and mRNA Destabilization

Translational inhibition occur commonly in animals where target cleavage is not always possible due to limited miRNA target complementarity as well as in plants showing almost perfect miRNA target complementarity (Zeng, Yi et al. 2003, Brodersen, Sakvarelidze-Achard et al. 2008). It is shown that overexpression of miR172 inhibit APETAL (AP2) protein accumulation in Arabidopsis and lead to loss of function in *SUPERNUMERARY BRACT (SNB)* gene in rice without affecting neither *AP2* RNA abundance nor SNB transcript levels, suggesting that miR172 acting through translational inhibition in Arabidopsis and rice(Aukerman and Sakai 2003, Zhu, Upadhyaya et al. 2009). Similarly, miR156/miR157 in Arabidopsis inhibits expression of its target gene *SPL3* only at protein level (Gandikota, Birkenbihl et al. 2007).

Similar to translational inhibition, mRNA destabilization is common in animals where complementarity between miRNA and its target mRNA is limited (Brennecke, Stark et al. 2005, Rogers and Chen 2013). In this process, miRNA RISC destabilizes target mRNA by deadenylation and decapping (Eulalio, Huntzinger et al. 2009, Ma, Cao et al. 2013). Recent studies reveal that translational repression precedes mRNA deadenylation and destabilization, which could indicate that translational repression is likely to be main action mechanism of animal miRNAs contrary to plant miRNAs (Ma, Cao et al. 2013).

1.1.2.3 DNA Methylation and Histone Modification

Epigenetic research signs that DNA methylation is another modulatory function of miRNAs, which is conducted at transcriptional level. Both animal and plant miRNAs are capable of this kind of transcriptional gene silencing (Khraiwesh, Arif et al. 2010, Wu, Zhou et al. 2010). To illustrate, pri-mir820 in rice produces two variants, which are 21 nucleotides long miR820.1 and 24 nucleotides long miR820.2. While short variant directly cleave its target, long variant miR820.2 is loaded to AGO4 protein and can direct DNA methylation around the target site within target loci (Wu, Zhou et al. 2010).

1.1.3 Role of miRNA Overexpression in Drought Stress Tolerance

Overexpression of a gene indicates elevated expression of a certain gene beyond its normal expression level. It has been utilized as a genetic tool since rise of yeast transformation techniques and genomic libraries (Beggs 1978, Carlson and Botstein 1982, Prelich 2012). Among numerous overexpressed genes, studies seeking for function of plant miRNAs in response to overexpression carries significant role for agricultural improvement.

MiR398 was the first plant miRNA correlated with stress tolerance, whose expression was down-regulated in *Arabidopsis thaliana* due to oxidative stress (Sunkar, Kapoor et al. 2006). After miR398, new plant miRNAs found to be involved in stress responses through miRNA sequencing and microarray analysis (Sunkar and Zhu 2004, Liu, Tian et al. 2008, Zhou, Wang et al. 2008, Lee, Yoo et al. 2010).

Role of miRNA overexpression for improvement of stress resistance have been verified in different plant species with diverse abiotic stress conditions (Zhang 2015). Overexpression of first identified plant miRNA, miR156, was found to represent improved heat stress tolerance in Arabidopsis (Stief, Altmann et al. 2014). Similarly, Ni, Hu et al. (2012) stated that overexpression of miR394 in Arabidopsis minimized water loss and provide drought resistance. Moreover, overexpressed miR402 in Arabidopsis enhanced seed germination in dehydration conditions (Kim, Kwak et al. 2010). In tomato, overexpression of miR169 caused enhanced drought tolerance (Zhang, Zou et al. 2011) while miR319 showed the parallel function in creeping bentgrass (Zhou, Li et al. 2013). Another overexpressed miRNA, miR408 was found to be important for chickpea in dehydration conditions (Hajyzadeh, Turktas et al. 2015).

1.1.4 miR156 and miR171

Among wide variety of plant microRNAs with diverse functions, miR156 and miR171 have agricultural significance for plant development and stress tolerance (Jones-Rhoades and Bartel 2004, Sunkar and Zhu 2004). Genomic analysis of miR156 revealed that regulatory

repertoire of miR156 is highly conserved throughout plant evolution (Arazi, Talmor-Neiman et al. 2005, Axtell, Snyder et al. 2007, Zhang, Ling et al. 2015). In rice, miR156 genes down-regulates *Squamosa Promoter Binding Protein Like* genes, which enhance development from juvenile to adult, flowering, leaf formation and vegetative phase chance (Wu and Poethig 2006, Schwarz, Grande et al. 2008, Xing, Salinas et al. 2010, Huijser and Schmid 2011, Zhang, Ling et al. 2015). In *Triticum aestivum*, miR156 is responsive to heat stress with up-regulation (Xin, Wang et al. 2010) and its overexpression multiplies plant biomass in switchgrass (Fu, Sunkar et al. 2012). Moreover, overexpression of miR156 elevates level of anthocyanin biosynthesis in Arabidopsis (Gou, Felippes et al. 2011), which was reported to minimize plant sensitivity to salt and drought stress (Cui, Shan et al. 2014). Role of miR156 in drought, salt and cold stress was revealed in many studies by high throughput data covering microarray and microRNA sequencing (Sunkar and Zhu 2004, Liu, Tian et al. 2008, Zhou, Wang et al. 2008, Lee, Yoo et al. 2010). Kantar and Lucas et al. (2011) reported that miR156 is responsive to drought stress in *Triticum dicoccoides* root tissue. Similar to miR156, miR171 is a drought responsive microRNA targeting SCL transcription factors acting on floral development, root pattern, light and giberellin signaling (Llave, Xie et al. 2002). In Arabidopsis, both miR156 and miR171 were shown to be up-regulated in drought conditions (Liu, Tian et al. 2008), while miR171 was down-regulated against drought stress in *Triticum dicoccoides* (Kantar, Lucas et al. 2011). Interestingly, miR171b is both up-regulated and down-regulated in different developmental stages of rice during drought stress (Zhou, Liu et al. 2010).

1.1.5 Targets of miR156 and miR171

Two main targets of miR156 and miR171 are Squamosa-promoter binding protein (SBP)-like transcription factors (Wu and Poethig 2006) and Scarecrow-like proteins (Llave, Xie et al.2002), respectively. Other target sequences of these two microRNAs detected via psRNATarget program were reported in Appendix J and Appendix K.

1.2 Drought as an Agricultural Threat

As a universally acknowledged truth, global climate change influences discernibly physical and biological systems as well as human and managed systems (Climate Change 2014 Synthesis Report). Ability of climate change to shift atmospheric conditions and environmental soils leads to adverse conditions for plant growth and development (Zhang 2015). According to global hydrological models (GHM), climate change is very likely to trigger not only regional but also global water deficiency dramatically (Schewe, Heinke et al. 2014). Kantar, Lucas et al. (2011) stated that drought is a major environmental stress factor globally affecting plant growth and development and drought stress leads to changes in physiological, biochemical and morphological structure of plants (Bray 1993, Aguirrezabal, Bouchier-Combaud et al. 2006, Mishra, Iannacone et al. 2012). Given the risk of climate change and possible insufficiency of water resources, it is imperative to guarantee sustainability of plants with nutritional and economic importance.

1.2.2 Influence of Drought Stress in Plants

According to Mahajan and Tuteja (2005), drought stress leads to disruption of bilayer structure of cell membrane causing exceptionally porous form as well as disorganized membrane proteins; eventually, membrane unity, selective permeability, enzyme activity and cellular metabolism are disturbed. Other physiological influence of drought on plants is reduction of cell division and growth rate due to decreased cyclin dependent kinase activity (Schuppler, He et al. 1998). Also water deficiency in plants adversely affect photosynthesis because of closure of stomata in order to retain available water in tissues (Mahajan and Tuteja 2005). Inhibition of photosynthesis naturally reduces carbohydrate synthesis, leading to arrested cell division. Moreover, insufficiency of water negatively affects generative organs by causing ovule abortion and pollen sterility (Barnabas, Jager et al. 2008).

1.2.3 Plant Adaptations against Drought Stress

The first protective response of plants against water deficiency is closure of stomata (Davies, Mansfield et al. 1990), which requires ions and metabolites under the control of abscisic acid (ABA). One of the adaptations against drought is reduction of leaf area in order to minimize transpiration. Similarly, cotton increase senescence and abscission rate of older leaves when subjected to drought, which is leaf area adjustment. Unlike leaf tissue, root sustains growth to draw more water from deeper soil layers (Mahajan and Tuteja 2005). Also, increasing the rigidity of cell wall is another plant adaptation to adjust water potential in tissues. Moreover, efficient removal of Reactive Oxygen Species (ROS) formed during photosynthesis and cellular respiration could provide drought tolerance for plants (Sairam and Saxena 2000). Plants could also preserve water reservoir by synthesis of osmolytes, sugar transporters and aquaporins, which are membrane proteins transporting water (Yoshiba, Kiyosue et al. 1997, Alexandersson, Fraysse et al. 2005) At molecular level, many genes identified either directly protecting plants against drought stress or regulating gene expression in stress response (Chen and Zhu 2004, Zhou, Wang et al. 2007).

1.3 Genus *Nicotiana*

As the fifth largest genus of *Solenaceae* family, *Nicotiana* is one of the most essential plants with global, agricultural and economic value. Currently, 86 species recorded in genus *Nicotiana* (Knapp, Chase et al. 2004); they are mainly grown in America and Australia with 75% and 25% rates, respectively. Members of *Nicotiana* family could be either annual or perennialwith tube shaped flowers and commonly broad leaf structure. Characteristic property of many Nicotiana species is production of defensive chemicals called alkaloids. Nicotine is the most famous alkaloid among other three; namely, nornicotine, anabasine and anatabine (Saitoh, Noma et al. 1985).

Genus *Nicotiana* has been thought to be evolved in east of Andes in southern South America (Clarkson, Knapp et al. 2004). Through evolution path, family members have shown variety in ploidy level and chromosome number. Allotetraploid *Nicotiana tabacum*was formed in the last 200.000 years (Wang and Bennetzen 2015), which has S and T genomes with 2n=48

chromosomes arised from polyploidization of diploid paternal donor *N. tomentosiformis* and diploid maternal donor *N. sylvestris* (Leitch, Hanson et al. 2008, Sierro, Battey et al. 2013). While *Nicotiana benthamiana* has B and S genome with 2n= 38 chromosomes with *Nicotiana* section *Noctiflorae* and *Nicotiana* section *Sylvestres* donors (Goodin, Zaitlin et al. 2008, Leitch, Hanson et al. 2008),*Nicotiana rustica*with 2n=48 chromosomes derived from U genome of *N. Undulata* and P genome of *Nicotiana* section *Paniculatae* (Chase, Knapp et al. 2003, Clarkson, Knapp et al. 2004, Yukawa, Tsudzuki et al. 2006).

1.3.1 Global Production, Agricultural and Economic Value

Among members of *Nicotiana* family, *Nicotiana tabacum*, *Nicotiana benthamiana* and *Nicotiana rustica* are the most popular species. Ni*cotiana tabacum* and *Nicotiana rustica* are cultivated mainly for tobacco production. *Nicotiana tabacum* is grown more than 30 countries. Among these, top five countries - Brazil, China, India, Turkey and United States - produces 2/3 of the world's tobacco (WHO, The Tobacco Atlas, 2004).

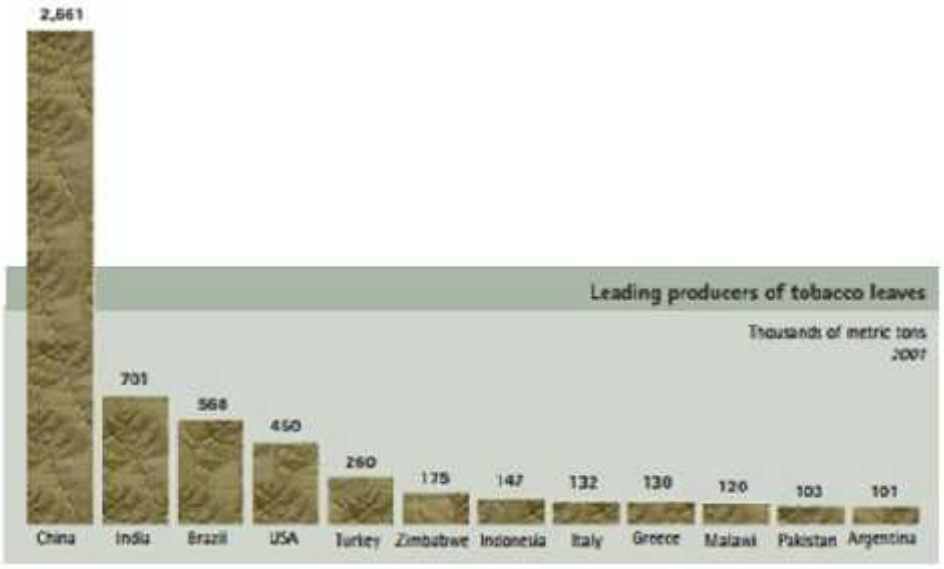


Figure 3 Global tobacco producing countries(WHO, The Tobacco Atlas, 2004).

Nicotiana rustica is also cultivated for mainly tobacco consumption. Unlike *Nicotiana tabacum*, *Nicotiana rustica* is consumed in smokeless way in the form of Maraş powder, named after common consumption in eastern Mediterranean part of Turkey, by mixing

powder of *Nicotiana rustica* leaves with ashes of vine stem, walnut, and oak (Sucakli, Ozkan et al. 2013).

On the other hand, *Nicotiana benthamiana* is an invaluable species for agricultural studies of plant pathogen interactions (Krenz, Bronikowski et al. 2015, Li, Tee et al. 2015, Liou, Hu et al. 2015, Sivanandam, Mathews et al. 2015) due to its vulnerability to wide variety of pathogens and convenience for virus induced gene silencing and transient protein expression methods (Bombarely, Rosli et al. 2012).

1.3.2. Genome Organization of Genus *Nicotiana*

First genome sequencing of *Nicotiana* genus was performed for Hicks Broad Leaf cultivar of *Nicotiana Tabacum* with the aim of sequencing open reading frames (ORFs) in 2007 (Wang and Bennetzen 2015). Methylation filtration method of Sanger sequencing technology could only covered a small proportion of the genome for that time; however, arise of Next Generation Sequencing enabled sequencing of whole genomes of *N. otophora*, *N. sylvestris*, *N. tomentosiformis*, *N. benthamiana* and three cultivars of *N. tabacum* (Bombarely, Rosli et al. 2012, Nakasugi, Crowhurst et al. 2013, Sierro, Battey et al. 2013, Sierro, Battey et al. 2014)

Estimated average genome size of *N. rustica* and *N.tabacum* is 4.5 Gb (Leitch, Hanson et al. 2008). Whenwhole genomes of three cultivars of *N.tabacum* has been sequenced, Sierro, Battey et al. (2014) reported that genome size of TN90 is ~ 4.4 Gb, K326 is ~ 4.6 Gb and Basma Xanthi (BX) is ~ 4.5 Gb. Among 134,694–188,510 transcripts for these cultivars, roles for more than 50,000 potential proteins have been predicted (Sierro, Battey et al. 2014). When it comes to ancestors, genome size of *N. sylvestris* and *N. tomentosiformis* is almost half of *Nicotiana tabacum* and most DNA sequences were stated as repetitive elements(Sierro, Battey et al. 2013). On the other hand, genome size of *Nicotiana benthamiana*, which is around 3.1 Gb, is smaller than *N.rustica* and *N.tabacum*.

1.4 *Agrobacterium tumefaciens*as a Genetic Engineer

Agrobacterium tumefaciens is a gram negative soil bacteria, well known with pathogenic characteristics due to large tumor inducing (Ti) plasmid and high potential to cause crown gall disease in diverse plant species (Gelvin 2003). Action mechanism of disease is delivery of virulent DNA molecule called transfer DNA (T-DNA) into genome of host plant via type IV secretion system (T4SS) (Alvarez-Martinez and Christie 2009), which results in expression of oncogenes and eventually tumor induction (Chilton, Drummond et al. 1977, Pitzschke and Hirt 2010, Krenek, Samajova et al. 2015).

Tumor induction in host plant depends not only on T-DNA region but also Vir region, which suppress innate immune system of the host plant. Unlike T-DNA, Vir region of Ti plasmid is not inserted to plant cell; instead, proteins encoded from Vir loci (virA, virB, virC, virD, virE, virF, virG and virH) aid T-DNA in transfer, nuclear targeting and integration to plant chromosome (Krenek, Samajova et al. 2015). However, expression of Vir genes mostly require wounded plant tissue in which environmental conditions are met; namely, sugar availability, low pH, limiting phosphate and particularly production of phenolics. (Winans 1991).When integrated T-DNA genes are expressed, tumor growth is supported as specific food source is provided to pathogen by biosynthesis of aminoacid-sugar conjugates called opines (Zechner, Lang et al. 2012). Vitality of opines lays in the fact that they provide high amount of nitrogen and energy, by which *Agrobacterium tumefaciens* becomes advantageous over other parasites (Chumakov 2013).

1.4.1 Action mechanism of T-DNA Delivery

Ability of DNA insertion into host genome depends on whether *Agrobacterium* has Ti plasmid or not. In other words, *Agrobacterium* strains that are lack of Ti plasmid are not virulent (Krenek, Samajova et al. 2015). For the virulent ones as indicated by Zechner, Lang et al. (2012), formation of wound on plant tissue leads to Ti plasmid Vir gene expression by induction of VirA and VirG. T-DNA region is excised at the right border (RB) and left borders (LB) from Ti plasmid by VirD2 relaxase and other relaxosomes (VirD1, VirC1, VirC2). Right and left borders of T-DNA region is important as they harbor genes encoding

proteins related with plant-type hormones and opine synthesis (Zupan, Muth et al. 2000). Excised T-DNA relaxase assembly is recruited to T4SS by binding proteins and VirC1. VirB2 pilin and VirB5 adhesin binds to plant cell receptor and VirD4 starts transfer of single stranded T-DNA bound to relaxase. VirE3, VirF, VirD5 and VirE2 are also transferred with T-DNA in order to coat the strand and provide protection. Delivery of T-DNA is followed by transport to nucleus, integration and finally transgene expression (Figure 4).

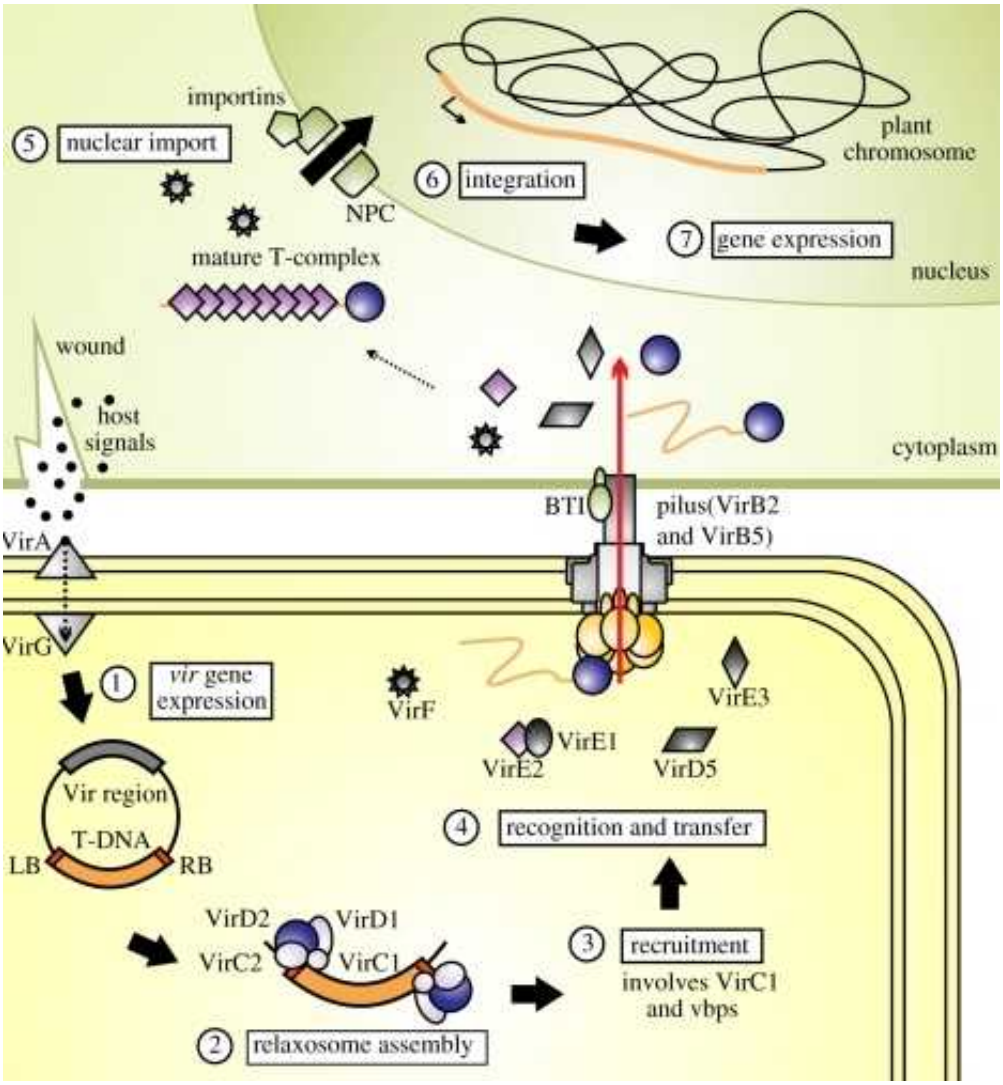


Figure4 T-DNA Delivery Stages of *Agrobacterium tumefaciens*(Zechner, Lang et al. 2012)

1.4.2 *Agrobacterium*-mediated studies

Since discovery of invaluable potential of virulent *Agrobacterium tumefaciens* to conduct genetic transformation studies, it has been utilized for diverse plant species. When *Agrobacterium*-mediated transformation was first applied on model plant organisms, it was found that transformation yield and success varies among different organisms. To illustrate, Arabidopsis is the best studies organism; yet, efficiency of Arabidopsis transformation via *Agrobacterium tumefaciens* is not successful as much as tobacco transformation (Wroblewski, Tomczak et al. 2005). Nevertheless, successfully transformed plant species could be categorized in a very broad repertoire; namely, model plants, cereal crops, legumes, nuts and fruits, vegetables, industrial and tropic plants, turf grasses, root plants, ornamental and medicinal plants (Ziemienowicz, 2014). Not only organism but also aim of transformation is in a wide spectrum including generation of biopharmaceuticals (Haq, Mason et al. 1995, Daniell, Streatfield et al. 2001, Dus Santos, Carrillo et al. 2005), biomonitoring toxic compounds (Hannink, Rosser et al. 2001, Dhankher, Li et al. 2002, Song, Sohn et al. 2003, Ellis, Sors et al. 2004, Cherian and Oliveira 2005), enhancement of crop productivity and nutritional content as well as reduction of agrochemical dependency (Gao, Hakimi et al. 2000, Peschen, Li et al. 2004, Wang, Xue et al. 2005).

1.5 Gateway Cloning System

Gateway cloning is a molecular gene cloning system to insert DNA sequences into vector systems for functional analysis and protein expression (Hartley, Temple et al. 2000, Walhout, Temple et al. 2000). It is based on integration of bacteriophage lambda into *E.coli*, which rises switch between *attB* site of *E.coli* and *attP* site of lambda chromosome (Guarante, Roberts et al. 1992). This conservative recombination event takes place thanks to two components; namely, recombination (*att*) sites and recombination enzyme mixes. Recombinant sites are 25 bp long *attB* site found in expression clone, 200 bp long *attP* site found in donor vector, 100 bp long *attL* site found in entry clone and 125 bp long *attR* site found in destination vector **(**Invitrogen Life Technologies, 2012).

1.5.1 BP and LR Recombination Reactions

Recombination reactions occur with the activity of clonase enzymes by binding to att sites, bringing together the target sites and cleaving these sites to attach DNA covalently. During BP reaction catalyzed by BP clonase, *attB* region carrying PCR product recombines with *attP*substrate, which is donor vector to form *attL* region containing entry clone. On the other hand, LR reaction catalyzed by LR clonase gives rise to recombination of *attL* containing entry clone with *attR* containing destination vector. This reaction results in formation of expression clone hosting *attB* region (Figure 5).

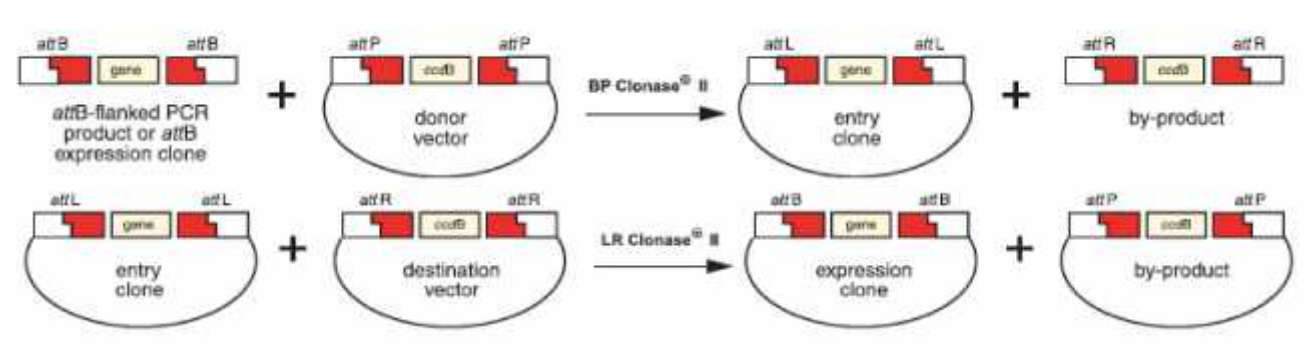


Figure 5 Representation of BP LR recombination reactions**(**Invitrogen Life Technologies, 2012)

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Material

In this study, fourteen wildtype *Nicotiana tabacum* cultivars and one wildtype *Nicotiana benthamiana* cultivar were used. Plant seeds were obtained from Ege Agricultural Research Institute and Ankara University Agricultural Faculty, which were GK Hakkari TR2084, Malatya TR4211, GK112 Bitlis TR42076, GK64 Sakarya TR42735, GK83 TR 42771, Sakarya TR42774, GK84 Sakarya TR42772, Reşatbey 97 TR68444, Kokulu İzmir TekelTE42986, GK 94 Sakarya TR42782, Ege97 TR68442, GK42 Muş TR42087, GK107 Bitlis TR42066, *Nicotiana benthamiana* and *Nicotiana tabacum* from Ankara University.

