GENOME-WIDE microRNA IDENTIFICATION IN DROUGHT TOLERANT WILD EMMER WHEAT

ASSESMENT OF ANTIMICROBIAL ACTIVITY OF SURFACE ACTIVE ANTIMIC AGENT ON RAW FRUIT AND VEGETABLE PACKAGING

by

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ABSTRACT-I

GENOME-WIDE microRNA IDENTIFICATION IN DROUGHT TOLERANT WILD EMMER WHEAT

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MSc Thesis, 2016
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Keywords: microRNA, drought, *Triticeae*, wheat, emmer wheat, *in silico* analysis, qRT-PCR

As the progenitor of A and B genomes of hexaploid bread wheat, wild emmer wheat (Triticum turgidum spp. dicoccoides) is the source of genes that are involved in several biotic and abiotic stress responses. It constitutes a rich genetic resource for the improvement of common wheat which has lost its stress resistance traits as a trade-off to high yield and quality due to years of cultivation and selection practices. Thus, untangling the genetic elements involved in the stress-response metabolism in the wild progenitors and exploitation of this genetic diversity holds great importance. microRNAs (miRNA) are small non-coding RNAs which post-transcriptionally regulate many vital cell metabolism pathways in plants across diverse tissues and stress conditions. miRNA mediated drought response of wheat have been investigated in a number of studies and the miRNA over-expression studies of other cereal species are giving promising results towards the development of drought tolerant crops. In this study, we utilized the 10.8 Gb whole genome shot-gun assembly of Zavitan, a highly drought tolerant wild emmer wheat accession to computationally identify its putative miRNAs, repeat-related fold-back structures (TE-miRs) and tRNAs. After constructing a wheat expression database, we have searched the identified miRNA precursors in it for the *in silico* expression evidence. Furthermore, expression of randomly selected mature miRNAs was demonstrated with RT-qPCR. To further investigate the involvement of these miRNAs in drought metabolism, we comparatively screened their stress induced expression profiles in root and leaf tissues across two different shock drought stress durations. In addition to exploring the miRNA repertoire of the wild emmer wheat, our results have delineated spatio-temporally changing drought-responsive miRNA profiles, bringing forth new miRNA gene candidates for future crop improvement studies.

ÖZET-I

KURAKLIĞA DİRENÇLİ YABANİ BUĞDAY GENOMUNDA mikroRNA TESBİTİ

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Yüksek Lisans Tezi, 2016

Prof. Dr. Hikmet Budak (Tez Danışmanı)

Anahtar kelimeler: mikroRNA, kuraklık, *Triticeae*, buğday, yabani emmer buğdayı, *in silico* analiz, qRT-PCR

Ekmeklik buğdayın A ve B genomları atası olan yabani emmer buğdayı (*Triticum turgidum* spp. *dicoccoides*) biyotik ve abiyotik stres yanıtında görev alan pek çok genin kaynağıdır. Emmer buğdayı genomu, senelerdir süregelen kültürleme ve seçilim uygulamalarının sonucu olarak stres-direnci genlerini kaybetmiş olan ekmeklik buğdayına yönelik geliştirme çalışmaları için zengin bir kaynak niteliğindedir. Bu nedenle tetraploid emmer buğdayının stres metabolizması ve bu metabolizmada görev alan elemanların anlaşılması büyük önem arz etmektedir. Küçük ve protein kodlamayan RNA türlerinden olan mikroRNA'lar hücre metabolizmasında post-transkripsiyonel regülasyonda görev alır ve birçok önemli yolağı kontrol ederler. Buğdayda mikroRNA aracılı kuraklık yanıtı ile ilgili çalışmalar mikroRNA'ya dayalı ekin geliştirme stratejileri için umut verici sonuçlar ortaya çıkarmıştır. Mevcut çalışmada kuraklığa dayanıklı tetraploid Zavitan emmer buğdayına ait 10.8 Gb boyutundaki genom sekansı kullanılarak potansiyel mikroRNA, tRNA ve genomic mikroRNA benzeri tekrar sekansları bulunmuştur. Prekürsör mikroRNA sekanslarının ekspresyonu, hexaploid ve tetraploid buğdaya ait ekspresyon verilerinden derlenerek hazırlanan ekspresyon veritabanında gösterilerek bilgisayar tabanlı olarak kanıtlanmıştır. Rastgele seçilen olgun mikroRNA'ların ekpresyonları RT-qPCR yöntemi ile deneysel olarak kanıtlanmıştır. Bu mikroRNAların kuraklık stresinde, farklı stres süreleri ve farklı dokulara göre değişen anlatım profilleri şok-kuraklık stresi uygulanan bitkilerin kök ve yaprak dokularında incelenmiştir. Bu çalışma, yabani emmer buğdayı genomuna ait mikroRNA repertuvarının tesbitinin yanı sıra doku ve stres süresi bazlı değişen mikroRNA profillerini tanımlayarak ileride yapılacak kuraklığa dayanıklı ekin geliştirme çalışmaları için önemli bir kaynak oluşturmuştur.

ABSTRACT-II

ASSESMENT OF ANTIMICROBIAL ACTIVITY OF SURFACE ACTIVE ANTIMIC AGENT ON RAW FRUIT AND VEGETABLE PACKAGING

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MSc Thesis, 2016
Prof. Dr. Hikmet Budak (Thesis Supervisor)

Keywords: Antimic, antimicrobial packaging, shelf life, raw fruits and vegetables

Post harvest preservation of the fresh fruits and vegetables is of great importance in terms of human health and economic losses caused by the food spoilage. Up until now, a variety of physical and chemical methods have been applied for the better preservation and longer shelf life, in addition to the newly popularized nanotechnology based antimicrobial packaging strategies. Antimic is a surface-active quaternary ammonium compound which covalently binds and covers the applied surfaces and exerts its antimicrobial activity on a wide spectrum of microorganisms. Its positively charged ammonium silane base is proposed to attract the negatively charged cell envelope of microorganism and disrupt their membrane integrity with the repeating units in its polymer chain. In the presented project, we have assessed the antimicrobial activity of Antimic-6000 on improving the post-harvest storage and shelf life of the raw fruits and vegetables. After the production of the packaging materials with Antimic-6000 coating, we surveyed their mechanical and physical characteristics. To determine the antimicrobial effect, we have performed microbiology tests on diverse food and packaging unit combinations. Statistical analysis revealed promising results for the Antimic-6000 mediated food preservation. Our study represents a preliminary work towards the use of novel and effective antimicrobial food packaging strategies.