2.1.2. Molecular Biology Kits

Molecular biology kits are listed in Appendix A.

2.1.3 Equipments

Equipments utilized in this study are listed in Appendix B.

2.2 Methods

2.2.1 Growth Conditions and Handling Techniques of Tobacco Plants

Sterilization and growth conditions of plants were applied according to protocol indicated by Zhang (Zhang, Henriques et al. 2006). First, seeds were subjected to surface sterilization via 70% ethanol treatment for 1 minute, followed by sterilization solution treatment containing 50% sodium hypochlorite, 50% sterile distilled water and 0.05% Tween for 10 minutes by vortexing suspension vigorously every 2 minutes. Seeds were then rinsed for 3 times with

sterile distilled water. After sterilization process, seeds were placed on sterile growth agar medium in petri dishes containing 4.3 gr/L MS (Murashige & Skoog) medium, 10 g/L sucrose, 0.5 g/L MES(2-(*N*-morpholino)ethanesulfonic acid), and 8gr/L plant agar with pH 5.7. Seeds in petri dishes were kept in dark at 4°C for 3 days to break seed dormancy. At the end of this period, petri dishes were transferred to growth chambers with 14/10 h light/dark photoperiod at 21°C. At the end of 3 weeks, tobacco seedlings in petri dishes were transferred to plastic magenta growth boxes containing growth agar medium. Finally, 5-week-old tobacco seedlings were transferred to torf and fertilizer mixture with a 3:1 composition ratio.

2.2.2 Preparation of miR156 and miR171 PCR Products

2.2.2.1 Design of Gateway Cloning Primers

Pre-miR156 and pre-miR171 sequences of Hicks Broad Leaf cultivar of *Nicotiana tabacum* (Tang, Wang et al. 2012) were downloaded from MIRBASE microRNA database (21st release, June, 2014 www.mirbase.org/). For whole genome sequences, TN90, K326 and BX cultivars of tobacco (Sierro, Battey et al. 2014), which were only available whole genome sequences belonging to *Nicotiana tabacum*, were downloaded from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). In order to determine locations of pre-miRNA sequences on whole genomes, Blast 2.2.25+ program was used. At the end of process, 17 of 20 sequences in whole genome showing 99% and 100% similarity with pre-miRNA sequences were used for primer design according to melting temperature and GC content criteria. Firstly, 18 nucleotide long template specific sequences were chosen in a distance of 3 to 10 nucleotide upstream or downstream of pre-miRNA sequence. Secondly, melting temperatures of primers were maximum 67°C and melting temperature difference between forward and reverse primer was no more than 2°C. Thirdly, last 5 nucleotide of 3' end and last 3 nucleotide of 3' end did not contain more than three Guanine or Cytosine and two Guanine or Cytosine, respectively. Also, GC content of primers was kept in a range of 40 to 60%. Finally, BP recombination providing *attB1* and *attB2* sites were attached to forward (5'GGGG-ACAAGTTTGTACAAAAAAGCAGGCT – template specific sequence -3') and reverse primers (5'GGGG-ACCACTTTGTACAAGAAAGCTGGGT – template specific sequence – 3') to clone into pDONR221 vectors via Gateway Cloning Systems.

2.2.2.2 DNA Isolation from Tobacco Cultivars

Three weeks old *Nicotiana tabacum* and *Nicotiana benthamiana* plants were used for DNA isolation by utilization of chemicals and protocol of Anatolia Genomic DNA Isolation Kit. 120 mg of tobacco leaves from each sample were used as plant tissue and homogenized by liquid nitrogen. Then, 600 µl LB1 Buffer was added to homogenized tissue samples in 1.5ml microcentrifuge tubes. Samples were incubated for 60 minutes at 65°C for cell lysis. After incubation, 300 µl of LB2 buffer was added and mixed by pulse – vortexing followed by incubation on ice for 10 minutes. Mixtures were centrifuged at 12.500 rpm for 5 minutes and supernatant was transferred to a new 1.5 ml microcentrifuge tube. 100% ethanol was added to mixtures and mixed by vortexing. Mixtures were applied to spin columns and centrifuged at 11.000 rpm for 1 minute. Flow – through in each tube was discarded, 450 µl of Buffer IR3 was added to spin columns and centrifuged at 11.000 rpm for 1 minute. After flow through was discarded, 450 µl of Buffer W4 was added and centrifuged again at 11.000 rpm for 1 minute. This step was repeated once more and empty columns were centrifuged at 12.500 rpm for 2 minutes to remove remaining wash solution. Then, collection tubes were discarded and spin columns were placed in clean 1.5 ml microcentrifuge tubes. After 2 minutes of waiting step to let evaporation of remaining ethanol in column, 50 µl of Buffer EL5 was added drop by drop to spin columns. Finally, columns were waited for 5 minutes and centrifuged at 12.500 rpm for 1 minute. Liquid flow through, which is DNA, was stored at -20°C.

2.2.2.3 Amplification of microRNA Sequences For Molecular Cloning

Lyophilized primers ordered from Midland Certified Reagent Company were prepared as 100µM stock solutions. Concentration of isolated plant DNA material was set to 50 ng/µl and amplification of DNA was achieved by Fermentas Taq Polymerase enzyme. Each PCR reaction mix was 20 µl in total volume consisting of 1 µl DNA, 2 µl Taq Buffer with KCl (10X), 1.6 µl dNTP, 1.6 µl $MgCl_2$ (25mM), 0.8 µl forward primer (10 µM), 0.8 µl reverse primer (10 µM), 0.4 µl Taq Polymerase (5u/µl) and 11.8 µl distilled water. PCR cycling conditions involved an initialization step at 95°C for 5 minutes, followed by 35 cycles of

denaturation at 95°C for 1 minute, annealing at 55 - 58°C for 1minute and extension at 72°C for 1 minute. A final elongation step was performed at 72°C for 10 minutes.

2.2.2.4 Gel Electrophoresis and Purification of PCR Products

PCR products were loaded in 1.5% agarose containing 0.5X TBE Buffer (108 g/L Tris, 55g/L Boric acid, 40 ml of 0.5 M Na_2EDTA, pH 8.0). PCR products were run in electrophoresis tank with 0.5X TBE buffer at 100 Volt for 40 minutes. Fermentas Thermo Scientific Low Range 50-1500bp SM1103 and Peqlab Low Range 25-700bp were used as DNA ladder.

Purification of PCR products by gel extraction was achieved by utilization of Qiagen QIAquick Gel Extraction Kit (Catalog no. 28706). All centrifugations were applied at 13.000 rpm. According to kit protocol, target region on gel was cut with sterile scalpel under UV and placed in 2 ml microcentrifuge tube. 3 volumes of QG buffer was added to 1volume of gel and incubated at 50°C for 10 min by vortexing every 2 minutes. After gel was dissolved completely, 1 gel volume of isopropanol was added. Spin column was placed in a 2ml microcentrifuge tube and mix was applied to spin column. After 1 minute long centrifugation, flow through was discarded; 0.5 ml QG Buffer was added and centrifugation was repeated. 0.75 ml PE Buffer was added to column. After centrifugation, flow through was discarded and empty column was centrifuged one more time. Then, column was placed into a clean 1.5 ml microcentrifuge tube. In order to elute DNA, 50 µl EB Buffer (10 mM Tris·Cl, pH 8.5) was added and column was incubated for 2 minutes at room temperature. Finally, column was centrifuged for 1 minute and eluted PCR product was sent to McLab Molecular Cloning Laboratories for sequencing.

2.2.3 Molecular Cloning of miR171 and miR156 Genes

2.2.3.1 Gateway Cloning Systems: BP and LR Reactions

Among DNA sequences, resulting highest identity with target miRNA sequences, TN90_1 mir156d, TN90_7 mir156b and TN90_7 mir171b titled PCR products were selected for BP reactions according to sequencing results. Tetracycline resistant, *attB* flanking region containing plasmid pEXP7-tet was used for BP reaction positive control whereas samples

without BP Clonase II enzyme were used for negative control. Kanamycin resistant plasmid pDONR221 was used as donor vector. BP and LR Reactions were conducted according to kit's user manual and protocol of Gateway Cloning Systems (Catalog no: 12535 – 029). Each reaction mix was composed of 5 µl PCR product, 1 µl pDONR221 vector and 2 µl TE Buffer (10 mM Tris-Cl, pH 7.5. 1 mM EDTA, pH: 8.0). While positive control mix was containing 2 µl pEXP7- tet vector, 1 µl pDONR221 vector and 5 µl TE Buffer, negative control tubes were containing 5 µl PCR product, 1 µl pDONR221 vector and 4 µl TE Buffer. 2 µl of BP Clonase II enzyme stored at -20°C was added to each reaction tube except negative control group. Then, all tubes were incubated at 25°C for 12 hours. After the incubation, reaction was ended by addition of 1 µl Proteinase K followed by an incubation step at 37°C for 10 minutes.

Clones obtained from BP reaction were sent to sequencing and successful recombination clones were chosen for LR recombination reaction. According to Gateway Cloning Systems kit protocol, 150 ng of BP clones were reacted with 1.4 µl Kanamycin resistant pEarleyGate103 destination vector and 4.6 µl TE Buffer. In positive control group, instead of BP clones, there was Kanamycin resistant pENTR-gus plasmid, which contains flanking regions providing recombination reaction. 2 µl LR Clonase II enzyme was added to all reaction tubes except negative control group. Again, all tubes were incubated at 25°C for 12 hours and reaction was ended by addition of 1 µl Proteinase K and incubation at 37°C for 10 minutes.

2.2.3.2 Transformation of Plasmids Utilized in BP and LR Reactions into *Escherichia coli* Cells

Plasmids that were carrying clones obtained from BP and LR reactions were selected. LR positive control pENTR-gus and negative control groups were transformed into *Escherichia coli* Tetracycline resistant OmniMAX 2-T1^{R} cells supplied with Gateway Cloning Systems (Catalog no 12535 – 029). On the other hand, tetracycline resistant BP Reaction positive control plasmid pEXP-tet was transformed into *Escherichia coli* DH5α competent cells which were not carrying tetracycline resistance gene, unlike *Escherichia coli* OmniMAX 2-T1^{R} cells. Transformation experiments were applied by Gateway Cloning Systems protocol (Catalog no 12535 – 029). To begin with, tubes containing *Escherichia coli* OmniMAX 2-T1^{R}

and *Escherichia coli* DH5α cells were thawed on ice, followed by addition of 1µl recombination reaction mix. After mixed gently without pipetting, tubes were incubated on ice for 30 minutes. At the end of incubation, tubes were transferred immediately to 42°C water bath, incubated for 30 seconds and placed back on ice for 2 minutes. Then, 250 µl SOC medium (Super Optimal Broth, 2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$,20 mM glucose) was added and tubes were incubated for an hour at37°Cwith shaking at 225 rpm. Then, each transformation mix was diluted with LB medium at 1:10 ratio. 20 and 100 µl of transformation mixes were spread on LB Agar plates containing antibiotics. Clones inserted in pDONR221 and pEarleyGate103 plasmids and LR positive as well as negative controls were spread on 50 µg/ml Kanamycin containing plates and 50 µg/ml Kanamycin with 30 µg/ml Chloramphenicol containing plates. BP reaction positive control was spread on 20 µg/ml Tetracycline containing plate. At the end of spreading, petri dishes were incubated at 37°Cfor 18 hours.

2.2.3.3 Plasmid DNA Isolation from Transformed *Escherichia coli* Cells

Plasmid DNA Isolation was performed according to the protocol of Thermo Scientific GeneJET Miniprep Kit (Kat no: #K0502). All centrifugations were performed at 12.000 rpm unless otherwise specified. Before starting the isolation process, single colonies obtained from transformation plates were transferred to 5 ml of LB medium and incubated at 37 °C for 16 hours. Then, liquid medium containing cells were centrifuged at 8000 rpm for 2 minutes. Pellet was resuspended in 250 µl Resuspension Solution and mixed until no cell clumps remained. Then, 250 µl of the Lysis Solution was added and mixed thoroughly by inverting the tube 6 times until solution becomes viscous. Next, 350 µl Neutralization Solution was added and mixed by inversion for 6 times. Each tube was centrifuged for 5 minutes to pellet cell debris and chromosomal DNA. Then, supernatant was transferred to spin columns and centrifuged for 1 minute. After flow through was discarded, 500 µl Wash Solution was added to spin columns and columns were centrifuged for 1 minute. Then, wash step was repeated and empty columns were centrifuged one more time to avoid residual plasmids. Spin columns were transferred to fresh microcentrifuge tubes and 50 µl Elution Buffer, previously warmed at 60°C, was added drop by drop to the center of spin columns. After a long incubation for 5 minutes at room temperature, columns were centrifuged for 2 minutes and purified plasmid DNA was stored for sequencing.

2.2.4 *Agrobacterium tumefaciens* Mediated Tobacco Transformation

2.2.4.1 Transformation of Destination Clones into *Agrobacterium tumefaciens* cells

According to sequencing results, three different clones were chosen for transformation into AGL1 strain of *Agrobacterium tumefaciens*. According to the protocol applied during transformation process (Wise, Liu et al. 2006), 100 µl competent AGL1 strain of *Agrobacterium tumefaciens* cell stocks (previously stored at -80°C)were thawed on ice and 1µg destination clone was added in each tube. Then, each tube was covered with aluminum foil and incubated in liquid nitrogen for 5 minutes. Next, each tube was incubated at 37°C in water bath in order to provide heat shock. At the end of incubation, cells were transferred to 10ml tubes containing 2ml LB liquid medium and incubated at 28°Cfor 3 hours with shaking at 140 rpm. Then, incubated cells were centrifuged at 4000 rpm for 5minutes and pellet cells were resuspended in 500 µl liquid LB medium. 20 and 100 µl of resuspended cells were spread on 100µg/ml Kanamycin and 200 µg/ml Carbenicillin containing agar plates. Finally, petri plates were incubated at 28°C for 15 hours.

2.2.4.2 Plasmid DNA Isolation from *Agrobacterium tumefaciens* cells

Plasmid DNA isolation from *Agrobacterium tumefaciens* cells was performed according to protocol followed in part 2.2.9. However, instead of using a 5 ml starting cell culture, 10 ml cell culture was used with *Agrobacterium tumefaciens* cells. Plasmids obtained from *Agrobacterium tumefaciens* cells were sent sequencing.

2.2.4.3 Growth of *Agrobacterium tumefaciens* Cell Culture as Infection Inoculum

Infection inoculum was prepared according to Kutty et al. 2011. Instead of using YEP medium recommended in protocol, inoculum preparation optimized by utilization of LB medium with minor modifications. First, AGL1 strain of *Agrobacterium tumefaciens* glycerol stock culture was streaked on solid LB medium containing 100 µg/ml Kanamycin and

200µg/ml Carbenicillin, and incubated at 28°C for 2 days. Single colony was isolated and inoculated in 2 ml LB medium with antibiotics in given concentrations. After culture was grown for 12 hours at 28°C, overnight culture was added into 30 ml liquid LB containing 100 µg/ml Kanamycin, 200 µg/ml Carbenicillin and 100 µM Acetosyringone. Since monocotyledon plants like tobacco plants are lack of Acetosyringone secretion after wounding unlike dicotyledons; Acetosyringone is added to LB medium to enhance transformation efficiency by allowing host-pathogen recognition. Cell culture was incubated until optical density reached 0.8 at 600 nm. At this point, cells were harvested for 15 minutes at 1000 g. Cell pellet was resuspended in 10ml liquid MS medium and 1ml of this resuspended culture was used at a time for infection of 5-10 leaves in 15 ml of MS medium.

2.2.4.4 Transformation of Tobacco Cultivars

Agrobacterium tumefaciens transformation of tobacco cultivars, growth of transformed plants and BASTA selection were all achieved by utilization of Kutty's protocol (Kutty et al. 2011). First, 5-6 weeks old seedlings with attached petiole were harvested. Then, tiny prics were made on leaf tissue using sterile scalpel for wounding effect so that *Agrobacterium tumefaciens* cells may head for the wounding area with chemotactic movement. Also, 1mm of leaves margins was cut and still petiole was intact. These leaf tissue samples were immersed in liquid pre-culture medium, which is a MS medium without plant agar. After incubation at room temperature for 2 days, tissue explants were immersed in infection medium composed of 15 ml liquid MS and 1ml bacterial inoculum as prepared in Section 2.2.4.3. Transformation process with bacterial suspension was carried out for 30 minutes at room temperature. After infection, leaf explants were blotted dry on sterile filter paper and placed on co-cultivation medium containing solid MS medium with contents explained in Section2.2.1with addition of 200 µM Acetosyringone, 4 mg/L BAP (6-benzylaminopurine) hormone for shoot development and 0.1 mg/L IBA (Indole -3- butyric acid) hormone for root development. Leaf explants were incubated on co-cultivation medium in dark at 22°C for 2 days.

Other than transformation group, control samples were obtained from each cultivar, which were not exposed to *Agrobacterium tumefaciens* transformation. These samples were cut and subjected to pre-culture medium like putative transgenic group. At the end of two days long

pre-culture period, control samples were cultured on MS agar plates containing 4 mg/L BAP and 0.1 mg/L IBA for four weeks under 16/8 hours light/dark photoperiod.

2.2.4.5 Growth Conditions of Transformed Plants and BASTA Selection

At the end of co-cultivation, explants were washed two times with sterile distilled water to remove bacterial residue. After dried on sterile filter paper, leaf explants were placed on Resting Medium containing MS solid medium with contents explained in 2.2.1 by addition of 4 mg/L BAP, 0.1 mg/L IBA and 400 mg/L Carbenicillin. After a week, explants were transferred to Selection Medium, which contains 1.5 mg/L BASTA in addition to Resting Medium. BASTA (phosphinothricin, ppt, glufosinate ammonium) is an herbicide providing selection of transformed cells rather than non-transformed ones. Unlike wild type tobacco plants, *Agrobacterium tumefaciens* cells were carrying bialaphos resistance (*bar*) gene, which is a resistance gene against BASTA herbicide. Therefore, only successfully transformed leaf explants were expected to survive when BASTA herbicide was applied. Leaf explants were sub-cultured every two weeks. After first two weeks, BASTA concentration was increased to 3mg/Lfrom 1.5 mg/L and Carbenicillin was discarded. All cultures were incubated for four weeks under 16/8 hours light/dark photoperiod at 25 °C.

2.2.5 Drought Stress in Wild Type Tobacco Cultivars

2.2.5.1 Preparation of PEG Infused Drought Stress Media

Drought stress application on wild type tobacco cultivars was conducted via utilization of 20% polyethylene glycol (PEG6000) addition to MS agar medium (van der Weele, Spollen et al. 2000, Verslues and Bray 2004, Kuzuoglu-Ozturk, Cebeci Yalcinkaya et al. 2012, Yin, Gao et al. 2014). Sucrose addition was avoided in order to stabilize water potential of the medium. For a single drought treatment, 0.108 g MS, 0.025 g MES and 0.75g plant agar in 50 ml distilled water (pH 5.7)was autoclaved for 15 minutes at 121°C, 15lb/sq and poured in magenta boxes. In another glass vial, 0.108 g MS and 0.025 g MES with 50ml distilled water was autoclaved for 15 minutes at 121°C, 15lb/sq. After autoclave, 20g PEG6000 was added in hot liquid MS medium and stirred until all PEG was dissolved and medium cooled. Cooled

PEG-MS liquid mixture was poured on solidified MS agar medium in magenta boxes and let stand for 16 hours for equilibration. Before transfer of plants onto PEG treated solid MS medium, liquid PEG mixture was poured off.

2.2.5.2 Application of Drought Stress in Tobacco Callus Tissues and Seedlings

6 weeks old wildtype *Nicotiana tabacum* and *Nicotiana benthamiana* seedlings as well as putative transformed tobacco callus tissues and non-transformed control callus group were subjected to 48 hours and 96 hours long drought stress treatment under 16/8 hours light/dark condition at 25 °C by transferring the tissues to MS medium which was previously treated with 20% PEG.

2.2.6 RNA Isolation

RNA isolation was performed with 150 mg leaf, root and shoot tissue of non-transformed wildtype tobacco seedlings, transformed and non-transformed callus tissues**.** First, samples were treated with liquid nitrogen in sterile mortar with pestle to bring in powder form. Then, powder tissue transferred to 2 ml eppendorf tube on ice and homogenized by addition of 1.4 ml TriReagent (Sigma). After vortex mixing, tubes were transferred to ice and 0.5 ml chloroform was added. Tubes were inverted up and down slowly and incubated for 7 minutes at room temperature. Following centrifugation at 12000 g for 15 minutes at 4°C, supernatant transferred to new eppendorf tube while avoiding interference of pellet. 0.5 ml isopropanol was added; tubes were inverted up and down by hand until mix homogenized. After incubation at 10 minutes at room temperature, samples were centrifuged at 12000 g for 10 minutes at 4°C. Supernatant discarded and pellet in each tube was washed with DEPC treated 1ml 75% ethanol. Samples were mixed and spinned at 7500g for 5 minutes at 4°C. Supernatant was discarded and pellet was let dry for 10 minutes. Finally, RNA pellets were dissolved in 30 µl DEPC treated sterile water which was incubated at 60°C since the beginning of isolation procedure. RNA integrity was verified by electrophoresis using 1.5% agarose gel.

2.2.7 Removal of DNA from RNA samples

Isolated RNA samples were subjected to DNAse treatment to avoid interference of any DNA strand by addition of 1 µl Fermentas RNAse free DNAse I (1U), 1 µl reaction buffer (10X) with $MgCl_2$ to 1 µg RNA. Reaction mix was completed to 10 µl by addition of DEPC treated sterile water and incubated at 37°C for 30 minutes. Then, 1 µl 50mM EDTA was added and each tube was incubated at 65 °C for 10 minutes. DNAse treatment protocol was applied according to manufacturer's (Fermentas RNAse free DNAse I Kit) instructions. DNAse treated samples were stored at -20°C.

2.2.8. cDNA Synthesis with Reverse Transcription Polymerase Chain Reaction

Stem loop reverse transcription primers for cDNA synthesis were designed by following instructions of Kramer (2011) and Varkonyi-Gasic, Wu et al. (2007). cDNA synthesis from isolated RNA samples was achieved by addition of 1µl RNA (100ng/µl) in reaction mix containing 1µl of stem loop RT primer (1µM) and 9 µl DEPC treated water. After incubation at 70°C for 5 minutes, reaction mix tubes were chilled on ice for 2 minutes. Following addition of 4 µl reaction buffer (5X), 2 µl dNTP mix (10mM) and 0.5 µl Ribolock RNAse inhibitor (40U/µl), whole mix is completed to 19 µl with addition of DEPC treated water and incubated at 37°C for 5 minutes. At the end of incubation, 1 µl Thermoscientific Reverse Aid Reverse Transcriptase enzyme (200 U) was added and reaction was started with following conditions: 30 minutes at 16°C, 30 seconds at 30°C for 60 cycles, 30 seconds at 42°C, 1 second for 50°C and 10 minutes at 70°C. Reaction resulted in 5ng/µl cDNA.

2.2.9.Determination of Gene Expression Levels with Quantitative Real Time Polymerase Reaction

Expression levels of miR156 and miR171 in putative transgenic and wild type tobacco samples were measured by Real Time PCRs. Forward and universal reverse primers were designed by following instructions of Kramer (2011) and Varkonyi-Gasic, Wu et al. (2007). Reactions were conducted by utilization of Roche LightCycler 480 Instrument II. Each reaction mix composed of triplets containing 1.5 ng cDNA, 0.4 µl of 10 µM forward primer,

0.4 µl of 10 µM universal reverse primer and 10 µl of 2X Quanta SYBR Green SuperMix. Samples were including reverse transcriptase neglecting negative controls and no RNA negative control. Reaction mix was completed to 20 µl in volume with nuclease free DEPC treated water. Quantitative real time PCR cycles were performed as follows: 95°C for 2 minutes, 40 cycles of 95°C for 5seconds, 60°C for 15 seconds, 70°C for 15 seconds followed by 95 °C for 1 minute and 55 °C for 1minute.

2.2.10 Data Analysis of qRT-PCR via LinRegPCR

Initial concentrations (N0) of miR156 and miR171 were obtained via utilization of LinRegPCR program (Ruijter, Ramakers et al. 2009). First, LC480 Conversion program was used in order to convert data obtained from Roche Light Cycler 480 into applicable excel file form for LinRegPCR. After excel is uploaded to LinRegPCR, monitoring chemistry was chosen as DNA binding dye, amplification as single stranded cDNA, data file format as Light Cycler 480. Data baseline corrected option was chosen as 'No' and baselines were determined. Amplicon groups and tissue groups were determined and output excel is saved from file tab by choosing 'compact+complete'. Initial concentrations of miR156 and miR171 were obtained from output file and comparison analysis was conducted accordingly.

3. RESULTS

3.1 Growth Stages of Tobacco Plants Used in Transformation Studies

Tobacco seeds were grown in growth conditions as stated in Section 2.2.1. Growth stages of tobacco plants were exemplified with cultivar GK42 Muş TR42087in Figure 6. Illustrated stages were one week, three weeks, five weeks and sixteen weeks old wild type tobacco plants.

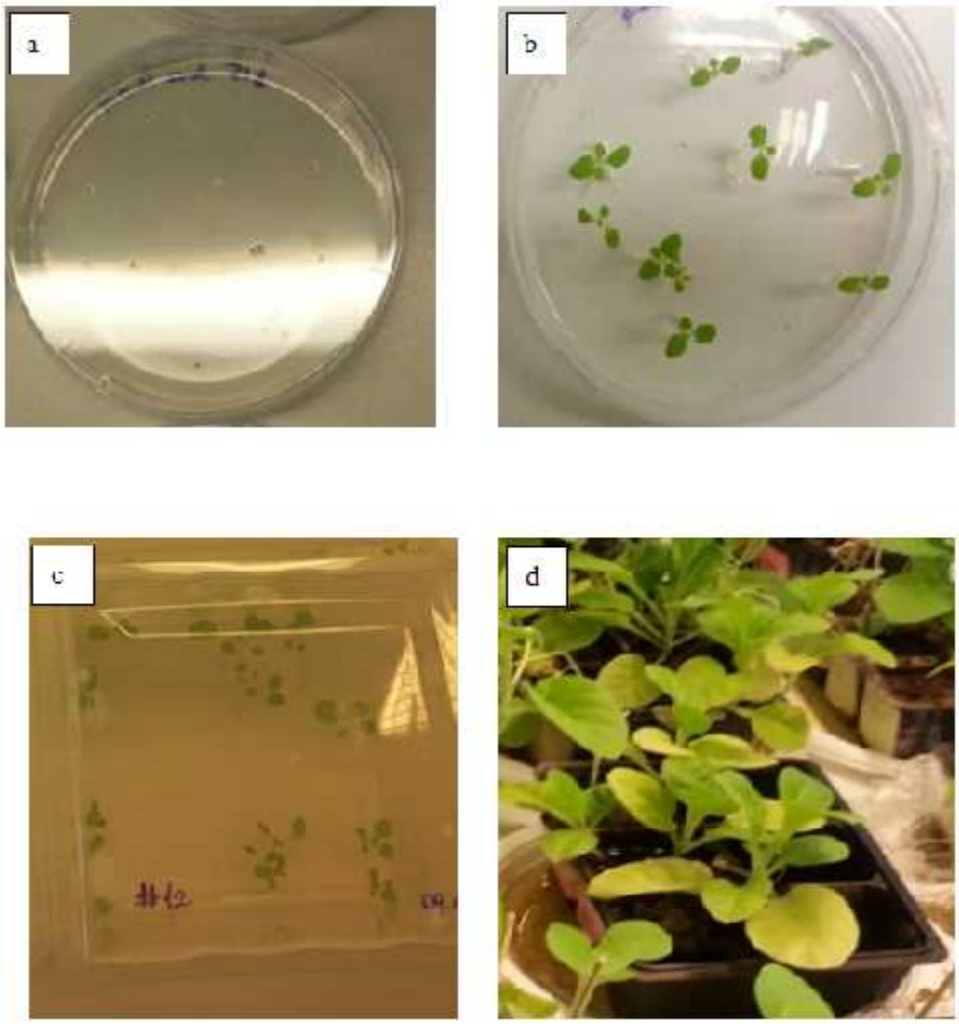


Figure 6 Developmental stages of tobacco cultivar GK42 Muş TR42087. a) 1 week b) 3 weeks c) 5 weeks d) 16 weeks old tobacco plants.

3.2 Gel Electrophoresis of PCR Products Chosen For Molecular Cloning

PCR product of DNA, isolated from 15 tobacco cultivars, were loaded on 1.5% agarose gel and subjected to 100Volt in electrophoresis tank. Distribution pattern of DNA fragments and200 bp long DNA fragment targeted to amplify is shown (Figure 7).

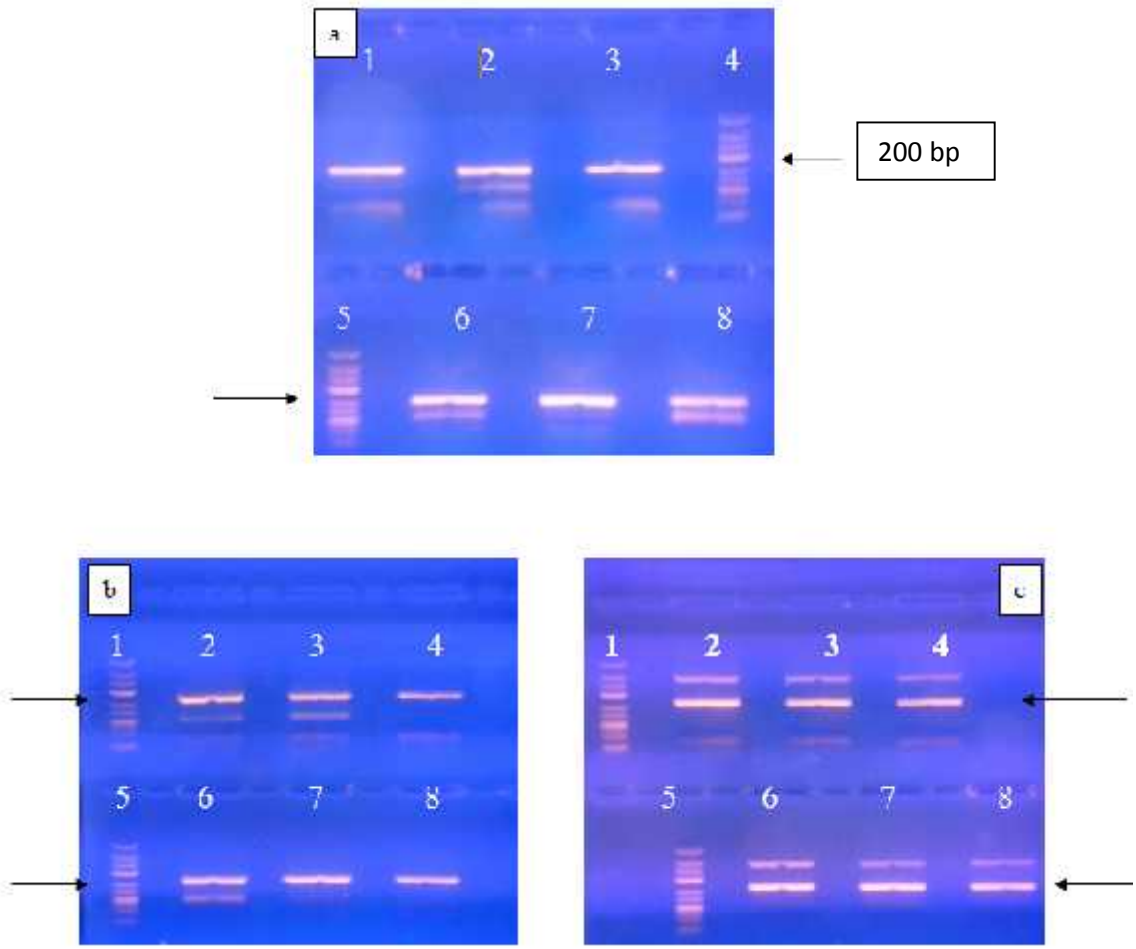


Figure 7 DNA samples of Nicotiana tabacum amplified with TN90_7 mir171b, TN90-7 mir156b and TN90-1 mir156d primers.(a) Tobacco cultivars amplified with primer TN90 -7 mir171b (1) GK83 TR 42771, (2) Sakarya TR42774, (3) GK84 Sakarya TR42772, (4, 5) Peqlab 25-700bp DNA ladder, (6) Reşatbey 97 TR68444, (7) Kokulu İzmir Tekel TE42986, (8) GK 94 Sakarya TR42782. (b, c) Tobacco cultivars amplified with primer TN90-7 mir156b and primer TN90-1 mir156d, respectively. (1)Peqlab 25-700bp DNA ladder, (2) GK83 TR 42771, (3) Sakarya TR42774, (4) GK84 Sakarya TR42772, (5) Peqlab 25-700bp DNA ladder, (6) Reşatbey 97 TR68444, (7) Kokulu İzmir Tekel TE42986, (8) GK 94 Sakarya TR42782.

3.3 Sequencing of PCR Products and Comparison with Pre-miRNA Gene Sequences

Gel pieces containing target DNA sequence was purified and sent for sequencing in order to verify that these sequences were pre-miRNA of miR156 and miR171 with *attB* flanking regions. By utilizing NCBI's Nucleotide BLAST program, Align Sequences Nucleotide BLAST option, sequencing results obtained from McLAB were blasted against stem loop miRNA sequences. Identity percentage of obtained sequences with TN90_1 pre-miR156d, TN90_7 pre-miR156b and TN90_7 pre-miR171b was found to be 98%, 99% and 99%, respectively (Figure 8). N was standing for nucleotide that could not be identified during sequencing process.

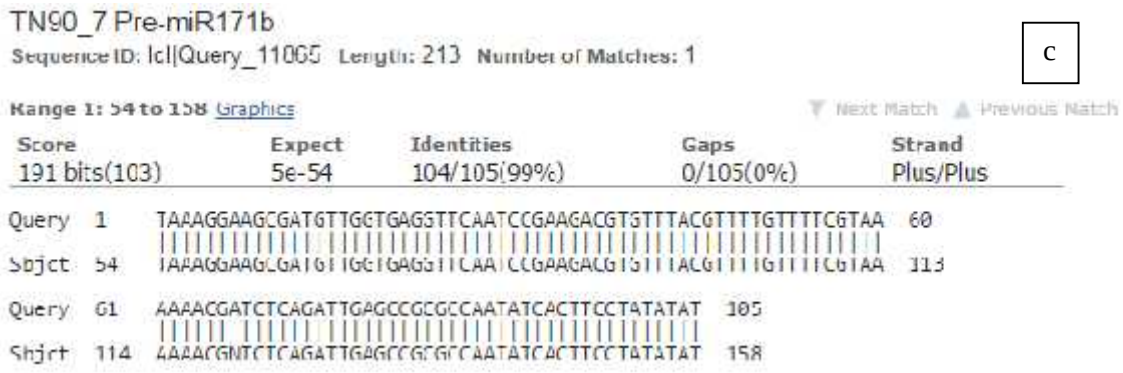


Figure 8 Percentage of identity and sequence comparison of designed sequences with pre-miRNA sequences a.TN90_1 Pre-miR156d b.TN90_7 Pre-miR156b c.TN90_7 Pre-miR171b.

3.4 Transformation of Cloning Products into *Escherichia coli* Cells

3.4.1 Transformation of BP Reaction Products into *Escherichia coli* Cells

Verification of plasmid transformation containing target sequences into *Escherichia coli* OmniMAX 2-T1^{R}cells was performed by Kanamycin antibiotic selection. As pDONR221 plasmid was resistant to Kanamycin but non-transformed *Escherichia coli* OmniMAX 2-T1^{R}cells was not, successful transformants were expected to grow in Kanamycin containing LB plates. As shown in Figure 9, negative control plate which containing E.coli2-T1^{R}cells lacking of pDONR221 did not grow while colony formation was observed in other plates (Figure 9a, 9b, 9c, 9e). Also, positive control containing *E.coli* DH5α cells transformed with pEXP-tet plasmid were found to be tetracycline resistance as expected (Figure 9d).

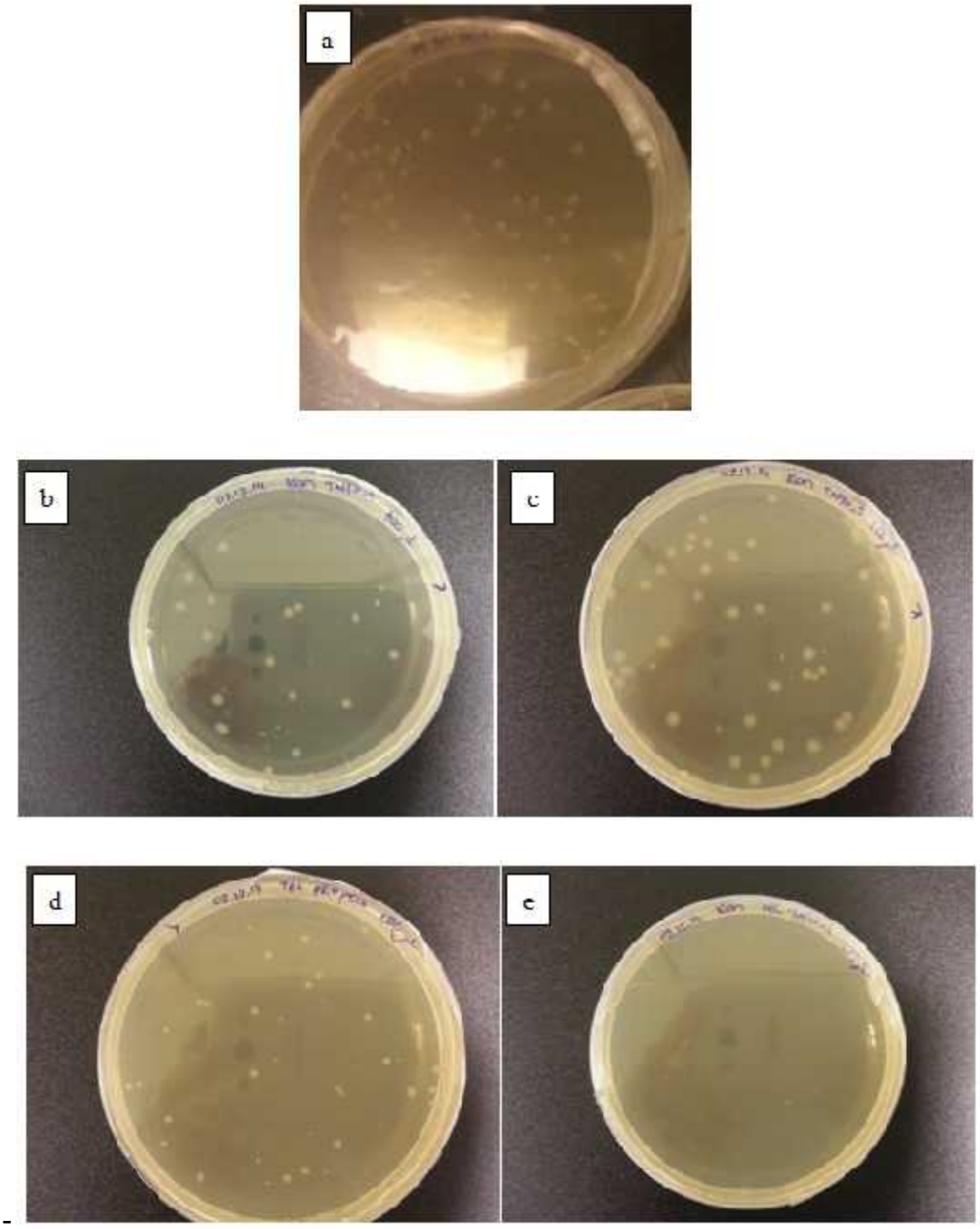
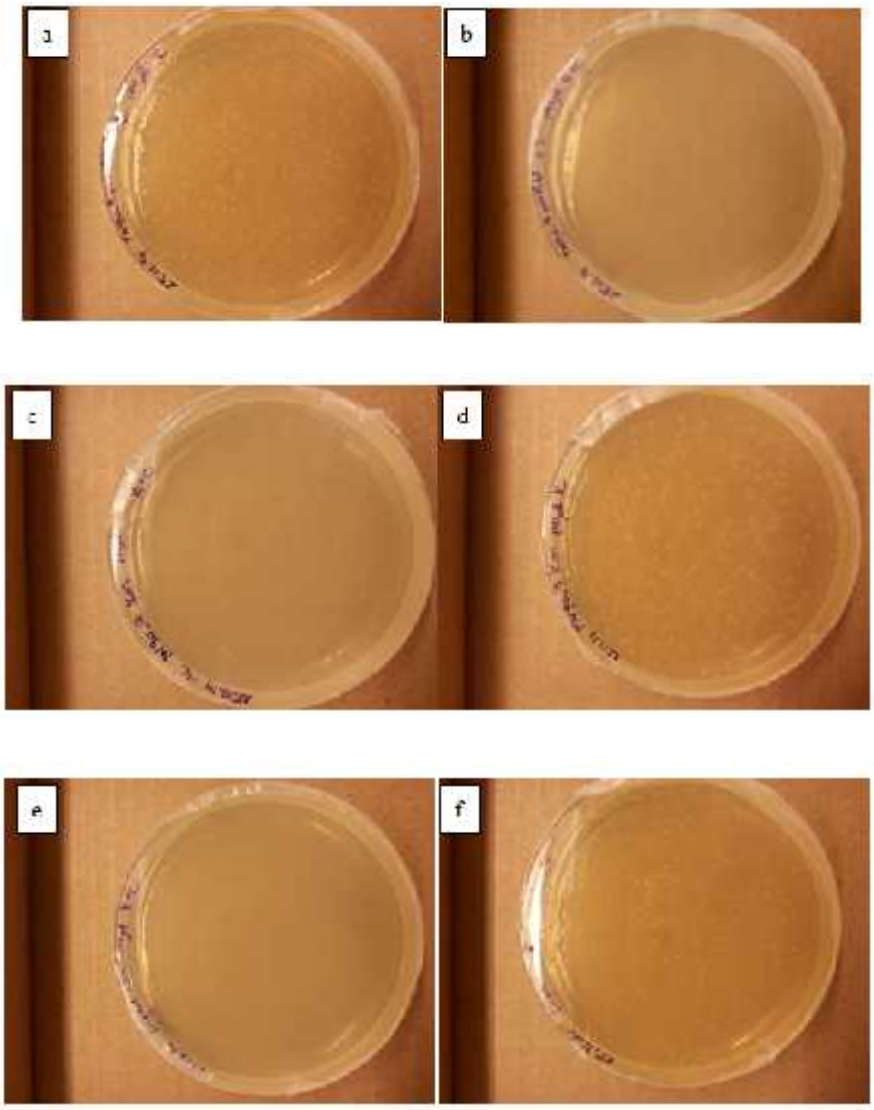


Figure 9 *Escherichia coli* colonies in antibiotic containing agar plates after transformation of BP reaction products.a. TN90_7 mir156b, Kanamycin, b. TN90_1 mir156d, Kanamycin c. TN90_7 miR171b, Kanamycin, d. pExp-tet positive control, Tetracyline, e. Negative control, Kanamycin.

3.4.2 Transformation of LR Reaction Products into *Escherichia coli* Cells

LB Agar plates of *E.coli* colonies transformed with plasmids containing LR reaction product were shown in Figure 10. The chloramphenicol resistance gene in pEarleyGate103 was replaced by the desired gene during LR reaction, which made possible counter-selection of destination clone with chloramphenicol. Correct destination clones should not be Chloramphenicol resistant but only should be Kanamycin resistant. Therefore, real transformants grew in Kanamycin containing plates but not in Kanamycin and Chloramphenicol plates (Figure 10).



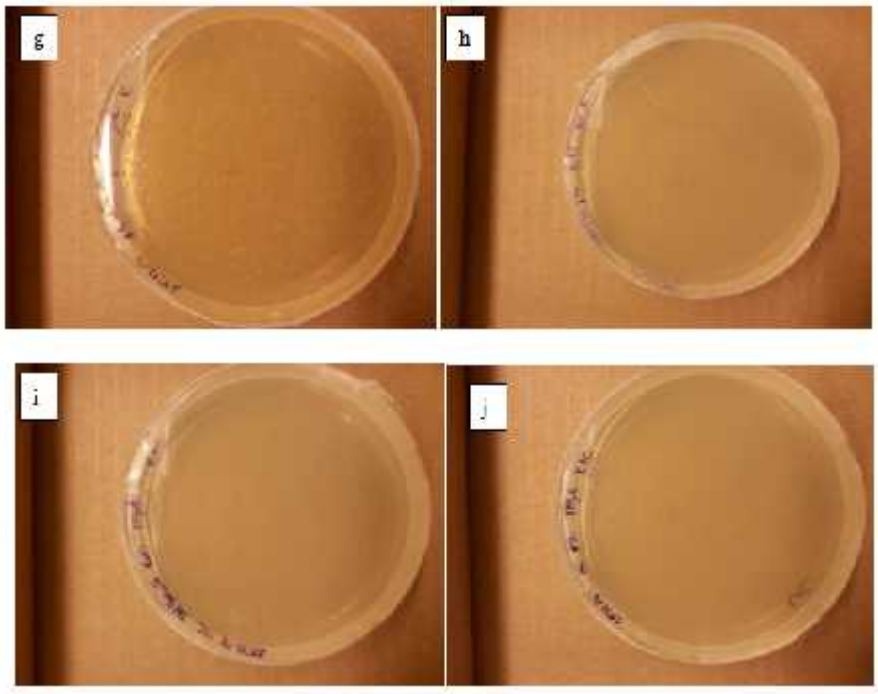


Figure 10 *Escherichia coli* colonies in antibiotic containing agar plates after transformation of LR reaction products. a) TN90_7 mir156d, Kanamycin, b) TN90_7 mir156d, Kanamycin and Chloramphenicol, c) TN90_7 mir171b Kanamycin and Chloramphenicol, d) TN90_7 mir171b, Kanamycin, e) TN90_1 mir156d, Kanamycin and Chloramphenicol f) TN90_1 mir156d, Kanamycin, g)100 µl pENTR-gus Kanamycin, h) pENTR-gus, Kanamycin and Chloramphenicol, i) Negative control, Kanamycin, j) Negative control, Kanamycin and Chloramphenicol.

3.5 Sequencing of LR Reaction Products and Comparison with Pre-miRNA Gene Sequences

Sequencing result of LR reaction products were blasted against original pre-miRNA sequences of miR156d, miR56b and miR171b. Percentage of sequence identity was found 100% for miR156d, 99% for miR156b and 98% for miR171b (Figure11a, 11b, 11c).

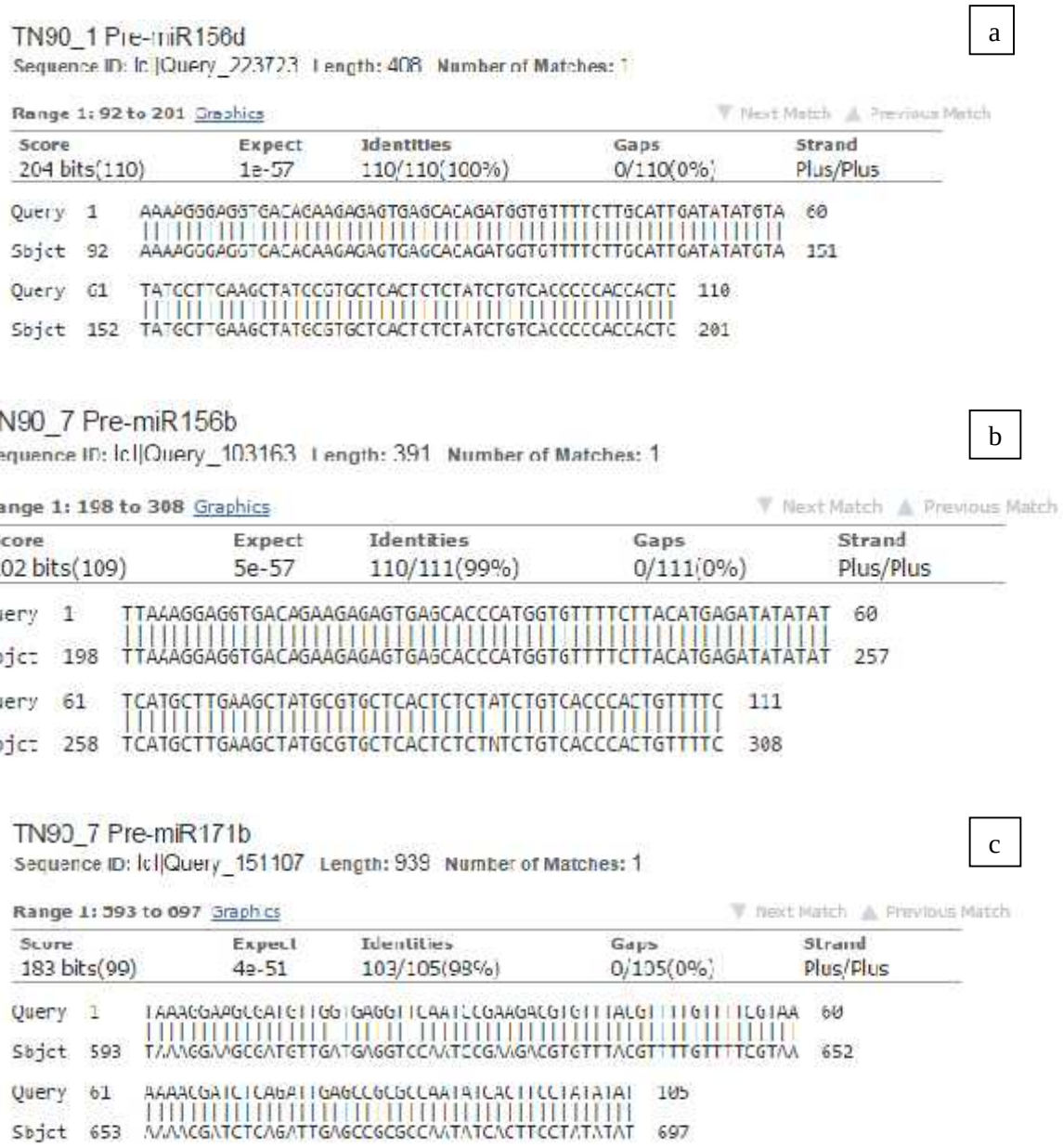


Figure 11 Percentage of identity and sequence comparison of sequences from LR reaction products with pre-miRNA sequences. a. TN90_1 Pre-miR156d, b.TN90_7 Pre-miR156b, c.TN90_7 Pre-miR171b.

3.6 Transformation of LR Reaction Products into *Agrobacterium tumefaciens* Cells

Plasmids isolated from successful transformants of LR reaction products were transformed into AGL1 strain of *Agrobacterium tumefaciens* cells as stated in Section 2.2.4.1. Transformed cells were incubated in LB agar plates containing 200 µg /ml Carbenicillin and 100 µg/ml Kanamycin and colony formation was observed as expected (Figure 12).

Carbenicillin resistance resulted from AGL1 strain of *Agrobacterium tumefaciens* cells and Kanamycin resistance resulted from plasmid pEarleyGate103.