ÖZET-II

YÜZEY AKTİF ANTİMİKROBİYAL ANTİMİC ÜRÜNÜNÜN YAŞ MEYVE VE SEBZE PAKETLEMEDE ETKİNLİĞİNİN DEĞERLENDİRİLMESİ

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Yüksek Lisans Tezi, 2016

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Anahtar kelimeler: Antimic, antimikrobiyal paketleme, raf ömrü, yaş sebze ve meyve.

Yaş meyve ve sebzelerin hasat sonrası korunumu gıda zehirlenmelerine bağlı hastalıkların ve çürümeye bağlı ekonomik kaybın önlenmesi açısından büyük önem arz etmektedir. Gıda ürünlerinin korunması için geçmişten beri süregelen fiziksel ve kimyasal koruma stratejilerinin yanısıra, nanoteknoloji tabanlı antimikrobiyal paket üretimi son zamanlarda popülerite kazanmıştır. Antimic, kovalent bağlar ile uygulandığı yüzeylere tutunan ve geniş spektrumda antimikrobiyal etki gösteren yüzey aktif bir kuaterner amonyum bileşiğidir. Antimic ürününün çalışma makenizmasının, yapısındaki amonyum ve silan gruplarının pozitif yükü sayesinde, mikroorganizmaların negatif yüklü hücre zarlarını yüzeye çekmesi ve tekrarlı polimer zinciri ile hücre zarının bütünlüğünü bozması şeklinde gerçekleştiği düşünülmektedir. Bu çalışmada Antimic-6000'in hasat sonrası taze sebze ve meyvelerin korunmasında ve raf ömrünü arttırmadaki etkinliği incelenmiştir. Antimic kaplanarak hazırlanan paketleme ürünlerinin fiziksel ve mekanik özellikleri test edildikten sonra faklı paket materyali ve meyve kombinasyonları önerilen optimum koşullarda depolanmış ve bu paketlerde muhafaza edilen ürünler üzerinde mikrobiyolojik testler yapılmıştır. Sonuçların istatistiksel analizi Antimi-6000'in bazı meyve/sebze ve paket kombinasyonlarında yaş meyve ve sebzelerin korunmasında olumlu etki gösterdiği görülmüştür. Bu çalışma yeni ve etkin antimikrobiyal paketleme stratejilerine yönelik bir ön çalışma niteliği teşkil etmektedir.

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ABBREVIATIONS-I

%GC	Guanine-Cytosine content
µL	Microliter
µM	Micromolar
ABA	Abscisic acid
AGO	Argonaute
BLAST	Basic Local Alignment Search Tool
bp	Basepair
cDNA	Complementary DNA
cm	Centimeter
Cq	Quantification cycle
CT	Threshold cycle
DCL1	Dicer-Like 1 protein
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DREB	Dehydration-Responsive Element-Binding protein
E	Efficiency
En/Spm	Suppressor-mutator Transposable element familiy
EST	Expressed Sequence Tag
FLcDNA	Full-length cDNA
g	Gram
Gb	Giga base
gDNA	Genomic DNA
GO	Gene Ontology
HD-ZIP	Homodomain-leucine zipper
HEN 1	HUA ENHANCER 1
hr	Hour

HST	HASTY
HYL1	HYPONASTIC LEAVES 1
ICCI	Institute for Cereal Crops
L	Liter
LINE	Llong Interspersed Nuclear Element
LTR	Long Terminal Repeat
m	Meter
M	Molar
MAS	Marker-Assisted Selection
Mb	Mega base
MFE	Minimum Free Energy
MFEI	Minimal Folding Free Energy İndex
mg	Milligram
MIRs	microRNA encoding genes
MITE	Miniature Inverted-repeat Transposable Elements
miRNA	microRNA
MPSS	Massively Parallel Signature Sequencing
mRNA	Messenger RNA
MS	Murashige and Skoog
MuDR	Mutator Transposable element familiy
NAC	NAM, ATAF, and CUC)
NCBI	National Center for Biotechnology Information
ncRNAs	Non coding RNAs
ng	Nanogram
NGS	Next Generation Sequencing
nt	Nucleotide
^{o}C	Celcius degree

ORF	Open Reading Frame
PGSB	Plant Genome and Systems Biology
PlantGDB	Plant Gene Data Base
Ppm	Parts per million
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
q-RT PCR	Quantitative-Real Time Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RIL	Recombinant Inbred Line
RISC	RNA-induced silence complex
RLM-RACE	RNA Ligation-Mediated Rapid Amplification of cDNA Ends
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA
RT	Reverse Transcription
RT-PCR	Reverse Transcription PCR
s	Second
SeC	Selenocystein
siRNA	Small interfering RNA
SPL	SQUAMOSA PROMOTER PROTEIN-LIKE
sRNAs	Small RNAs
SSR	Simple Sequence Repeat
Sup	Suppressor tRNA sequences
TcMar	TcMariner Transposable element familiy
TE-miR	Repeat/transposable element-related hairpins

tRNA	Transfer RNA
tRNA	Translational RNA
WEWseq	The International Wild Emmer Wheat genome sequencing consortium

ABBREVIATIONS-II

µm	Micromolars
^{0}C	Celcius
A&I packaging	Active and intelligent packaging
AATCC	American Association of Textile Chemists and Colorists
AFM	Atomic Force Microscopy
ANOVA	Analysis of variance
APC	Aerobic Plate Count agar
CBPs	Chlorine by-products
CFSAN	Center for Food Safety and Applied Nutrition
Cm	Centimeters
cyc	Cycloheximide
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EPA	United States Environmental Protection Agency
EU	European Union
FDA	The United States Food and Drug Administration
FTIR	Fourier Transform Infrared Spectroscopy
HPP	High Pressure Processing
IMS	Immunologic Magnetic Separation
IPA	Isopropyl alcohol
ISO	International Organization for Standardization

LSD	Least Significant Difference
MAS	Modified Atmosphere Packaging
MIC	Minimal Inhibitory Concentration
mL	Milliliters
MPa	Megapascal
OD	Optical Density
OFAS	The Office of Food Additive Safety
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PHMB	Polyhexamethylene biguanide chloride
PU	Polyurethane
PVC	Polyvinyl chloride
QACs	Quaternary ammonium compounds
Rmp	Revolutions per minute
SPC	Standard Plate Count agar
ss	Streptomycin sulphade
TEM	Transmission Electron Microscopy
TSA	Tryptic Soy Agar
TSP	Trisodium phosphate
UV	Ultraviolet

FORMULAS-I

FORMULAS-II

CHAPTER I

1. INTRODUCTION-I

1.1 Drought as a major abiotic stress

Drought stress, as a major abiotic stress which is affecting the wheat growth was reviewed by Budak et al. in detail (Budak, Kantar, Bulut, & Akpinar, 2015). As a major abiotic stress condition, drought causes severe yield losses in cereals worldwide. Estimated numbers reflect the regular drought exposure in the 50% of the wheat growing areas (Peleg et al., 2009). Extensive exploration of drought response at the molecular level revealed that miRNAs are involved in a variety of drought related cellular pathways, including abscisic acid (ABA) response, auxin signaling, osmo protection, antioxidant defense, cell growth, photosynthesis and respiration. The expression profiles of rice (Barrera-Figueroa et al., 2012), barley (Lv et al., 2012), maize (Y. Ding, Tao, & Zhu, 2013), and *Triticum* (M Kantar, Lucas, & Budak, 2011) suggested a complex and dynamic drought induced miRNA regulation that can differ among the members of the same miRNA family, such as miR169g in rice, the only member of its family induced by drought (Y. Ding et al., 2013) . A comprehensive study revealed that two-thirds of all known or predicted rice miRNAs have drought-responsive targets, indicating that a considerable portion of the overall rice miRNA repertoire is involved in drought signaling (Shaik & Ramakrishna, 2012).Shared motifs found in the upstream sequences of stress-responsive miRNA genes are especially significant, pointing out to co-regulation of different miRNAs. In a recent report, six such stress-related elements (M1–M6, as referred in the report) were reported to be present in the promoters of genes encoding three different drought responsive miRNAs (miR166k, miR393 and miR397b) (Devi et al., 2013). Additionally, some genes encoding drought responsive miRNAs were found to harbor motifs responsive to ABA (e.g. ABA responsive element, MYB-binding site, MYC-binding site, Motif lib, CE3), and/or drought responsive element (DREB), suggesting their regulation through either or both ABA-dependent or independent pathways (Covarrubias & Reyes, 2010; Devi et al., 2013). However, motif content and composition alone does not necessarily account for all cases of differential miRNA expression profiles. In a recent study, miR408 expression was observed to remain steady in drought tolerant rice cultivars in response to dehydration, while reduced in sensitive cultivar. The contrasting expression profiles of miR408 were later linked to distinct expression profiles of squamosa promoter-binding-like9

transcription factor regulating miR408 expression, although the promoter sequences of both genes in different cultivars were identical (Mutum et al., 2013).

1.2 Employing Wild Wheat Relatives towards Wheat Improvement

Domestication of cereals, followed by years of cultivation, and breeding has considerably narrowed gene pools of today's elite cultivars. Common agricultural practices favor growing cereals under tightly controlled conditions. These practices introduce an artificial selection pressure for yield, which eradicates genetic diversity in the long run, and leads to the loss of valuable alleles for abiotic and biotic stress tolerance. Few agronomic traits are controlled by single gene or isolated biological pathways. Cellular responses against stress conditions usually involve intermingled, complex networks of gene interactions that are regulated at multiple levels. Therefore, understanding the molecular basis of stress responses in cereal is highly challenging but also, crucial.

Utilization of the knowledge coming from the ancestors of the bread wheat is an integral research focus for the elucidation of these intermingled ancient stress response pathways. This approach both overcomes the difficulties of producing and analyzing the hexaploid and highly repetitive bread wheat genome sequence and also beats the dust out of the ancient stress response mechanisms of the wild relatives which are dulled in the modern wheat. Therefore genome donors of wheat are under extensive scientific scrutiny as comparatively easier targets in terms of genome and transcriptome scale examinations. Consisting of three different genome sets; AA, BB and DD, allohexaploid bread wheat genome was formed through two polyploidization events involving the three diploid members of the Triticeae tribe. Although the genetic studies referred *Triticum urartu* (AA) as the A genome, *Aegilops tauschii* (DD) and the D genome and a close relative of *Aegilops speltoides* (SS) as the B genome donor, neither chronology of the allopolyploidization events nor the divergence dates of the phylogenic branches in the tribe has adequate scientific evidence as yet (Marcussen, Sandve, Heier, Spannagl, et al., 2014). Allotetraploid (2n=4x) emmer wheat consisting of AABB is the more recent progenitor of both bread wheat and modern durum wheat and its origin believed to date back in few hundred thousand years (Huang et al., 2002).

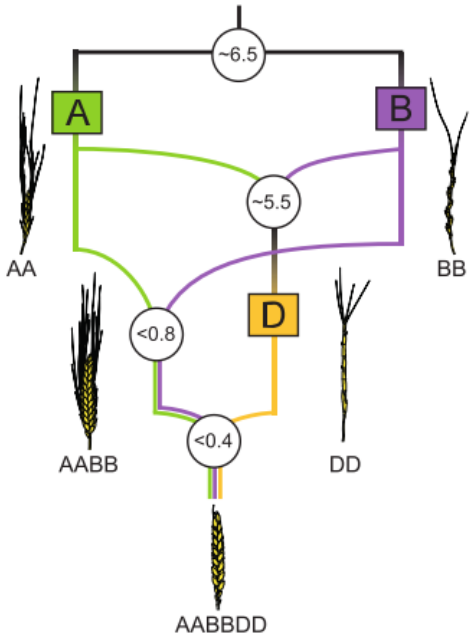

Figure 1: Recently proposed model of the bread wheat genome evolution (Marcussen, Sandve, Heier, Spannagl, et al., 2014)

The recently proposed phylogenetic history suggests the D genome formation by the hybridization between ancient A and B genome progenitors (approximately 6.5 million years ago) and AABB genome formation by the allopolyploidization (less than 0.8 million years ago) between the same lineages. A third hybridization and allopolyploidization event led the formation of hexaploid bread wheat around 0.4 million years ago.

Though hexaploid bread wheat has been adapted to the well watered and controlled growth conditions, its genome progenitor is still continuing to grow in its geographical origin; Fertile Crescent and possessing its resistance genes and response pathway elements against varying unfavorable conditions. The ease of crossing hexaploid and wild tetraploid wheat makes the tetraploid wheat a resourceful genomic tool for crop improvement studies.