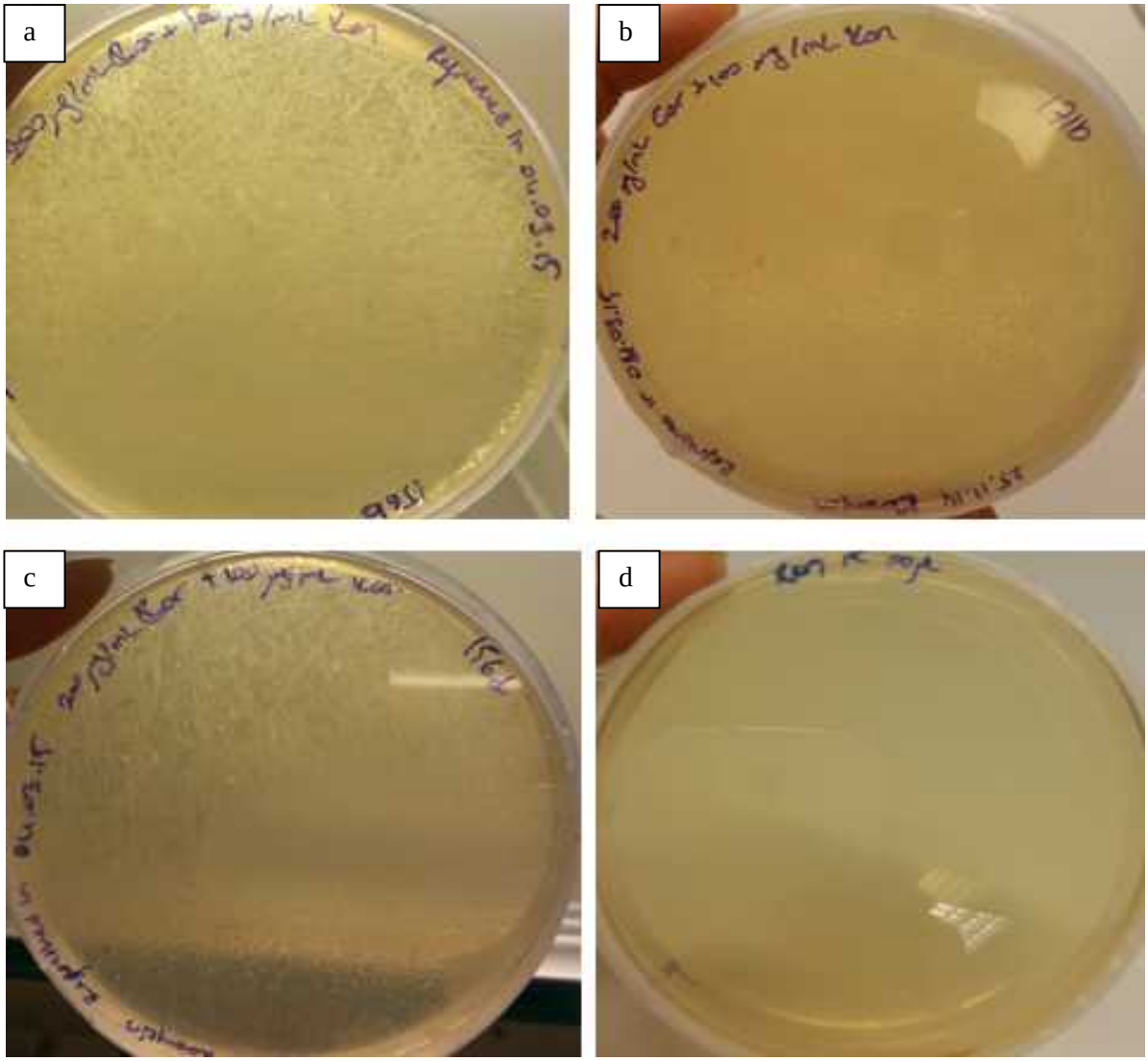


Figure 12 AGL1 strain of *Agrobacterium tumefaciens* cells in LB agar plates with 200 µg/ml Carbenicillin and 100 µg/ml Kanamycin. a.TN90_1 miR156b, b.TN90_7 miR171b, c. TN90_1 miR156d, d.Negative control.

3.7 Sequencing of LR Reaction Products from *Agrobacterium tumefaciens*and Comparison with Pre-miRNA Sequences

Plasmid DNA containing TN90_7 mir156b, TN90_7 mir171b and TN90_1 mir156d clones were sent to Mclab for sequencing in order to validate the transformation to *Agrobacterium*

*tumefaciens*cells. According to sequencing results (Figure 13), TN90_1 mir156d and TN90_7 mir171b showed 100% query coverage and 98% identity while TN90_7 mir156b showed no significant similarity; in other words, sequencing results obtained from forward and reverse complementary of reverse primer didn't overlap according to sequencing results. Therefore, TN90_7 mir171b and TN90_1 mir156d were used for the following steps.

Figure 13Percentage of identity and sequence comparison of sequences from LR reaction products isolated from AGL1 strain of *Agrobacterium tumefaciens* cells. a. TN90_1 Pre-miR156d, b.TN90_7 Pre-miR171b.

3.8 Generation of Putative Transgenic Tobacco Plants

3.8.1 Incubation in Pre-Culture Medium

Leaf with tiny pricks and leaf disks in pre-culture medium are shown in Figure 14. Tiny pricks and pre-culture medium were utilized to enhance transformation efficiency.

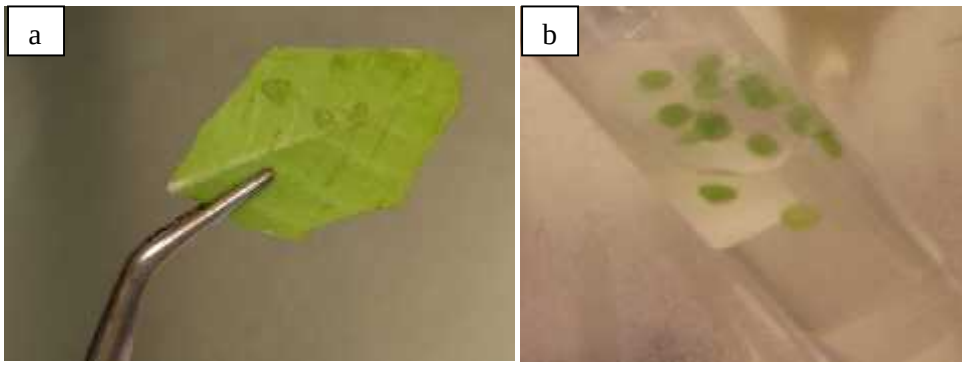


Figure 14 Pre-culture medium. a.Wounded leaf tissue of cultivar GK 94 Sakarya TR42782, b. Leaf tissue of cultivarGK42 Muş TR42087 in pre-culture medium.

3.8.2*Agrobacterium tumefaciens* Infection of Tobacco Leaves

Leaf explants incubated in pre-culture medium were exposed to infection medium containing AGL1 strain of *Agrobacterium tumefaciens* cells for 30 minutes (Figure 15).

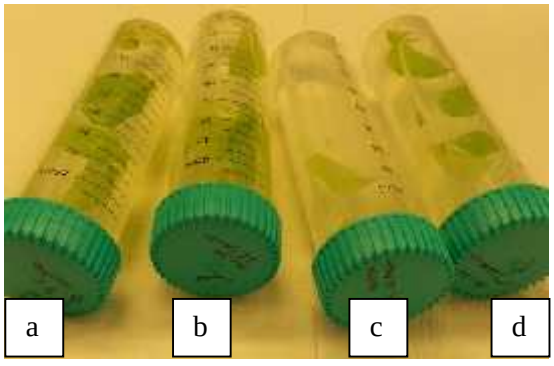


Figure 15Leaf explants in infection medium. a. GK Hakkari TR2084, b. Malatya TR4211, c. GK112 Bitlis TR42076, d. GK84 Sakarya TR42772.

3.8.3 Co-cultivation of Infected Leaf Tissues

After incubation in infection medium, leaf explants were placed on co-cultivation medium for two days at dark for the infection of *Agrobacterium tumefaciens* cells (Figure 16).

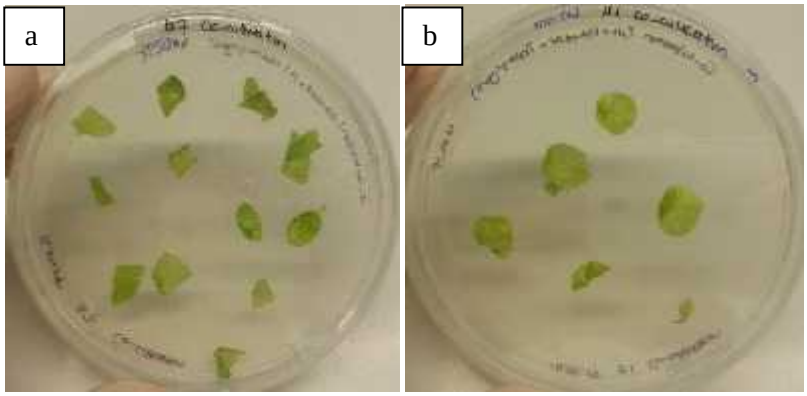


Figure 16 Leaf explants in co-cultivation medium. a. Cultivar GK84 Sakarya TR42772, b. GK Hakkari TR2084.

3.8.4 Resting Period of Transformed Leaf Tissues

At the end of incubation at co-cultivation medium, plants were transferred to resting medium (Figure 17).

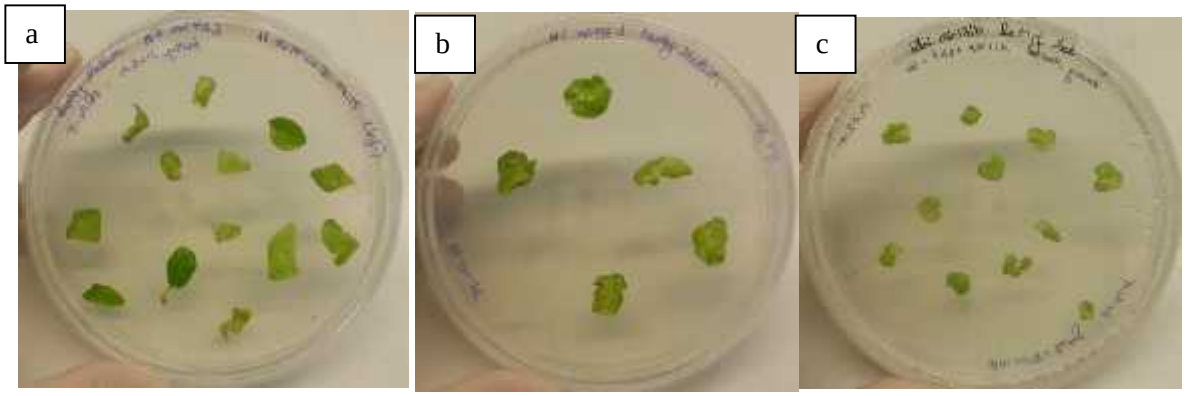


Figure 17 Leaf explants in resting medium. a. Cultivar GK84 Sakarya TR42772, b. GK Hakkari TR2084, c. *Nicotiana benthamiana* A.U.

3.8.5 Selection of Putative Transgenic Lines

In order to select putative transgenic plants, leaf explants were exposed to selection medium containing herbicide BASTA phosphinothricin (PPT). Transformed cells (tissues) hosting gene *'bar'* showed resistance to BASTA keeping their healthy form or showed recovery after a short period of yellow color state while color of non-transformed ones turned into yellow and these plants eventually died (Figure 18). In order to validate killing activity of herbicide concentration, 3mg/L BASTA was applied for non-transformed control leaf tissues and necrosis was observed (Figure 19). Putative transgenic cultivars surviving from herbicide selection were miR156 and miR171 transformants of Malatya TR4211, GK83 TR42771, GK84 Sakarya TR42772 and GK107 Bitlis TR42066.

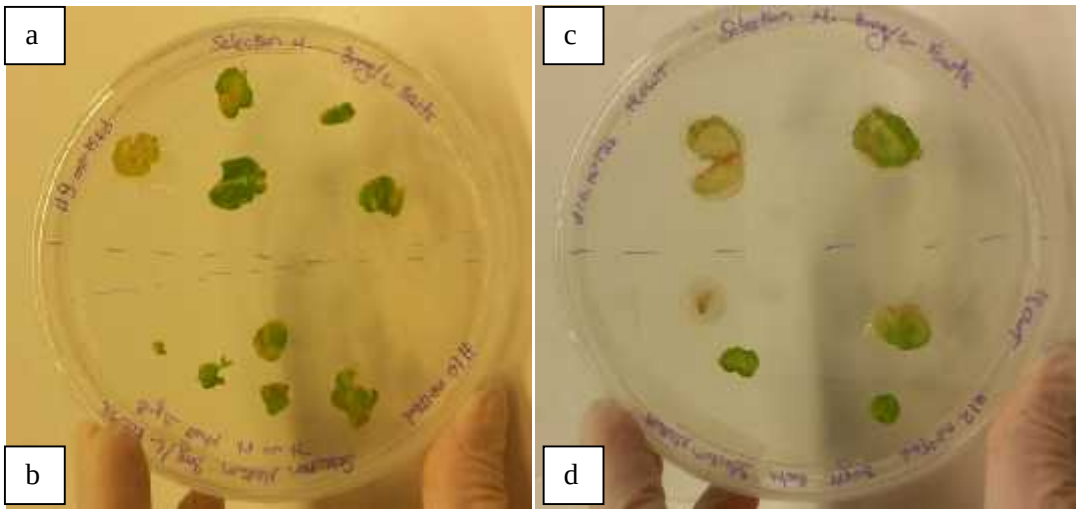


Figure 18 Second week of 3mg/L BASTA (PPT) selection on transformed tobacco leaf explants. a. Kokulu İzmir Tekel TE42986 miR156, b. GK 94 Sakarya TR42782 miR156, c. GK 94 Sakarya TR42782 miR171, d.GK42 Muş TR42087 miR156.

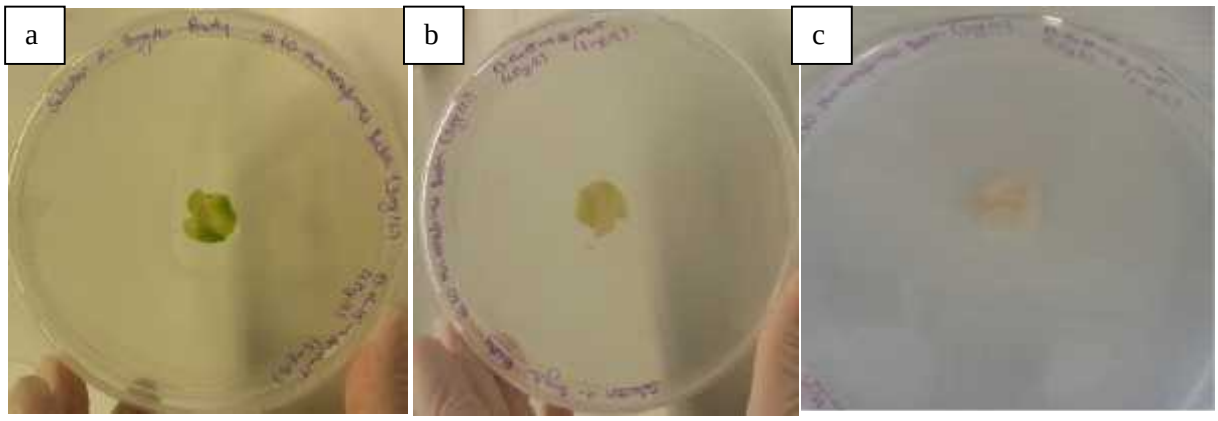


Figure 19BASTA selection on nontransformed tobacco cultivar GK 94 Sakarya TR42782.a. One week after BASTA application, b. Two weeks after BASTA application, c. Four weeks after BASTA application.

3.9 Callus Formation

3.9.1 Callus Formation in Non-transgenic Control Cultivars

Callus formation in leaf explants of non-tranformed control groups were shown below (Figure 20).

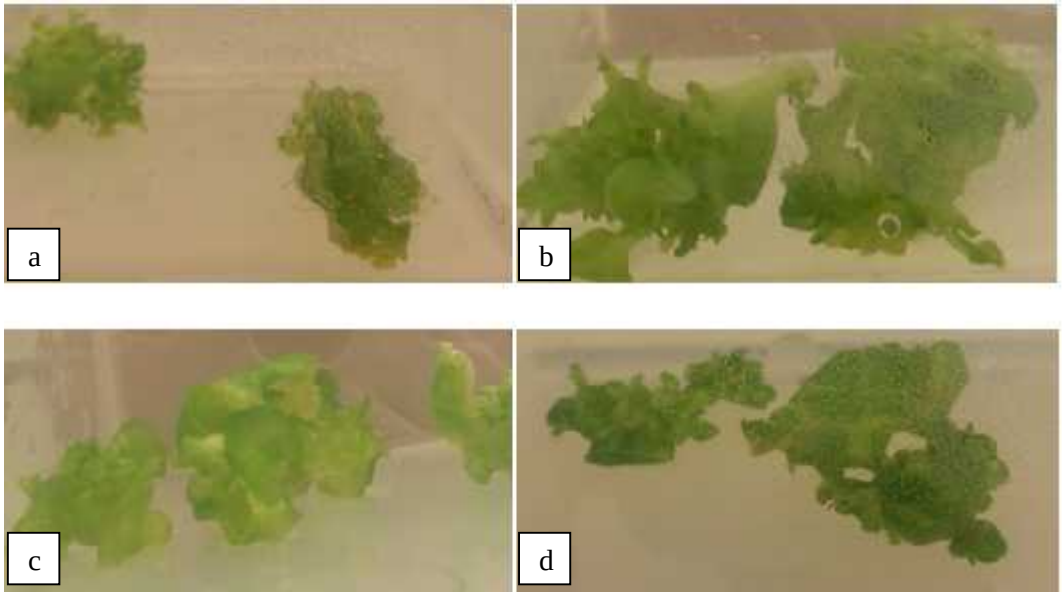


Figure 20 Three weeks old non-transformed control callus tissue. a. Malatya TR4211, b. GK83 TR 42771, c. GK84 Sakarya TR 42772, d.GK107 Bitlis TR42066.

3.9.2 Callus Formation in Putative Transgenic Cultivars

Callus formation in leaf explants of putative transgenic groups containing miR156 and miR171 were shown below (Figure 21).

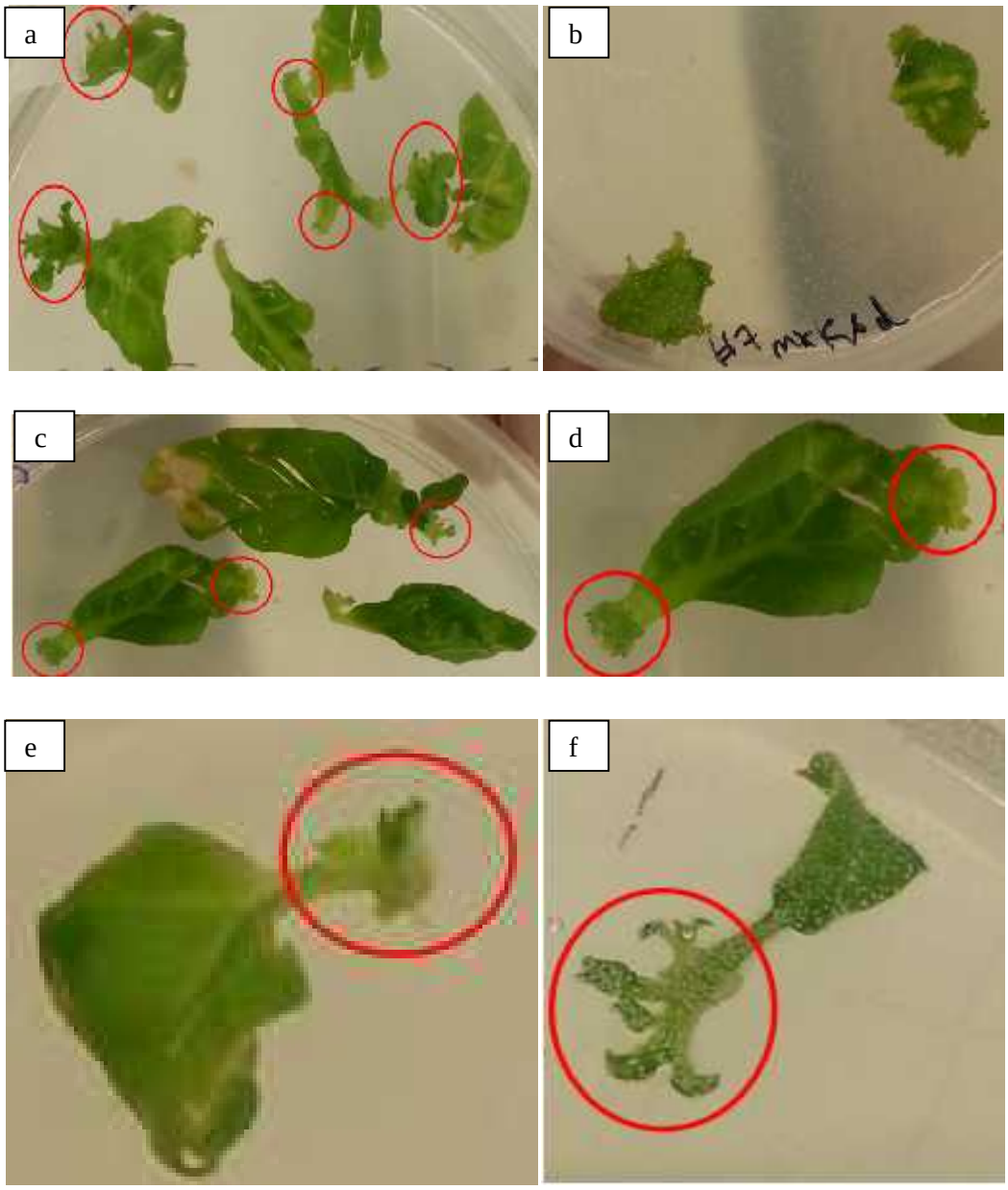


Figure 21 Callus tissue formations in putative transgenic cultivars. a. GK83 TR 42771 mir156d, b. GK84 Sakarya TR42772 mir156d, c. GK84 Sakarya TR42772 mir171b, d. GK84 Sakarya TR42772 mir171bfocused,e. GK107 Bitlis TR42066 miR171b, f. GK107 Bitlis TR42066 mir156d.

3.10Exposure of Tissue Samples to Drought Stress

Wildtype tobacco seedlings as well as callus tissue obtained from leaf explants of transformed and control cultivar groups were subjected to two day long and four day long drought stress as represented for cultivar GK84 Sakarya TR42772 in Figure 22 and Figure 23.

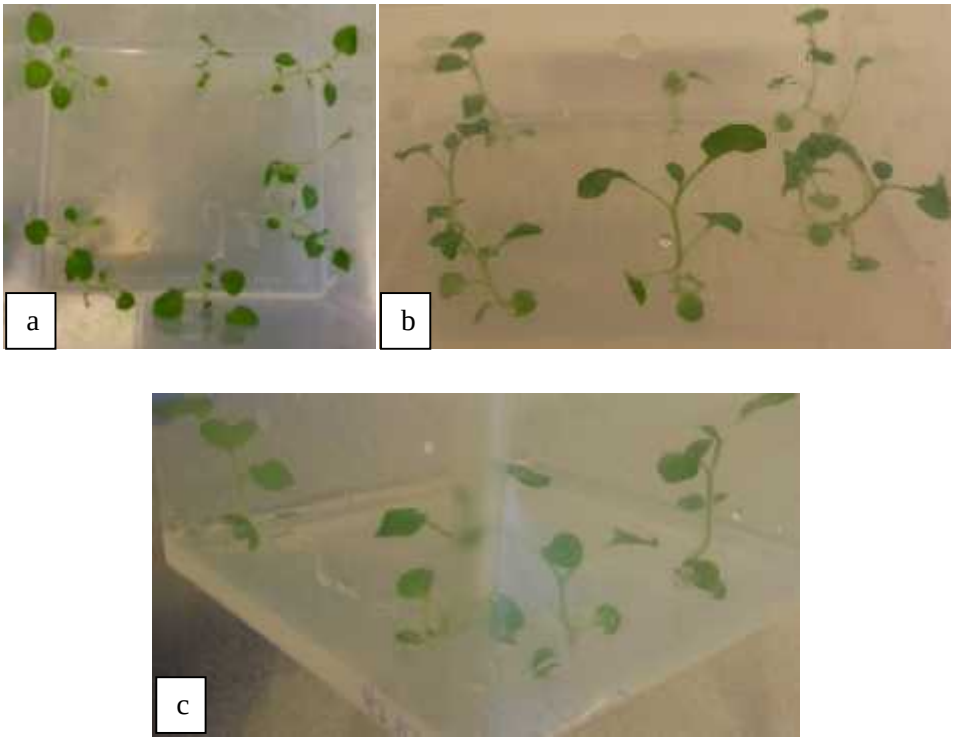


Figure 22 Wild type cultivar GK84 Sakarya TR42772 in drought stress medium. a. No stress, b. Two day long stress, c. Four day long stress.

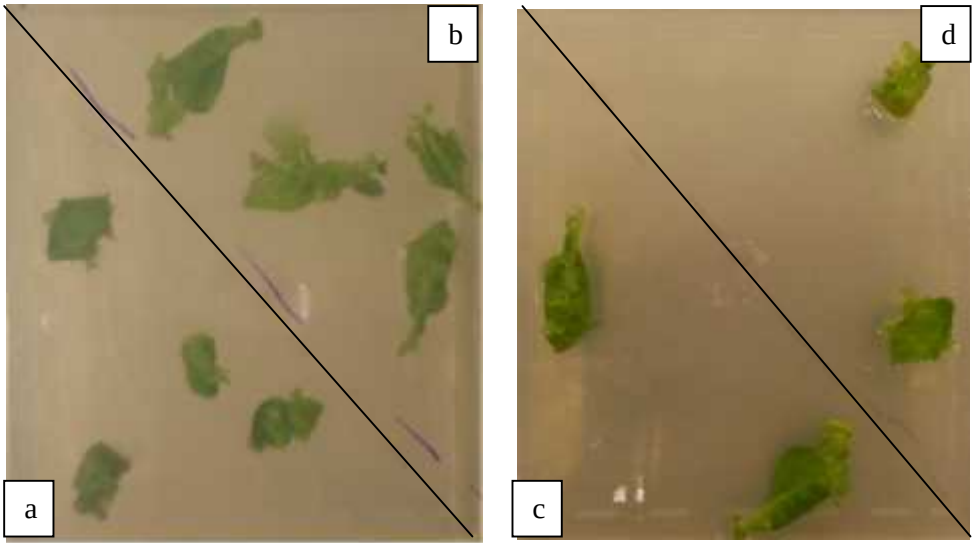


Figure 23 Transformed callus tissue of GK84 Sakarya TR42772 in drought stress medium. a. miR156 putative transgenic in two day long stress. b. miR171putative transgenic in two day long stress. c. miR156putative transgenic in four day long stress. d. miR171putative transgenic in four day long stress.

3.11Drought Stress Response of miR156 and miR171 in Wildtype Tobacco Seedlings

3.11.1.Initial Concentration of miR156 in Wildtype Tobacco Seedlings

Initial concentrations of miR156 in each cultivar with respect to stress conditions were compared (Figure 24). According to analysis, initial concentration of miR156 in Cultivar GK84 Sakarya TR42772, GK42 Muş TR42087, *Nicotiana benthamiana from* A.U. and *Nicotiana tabacum* from Ankara University was found higher in two day long drought stress samples compared to nonstress group. Similarly, initial concentration of miR156 in four day long stress groups ofGK84 Sakarya TR42772, GK42 Muş TR42087 and *Nicotiana tabacum* from Ankara University was found higher than nonstress group. Conversely, nonstress group of GK 94 Sakarya TR42782 showed higher initial concentration of miR156 compared to two days long and four days long stress applied groups. Data for *Nicotiana benthamiana* with four day long stress conditions could not be obtained.

Also, in comparison among cultivars without stress application, highest initial concentration was belonging to GK94 Sakarya TR42782 (Figure 24). Highest initial concentration

responding to two and four day long stress application was detected in GK42 Muş 42087 (Figure 24).

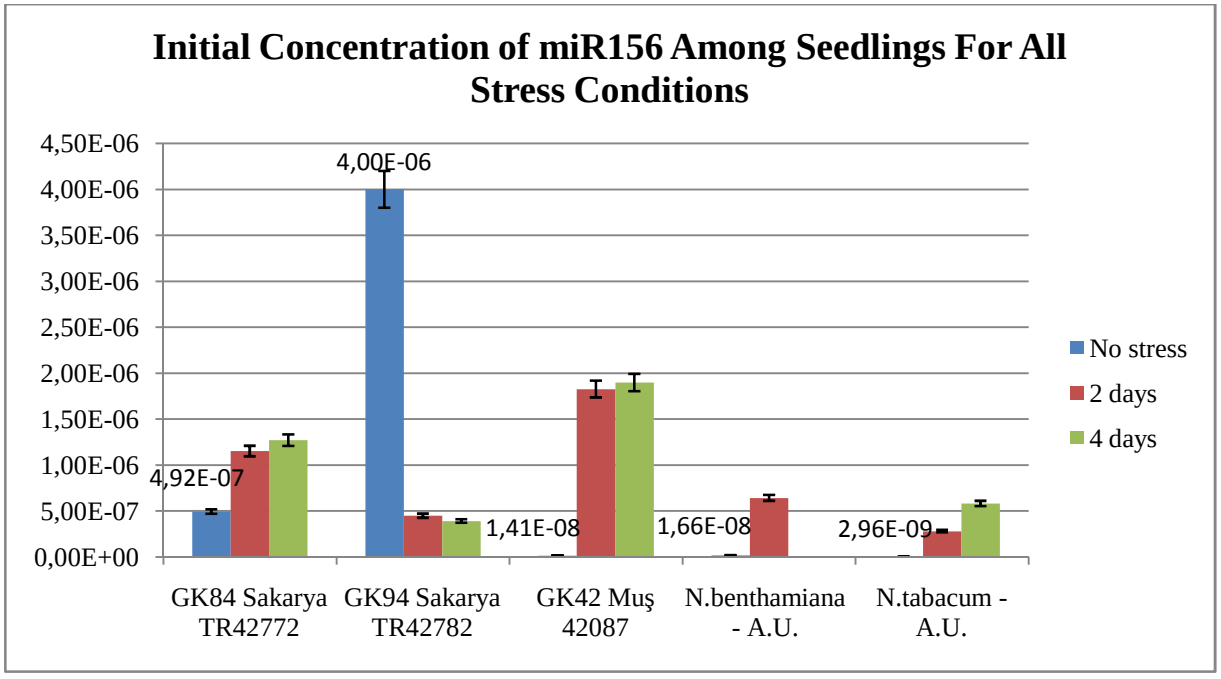


Figure 24 Initial concentrations (N0) of miR156 among seedlings for all stress conditions.

3.11.2. Relative Quantification of miR156 in Wild Type Tobacco Seedlings

Relative quantification of miR156 indicates ratio of no stress concentration to two day long and four day long stress conditions. Relative quantification of miR156 among cultivars except GK 94 SakaryaTR42782 resulted in increase of concentration level from two day long stress condition to four day long stress condition (Figure 25).

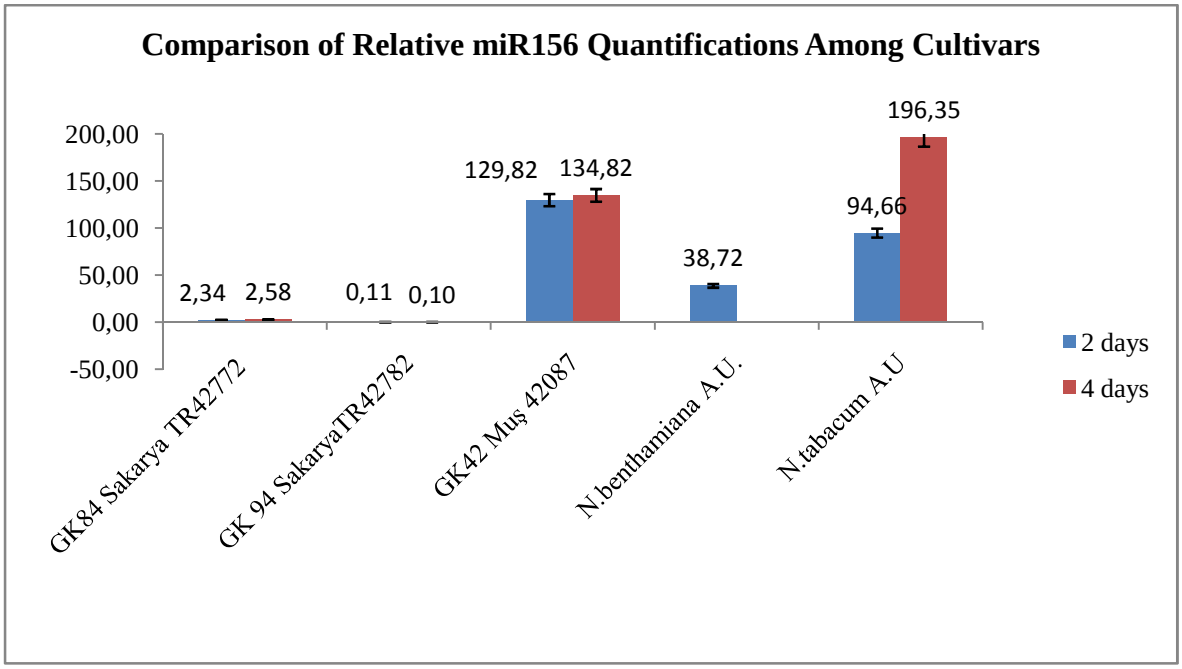


Figure 25 Relative quantification of miR156 among seedling cultivars.

3.11.3.Initial Concentration of miR171 in Wild Type Tobacco Seedlings

Similar to miR156 expression analysis, miR171 expression analysis was conducted with 8 weeks old non-transformed plant seedling. According to quantitative Real Time PCR results, initial concentration of miR171 in GK84 Sakarya TR42772 increased from no stress to two day long stress period and decreased in four day long stress period (Figure 26). MiR171 concentration in GK 94 Sakarya TR42782 showed tiny rate of decrease from no stress state to two day long stress state and then increased in four day long stress application. GK42 Muş TR42087 showed decrease in miR171 concentration from no stress to two and four stress period (Figure 26). MiR171 concentration in *Nicotiana benthamiana* from Ankara University increased in two day long stress and decreased in four day long stress which is exactly opposite pattern of *Nicotiana tabacum* from Ankara University.

Among cultivars without stress application and two day long stress exposure, *N. benthamiana* showed highest miR171 concentration while *N.tabacum* showed the lowest one. In four day long stress exposure, GK94 Sakarya TR42782 showed highest miR171 concentration while *N.tabacum* from Ankara University resulted in the lowest miR171 concentration, again (Figure 26).

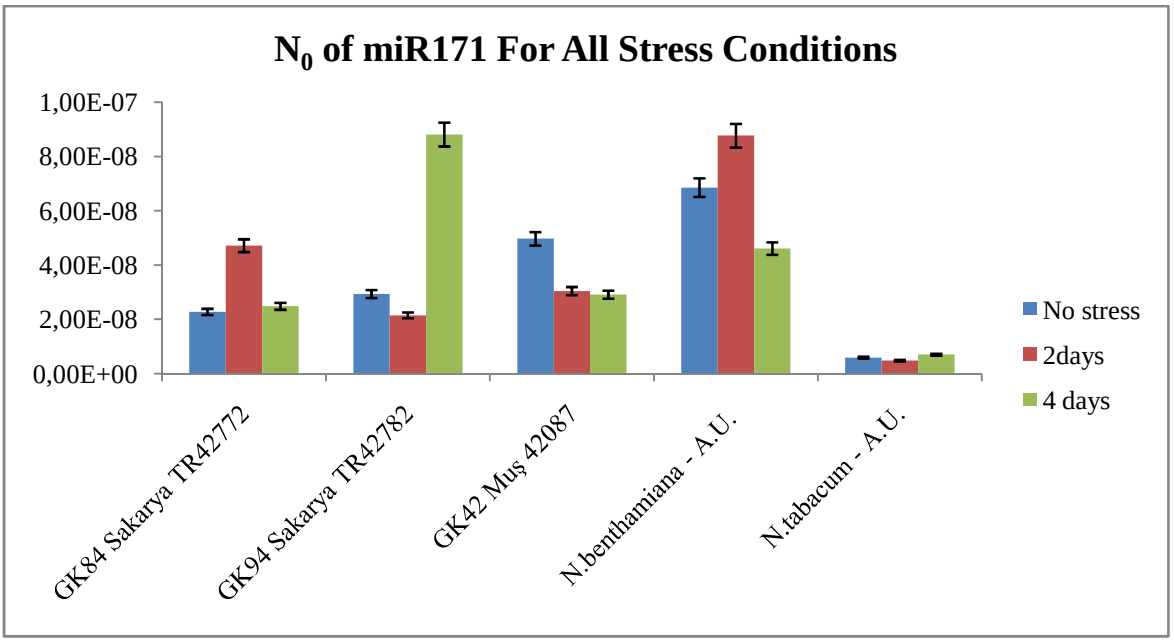


Figure 26 Comparison of initial miR171 concentrations of tobacco cultivars in stress conditions.

3.11.4 Relative Quantification of miR171 in Wild Type Tobacco Seedlings

Among relative quantifications of miR171 in two day long and four day long stress conditions relative to no stress condition, there is no certain pattern of increase or decrease is obtained (Figure 27).

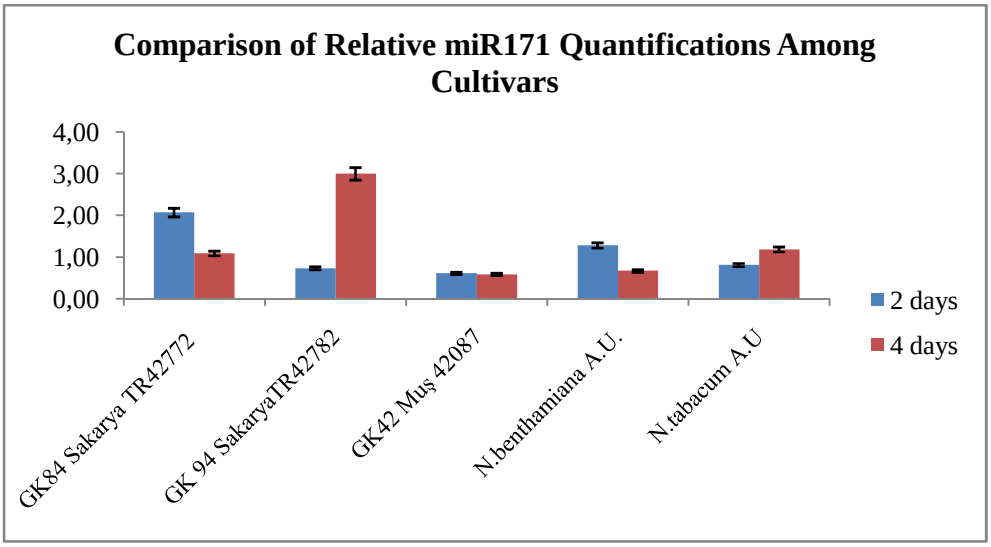


Figure 27 miR171 quantifications of wild type tobacco seedlings relative to no stress concentrations.

3.12. Drought Stress Response of miR156 and miR171 in Control and Transformed Tobacco Callus Tissue

3.12.1 Relative Quantification of miR156 in Control and Transformed Tobacco Callus Tissue

Quantification of miR156 putative transgenics relative to control groups showed that miR156 expression levels decreased in four day long stress application compared to two day long stress period. For all cultivars except GK83 TR 42771, miR156quantification of putative transgenics were lower than control groups as ratio of initial concentration of putative transgenics to control is below 1.0 for those cultivars (Figure 28). Data for miR156 quantification of putative transgenics GK107 Bitlis TR42066 relative to control group in four day long stress period could not be obtained.

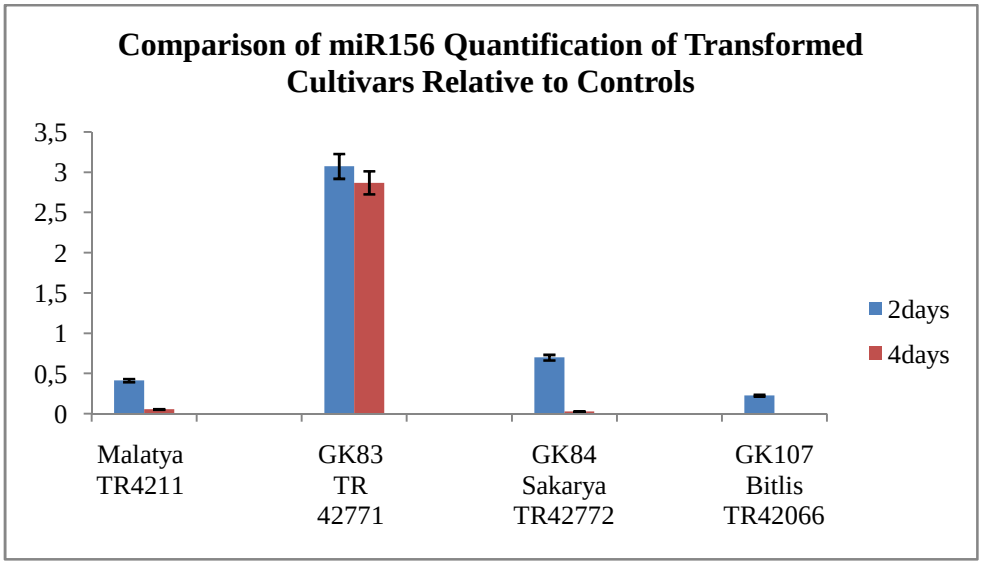


Figure 28 MiR156 relativequantifications of transformed callus in different drought stress conditions.

3.12.2. Relative Quantification of miR171 in Control and Transformed Tobacco Callus Tissue

Results of relative quantification of miR171 among putative transgenic cultivars showed that expression of miR171 increased from two days to four says stress period for putative transgenic callus tissues of Malatya TR4211, GK83 TR 42771 and GK107 Bitlis TR42066 (Figure 29). On the other hand, miR171 quantification of putative transgenic GK84 Sakarya TR42772 relative to control is decreased from two days long to four days long stress application (Figure 29).

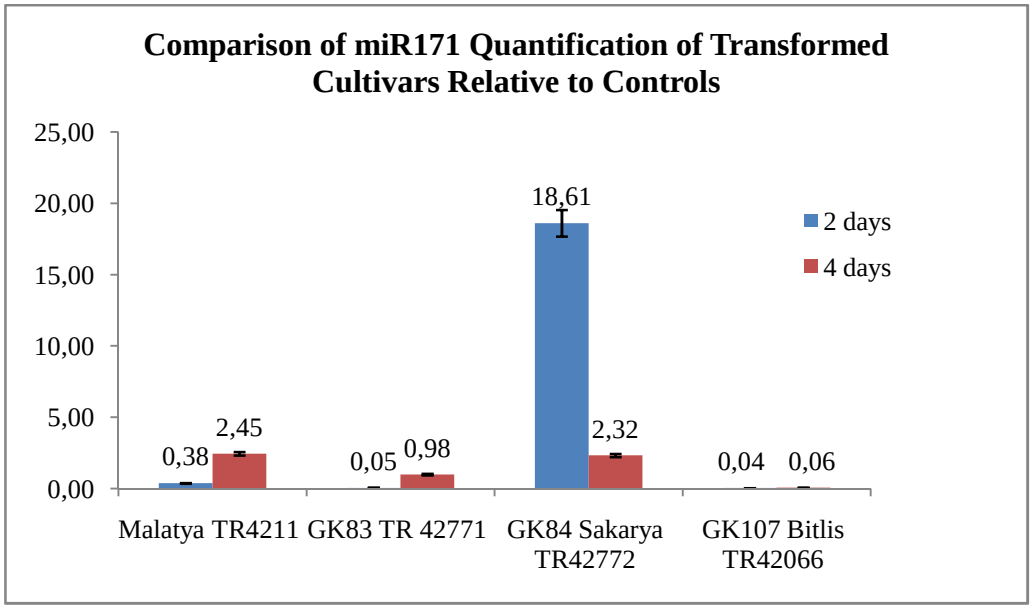


Figure 29MiR171 relative quantifications of transformed callus in different drought stress conditions.

3.13. Comparison of miR156 and miR171 Responses in Two Different Developmental Stage

Initial concentration of miR156 was found lower for wildtype callus tissue compared to eight week old wildtype seedling tissue although both show increase of response from two days long stress to four days long stress. On the other hand, initial concentration of miR171 for both wildtype callus and seedlings was found very low compared to miR156 concentrations. Yet, the result showed that higher concentration in seedling tissue compared to callus tissue is conserved for miR171, too.

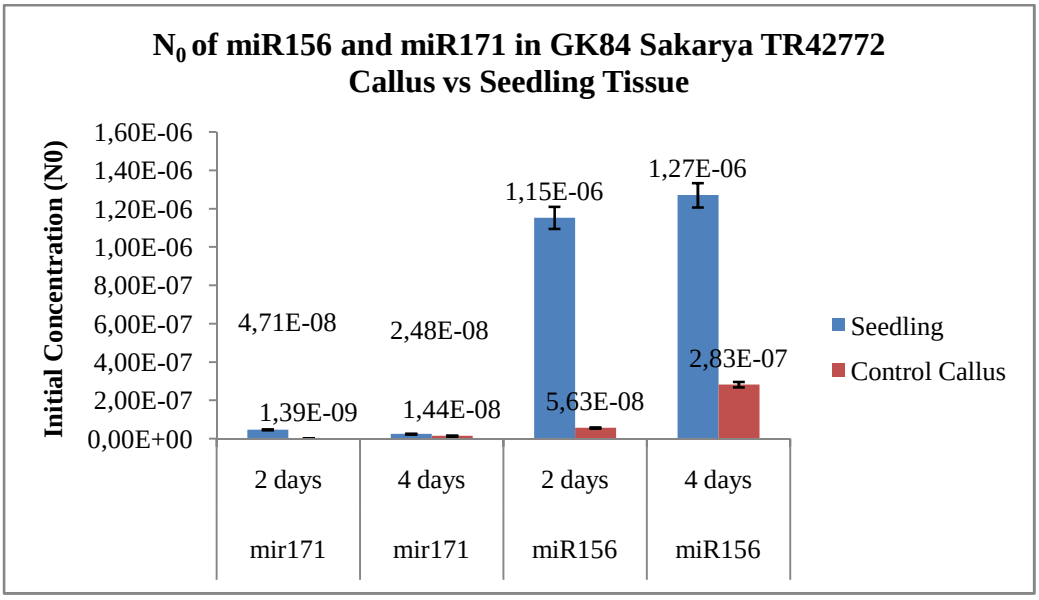


Figure 30 Initial concentrations of miR156 and miR171 for two different developmental stages of GK84 Sakarya TR42772.

3.14. Overall Comparison of miR156 and miR171 Relative Quantifications for Transformed Tissues

Among putative transfgenic cultivars, callus ofGK84 Sakarya TR42772 subjected to two days long stress resulted in the most drastic quantification of miR171. Relative quantifications of miR171 in Malatya TR4211 and GK84 Sakarya TR42772 cultivars with relative quantifications of miR156 in GK83 TR 42771 were found very close to each other (Figure 31).

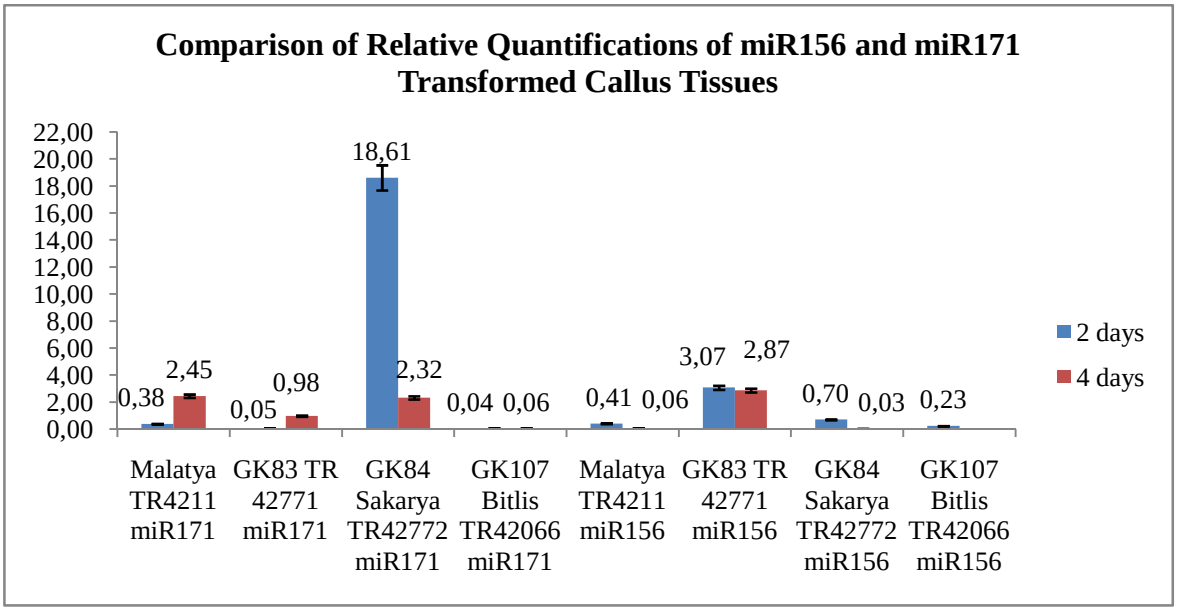


Figure 31 Comparison of relative quantifications of miR156 and miR171 in transformedcallus tissues.

4. DISCUSSION

MiR156 and miR171 are important microRNAs that are considered as having significant role in abiotic stress conditions. This study focuses on response of these microRNAs in tobacco cultivars exposed to drought stress conditions. Tobacco is a widely studied organism in plant biotechnology and genetics; yet, response of these two miRNAs in tobacco against drought stress has not been studied until our report.

Agrobacterium tumefaciens transformation caused loss of cultivars except Malatya TR4211, GK83 TR42771, GK84 Sakarya TR42772 and GK107 Bitlis TR42066. Loss of cultivars was due to the presence of *Agrobacterium tumefaciens* cells after co-cultivation medium. Transfer of leaf explants from co-cultivation to resting medium was involving a wash step of leaf explants with sterile distilled water in order to remove *Agrobacterium tumefaicens* cells from the surface of leaf explants. However, distilled water was most likely not enough and wash step could have been successful if the explants were washed with antibiotic containing water.

In our study, miR156 was up-regulated in all wild type cultivars except GK 94 SakaryaTR42782 cultivar of tobacco for all drought stress conditions. Similar to our findings, miR156 in *Arabidopsis thaliana* (Liu, Tian et al. 2008), barley (Kantar, Unver et al. 2010), *Triticum dicoccoides* (Kantar, Lucas et al. 2011), *Populus tomentosa* (Ren, Chen et al. 2012), *Prunus persica* (Eldem, Celikkol Akcay et al. 2012) were found to be up-regulated in drought stress while it is down-regulated in rice (Zhou, Liu et al. 2010). Among five wild type tobacco cultivars subjected to drought stress, GK Muş 42087 and *N.tabacum* A.U. resulted in highest fold change of miR156 level.