1.3 Wild Emmer Wheat and miRNA-mediated Drought Response

The wild emmer wheat *Triticum turgidum* spp. *dicoccoides* is the ancestor of both hexaploid bread wheat and tetraploid durum wheat. The crosses between both species result into fertile progenies. It has a very rich allelic repertoire in terms of stress resistance and some of the defined cultivars resemble high yield and performance even under severe drought conditions

(Budak, Kantar, & Yucebilgili Kurtoglu, 2013). However, similar to many other morpho-physiological traits, drought is not a qualitative trait and rather, controlled by multiple factors complicating its manipulation (Peleg et al., 2009). Also, 'linkage drag' phenomena which is the co-transfer of the unwanted chromosomal segments; hence, genes, during introgression complicates the crossing based approaches further (Ani Akpinar et al., 2015). Therefore delineation of the genes, gene complexes and post-transcriptional regulatory elements in wild emmer wheat is of great significance. A number of studies on the drought stress-responsive metabolism of tolerant and susceptible genotypes of wild emmer wheat revealed important genes and transcripts and linked some of those elements to known wheat drought response related pathways (Bala Ani Akpinar, Kantar, & Budak, 2015; Ergen & Budak, 2009; Ergen, Thimmapuram, Bohnert, & Budak, 2009a; Krugman et al., 2010; Peleg et al., 2009). Also, the results of these experiments showed some of the genes' up-regulated expression in the tolerant genotypes, highlighting a genotype based differentiation in the executed response. Identified drought responsive genes were biased in the high number of transcription factors and transcription factor binding proteins. A detailed review of the drought response regulation in *T. dicoccoides* have been provided by Budak et al. (Budak et al., 2013).

The first and only microRNA mediated drought response studies on wild emmer wheat have been carried out by our group with microarray and RNA sequencing based strategies (Bala Ani Akpinar, Kantar, et al., 2015; Melda Kantar, Lucas, & Budak, 2011). First study was performed with the root and leaf tissues of two drought tolerant wild emmer wheat cultivars that have been subjected to 4h and 8h shock drought stress at the four leaf stage. Hybridization of the total RNA from control and stress samples to a plant miRNA specific microarray has identified 250 and 438 microRNAs respectively. Among these, 13 miRNA was drought responsive and results have revealed spatio-temporally changing expression patterns of the miRNAs in addition to the diverse expression patterns of different members of the same miRNA family (Melda Kantar et al., 2011). The 2016 RNA sequencing study subjected one *T. durum* cultivar and two *T. dicoccoides* genotypes that are contrasting in their drought tolerance to slow drought stress (9 days). Homology based *in silico* miRNA prediction by utilizing the RNA seq assembly of one month old root tissues has identified some microRNA precursors that are specifically expressed in the tolerant cultivars' stress response (Bala Ani Akpinar, Kantar, et al., 2015).

Recognition of the wild emmer wheat genome as a convenient tool for the crop improvement studies has led the establishment of The International Wild Emmer Wheat Genome

Sequencing consortium (WEWseq http://wewseq.wix.com/consortium). The extensive efforts of the consortium have borne fruit with the assembly of 10.8 Gb whole genome sequence of the *Triticum dicoccoides* Zavitan accession. Belonging to the *judaicum* sub-population of the T. dicoccoides population grown in the western Fertile Crescent, Zavitan is a drought tolerant, accession (Avni et al., 2014). Scrutinizing this data for the identification of stress related gene identification is an immediate task for the wheat researchers. Along with the two mentioned studies, present research expands our knowledge on the microRNA-mediated spatio-temporal drought response in wild emmer wheat.

1.4 microRNA Biogenesis

As reviewed by Budak et al., miRNAs are endogenous small RNAs (sRNAs) which are central to post-transcriptional RNA mediated silencing of genes (Budak, Kantar, et al., 2015). miRNA biogenesis begins with the transcription of the long primary transcripts (pri-miRNA) from miRNA genes (MIR), which are processed into smaller stem-loop precursors (pre-miRNA) and subsequently, into mature miRNAs. miRNAs exert their effects on gene regulation following their loading onto the RNA-induced ribonucleoprotein silencing complex (RISC) by directing it to the target complementary mRNA. Highly complex mechanisms of miRNA biogenesis and miRNA mediated gene silencing are reviewed in detail elsewhere (Figure 2) (Ul Hussain, 2012).