Unlike miR156, fold change of initial concentration in miR171 in drought stress was diverse among tobacco cultivars in our study. Zhou, Liu et al. (2010) previously stated that drought stress leads to down-regulation of miR171 in rice (*Oryza sativa*), *Populus trichocarpa* and*Medicago truncatula*. Kantar, Lucas et al. (2011) also found that miR171 was down-regulated against drought stress in *Triticum dicoccoides*. With a small fold change, we also found that miR171b was down-regulated in two days long stress group of GK94 Sakarya

TR42772, GK42 Muş TR42087, *N.tabacum* A.U and four days long stress group of GK42 Muş TR42087 and *N.benthamiana* A.U. On the other hand, Zhou, Liu et al. (2010) found that miR171b was up-regulated in *Selaginella moellendorffi* against drought stress. This is parallel to our finding, which is threefold up-regulation of miR171b in wild type GK94 Sakarya TR42772 in four days stress condition and twofold up-regulation in wild type GK84 Sakarya TR42772 in two days long stress condition. Up-regulation of miR171 as a drought stress response was also previously stated for *Arabidopsis thaliana* (Liu, Tian et al. 2008). MiR171 expression level of four day long stress group of GK84 Sakarya TR42772 and *Nicotiana tabacum* A.U. as well as two days long stress group of *Nicotiana benthamiana* A.U. were almost remained unchanged in our study.

In the second part of the project, which is detection of drought stress response of overexpressed tobacco cultivars with miR156 and miR171, putative transgenic and control groups of Malatya TR4211, GK83 TR 42771, GK84 Sakarya TR42772 and GK107 Bitlis TR42066 were utilized. Putative transgenic tobacco cultivars showed retarded callus formation compared with nontrangenic tobacco cultivars. Moreover, nontransgenic leaf explants completely formed callus while putative transgenics formed incomplete callus tissue on leaf explants. This might have been attributed to BASTA herbicide and antibiotic applications that caused growth retardation in putative transgenic leaf explants.

Fold change of miR156 in putative transgenic cultivars was found below one relative to control groups for Malatya TR4211, GK84 Sakarya TR42772 and GK107 Bitlis TR42066. In other words, initial concentration of miR156 of putatively transformed group of these three cultivars in two days and four days stress application never exceeded initial concentration of nontransformed plants. Among putatively transformed cultivars, only GK83 TR 42771resulted in higher miR156 concentration level in putatively transformed ones compared to control group. Although miR156 concentration ratio of putatively transformed GK83 TR 42771to control GK83 TR 42771never decrease to below one, it is significant that this cultivar also showed lower fold change in four day long stress condition than two days long stress conditions. Among all putatively transformed cultivars tested with miR156 concentration levels, fold change was found higher in two days long stress group than four days long stress group.

Interestingly, fold change of miR171 in putatively transformed cultivars were more diversified compared to fold change of miR156. To illustrate, two days long stress application led to lower concentration of miR171 in transformed Malatya TR4211, GK83 TR 42771 and GK107 Bitlis TR42066 compared to nontransformed groups of these cultivars, giving rise to a concentration ratio lower than one, indicating that miR171 might be down-regulated among putatively transformed of these cultivars. On the other hand, GK84 Sakarya TR42772 showed significant miR171 concentration difference between putatively transformed and nontransformed groups in two days long stress condition, which was eighteen-fold higher in putatively transformed group than nontransformed group. Surprisingly, this ratio fell to twofold in four days long stress condition for the same cultivar. Conversely, four day long stress led to increase in miR171 concentration ratio of putatively transformed groups to control groups among Malatya TR4211, GK83 TR 42771 and GK107 Bitlis TR42066. Although this change was tiny for GK107 Bitlis TR42066 and kept this ratio below one, it was enough to exceed miR171 concentration ratio of putatively transformed ones to control group above two for cultivar Malatya TR4211. Similarly, four day long stress application gave rise to higher change in miR171 concentration than two day long stress for cultivar GK83 TR 42771; yet, the ratio of miR171 concentration of putatively transformed to control group could hardly get close to one. Among these four cultivars mentioned above, GK84 Sakarya TR42772 gave the most striking result for change in miR171 level.

In this study, there was a common cultivar named GK84 Sakarya TR42772 among wild type seedling cultivars, nontransformed callus tissue and putatively transformed callus tissue. Relative miRNA quantifications of this cultivar compared by checking the ratio of initial miR156 and miR171 concentrations of control callus tissue to wildtype seedlings in two days long and four days long stress conditions. According to this comparison, initial concentration of miR156 in wild type seedling was found fifty times higher than miR156 in control callus tissues for two days long stress condition. Also, miR156 in wild type seedling was found four times higher than miR156 in control callus tissues for four days long stress condition. On the other hand, initial concentration of miR171 in seedling and callus group was found much lower than miR156. For two days long stress condition, initial concentration of miR171 was almost higher in seedling than miR171 initial concentration in callus tissues. However, this difference is not easily noticeable on graph as both values are really small compared to miR156 concentrations. On the other hand, four days long stress application did not result in a huge difference of miR171 concentration between seedling and callus tissue. The ratio of

miR171 concentration between these two was found two. In other words, miR171 concentration in seedling was found two fold higher than miR171 concentration in callus tissue.

Among putatively transformed cultivars subjected to two days long stress and four days long stress conditions, overexpression of miR171 could be achieved in GK84 Sakarya TR42772 cultivar as miR171 concentration level of putatively transformed callus tissues of this cultivar was found higher than control callus tissues of GK84 Sakarya TR42772. Similarly, GK83 TR42771 cultivar could be stated as miR156-overexpressed as miR156 concentration in putatively transformed callus tissues of GK83 TR42771 was found higher than control callus tissues of GK83 TR42771 for both two days long and four days long stress exposure.

Studies seeking for role of microRNAs in stress response promise utilization of these miRNAs for development of stress tolerant plants. As a future perspective, our study could be maintained by conducting development of putatively transformed callus tissues to mature plants and studying survival capability of these miR156-overexpressed and miR171-overexpressed cultivars under drought stress conditions.

5. CONCLUSION

microRNAs (miRNAs) are small single stranded RNA molecules with vital roles to regulate mRNAs. In plants, miR156 and miR171 are among these small molecules with agricultural significance for plant development and abiotic stress tolerance. As a major abiotic stress factor, drought affects plant growth and development by modifications in physiological, biochemical and morphological structure of plants including the ones with global, agricultural and economic value. Among these plants, *Nicotiana* is the fifth largest genus of *Solenaceae* family.

This study covers miR156 and miR171 quantification responding to drought stress conditions in different Turkish tobacco cultivars. In the first part of the study, wild type seedlings of GK42 Muş 42087 and *Nicotiana tabacum* from Ankara University were found most responsive cultivars with respect to miR156 and GK94 Sakarya TR42782 with respect to miR171. In the second part of the study, putatively transformed callus of GK84 Sakarya TR42772 was found to be miR171b-overexpressed and putatively transformed callus of GK83 TR42771 was found to be miR156-overexpressed.

Putative transgenic cultivars reported in this study could be an important potential for arise of drought tolerant tobacco plants.Novelty of this study is the fact that it represents the first data on drought response of miR156 and miR171 in tobacco plants, which could be utilized to elucidate the molecular mechanisms of drought stress and the roles of miR156 and miR171 in tobacco.

APPENDIX A

Molecular Biology Kits

Genomic DNA Isolation Kit	Anatolia	XPD002
Gel Extraction Kit	Qiagen QIAquick	28706
Gateway Cloning Systems	Invitrogen	12535 – 029
Plasmid DNA Isolation Kit	Thermo Scientific GeneJET	K0502

APPENDIX B

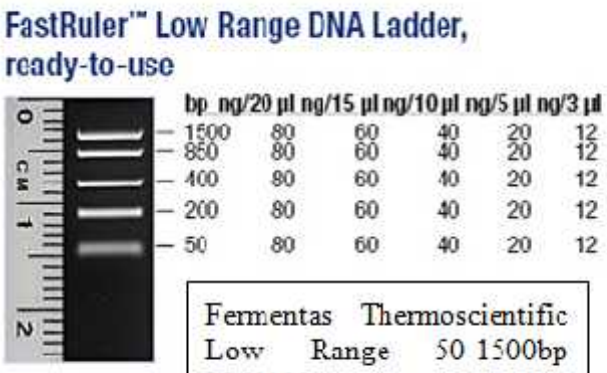
Equipments

Autoclave:	Hirayama, Hiclave HV-110, JAPAN
	Nüve 0T 032, TÜRKİYE
Balance:	Sartorius, BP221S, GERMANY
	Schimadzu, Libror EB-3 200 HU, JAPAN
Centrifuge:	Microfuge 18 Centrifuge Beckman Coulter, USA
	Kendro Lab. Prod., Heraeus Multifuge 3S-R, GERMANY
	Kendro Lab. Prod., Sorvall RC5C Plus, USA
	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
Deepfreeze:	-20 °C Bosch, TURKEY
	-80 °C Thermo electron corporation, USA
Distilled Water:	Millipore, Elix-S, FRANCE
	Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Labnet Gel XL Ultra V-2, USA
	Biogen Inc., USA
	Biorad Inc., USA
Fiter papers:	Whatman General Purpose Filtration Paper
	WHASE1141, Sigma, MO, USA
Gel Documentation:	Biorad Universal Hood II F1-F2 Fuses Type T2A, USA
	Biorad, UV-Transilluminator 2000, USA
Glassine crossing bags:	Focus Packaging & Design Ltd, North Lincolnshire, UK
Growth chamber:	Digitech DG12, Ankara, TURKEY
Heating block:	HDV Life Sciences, AUSTRIA
	Thermostat Bio TDB-100, LATVIA
Ice Machine:	Scotsman Inc., AF20, USA
Incubator:	Innova 4330, USA
	Memmert, Modell 300, GERMANY

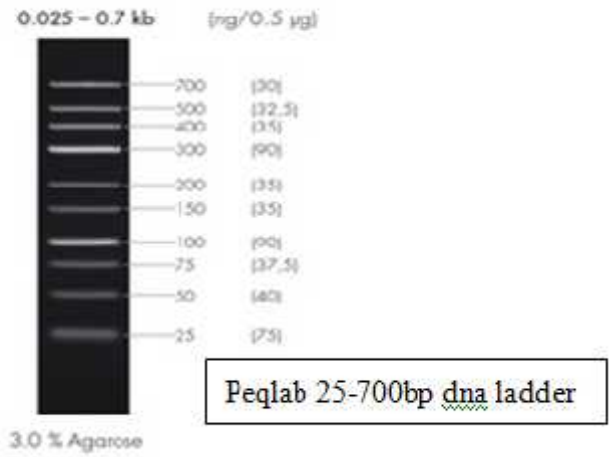
	Memmert, Modell 600, GERMANY
Laminar Flow:	Holten LaminAir Model 1.8 82034000, DENMARK
	Heraeus, Modell HS 12, GERMANY
Magnetic Stirrer:	VELP Scientifica, ITALY
Microliter Pipette:	Gilson, Pipetman, FRANCE
	Eppendorf, GERMANY
Microwave Oven:	Bosch, TÜRKİYE
Nitrogen tanks:	Linde Industrial Gases, TURKEY
Oven:	Memmert D06062 Modell 600, GERMANY
pH Meter:	WTW, pH540, GLP MultiCal, GERMANY
Power Supply:	Biorad, PowerPac 300, USA
Real-Time PCR:	Roche LightCycler 480 Instrument II
Refrigerator: +4 oC	Bosh, TÜRKİYE
Shaker:	Forma Scientific, Orbital Shaker 4520, USA
	GFL, Shaker 3011, USA
	New Brunswick Sci., InnovaTM 4330, USA
	New Brunswick Scientific Excells E24, USA
Spectrophotometer:	Amersham Biosciences Ultraspec 2100 pro, USA
	Nanodrop, ND-1000, USA
Sterilizer:	Steri 350, Simon Keller Ltd., SWITZERLAND
Thermocyler:	Eppendorf, Mastercycler Gradient, GERMANY
	Biorad Gradient Cycler DNA Engine, USA
Vortex Mixer:	VELP Scientifica 2X3, ITALY
Water bath:	Memmert, GERMANY

APPENDIX C

DNA Ladders



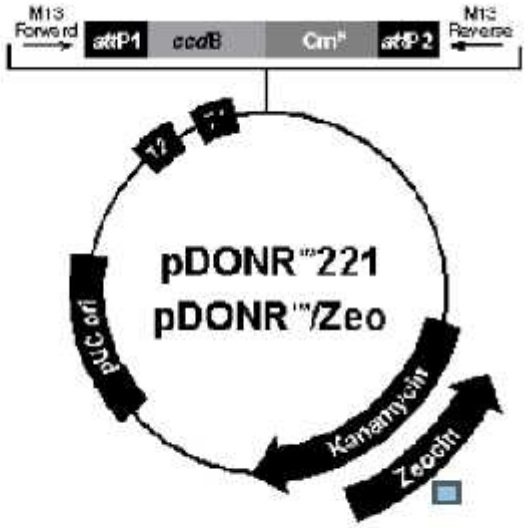
Fermentas Thermoscientific Low Range 50 1500bp SM1103



Peqlab 25-700bp dna ladder

APPENDIX D

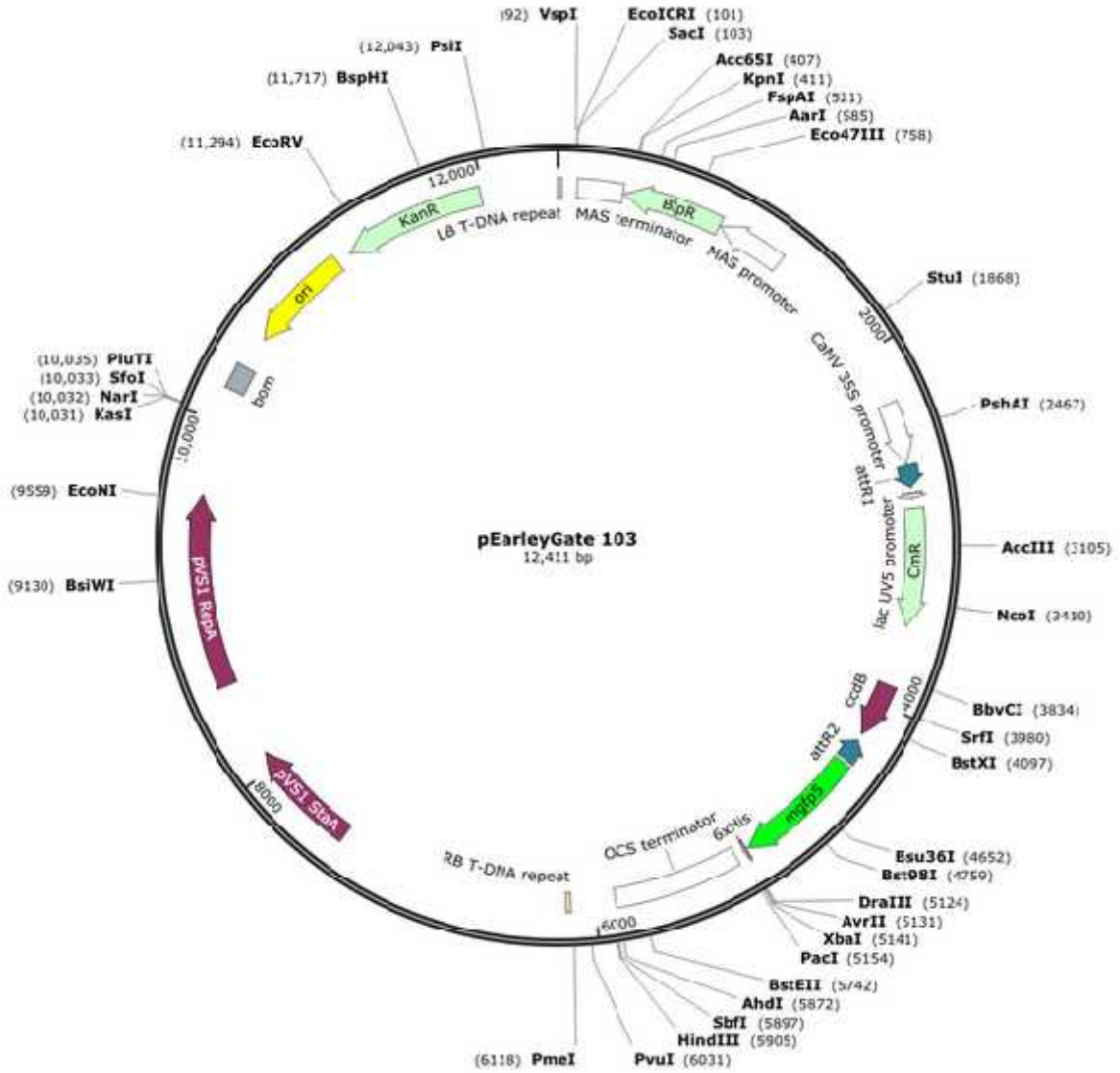
Vector Map of Pdonr 221



Comments for:	pDONR™221 4762 nucleotides	pDONR™/Zeo 4291 nucleotides
rrnB T2 transcription termination sequence (c):	268-295	268-295
rrnB T1 transcription termination sequence (c):	427-470	427-470
M13 Forward (-20) priming site:	537-552	537-552
attP1:	570-801	570-801
ccdB gene (c):	1197-1502	1197-1502
Chloramphenicol resistance gene (c):	1847-2506	1847-2506
attP2 (c):	2754-2905	2754-2905
M13 Reverse priming site:	3027-3043	3027-3043
Kanamycin resistance gene:	3155-3965	---
EM7 promoter (c):	---	3488-3552
Zeocin resistance gene (c):	---	3111-3485
pUC origin:	4066-4759	3615-4288
(c) = complementary strand		

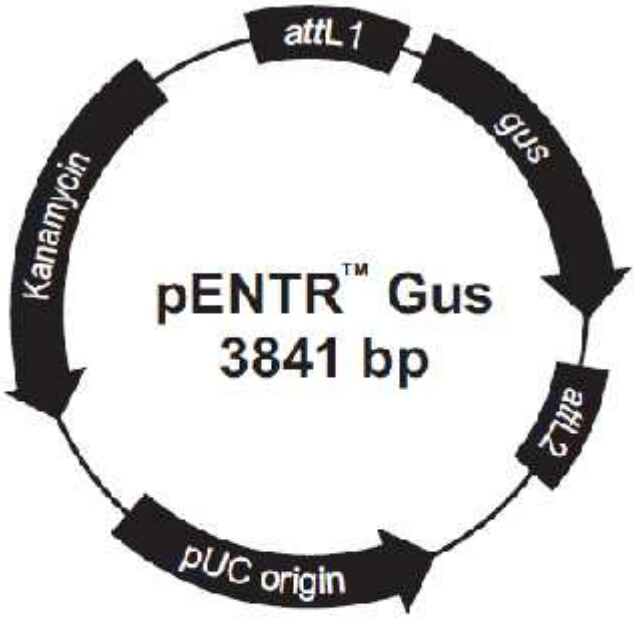
APPENDIX E

Vector Map of pEarleyGate 103



APPENDIX F

Vector Map of pENTR Gus



Comments for pENTR™ Gus
3841 nucleotides

*att*L1: bases 99-198 (complementary strand)
gus gene: bases 228-2039
*att*L2: bases 2041-2140
pUC origin: bases 2200-2873 (C)
Kanamycin resistance gene: bases 2990-3805 (C)

C = complementary strand

APPENDIX G

Percentage of sequence identity between pre-miRNA sequences of Hicks Broad Leaf and tobacco cultivars with known whole genome hosting those miRNAs

Tobacco cultivars with whole genome and contigs hosting miRNAs	miRNA Hicks Broad Leaf	Percentage of Sequence Identity
TN90 - 1	mir156d	100
TN90 -2	mir156g	100
TN90 -5	mir156e	99.04
TN90 -7	mir156d	100
TN90 -8	mir156i	100
TN90 -2	mir171c	100
TN90 -4	mir171a	100
TN90 -4	mir171c	100
TN90-7	mir171b	100
K326-3	mir156e	100
K326-4	mir156c	100
K326-6	mir171b	100
K326-8	mir171a	100
K326-8	mir171c	100
BX-3	mir156e	99.04
BX-5	mir156b	100
BX-7	mir156g	100
BX-6	mir171c	100
BX-6	mir171b	100
BX-8	mir171a	100

APPENDIX H

Pre-miR156 and pre-miR171 sequences of tobacco cultivar Hicks Broad Leaf

nta-MIR171a	AUACACGAGAUAUUGGUGCGGUUCAAUGAGAAAGCAGUACUCGACAAA UUUUGACUCUACUUUUUGAUUGAGCCGUGCCAAUAUCACGUGUAGCA
nta-MIR171b	UAAAGGAAGCGAUGUUGGUGAGGUUCAAUCCGAAGACGUGUUUACGU UUUGUUUUCGUAAAAAACGAUCUCAGAUUGAGCCGCGCCAAUAUCACU UCCUAUAUAU
nta-MIR171c	ACAGUAACUCAGGUAUUGGCCUGGUUCACUCAGACAACAAGAUGAAAC UAUUUGAAUAUUGAAUGGUGGAGUUUUGGUUUGAUUGAGCCGUGCCA AUAUCUCAGUUUCUC
nta-MIR156a	AUGUGAUAGGUGACAGAAGAGAGUGAGCACACAUGGUGUUUUCUUGC AUAUGAUAUACUUGAACCUAUGCGUGCUCACUGCUCUAUCUGUCACCC CCACACUCU
nta-MIR156b	UUAAAGGAGGUGACAGAAGAGAGUGAGCACCCAUGGUGUUUUCUUACA UGAGAUAUAUAUUCAUGCUUGAAGCUAUGCGUGCUCACUCUCUAUCUG UCACCCACUGUUUUC
nta-MIR156c	AGAAUCAAGACUGACAGAAGAGAGUGAGCACACGCAGGCAAUUGUAUA GAGAUUAUAUUACUGUCAUUUGCGUGUGCUCACUUCUCUUGCUGUCAU CUCUCUCUAUA
nta-MIR156d	AAAAGGGAGGUGACAGAAGAGAGUGAGCACAGAUGGUGUUUUCUUGC AUUGAUAUAUGUAUAUGCUUGAAGCUAUGCGUGCUCACUCUCUAUCUG UCACCCCCACCACUC
nta-MIR156e	UAAAGGGAGGUGACAGAAGAGAGUGAGCACACAUGGUAUUUUCUUGC AUAUGAUAUGCUUGAAGCUAUGCGUGCUCACUGCUCUAUCUGUCACCC CCCACCACU
nta-MIR156f	UUAAAGGAGGUGACAGAAGAGAAUGAGCACACAUGGUGUUUUCUUAC AUGAGAUAUAUAUAUUCAUGCUUGAAACUAUGCGUGCUCACUCUCUAU CUGUCACCCACUUUCUUU
nta-MIR156g	GUGAGAUUGUUGACAGAAGAUAGAGAGCACAGAUGAUGAUCAUGCUG CUAAAUCUGGGAUUGGAGAGGGCACUGAAUCAAUUAAACUGCAAAAAA AAAAAGCAUCUCAAUUCAUUUGUGCUCUCUAUGCUUCCGUCAUCACCU UCACCG
nta-MIR156h	GGAGAUGCUGUUGACAGAAGAUAGAGAGCACAGAUGAUGAUAAGAUG CUAAUUGGAAGCUUUCUGCACCUUAAUCCUUUGUGCUCUCUAGUCUUC UGUCAUCAUCCUCAGCC
nta-MIR156i	UGAUAAUUGUUGACAGAAGAUAGAGAGCACUGAUGAUGAUAUGCUAA UUAAAUUUGUGCAGCAAAAGCAUCUCAAUUCAUUUGUGCUCUCUAUGC UUCUGUCAUCACCUUCAGCCA
nta-MIR156j	UGACAAUUGUUGACAGAAGAUAGAGAGCACUAAUGAUGAUAUGCUAA GUAAAUGUAGGGGCAAAAGCAUCUCAAUUCAUUUGUGCUCUCUAUGCU UCUGUCAUCACCUUCGGCCA

APPENDIX I

Forward Primer Sequences Utilized for Gateway Cloning Systems

Cultivar_contig_miRNA	Forward Primer Sequence
BX_5_mir156b	GGGGACAAGTTTGTACAAAAAAGCAGGCTGAGAACGTCTTAGTCCTA
BX_3_mir156e	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGGTTAACGACATAAAGG
BX_7_mir156g	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGAAGAAGGGCAAGAGTC
BX_8_mir171a	GGGGACAAGTTTGTACAAAAAAGCAGGCTGTTGAAGTTGTAGTCGGA
BX_6_mir171b	GGGGACAAGTTTGTACAAAAAAGCAGGCTCACCATGTCAATTATGTC
K326_3_mir156e	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGGTTAACGACATAAAGG
K326_8_mir171a	GGGGACAAGTTTGTACAAAAAAGCAGGCTGTTGAAGTTGTAGTCGGA
K326_6_mir171b	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTTCACCATGCCAATTAT
TN90_7_mir156b	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAACGAGAACGTCTTAGT
TN90_1_mir156d	GGGGACAAGTTTGTACAAAAAAGCAGGCTGTAGATCCAGGTTAACTA
TN90_5_mir156e	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTAACGACATAAAGGGGT
TN90_8_mir156i	GGGGACAAGTTTGTACAAAAAAGCAGGCTAATGAGTATAGAGGAAGT
TN90_4_mir171a	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAAGGATGGATATTGTTG
TN90_7_mir171b	GGGGACAAGTTTGTACAAAAAAGCAGGCTCATGCCAATTATGTCAAG
TN90_2_mir171c	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCCTCTTTCTGGTAGATA
TN90_4_mir171c	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGCAGATAATCACATACT