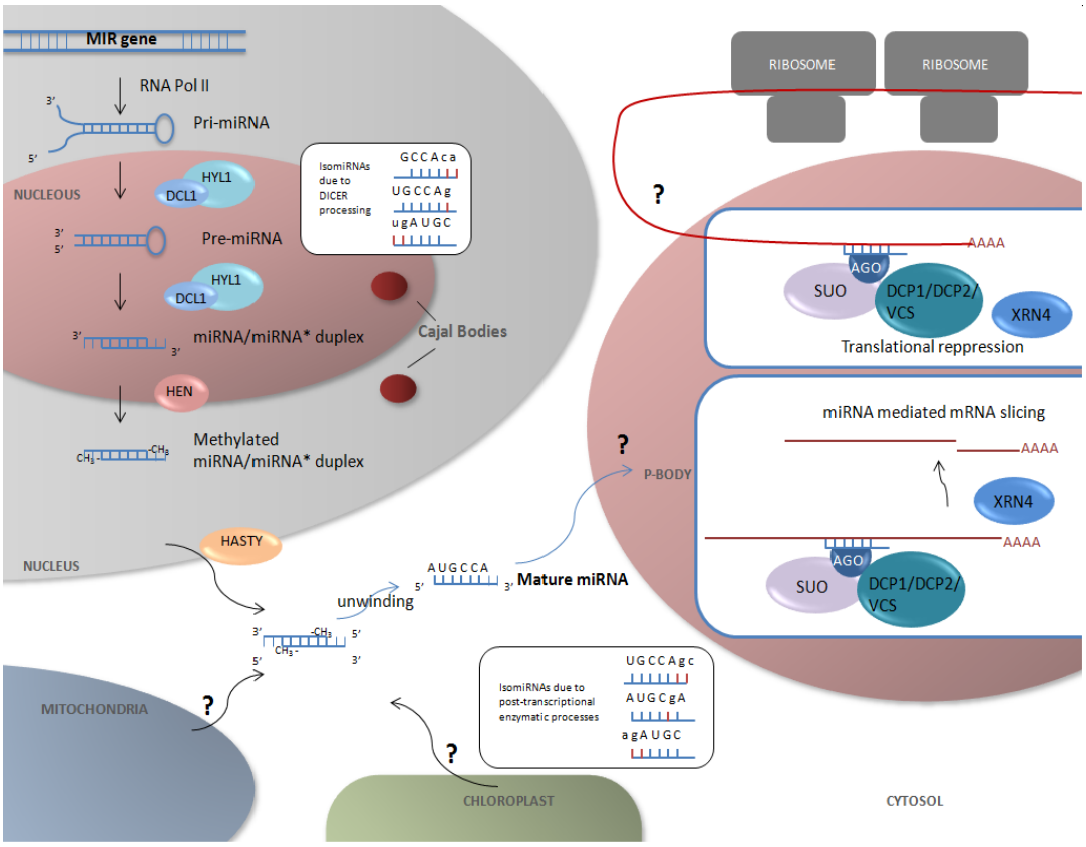

Figure 2: Basic miRNA regulatory network model in plant cells (Budak, Kantar, et al., 2015)

While a general framework of miRNA biogenesis and miRNA mediated gene silencing is relatively well-established, ongoing research continues to extend this general view and reveal new factors affecting miRNA metabolism and function. The mechanisms regulating MIR transcription and subsequent processing, as well as, factors affecting miRNA incorporation to alternative Argonaute proteins (AGOs) and miRNA turnover may carry additional functional significance. For instance, translational inhibition, which may enable plants to rapidly change their responses under transient stress conditions is increasingly considered to contribute to miRNA mediated gene silencing. A closer look into the recent evidence pointing out to transcriptional regulation of MIR loci, subsequent alternative splicing and phosphor-regulation of micro-processor complex components may lead to the detection of alternative biogenesis routes and uncover novel mechanisms linking miRNA metabolism and function to cellular stress responses (K Rogers & Chen, 2012; Kestrel Rogers & Chen, 2013). Indeed, heat stress was found to induce splicing of introns hosting two heat responsive miRNAs (miR160a, miR5175a) in barley (Kruszka et al., 2014).Changes in gene expression induced by various factors are regulated both at the transcriptional and post-transcriptional levels. As well-established trans-regulatory mediators of post-transcriptional regulation, miRNAs are

known to target a variety of transcription factors, acting in accordance with the transcriptional control on gene expression. miRNAs are encoded as independent transcription units by their own genes at diverse intergenic or genic locations and MIR trancription is subjected to control at the transcriptional level, similar to protein coding genes (Budak, Kantar, et al., 2015).

1.5 Utilization of miRNAs in the development of stress tolerant crops

Recent advances in molecular biology as well as functional and comparative tools offer new opportunities for crop improvement through molecular approaches. Development of stress tolerant crops can be achieved by the introduction of stress-related components through breeding or transgenic approaches. Breeding has played an important role in the past century for enhancing stress tolerance of several crops. Heterosis is the phenomenon in which the progeny of two inbred lines exhibits superior agronomic performance compared to either of the parents. It has been widely used inbreeding programs and plays a significant role in increasing yield, improving quality and enhancing stress tolerance. Recently, several lines of evidence have suggested roles for miRNAs in molecular mechanisms underlying heterosis. Compared to the parental lines, hybrids exhibited a general trend of miRNA repression in maize (D. Ding et al., 2012; P. Zhao et al., 2014) and rice (Chen et al., 2010). An overall reduction in miRNA expression is speculated to result in increased diversity or abundance of transcripts that are involved in heterosis. Future research defining and elaborating the involvement of miRNAs in these mechanisms will provide a better understanding of the molecular basis of heterosis, which can then be utilized in breeding efforts. In addition to conventional breeding approaches, molecular breeding has emerged to facilitate and fasten crop improvement programs. Particularly, Marker-Assisted Selection (MAS) utilizes molecular markers linked to traits of interest, to screen and select plants with improved characteristics. Recent increases in sequence availability sequence availability have provided a rich source to design several high quality genetic markers for breeding through MAS, one of which is Simple Sequence Repeats (SSRs). Very recently, miR-SSR markers were mined from salt-responsive miRNA genes of rice. These markers were experimentally validated in discriminating two rice panels with distinct degrees of tolerance to salinity (Mondal & Ganie, 2014). The discovery of similar miRNA-linked markers and the application of these miR-SSRs to other stress treatments can provide novel resources for molecular breeding programs.

Transgenics is another methodology for ongoing breeding pro-grams, with the advantage of transferring only the desired genes from one species to another. Potentially, this allows the transfer of a single locus associated with stress tolerance into the recipient crop, thereby minimizing yield losses due to linkage drag. Therefore, improved transgenic strategies are currently being developed. Stress-related candidate miRNAs that hold potential for crop improvement are accumulating through intensive stress-related miRNA research, which can be utilized for crop improvement with the use of transgenics. As well as being promising targets of transgenic modification, detailed knowledge on miRNAs has also enabled the development of advanced transgenic techniques, silencing through artificial miRNAs and target mimicry (Budak, Kantar, et al., 2015).

1.6 Computational and wet-lab methods to identify microRNA

The techniques for miRNA identification are becoming progressively sophisticated as the desire to understand their physiological roles in cellular processes, including abiotic and biotic stress responses increases. One of the conventional techniques for miRNA identification has been forward genetic screening, which has been employed for the identification of miR164c responsible for the extra petal phenotype of mutant Arabidopsis thaliana plants (Baker, Sieber, Wellmer, & Meyerowitz, 2005). Forward genetic screening has the advantage of providing information on the particular miRNA function. However, this approach is costly and time consuming, and has resulted in the discovery of only a limited number of miRNAs. In the past, most miRNAs were identified through traditional Sanger sequencing subsequent to size fractionation of small sequences and their ligation into cloning vectors. sRNA cloning was one of the most initially used (Llave, Kasschau, Rector, & Carrington, 2002) and effective methodology to identify miRNAs and has been the basis of numerous publications in the past years. Later, the advent of high-throughput sequencing technologies, eliminated the need for cloning prior to sequencing. Deep-sequencing of sRNA libraries and another high-throughput transcriptomics technique, hybridization-based microarray platforms were implemented for miRNA detection, providing an additional advantage of comparing components of experimentally generated high-throughput sRNA data interpretation. They are also extensively utilized as principal and less resource-intensive strategies for miRNA discovery in species where large-scale genome or transcriptome sequence information is

available. While these methods are automated and new bioinformatics tools are developed to process high-throughput data with high specificity, sequence datasets of many plants, which may serve as comprehensive inputs for miRNA prediction, are also swiftly accumulating.