Reverse Primer Sequences Utilized for Gateway Cloning Systems

Cultivar_contig_miRNA	Reverse Primer Sequence
BX_5_mir156b	GGGGACCACTTTGTACAAGAAAGCTGGGTAGGTCTAATGAGAGAAAG
BX_3_mir156e	GGGGACCACTTTGTACAAGAAAGCTGGGTACAGATGCGCATATATAC
BX_7_mir156g	GGGGACCACTTTGTACAAGAAAGCTGGGTGGAAGAAAGAGAGCGAAA
BX_8_mir171a	GGGGACCACTTTGTACAAGAAAGCTGGGTTGGTGATGGAGAATTAAA
BX_6_mir171b	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATGGACAAGAAAAAT
K326_3_mir156e	GGGGACCACTTTGTACAAGAAAGCTGGGTAACAGAAAGAAATCCACA
K326_8_mir171a	GGGGACCACTTTGTACAAGAAAGCTGGGTGGTGATGGAGAATTAAAT
K326_6_mir171b	GGGGACCACTTTGTACAAGAAAGCTGGGTATAAATGAAATAGTACGT
TN90_7_mir156b	GGGGACCACTTTGTACAAGAAAGCTGGGTAGGTCTAATGAGAGAAAG
TN90_1_mir156d	GGGGACCACTTTGTACAAGAAAGCTGGGTAAATCTAGAGAGAGAAGA
TN90_5_mir156e	GGGGACCACTTTGTACAAGAAAGCTGGGTAAAGAAATCCACAGAGAG
TN90_8_mir156i	GGGGACCACTTTGTACAAGAAAGCTGGGTAGATGAAGTTAAGAGAAG
TN90_4_mir171a	GGGGACCACTTTGTACAAGAAAGCTGGGTGGTGATGGAGAATTAAAT
TN90_7_mir171b	GGGGACCACTTTGTACAAGAAAGCTGGGTGATAAATGAAATAGTACG
TN90_2_mir171c	GGGGACCACTTTGTACAAGAAAGCTGGGTTTAAATTATGCAGACATG
TN90_4_mir171c	GGGGACCACTTTGTACAAGAAAGCTGGGTTAAATTATGCAGACATGG

APPENDIX J

Targets of miR156d

Ath-miR156d Target_Acc.	Target_aligned_fragment	Inhibition	Target_Desc.
chr2_14310001_14313400_FORWARD	UUGCUUACUCUCUUCUGUCA	Cleavage	AT2G33820.1 chr2:14313369-14315369 REVERSE; AT2G33810.1 chr2:14312077-14313148 FORWARD; AT2G33815.1 chr2:14312084-14313129 REVERSE; [PFAM] 2577-2717 PF03110.7 SBP domain;
chr1_9504001_9507400_REVERSE	GUGCUCUCUCUCUUCUGUCA	Cleavage	AT1G27370.1 chr1:9505188-9508267 REVERSE; AT1G27370.2 chr1:9505189-9508468 REVERSE; AT1G27370.3 chr1:9505189-9507315 REVERSE; AT1G27370.4 chr1:9505189-9508309 REVERSE; [PFAM]
chr2_17595001_17598400_FORWARD	GUGCUCUCUCUCUUCUGUCA	Cleavage	AT2G42200.1 chr2:17594485-17596708 FORWARD; AT2G42210.1 chr2:17597269-17598930 FORWARD; AT2G42210.2 chr2:17597354-17598930 FORWARD; AT2G42210.3 chr2:17597282-17598930 FORWARD; AT2G42210.4 chr2:17597291-17598930 FORWARD; [PFAM] 674-772 PF03110.7 SBP domain;
chr5_17376001_17379400_REVERSE	GUGCUCUCUCUCUUCUGUCA	Cleavage	AT5G43270.2 chr5:17377560-17381001 REVERSE; AT5G43270.3 chr5:17377560-17380191 REVERSE; AT5G43270.1 chr5:17377529-17380201 REVERSE; [PFAM]
chr1_26010001_26013400_FORWARD	GUGCUCUCUCUCUUCUGUCA	Cleavage	AT1G69180.1 chr1:26011128-26012722 REVERSE; AT1G69170.1 chr1:26008731-26010926 FORWARD; [PFAM]
chr3_21453001_21456400_REVERSE	GUGCUCUCUCUCUUCUGUCA	Cleavage	AT3G57920.1 chr3:21455298-21457012 REVERSE; AT3G57900.1 chr3:21453258-21453551 REVERSE; AT3G57910.1 chr3:21453725-21455288 FORWARD; [PFAM] 146-235 PF03110.7 SBP domain;
chr3_10755001_10758400_FORWARD	GUGCUCACUUUCUUUUGUUA	Cleavage	AT3G28690.1 chr3:10756276-10759105 FORWARD; AT3G28690.2 chr3:10756725-10759105 FORWARD; [PFAM]
chr5_20598001_20601400_REVERSE	GUGCUCUCUCUCUUCUGUCA	Cleavage	AT5G50570.1 chr5:20599309-20601785 REVERSE; AT5G50565.1 chr5:20598068-20599052 FORWARD; AT5G50570.2 chr5:20599309-20601106 REVERSE; [PFAM] 684-824 PF03110.7 SBP domain;
chr1_9501001_9504400_FORWARD	GUGCUCUCUCUCUUCUGUCA	Cleavage	AT1G27360.1 chr1:9501958-9503856 FORWARD; AT1G27360.2 chr1:9501064-9503856 FORWARD; AT1G27360.3 chr1:9501764-9503856 FORWARD; AT1G27360.4 chr1:9501808-9503856 FORWARD; [PFAM] 1645-1788 PF03110.7 SBP domain;
chr5_20631001_20634400_REVERSE	GUGCUCUCUCUCUUCUGUCA	Cleavage	AT5G50665.1 chr5:20631410-20632394 FORWARD; AT5G50670.1 chr5:20632930-20635065 REVERSE; [PFAM] 342-482 PF03110.7 SBP domain;
chr1_19809001_1981240	CUGCUCUCUCUCUUCU	Cleavage	AT1G53160.2 chr1:19810087-19811276 FORWARD; AT1G53160.1 chr1:19810089-19810969 FORWARD;

0_FORWARD	GUCA		[PFAM]
chr3_19275001_19278400_FORWARD	AAGCUCAUUUUCUUCUGUCA	Cleavage	AT3G51910.1 chr3:19276272-19277597 FORWARD; [PFAM] 1359-1535 PF00447.10 HSF-type DNA-binding;
chr1_17889001_17892400_REVERSE	GUGCUCGUUUUCUUCUGUUU	Cleavage	AT1G48405.1 chr1:17888313-17889282 REVERSE; AT1G48410.1 chr1:17889766-17896254 REVERSE; AT1G48410.2 chr1:17889766-17896254 REVERSE; [PFAM]
chr1_5172001_5175400_REVERSE	CUGCUCACUCUUUUCUCUCA	Cleavage	AT1G15020.1 chr1:5173092-5176215 REVERSE; AT1G15020.2 chr1:5173120-5176218 REVERSE; [PFAM]
chr1_18030001_18033400_FORWARD	GUGCUCUCUAUCUUCUGUCA	Translation	AT1G48742.1 chr1:18030506-18030677 REVERSE; [PFAM]
chr3_6243001_6246400_FORWARD	GUGCUCUCUAUCUUCUGUCA	Translation	AT3G18217.1 chr3:6244535-6244699 REVERSE; [PFAM]
chr4_16536001_16539400_FORWARD	GUGUUCUCUUUCUUCUGUUA	Cleavage	AT4G34620.1 chr4:16534860-16536176 REVERSE; AT4G34640.1 chr4:16538287-16541931 FORWARD; AT4G34630.1 chr4:16536870-16537751 REVERSE; [PFAM]
chr3_1887001_1890400_REVERSE	GAGUUCACUUUCUUCUGUUA	Cleavage	AT3G06250.1 chr3:1888842-1892781 REVERSE; AT3G06240.1 chr3:1887049-1888756 FORWARD; [PFAM]
chr4_16533001_16536400_FORWARD	GUGUUCUCUUUCUUCUGUUA	Cleavage	AT4G34620.1 chr4:16534860-16536176 REVERSE; [PFAM] 2969-3073 PF00886.12 Ribosomal protein S16;
chr3_5139001_5142400_REVERSE	CCGCUCUCUCUCUUCUGUCA	Cleavage	AT3G15270.1 chr3:5140372-5141353 REVERSE; AT3G15260.1 chr3:5138693-5140382 FORWARD; [PFAM]
chr3_204001_207400_FORWARD	CUGAUCACUUUCUUUUGUCA	Cleavage	AT3G01516.1 chr3:207343-208093 FORWARD; [PFAM]
chr3_201001_204400_FORWARD	CUGAUCACUUUCUUUUGUCA	Cleavage	AT3G01513.1 chr3:201927-202747 REVERSE; AT3G01510.1 chr3:198686-201749 REVERSE; [PFAM]
chr1_26340001_26343400_FORWARD	UUGCUCAUACUUUUCUGUCA	Cleavage	AT1G69930.1 chr1:26341160-26342458 REVERSE; [PFAM]
chr1_5691001_5694400_FORWARD	GUGUUCACUCUCCUCUGUUU	Cleavage	AT1G16650.1 chr1:5687936-5691191 FORWARD; [PFAM]
chr5_24693001_24696400_REVERSE	GAGCUUCCUCUCUUCUGUCA	Cleavage	AT5G61380.1 chr5:24692313-24695776 FORWARD; AT5G61390.1 chr5:24695888-24698170 REVERSE; [PFAM] 1131-1259 PF06203.7 CCT motif;
chr2_16860001_16863400_FORWAR	GUACUCACUCUCUUCUUUCG	Cleavage	intergenic [PFAM]

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chr2_18021001_18024400_REVERSE	GUGCUCUCUCUUUUCUAUCA	Cleavage	AT2G43370.1 chr2:18020447-18022531 FORWARD; [PFAM]
chr4_12768001_12771400_REVERSE	GUGCACAUUUUCUUUUGUUA	Cleavage	AT4G24780.1 chr4:12770341-12772343 REVERSE; AT4G24770.1 chr4:12766040-12768050 REVERSE; [PFAM]
chr1_22725001_22728400_REVERSE	GUUCUCACUCUCUUCUCUUA	Cleavage	AT1G61580.1 chr1:22724357-22726817 REVERSE; AT1G61590.1 chr1:22727166-22729739 REVERSE; [PFAM]
chr1_16914001_16917400_REVERSE	GUGAUUACUCUCUUUUGUUG	Cleavage	AT1G44780.1 chr1:16912148-16914710 FORWARD; AT1G44800.1 chr1:16916449-16919437 REVERSE; AT1G44790.1 chr1:16914594-16916104 REVERSE; AT1G44780.2 chr1:16912148-16914710 FORWARD; [PFAM]
chr2_9312001_9315400_FORWARD	GGGUUCAAUCUCUUCUGUCG	Cleavage	AT2G21830.1 chr2:9310793-9312977 FORWARD; AT2G21840.1 chr2:9314140-9317138 REVERSE; [PFAM]
chr1_7554001_7557400_REVERSE	GUGCUGAUACUCUUUUGUCA	Cleavage	AT1G21560.1 chr1:7554829-7557014 FORWARD; AT1G21570.1 chr1:7557251-7560272 REVERSE; [PFAM]
chr2_9315001_9318400_REVERSE	GGGUUCAAUCUCUUCUGUCG	Cleavage	AT2G21840.1 chr2:9314140-9317138 REVERSE; [PFAM]
chr2_9117001_9120400_REVERSE	GUGAUUACUGUCUUUUGUCA	Translation	AT2G21270.1 chr2:9114629-9117342 FORWARD; AT2G21290.1 chr2:9119937-9120411 FORWARD; AT2G21280.1 chr2:9117394-9119788 REVERSE; AT2G21270.2 chr2:9114700-9117342 FORWARD; AT2G21270.3 chr2:9114647-9117342 FORWARD; [PFAM] 2669-2812 PF08338.4 Domain of unknown function (DUF1731);

Bdi-miR156d Target_Acc.	**Target_aligned_fragment**	**Inhibition**	**Target_Desc.**
TC12901	UCACAUAUAGAGCAGUGGGC	Cleavage	weakly similar to UniRef100_Q10Q73 Cluster: Expressed protein; n=1; Oryza sativa Japonica Group|Rep: Expressed protein - Oryza sativa subsp. japonica (Rice), partial (21%)

Hvu-miR156d Target_Acc.	**Target_aligned_fragment**	**Inhibition**	**Target_Desc.**
TC245963	UGUGCUCUCUCUCUUCUGUCA	Cleavage	similar to UniRef100_Q6YZE8 Cluster: Squamosa promoter-binding-like protein 16; n=1; Oryza sativa Japonica Group|Rep: Squamosa promoter-binding-like protein 16 - Oryza sativa subsp. japonica (Rice), partial (45%)

TC265421	UGUGCUCU CUCUCUUC UGUCA	Cleavage	similar to UniRef100_Q0J0K1 Cluster: Squamosa promoter-binding-like protein 18; n=2; Oryza sativa Japonica Group|Rep: Squamosa promoter-binding-like protein 18 - Oryza sativa subsp. japonica (Rice), partial (32%)
CA032492	UGUGCUCU CUCUCUUC UGUCA	Cleavage	similar to UniRef100_A3C057 Cluster: Squamosa promoter-binding-like protein 17; n=1; Oryza sativa Japonica Group|Rep: Squamosa promoter-binding-like protein 17 - Oryza sativa subsp. japonica (Rice), partial (36%)
BF258419	UGUGCUCU CUCUCUUC UGUCA	Cleavage	similar to UniRef100_A4GXB6 Cluster: Promoter binding protein; n=1; Gossypium hirsutum|Rep: Promoter binding protein - Gossypium hirsutum (Upland cotton) (Gossypium mexicanum), partial (27%)
EX598089	UGUGCUCU CUCUCUUC UGUCA	Cleavage	similar to UniRef100_Q0J0K1 Cluster: Squamosa promoter-binding-like protein 18; n=2; Oryza sativa Japonica Group|Rep: Squamosa promoter-binding-like protein 18 - Oryza sativa subsp. japonica (Rice), partial (17%)
TC245020	GUGCUCUC UCUCUUCU GUCA	Cleavage	similar to UniRef100_Q6H509-2 Cluster: Isoform 2 of Q6H509 ; n=1; Oryza sativa Japonica Group|Rep: Isoform 2 of Q6H509 - Oryza sativa subsp. japonica (Rice), partial (36%)
TC252995	AUGCUCCC UCUCUUCU GUCA	Cleavage	similar to UniRef100_Q6Z461 Cluster: Squamosa promoter-binding-like protein 13; n=3; Oryza sativa|Rep: Squamosa promoter-binding-like protein 13 - Oryza sativa subsp. japonica (Rice), partial (61%)
TC246441	AUGCUCUC UCUCUUCU GUCA	Cleavage	similar to UniRef100_A2X0Q6 Cluster: Squamosa promoter-binding-like protein 3; n=2; Oryza sativa|Rep: Squamosa promoter-binding-like protein 3 - Oryza sativa subsp. indica (Rice), partial (26%)
TC241815	UGAGCUCA CUCUUUUU UGUUG	Cleavage	weakly similar to UniRef100_A7QHY8 Cluster: Chromosome chr17 scaffold_101, whole genome shotgun sequence; n=1; Vitis vinifera|Rep: Chromosome chr17 scaffold_101, whole genome shotgun sequence - Vitis vinifera (Grape), partial (39%)
TC270251	UGCACUCA UUCUUUUC UGUCA	Cleavage	similar to UniRef100_A7NXS2 Cluster: Chromosome chr5 scaffold_2, whole genome shotgun sequence; n=1; Vitis vinifera|Rep: Chromosome chr5 scaffold_2, whole genome shotgun sequence - Vitis vinifera (Grape), partial (22%)
BF630636	ACGCUCCC UCUCUUCU GUCA	Cleavage	homologue to UniRef100_Q5U8L4 Cluster: SBP transcription factor; n=1; Gossypium hirsutum|Rep: SBP transcription factor - Gossypium hirsutum (Upland cotton) (Gossypium mexicanum), partial (30%)
BM368509	UGCACUCA UUCUUUUC UGUCA	Cleavage	
TC279246	GUACUCAU UUGCUUCU GUCA	Translation	similar to UniRef100_Q5VN19 Cluster: Mitogen-activated protein kinase 11; n=2; Oryza sativa Japonica Group|Rep: Mitogen-activated protein kinase

			11 - Oryza sativa subsp. japonica (Rice), partial (42%)
BF628940	GUGCUCAC CCUCUCUC UGUCA	Cleavage	homologue to UniRef100_A5NML4 Cluster: Sensor protein; n=1; Methylobacterium sp. 4-46|Rep: Sensor protein - Methylobacterium sp., partial (1%)

Nta-miR156d Target_Acc.	**Target_aligned_fragment**	**Inhibition**	**Target_Desc.**
SGN-U384021	GUGCUCUC UCUCUUCU GUCA	Cleavage	Nicotiana tabacum #1 [1 ESTs aligned] arabidopsis/peptide: | Symbol: None | squamosa promoter-binding protein-like 9 (SPL9), identical to squamosa promoter binding protein-like 9 (Arabidopsis thaliana) GI:5931673; contains Pfam profile PF03110: SBP domain | chr2:17594485-17596708 FORWARD | Aliases: T24P15.11, T24P15_11(evalue: 4e-44, score=175) genbank/nr: gi|5931641|emb|CAB56569.1| squamosa promoter binding protein-homologue 4 [Antirrhinum majus] (evalue: 3e-68, score=261) [PFAM] 313-537 PF03110.7 SBP domain;
SGN-U367182	UUGCUCUC UCUCUUCU GUCA	Cleavage	Nicotiana tabacum #1 [2 ESTs aligned] arabidopsis/peptide: | Symbol: None | squamosa promoter-binding protein-like 4 (SPL4), nearly identical to squamosa promoter binding protein-like 4 (Arabidopsis thaliana) GI:5931657; contains Pfam profile PF03110: SBP domain | chr1:19810087-19811276 FORWARD | Aliases: None(evalue: 1e-35, score=147) genbank/nr: gi|62856979|gb|AAY16440.1| squamosa promoter binding-like protein [Betula platyphylla] (evalue: 4e-52, score=208) [PFAM] 296-526 PF03110.7 SBP domain;
SGN-U379394	UUGCUCUC UCUCUUCU GUCA	Cleavage	Nicotiana tabacum #1 [1 ESTs aligned] arabidopsis/peptide: | Symbol: None | squamosa promoter-binding protein-like 5 (SPL5), identical to squamosa promoter binding protein-like 5 (Arabidopsis thaliana) GI:5931629; contains Pfam profile PF03110: SBP domain | chr3:5140396-5141353 REVERSE | Aliases: K7L4.7(evalue: 2e-33, score=139) genbank/nr: gi|62856979|gb|AAY16440.1| squamosa promoter binding-like protein [Betula platyphylla] (evalue: 6e-38, score=160) [PFAM] 207-428 PF03110.7 SBP domain;
SGN-U385631	AUGCUUAC UCUCUUCA GUCA	Cleavage	Nicotiana tabacum #1 [1 ESTs aligned] arabidopsis/peptide: | Symbol: None | squamosa promoter-binding protein-like 5 (SPL5), identical to squamosa promoter binding protein-like 5 (Arabidopsis thaliana) GI:5931629; contains Pfam profile PF03110: SBP domain | chr3:5140396-5141353 REVERSE | Aliases: K7L4.7(evalue: 1e-30, score=129) genbank/nr: gi|62856979|gb|AAY16440.1| squamosa promoter binding-like protein [Betula platyphylla] (evalue: 3e-30, score=135) [PFAM] 145-366 PF03110.7 SBP domain;

SGN-U370298	GUUCUUAUUCUUUUCUGUUA	Cleavage	Nicotiana tabacum #1 [4 ESTs aligned] arabidopsis/peptide: | Symbol: None | 1-phosphatidylinositol phosphodiesterase-related, contains weak similarity to 1-phosphatidylinositol phosphodiesterase precursor (EC 4.6.1.13) (Phosphatidylinositol diacylglycerol-lyase) (Phosphatidylinositol- specific phospholipase C) (PI-PLC). (Swiss-Prot:P34024) (Listeria monocytogenes) | chr4:18074469-18075759 REVERSE | Aliases: T9A14.2(evalue: 3e-139, score=491) genbank/nr: gi|15234016|ref|NP_195581.1| expressed protein [Arabidopsis thaliana] gi|7485885|pir||T05697 hypothetical protein F20M13.250 - Arabidopsis thaliana gi|4467156|emb|CAB37525.1| putative protein [Arabidopsis thaliana] gi|7270852|emb|CAB80533.1| putative protein [Arabidopsis thaliana] gi|17979229|gb|AAL49931.1| AT4g38690/F20M13_250 [Arabidopsis thaliana] gi|20147101|gb|AAM10267.1| AT4g38690/F20M13_250 [Arabidopsis thaliana](evalue: 3e-137, score=491) [PFAM]

Osa-miR156d Target_Acc.	**Target_aligned_fragment**	**Inhibition**	**Target_Desc.**
TC492476	GUGCUCUCUCUCUUCUGUCA	Cleavage	UniRef100_Q6YZE8 Cluster: Squamosa promoter-binding-like protein 16; n=1; Oryza sativa Japonica Group|Rep: Squamosa promoter-binding-like protein 16 - Oryza sativa subsp. japonica (Rice), complete
TC493885	GUGCUCUCUCUCUUCUGUCA	Cleavage	UniRef100_Q7EXZ2 Cluster: Squamosa promoter-binding-like protein 14; n=1; Oryza sativa Japonica Group|Rep: Squamosa promoter-binding-like protein 14 - Oryza sativa subsp. japonica (Rice), complete
TC503310	GUGCUCUCUCUCUUCUGUCA	Cleavage	UniRef100_Q6H509 Cluster: Squamosa promoter-binding-like protein 4; n=1; Oryza sativa Japonica Group|Rep: Squamosa promoter-binding-like protein 4 - Oryza sativa subsp. japonica (Rice), complete
TC496959	GUGCUCUCUCUCUUCUGUCA	Cleavage	UniRef100_Q653Z5 Cluster: Squamosa promoter-binding-like protein 11; n=1; Oryza sativa Japonica Group|Rep: Squamosa promoter-binding-like protein 11 - Oryza sativa subsp. japonica (Rice), complete
TC498553	GUGCUCUCUCUCUUCUGUCA	Cleavage	UniRef100_Q0JGI1 Cluster: Squamosa promoter-binding-like protein 2; n=1; Oryza sativa Japonica Group|Rep: Squamosa promoter-binding-like protein 2 - Oryza sativa subsp. japonica (Rice), partial (75%)
TC491943	GUGCUCUCUCUCUUCUGUCA	Cleavage	UniRef100_Q0J0K1 Cluster: Squamosa promoter-binding-like protein 18; n=2; Oryza sativa Japonica Group|Rep: Squamosa promoter-binding-like protein 18 - Oryza sativa subsp. japonica (Rice), complete
CK063063	GUGCUCUCUCUCUUCUGUCA	Cleavage	homologue to UniRef100_A2X0Q6 Cluster: Squamosa promoter-binding-like protein 3; n=2; Oryza sativa|Rep: Squamosa promoter-binding-like protein 3 - Oryza sativa subsp. indica (Rice), partial

			(18%)
CK074762	GUGCUCUC UCUCUUCU GUCA	Cleavage	UniRef100_A3C057 Cluster: Squamosa promoter-binding-like protein 17; n=1; Oryza sativa Japonica Group|Rep: Squamosa promoter-binding-like protein 17 - Oryza sativa subsp. japonica (Rice), partial (34%)
TC545406	GAGCUCAC UCUCUUCU GUCA	Cleavage	
TC506566	AUGCUCUC UCUCUUCU GUCA	Cleavage	UniRef100_A2X0Q6 Cluster: Squamosa promoter-binding-like protein 3; n=2; Oryza sativa|Rep: Squamosa promoter-binding-like protein 3 - Oryza sativa subsp. indica (Rice), partial (52%)
TC515668	AUGCUCCC UCUCUUCU GUCA	Cleavage	UniRef100_Q6Z461 Cluster: Squamosa promoter-binding-like protein 13; n=3; Oryza sativa|Rep: Squamosa promoter-binding-like protein 13 - Oryza sativa subsp. japonica (Rice), complete
TC527460	AUGCUCCC UCUCUUCU GUCA	Cleavage	UniRef100_Q6Z461 Cluster: Squamosa promoter-binding-like protein 13; n=3; Oryza sativa|Rep: Squamosa promoter-binding-like protein 13 - Oryza sativa subsp. japonica (Rice), partial (54%)
CR279709	AUGCUCUC UCUCUUCU GUCG	Cleavage	
CA753299	GUGUUUAU UCUCUUCU GUUC	Cleavage	similar to UniRef100_Q9TUG4 Cluster: Dopamine receptor D4; n=1; Hylobates muelleri|Rep: Dopamine receptor D4 - Hylobates muelleri (Mueller's gibbon), partial (14%)
TC508767	GUCUUCGU UCUCUUCU GUCA	Cleavage	
CT860049	GUGCUCGC UCUCGUUU GUCU	Cleavage	similar to UniRef100_Q0IZZ0 Cluster: Os09g0540900 protein; n=1; Oryza sativa Japonica Group|Rep: Os09g0540900 protein - Oryza sativa subsp. japonica (Rice), partial (11%)
TC513071	GUGACCAC UCUUUUCU GUCA	Cleavage	UniRef100_Q53NQ8 Cluster: At1g34320; n=1; Oryza sativa Japonica Group|Rep: At1g34320 - Oryza sativa subsp. japonica (Rice), partial (21%)
TC553750	GUACUCCC UUUUUUCU GUCA	Cleavage	UniRef100_Q0J6T2 Cluster: Os08g0270500 protein; n=1; Oryza sativa Japonica Group|Rep: Os08g0270500 protein - Oryza sativa subsp. japonica (Rice), complete
TC514408	GUAGUCGC UCUUUUCU GUCA	Cleavage	homologue to UniRef100_Q0J0F3 Cluster: Os09g0513100 protein; n=1; Oryza sativa Japonica Group|Rep: Os09g0513100 protein - Oryza sativa subsp. japonica (Rice), complete

Zma-miR156d Target_Acc.	**Target_aligned_fragment**	**Inhibition**	**Target_Desc.**
GRMZM2G	GUGCUCUC	Cleavage	seq=cdna; coord=8:161522745..161525837:-1;