Table 1: Pros and cons of different miRNA identification approaches.

miRNA Identificaiton Method	**Pros**	**Cons**	
Forward screening	Gives functional information	Time consuming Costly	Should be complemented with bioinformatics analysis (i.e pre-miRNA stem-loop verification)
Microarrays	Useful when sequence information is limited	Only the miRNA homologous could be found Not exact miRNA sequence	
RNA-sequencing	Selective Precise Suitable for novel miRNA discovery	Resource intensive Limeted to only few samples	
Bioinformatics tools	Less resource intensive	Only when large-scale sequencing information is available	Should be verified through experimental procedures

Widespread application of next generation sequencing (NGS) technologies has highly contributed to miRNA identification, boosting related studies, as reviewed elsewhere (Budak, Khan, & Kantar, 2014a; Pais, Moxon, Dalmay, & Vincent Moulton, 2011). Hence, our knowledge on the miRNA repertoires of plants, including cereals, is rapidly growing

(Marcussen, Sandve, Heier, Pfeifer, et al., 2014). These powerful technologies are able to capture extensive collections of genome-wide or transcriptome-wide miRNAs. However, functional validation of miRNAs identified through computational analysis of the NGS data still require further experimental evidence through additional procedures, such as quantitative real time PCR (qRT-PCR),northern blotting, RNA gel blots, or splinted-ligation based detection. Additionally, in order to place the miRNAs in a broader context and understand their functional relevance, knowledge about their respective target(s) is of utmost importance. Thus, miRNA targets have also been identified in most of the miRNA studies either through computational approaches (web-based tools like psRNATarget, plantgrn.noble.org/psRNATarget; Cleave-Land (also uses degradome sequencing data), http://axtell-lab-psu.weebly.com/cleaveland.html), and/or experimental methods such as RNA Ligation-Mediated Rapid Amplification of cDNA Ends (RLM-RACE) or its high-throughput application, 'degradome sequencing'. miRNAs and their targets have been a hot topic of research in the last decade and public miRNA databases including miR-Base (http://mirbase.org/) are rapidly expanding. Still, research on miRNA sequence variants has been lagging behind (Budak, Kantar, et al., 2015).

1.7 microRNA evolution and TE-MIRs

For the elucidation of the miRNA origins, an indirect source of information has been the distribution and comparison of the conserved and non-conserved paralogous and orthologous miRNAs (Figure 3) in delegates of the divergent evolutionary branches. Notwithstanding, scarcity of the number of sequenced plant genomes and the unequal taxonomic sampling hinders a kingdom wide conclusion of miRNA origins (Taylor, Tarver, Hiscock, & Donoghue, 2014). Widening the first findings in the field, employment of deep sequencing for small RNA identification purposes have revealed a myriad of non-conserved plant miRNAs and spatio-temporal and interchangeable expression patterns of the conserved miRNAs between different species under stress conditions (Budak, Khan, & Kantar, 2014b; Jeong & Green, 2013; Kestrel Rogers & Chen, 2013; B. Zhang, 2015) emphasizing the diverse network-forming physiology and stress conditions they experience. In the following section hitherto hypotheses proposed for the microRNA evolution with a special focus on plant abiotic stress responses have been outlined.

First hypothesis for the origin of miRNA genes was proposed by Allen et al. based on the observation that newly emerged "young" miRNAs (Budak, Bulut, Kantar, & Alptekin, 2015) showing extended sequence homology to their targets aside from the mature miRNA region. According to this, an inverted duplication event (head-to-head or tail-to-tail orientation / with or without the promoter sequence) results into the formation of a fold-back transcript which can be recognized by the DCL enzymes and form small interfering RNAs that may negatively regulate the expression of the founder gene and eventually adapt to the miRNA machinery through changes in the secondary structure via mutational drift. After the formation of the unique target specificity subsequent to duplication, sequences flanking the mature miRNA region change over evolutionary trajectory. If the inverted duplication event includes one of the 'family domain' of the founder gene, resulted miRNA may orchestrate an extensive regulatory network which include different family members of the founder gene (Allen et al., 2004). Targeting of several Auxin response factors by miR167 and miR160, HD-ZIP transcription factors by miR166, NAC family transcription factors by miR164 and MYB family transcription factors by miR159 in soybean (Song et al., 2011) and the miR824 targeting MADS box genes which are recently shown to be involved in the drought stress response in *Brachypodium distachyon* and rice indicates the convenience and importance of this mechanism in response to a myriad of abiotic stresses (Arora et al., 2007; Kutter, Schöb, Stadler, Meins, & Si-Ammour, 2007; Wei et al., 2014). While most of miRNA evolved through this route targets its founder gene, accumulated mutations may bring forth the silencing of transcripts from unrelated loci as in the case of miR856 that targets both the founder gene *ZAT1* and a novel gene *CHX18* (Fahlgren et al., 2007; Felippes, Schneeberger, Dezulian, Huson, & Weigel, 2008).

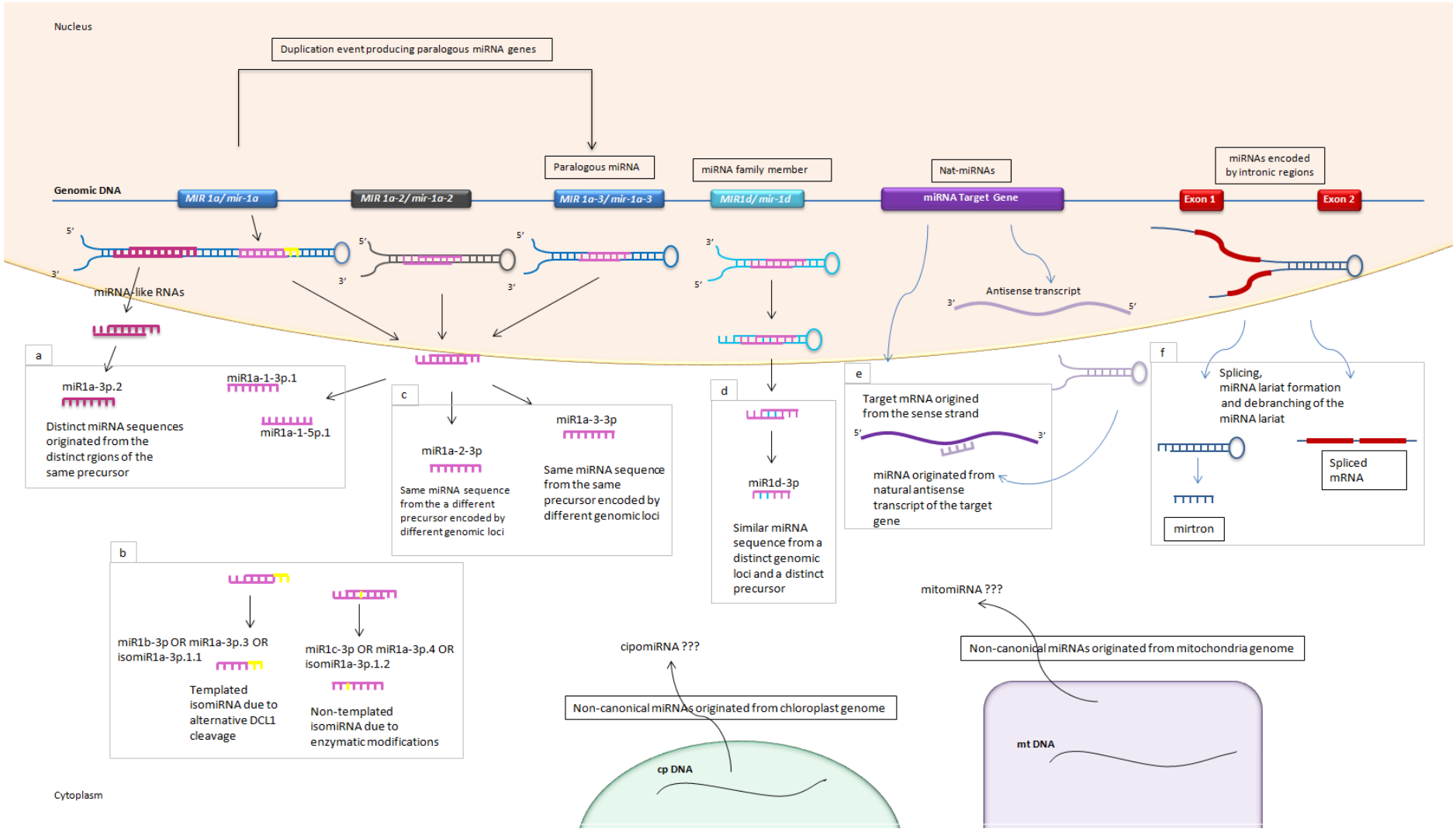

Figure 3: A simplified diagram of canonical and non-canonical miRNA origins (Budak, Bulut, et al., 2015)

Maher et al. surveyed the genome-wide, segmental and tandem duplications in *Arabidopsis thaliana* genome to reveal the evolution and expansion of miRNA gene families if it occurs in a similar fashion with protein-coding gene family evolution. They characterized tandemly duplicated miRNA family members that are physically linked (approximately by 1.9 kb) by analyzing non-coding flanking sequences, and miRNAs resulted from large duplication events by comparing the protein coding regions surrounding them (from same family, differing families and random genomic locations). They showed the intra- and inter-chromosomal duplication, subsequent inversion and rearrangement events that spawn miR159a and miR159b in chromosome 1 (estimated as dating back to 30 myr according to synonymous substitution and duplication event analysis) and miR159c reside on the chromosome 2. However miR159c could not be framed in this evolutionary timescale because of lacking sequence similarity in the flanking regions (Maher, Stein, & Ware, 2006). Both the sequential variation and its crucial role in plant stress response by targeting the MYB transcription factors indicate the antiquity of this family putting forward miR159c as the founder miRNA gene (Ambawat, Sharma, Yadav, & Yadav, 2013; Baldoni, Genga, & Cominelli, 2015).

After the identification of tandemly duplicated miRNAs, MPSS (Massively Parallel Signature Sequencing) analysis revealed polycistronic expression of some of them evident by their identical downstream MPSS signatures (Maher et al., 2006). Orchestration and dynamic alteration of the essential cellular pathways are vital especially during plants stress metabolism. Post-transcriptional silencing exerted by miRNAs comes into prominence in stress responses as this mechanism provides an immediate response. This immediate response can be intensified by the dosage increasing effect of tandemly duplicated miRNAs on the same strand of DNA, which are expressed as polycistronic transcription units (Merchan, Boualem, Crespi, & Frugier, 2009; J. Sun, Zhou, Mao, & Li, 2012).

Aside from being unwanted parasites that are epigenetically silenced through the action of repeat associated siRNAs (Slotkin & Martienssen, 2007), transposable elements (TEs) shape the eukaryotic genomes by mediating translocations and duplications. As most of them have translational regulation and splicing signals, integration of a transposable element into a protein coding gene may alter the expression of the gene and create new splice variants of the resulted protein (Kazazian, 2004). miRNAs that neighboring the transposable element regions and some bona fide miRNAs that possess identical sequences to the TEs concluded that some miRNAs are formed as a result of transposable element activity as they shape the genome in

the evolutionary course (Y. Li, Li, Xia, & Jin, 2011; Piriyapongsa & Jordan, 2008). Li et al. surveyed the previously annotated rice miRNAs in miRBase and select out the ones that are homologous or identical to transposable elements as TE-miRs. Not surprisingly, majority of them were derived from Miniature Inverted-repeat Transposable Elements (MITEs) due to their palindromic nature, other TE-miRs are proposed to be formed through juxtaposition of inverted copies of the same TE (Y. Li et al., 2011). They mostly positioned in the genic regions of the genome consistent with the high MITE insetion rates in genic regions (Mao, 2000; Tikhonov et al., 1999; Q. Zhang, Arbuckle, & Wessler, 2000). Expression analysis showed that while some of them were bona fide TE-miRs, some resembled the transition forms from TEs to real miRNA genes through siRNA path as they processed as 24 nt mature sequences. This founding supported the idea that young miRNA genes produce heterogenous small RNA populations and may encode for both miRNA and siRNA (Y. Li et al., 2011; Piriyapongsa & Jordan, 2008; Rajagopalan, Vaucheret, Trejo, & Bartel, 2006). These transition forms might incorporate into either siRNA or miRNA machinery depending on the transcription units they have harbored under (RNA polymerase type selectivity) and selective pressure created by the environmental stresses and invading genomic elements. In a recent study, small RNAs accompanied by high levels of miRNA* reads with anomalous putative hairpin structures in deep sequencing libraries have been categorized as "miRNA like siRNA loci" and they might be the reason of this mentioned transformation (Bertolini et al., 2013). Despite TE insertion is deleterious for the CDS most of the time; they might create alternative ORFs with the transcriptional regulatory units they harbor and multi-gene targeting profile of some miRNAs may be explained by their TE origins as a result of coding sequence domestication of the cognate transposable element (Y. Li et al., 2011).