371033_T01	UCUCUUCU GUCA		parent_gene=GRMZM2G371033
GRMZM5G 806833_T01	GUGCUCUC UCUCUUCU GUCA	Cleavage	seq=cdna; coord=7:136328788..136330155:-1; parent_gene=GRMZM5G806833
GRMZM2G 106798_T03	GUGCUCUC UCUCUUCU GUCA	Cleavage	seq=cdna; coord=4:17489205..17501119:1; parent_gene=GRMZM2G106798
GRMZM2G 106798_T02	GUGCUCUC UCUCUUCU GUCA	Cleavage	seq=cdna; coord=4:17489205..17501119:1; parent_gene=GRMZM2G106798
GRMZM2G 126018_T02	GUGCUCUC UCUCUUCU GUCA	Cleavage	seq=cdna; coord=2:189838915..189842817:-1; parent_gene=GRMZM2G126018
GRMZM2G 106798_T01	GUGCUCUC UCUCUUCU GUCA	Cleavage	seq=cdna; coord=4:17489205..17501119:1; parent_gene=GRMZM2G106798
GRMZM2G 126018_T01	GUGCUCUC UCUCUUCU GUCA	Cleavage	seq=cdna; coord=2:189838793..189842695:-1; parent_gene=GRMZM2G126018
GRMZM2G 307588_T01	GUGCUCUC UCUCUUCU GUCA	Cleavage	seq=cdna; coord=7:133170018..133173665:-1; parent_gene=GRMZM2G307588
AC233751.1 _FGT002	GUGCUCUC UCUCUUCU GUCA	Cleavage	seq=cdna; coord=4:44780143..44784469:-1; parent_gene=AC233751.1_FG002
GRMZM2G 460544_T01	GUGCUCUC UCUCUUCU GUCA	Cleavage	seq=cdna; coord=4:199278823..199283117:-1; parent_gene=GRMZM2G460544
GRMZM2G 097275_T04	GUGCUCUC UCUCUUCU GUCA	Cleavage	seq=cdna; coord=5:77940666..77945477:1; parent_gene=GRMZM2G097275
GRMZM2G 097275_T01	GUGCUCUC UCUCUUCU GUCA	Cleavage	seq=cdna; coord=5:77940666..77945714:1; parent_gene=GRMZM2G097275
GRMZM2G 061734_T01	GUGCUCUC UCUCUUCU GUCA	Cleavage	seq=cdna; coord=2:192157754..192162802:1; parent_gene=GRMZM2G061734
GRMZM2G 097275_T03	GUGCUCUC UCUCUUCU GUCA	Cleavage	seq=cdna; coord=5:77940666..77945676:1; parent_gene=GRMZM2G097275
GRMZM2G 097275_T02	GUGCUCUC UCUCUUCU GUCA	Cleavage	seq=cdna; coord=5:77940666..77945676:1; parent_gene=GRMZM2G097275
GRMZM5G 878561_T01	GUGCUCUC UCUCUUCU GUCA	Cleavage	seq=cdna; coord=3:159377754..159382446:-1; parent_gene=GRMZM5G878561
GRMZM2G 113779_T01	AUGCUCUC UCUCUUCU GUCA	Cleavage	seq=cdna; coord=2:201971116..201972947:-1; parent_gene=GRMZM2G113779
GRMZM2G 067624_T02	AUGCUCUC UCUCUUCU GUCA	Cleavage	seq=cdna; coord=7:151217710..151219397:-1; parent_gene=GRMZM2G067624

GRMZM2G044697_T10	AUGCUUACUCUUUUCUGUUA	Cleavage	seq=cdna; coord=3:19250256..19252626:1; parent_gene=GRMZM2G044697
GRMZM2G044697_T09	AUGCUUACUCUUUUCUGUUA	Cleavage	seq=cdna; coord=3:19250188..19252626:1; parent_gene=GRMZM2G044697
GRMZM2G156621_T01	AUGCUCUCUCUCUUCUGUCA	Cleavage	seq=cdna; coord=5:131187462..131190979:-1; parent_gene=GRMZM2G156621
GRMZM2G067624_T01	AUGCUCUCUCUCUUCUGUCA	Cleavage	seq=cdna; coord=7:151217709..151219397:-1; parent_gene=GRMZM2G067624
GRMZM2G126827_T01	AUGCUCUCUCUCUUCUGUCA	Cleavage	seq=cdna; coord=5:86783062..86786647:1; parent_gene=GRMZM2G126827
GRMZM2G133959_T04	AUGCUAACUCUCUUCUGUUA	Cleavage	seq=cdna; coord=8:157409381..157412926:1; parent_gene=GRMZM2G133959
GRMZM2G096352_T03	GUGAGCACUCUCUUUUGUCG	Cleavage	seq=cdna; coord=9:149044810..149049135:-1; parent_gene=GRMZM2G096352
GRMZM2G111204_T01	UGCUCAGCUCUCUUCUGUUG	Cleavage	seq=cdna; coord=2:174677185..174683559:-1; parent_gene=GRMZM2G111204

APPENDIX K

Targets of miR171b

Ath-miR171b Target_Acc.	Target_aligned_fragment	Inhibition	Target_Desc.
chr4_57001_60400_FORWARD	GAUUGAGCCGCGCCAAUAUCC	Cleavage	AT4G00150.1 chr4:57199-59286 REVERSE; [PFAM]
chr3_22422001_2242540_FORWARD	GAUUGAGCCGCGCCAAUAUCC	Cleavage	AT3G60630.1 chr3:22421340-22423583 REVERSE; [PFAM]
chr2_18624001_1862740_FORWARD	GAUUGAGCCGCGCCAAUAUCC	Cleavage	AT2G45160.1 chr2:18624937-18627225 REVERSE; [PFAM]
chr3_18156001_1815940_FORWARD	AUUGAAUUGCAUUAAUAUUA	Cleavage	AT3G48950.1 chr3:18159002-18160972 FORWARD; [PFAM]
chr3_16875001_1687840_REVERSE	GUUGAACGGCGCUAAUAUUU	Cleavage	AT3G45870.1 chr3:16878187-16879911 FORWARD; AT3G45860.1 chr3:16874386-16877026 REVERSE; AT3G45870.2 chr3:16878187-16879911 FORWARD; [PFAM]
chr4_11658001_1166140_REVERSE	AUUGAAUCGUACCAAUGUCU	Cleavage	AT4G22000.1 chr4:11659650-11660916 REVERSE; AT4G21990.1 chr4:11657020-11659117 REVERSE; [PFAM]
chr2_4020001_4023400_FORWARD	GACUGAACCGAACUGAUAUUU	Translation	AT2G10440.1 chr2:4020835-4025129 REVERSE; [PFAM]
chr3_11754001_1175740_FORWARD	GACUGAACCGGACUGAUAUUU	Translation	intergenic [PFAM]
chr5_20553001_2055640_FORWARD	GAUUGAACCUCGGUAAUGUCU	Translation	AT5G50430.1 chr5:20551399-20554343 REVERSE; AT5G50440.1 chr5:20554751-20556169 REVERSE; AT5G50430.2 chr5:20551399-20554297 REVERSE; AT5G50430.3 chr5:20551399-20554343 REVERSE; [PFAM]
chr4_8304001_8307400_FORWARD	CUUGAAUGGUACUAAUAUCU	Cleavage	AT4G14430.1 chr4:8304596-8305701 REVERSE; AT4G14440.1 chr4:8306741-8307749 REVERSE; [PFAM]
chr1_3849001_3852400_REVERSE	AUUGAACCUUAUUAAUAUCG	Cleavage	AT1G11430.1 chr1:3847243-3849162 FORWARD; AT1G11440.1 chr1:3849360-3850662 FORWARD; [PFAM]
chr4_10935001_1093840_FORWAR	CUUGGGCAGCACUAAUAUCU	Cleavage	AT4G20250.1 chr4:10938000-10940538 REVERSE; [PFAM]

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chr1_16236001_16239400_FORWARD	AUUGAAACACACUAAUAUUU	Cleavage	intergenic [PFAM] 2230-2352 PF10557.2 Cullin protein neddylation domain;
chr5_834001_837400_FORWARD	AUUGUAUCUCACUAAUAUCU	Cleavage	AT5G03380.1 chr5:832103-834411 REVERSE; AT5G03390.1 chr5:834987-836750 FORWARD; AT5G03380.2 chr5:832103-834284 REVERSE; [PFAM]
chr3_5022001_5025400_FORWARD	AUUGAACCUUGUUAAUGUCU	Cleavage	AT3G14940.1 chr3:5025245-5029716 FORWARD; AT3G14930.1 chr3:5020577-5022966 FORWARD; AT3G14930.2 chr3:5020577-5022937 FORWARD; AT3G14930.3 chr3:5020598-5022966 FORWARD; [PFAM]
chr5_25509001_2551240_FORWARD	AUUGGAUCUCACUAAUGUUU	Cleavage	AT5G63700.1 chr5:25511160-25514858 REVERSE; AT5G63690.1 chr5:25510090-25510509 REVERSE; AT5G63680.1 chr5:25507299-25509978 FORWARD; [PFAM] 4-300 PF00224.14 Pyruvate kinase, barrel domain;
chr5_4749001_4752400_FORWARD	AUUGAACCGCUCAAAUAUUU	Translation	intergenic [PFAM]
chr3_18954001_1895740_REVERSE	AUUCAACCACAUUAAUAUCU	Cleavage	AT3G50990.1 chr3:18954116-18955792 FORWARD; AT3G51000.1 chr3:18956006-18957602 REVERSE; [PFAM]
chr1_27321001_2732440_FORWARD	AUUGAACCGAACUGAUAACU	Translation	AT1G72550.1 chr1:27323350-27327699 REVERSE; AT1G72550.2 chr1:27323413-27327677 REVERSE; [PFAM]
chr2_7416001_7419400_FORWARD	AUUGAGCUACAUUAAUAUUU	Cleavage	AT2G17050.1 chr2:7417917-7422692 REVERSE; [PFAM]
chr1_11190001_1119340_FORWARD	AUUGGACCUUACUGAUAUUU	Cleavage	AT1G31290.1 chr1:11188274-11192298 FORWARD; [PFAM]
chr4_18036001_1803940_REVERSE	AUUUAAUCGUAUUAGUAUCU	Cleavage	AT4G38590.1 chr4:18036086-18040922 FORWARD; [PFAM]
chr5_21162001_2116540_FORWARD	AUUGAACUGUAUCUAAUAUCU	Translation	intergenic [PFAM]
chr5_21165001_2116840_FORWARD	AUUGAACUGUAUCUAAUAUCU	Translation	intergenic [PFAM]
chr1_16770001_1677340_REVERSE	AUAGAACCGAACUAAUAUCG	Translation	AT1G44100.1 chr1:16766845-16770125 REVERSE; [PFAM]

chr1_27381001_27384400_FORWARD	AUUGAACCAAACUAAUAUCG	Translation	AT1G72740.1 chr1:27383956-27386586 REVERSE; AT1G72730.1 chr1:27381460-27383844 REVERSE; AT1G72720.1 chr1:27380417-27381055 REVERSE; AT1G72740.2 chr1:27383956-27386586 REVERSE; [PFAM] 822-1028 PF00271.24 Helicase conserved C-terminal domain;
chr1_27552001_27555400_REVERSE	AUGGAAUGGCACUGAUAUCU	Cleavage	AT1G73270.1 chr1:27553067-27556178 REVERSE; [PFAM]
chr1_4473001_4476400_REVERSE	AAUGAAACGCAUUAAUAUUU	Cleavage	AT1G13120.1 chr1:4469179-4473211 REVERSE; AT1G13130.1 chr1:4474718-4477939 FORWARD; [PFAM]
chr1_27606001_27609400_FORWARD	GUUAAACUGCUCUAAUAUCU	Translation	AT1G73410.1 chr1:27605293-27606978 FORWARD; AT1G73430.1 chr1:27607316-27614729 FORWARD; [PFAM]
chr1_27609001_27612400_FORWARD	GUUAAACUGCUCUAAUAUCU	Translation	intergenic [PFAM]
chr5_25704001_25707400_REVERSE	AUUAAUCUGCACUAAUAUUU	Cleavage	intergenic [PFAM]
chr5_14256001_14259400_FORWARD	AUGGAAUGGCACUGAUAUCU	Cleavage	AT5G36180.1 chr5:14256258-14259365 FORWARD; [PFAM] 666-812 PF00450.15 Serine carboxypeptidase;
chr4_9753001_9756400_REVERSE	ACUAAACCGUACUAAUAUUU	Cleavage	AT4G17490.1 chr4:9752836-9753879 REVERSE; [PFAM]
chr1_27564001_27567400_REVERSE	AUGGAAUGGCACUGAUAUCU	Cleavage	AT1G73300.1 chr1:27563334-27565709 REVERSE; AT1G73310.1 chr1:27566476-27568838 REVERSE; [PFAM]

Bdi-miR171b Target_Acc.	**Target_aligned_fragment**	**Inhibition**	**Target_Desc.**
TC1863	AUAUUGAUAUCGCUCAAUCA	Translation	
TC14158	AUAUUGAUAUCGCUCAAUCA	Translation	
TC7428	AUACUGGCACAGAUCAAUCA	Translation	weakly similar to UniRef100_Q55DM1 Cluster: BEACH domain-containing protein; n=1; Dictyostelium discoideum|Rep: BEACH domain-containing protein - Dictyostelium discoideum (Slime mold), partial (4%)

Hvu-miR171b Target_Acc.	Target_aligned_fragment	Inhibition	Target_Desc.
CD054872	AUACUGGCACAGAUCAAUCA	Translation	weakly similar to UniRef100_O35242 Cluster: Protein FAN; n=1; Mus musculus|Rep: Protein FAN - Mus musculus (Mouse), partial (7%)

Nta-miR171b Target_Acc.	Target_aligned_fragment	Inhibition	Target_Desc.
TC134811	AAUGAUAUUGGCGCGGCUCAA	Cleavage	similar to UniRef100_Q8LL10 Cluster: Hairy meristem; n=1; Petunia x hybrida|Rep: Hairy meristem - Petunia hybrida (Petunia), partial (40%)
TC127385	AGGGAUAUUGGCGCGGCUCAA	Cleavage	similar to UniRef100_A7PYF4 Cluster: Chromosome chr15 scaffold_37, whole genome shotgun sequence; n=1; Vitis vinifera|Rep: Chromosome chr15 scaffold_37, whole genome shotgun sequence - Vitis vinifera (Grape), partial (44%)
EB427599	AGUGAUAUUGGUUCGGCUCAA	Translation	similar to UniRef100_A7NVA0 Cluster: Chromosome chr18 scaffold_1, whole genome shotgun sequence; n=1; Vitis vinifera|Rep: Chromosome chr18 scaffold_1, whole genome shotgun sequence - Vitis vinifera (Grape), partial (47%)
TC149774	AGUGAUAUUGGUUCGGCUCAG	Translation	similar to UniRef100_Q2PEG7 Cluster: Transcription initiator for nodulation; n=2; Lotus japonicus|Rep: Transcription initiator for nodulation - Lotus japonicus, partial (16%)
FG174873	GUGAUACUGGCACGGCUCAA	Translation	similar to UniRef100_Q9FYW2 Cluster: BAC19.14; n=1; Solanum lycopersicum|Rep: BAC19.14 - Solanum lycopersicum (Tomato) (Lycopersicon esculentum), partial (41%)

Osa-miR171b Target_Acc.	Target_aligned_fragment	Inhibition	Target_Desc.
chr10_20907001_20910400_REVERSE	GAUAUUGGCACGGCUCAAUCA	Cleavage	intergenic [PFAM]
chr06_25890001_25893400_REVERSE	GAUAUUGGCGCGGCUCAAUCA	Cleavage	intergenic [PFAM]
chr06_25887001_25890400_REVERSE	GAUAUUGGCGCGGCUCAAUCA	Cleavage	intergenic [PFAM]
chr02_26835001_268384	GAUAUUGGCGCGGCUC	Cleavage	LOC_Os02g44360.1 chr02:26835716-26838462 FORWARD; [PFAM]

00_FORWARD	AAUCA		
chr06_363001_366400_FORWARD	GAUAUUGGCGCGGCUCAAUCA	Cleavage	LOC_Os06g01620.1 chr06:364301-366066 FORWARD; [PFAM]
chr03_33105001_33108400_FORWARD	AUAUUGGUAUGGCUCAGUCA	Cleavage	intergenic [PFAM]
chr08_982501_9828400_REVERSE	AUAUUGGCACGGCUUGAUCU	Cleavage	LOC_Os08g16130.1 chr08:9824643-9827708 REVERSE; [PFAM]
chr03_21144001_21147400_REVERSE	GAUAUUGACGCGGCUCAAUCC	Cleavage	LOC_Os03g38170.1 chr03:21145945-21153622 FORWARD; [PFAM]
chr09_22050001_22053400_REVERSE	GGUAUUGGCAUUGCUCAAUUA	Translation	intergenic [PFAM]
chr05_16437001_16440400_FORWARD	AUAUUUGCACGGUUCGAUCA	Cleavage	LOC_Os05g28200.1 chr05:16433929-16439703 FORWARD; LOC_Os05g28200.2 chr05:16433929-16439703 FORWARD; [PFAM]
chr05_20352001_20355400_FORWARD	GAUAUUAGCACGGUUCAAUUU	Cleavage	LOC_Os05g34460.1 chr05:20350211-20354764 FORWARD; LOC_Os05g34470.2 chr05:20355189-20358236 FORWARD; LOC_Os05g34470.1 chr05:20355189-20358236 FORWARD; [PFAM]
chr09_330601_3309400_FORWARD	GAUAUUGGCACUGUUUAGUCG	Translation	LOC_Os09g06890.1 chr09:3302872-3306522 FORWARD; [PFAM]
chr07_329101_3294400_REVERSE	GAGGUUGGCACGGUUCGGUCA	Cleavage	LOC_Os07g06740.3 chr07:3286778-3291591 REVERSE; LOC_Os07g06740.2 chr07:3286778-3291591 REVERSE; LOC_Os07g06740.1 chr07:3286778-3291591 REVERSE; [PFAM]
chr10_20907001_20910400_FORWARD	GAUGUUGGCCCGGCUCACUCA	Cleavage	intergenic [PFAM]
chr09_330901_3312400_FORWARD	GAUAUUGGCACUGUUUAGUCG	Translation	intergenic [PFAM]
chr10_342901_3432400_FORWARD	GAUAUUGGAACGGUUAAAUCA	Cleavage	LOC_Os10g06640.1 chr10:3430659-3435076 FORWARD; [PFAM]
chr09_372001_375400_REVERSE	GAGGUUGGCACGGUUCAGUCG	Cleavage	LOC_Os09g01520.1 chr09:372351-374795 FORWARD; [PFAM]
chr01_35730001_35733400_FORWA	AUAUUGGUAUGGCUCAGUUU	Cleavage	LOC_Os01g61160.1 chr01:35729644-35732158 FORWARD; [PFAM]

RD			
chr01_38358001_38361400_REVERSE	AUAUUGGUACAGUUUAAUCA	Translation	LOC_Os01g65530.1 chr01:38358347-38362706 FORWARD; [PFAM]
chr05_21834001_21837400_FORWARD	AUAUUGGUACGGUUCAUUUA	Cleavage	LOC_Os05g37450.1 chr05:21831474-21834195 REVERSE; LOC_Os05g37460.1 chr05:21836704-21837732 REVERSE; [PFAM]
chr12_18957001_18960400_REVERSE	GAUAAUGGCAUGAUUCAAUCA	Translation	LOC_Os12g31560.1 chr12:18956069-18958083 FORWARD; [PFAM]
chr02_15771001_15774400_FORWARD	GAUAUAGGCCCGGCUCAAUUG	Cleavage	intergenic [PFAM]
chr07_630001_633400_FORWARD	UUAUUAGCACGGUUCAAUUA	Cleavage	LOC_Os07g02060.1 chr07:628861-632615 REVERSE; [PFAM]
chr07_633001_636400_FORWARD	UUAUUAGCACGGUUCAAUUA	Cleavage	intergenic [PFAM]
chr04_16458001_16461400_FORWARD	ACAUUGUCAUGGCUCAAUCA	Cleavage	LOC_Os04g28180.1 chr04:16458311-16460994 REVERSE; [PFAM]
chr01_2163001_2166400_FORWARD	AUAUCGGCACAGCUCGAUCA	Translation	intergenic [PFAM]
chr11_1563001_1566400_REVERSE	AUAUUGGCAUGUUUCGAUUA	Translation	LOC_Os11g03910.1 chr11:1561203-1564097 FORWARD; LOC_Os11g03920.1 chr11:1564342-1565323 REVERSE; [PFAM]
chr05_726901_7272400_REVERSE	GUAUUGUUAUGGUUCAAUCA	Cleavage	LOC_Os05g12700.1 chr05:7267943-7271166 REVERSE; [PFAM]
chr09_562201_5625400_REVERSE	AUAUUGGUUUGGUUCAAUUA	Cleavage	LOC_Os09g10320.1 chr09:5623093-5623416 FORWARD; LOC_Os09g10330.1 chr09:5624475-5625305 FORWARD; [PFAM]
chr08_26181001_26184400_FORWARD	GUGUUGGCCUGGCUCAAUUA	Cleavage	LOC_Os08g41690.1 chr08:26181912-26184994 REVERSE; [PFAM]
chr01_2166001_2169400_FORWARD	AUAUCGGCACAGCUCGAUCA	Translation	intergenic [PFAM]
chr11_18585001_18588400_FORWARD	AUAUAGGCACGGCUUUAUCA	Cleavage	LOC_Os11g32300.1 chr11:18584311-18586051 REVERSE; LOC_Os11g32310.1 chr11:18586830-18587222 REVERSE; [PFAM]
chr05_1860001_1863400	AUAAUGACAUGGCUCA	Cleavage	LOC_Os05g04180.1 chr05:1863062-1867285 REVERSE; [PFAM]

_FORWARD	AUCA		
chr09_3567001_3570400_REVERSE	AUGUUCGCACGGCUCGAUUG	Cleavage	intergenic [PFAM]
chr09_3570001_3573400_REVERSE	AUGUUCGCACGGCUCGAUUG	Cleavage	intergenic [PFAM]
chr04_21240001_21243400_REVERSE	GAUAUUGGCACUUGUCAAUCA	Translation	LOC_Os04g35260.2 chr04:21241121-21258041 REVERSE; LOC_Os04g35260.1 chr04:21241121-21258041 REVERSE; [PFAM]
chr04_21243001_21246400_REVERSE	GAUAUUGGCACUUGUCAAUCA	Translation	intergenic [PFAM]
chr10_14895001_14898400_REVERSE	GAUAUUGUCAAUGCUCAAUCA	Translation	LOC_Os10g29250.1 chr10:14895253-14895597 FORWARD; [PFAM]
chr07_21000001_21003400_REVERSE	AUAAUGGAACGGCUCAAUCU	Cleavage	LOC_Os07g35060.2 chr07:21001135-21003053 FORWARD; LOC_Os07g35060.1 chr07:21001135-21003053 FORWARD; [PFAM]
chr07_20997001_21000400_REVERSE	AUAAUGGAACGGCUCAAUCU	Cleavage	LOC_Os07g35050.1 chr07:20997635-20999614 FORWARD; [PFAM]
chr04_28035001_28038400_REVERSE	GUAUUUGCACAGCUCAAUUA	Translation	intergenic [PFAM]
chr01_25680001_25683400_FORWARD	AUAUUGGAGGGGCUCAAUUA	Translation	LOC_Os01g44220.7 chr01:25680617-25689032 FORWARD; LOC_Os01g44220.5 chr01:25680617-25689032 FORWARD; LOC_Os01g44220.3 chr01:25680617-25689032 FORWARD; LOC_Os01g44220.4 chr01:25680617-25689032 FORWARD; LOC_Os01g44220.1 chr01:25680617-25689032 FORWARD; LOC_Os01g44220.2 chr01:25680617-25689032 FORWARD; LOC_Os01g44220.6 chr01:25680617-25689032 FORWARD; [PFAM]
chr06_1992001_1995400_FORWARD	AGAUUUGCACGGCUCGAUCG	Cleavage	LOC_Os06g04580.1 chr06:1989030-1992736 FORWARD; [PFAM]
chr09_1338001_1341400_REVERSE	AUAUUGGCAUAAUUCAAUCA	Translation	LOC_Os09g02860.1 chr09:1340563-1341235 FORWARD; [PFAM]
chr08_11778001_11781400_FORWARD	AUAAUGGCAUGGAUCAAUUA	Cleavage	LOC_Os08g19694.1 chr08:11779187-11791873 REVERSE; [PFAM]
chr06_23139	GUAUGGGC	Cleavage	LOC_Os06g38980.1 chr06:23134706-23140106

001_23142400_REVERSE	ACGGGUCGAUCA		REVERSE; [PFAM]

Zma-miR171b Target_Acc.	**Target_aligned_fragment**	**Inhibition**	**Target_Desc.**
GRMZM2G174830_T04	GAUUGAACCAUGCUAAUAUG	Translation	seq=cdna; coord=10:24963538..24969021:-1; parent_gene=GRMZM2G174830
GRMZM2G174830_T02	GAUUGAACCAUGCUAAUAUG	Translation	seq=cdna; coord=10:24963522..24969175:-1; parent_gene=GRMZM2G174830
GRMZM2G174830_T03	GAUUGAACCAUGCUAAUAUG	Translation	seq=cdna; coord=10:24963537..24969175:-1; parent_gene=GRMZM2G174830
GRMZM2G174830_T01	GAUUGAACCAUGCUAAUAUG	Translation	seq=cdna; coord=10:24963522..24969175:-1; parent_gene=GRMZM2G174830
chr03_11064001_11067400_FORWARD	AUAUAGGUACGGAUCAAUCG	Cleavage	intergenic [PFAM]
chr01_25677001_25680400_FORWARD	AUAUUGGAGGGGCUCAAUUA	Translation	LOC_Os01g44210.1 chr01:25677469-25678396 REVERSE; [PFAM]
chr09_1335001_1338400_REVERSE	AUAUUGGCAUAAUUCAAUCA	Translation	LOC_Os09g02840.1 chr09:1331084-1336147 FORWARD; LOC_Os09g02850.1 chr09:1336310-1337153 FORWARD; [PFAM]
chr11_5910001_5913400_FORWARD	AUAUUGCUGCAGCUCAAUCA	Translation	LOC_Os11g10770.1 chr11:5912443-5914497 REVERSE; [PFAM]

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