Excluding the duplication and inversion events, an additional miRNA evolution route have been proposed as fortuitous arose of some miRNA genes from self-complementary fold-back sequences scattered through the plant genomes after being captured under transcriptional regulatory units. Contrary to inverted duplication hypothesis, some of the species specific MIRs in Arabidopsis thaliana failed to resemble similar sequences in the genome as their founder locus. Supporting this random formation, the syntheny analysis of the flanking protein coding sequences of the miRNA gene in its close relative Arabidopsis lyrata revealed the absence of similar fold back structures in between these orthologous gene blocks (Felippes et al., 2008). This hypothesis suggests spontaneous arrangement for the miRNA-target pairs that may be fixed through co-evolution under positive selection pressure. However this mechanism

is more suitable to explain animal miRNA-target pairing as the targeting does not require a near-perfect match like in plant miRNAs.

2. MATERIALS AND METHODS-I

2.1 Materials

2.1.1 Chemicals, Enzymes and Molecular Biology Kits

The enzymes, chemicals and molecular biology kits that are used through this study are listed in Appendix-I A.

2.1.2 Equipment

Equipments used through this study are listed in Appendix-I B.

2.1.3 Plant Material

The seeds of wild emmer wheat Zavitan accession were obtained from Dr. Assaf Distelfeld (Institute for Cereal Crops (ICCI), Tel Aviv University). TR39477 wild emmer wheat line and AL8/78 *Aegilops tauschii* accession seeds in Sabanci University were also used for the miRNA housekeeping gene investigation.

2.2 Methods

2.2.1 Computational Identification of *Triticum trugidum* spp. *dicoccoides* microRNAs

2.2.1.1 Sequence Datasets

2.2.1.1.1 Triticum turgidum spp. dicoccoides var. Zavitan Genome sequence

The 10.8 Gb chromosome anchored (Mascher et al., 2013) genomic scaffolds (NRGene assembly program http://nrgene.com/) of paired-end (100x coverage) and mate-pair (80x coverage) whole genome shotgun sequence of *Triticum turgidum* spp. *dicoccoides* var. Zavitan was kindly provided by Assaf Distelfeld (Institute for Cereal Crops (ICCI), Tel Aviv University).

Table 2: Features of Zavitan genome assembly produced by Distelfeld et al.

Assembly size	**10.8 Gb**
Scaffolds anchored to the genetic map	10.3 Gb
Gap size (N's)	~ 4%
Number of scaffolds	245,488
N50	6.8 Mb
N50 # of scaffolds	415
N90	1 Mb
N90 # of scaffolds	1911

2.2.1.1.2. Plant miRNA Reference Dataset

Mature miRNA sequences of 67 different plant species (*Viridiplantae*), were downloaded from miRBase release 21 (June 2014) (http://mirbase.org/). In cases where multiple miRNAs had the same mature miRNA sequence, only one was retained. The resulting dataset containing 4,801 unique mature miRNA sequences was used as a query in Zavitan miRNA prediction (**plantmiRNAs.txt.fsa).**

2.2.1.2 Homology-based *In silico* microRNA Identification

Homology based microRNA prediction (Figure 4) was employed using two in-house Perl scripts: SUmirFind and SUmirFold17, described in detail in our group's previous publications (Bala Ani Akpinar, Kantar, et al., 2015; Melda Kantar et al., 2012; Kurtoglu et al., 2014; Kurtoglu, Kantar, Lucas, & Budak, 2013; Lucas & Budak, 2012). SUmirFind script utilizes BLAST+ stand-alone toolkit, version 2.2.31 for the first step of *in silico* homology-based miRNA identification, and the mismatch criteria was set as <3.

The results table that was generated by the SumiRFind.pl script contained 64,731 and when the **plantmiRNAs.txt.fsa** file used as query. Resulted hits were filtered to get rid of the microRNA sequences that gave hit to the same region of the same scaffold with differing start or end positions by;

1- Removing duplicate sequences according to subjectID (column 2 of the table), sstart (column 9) and send (column 10)

```
$ awk '!x[$2,$9,$10]++' FS='\t' plantmiRNAs.txt.fs.results.tbl > filtered1.tbl
```

2- Removing duplicate sequences according to subjectID and sstart

   $ awk '!x[$2,$9]++' FS='\t' filtered1.tbl > filtered2.tbl

3- Removing duplicate sequences according to subjectID and send

   $ awk '!x[$2,$9]++' FS='\t' filtered2.tbl > filtered3.tbl

After the filtering step filtered3.tbl file containing 59,002 hits used for the secondary structure generation of putative miRNA precursors with SumiRFold17.pl script (Lucas & Budak, 2012). SumiRFold17.pl script utilizes RNA structure prediction algorithm mfold version 3.6 (Zuker, 2003) to generate all possible miRNA stem-loop structures with their minimum folding energies (MFE). SumiRFold predicts the secondary structure of the miRNA stem-loop and sorts out the ones that pass the secondary structure and miRNA-miRNA* base pairing criteria. After generation of pre-miRNA hairpins, these structures further examined for the previously defined pre-miRNA structure criteria with the in house python script: mirScreen.py.

2.2.2 Identification of repeat related and non-repeat related putative *Triticum trugidum* spp. *dicoccoides* microRNAs

Precursor sequences of putative microRNAs were analyzed for their repeat content. For this purpose unique pre-miRNA sequences were masked with RepeatMasker software version 4.0.6 (www.repeatmasker.org) (in the –q mode), against PGSB Repeat Poaceae Database (Nussbaumer et al., 2013) which involves 34,135 repeat element records. Pre-miRNAs which are covered by repeat elements by 50% or more are denoted as repeat/transposable element-related hairpins (TE-MIRs). The repeat elements that gave hit to the putative precursory sequences were grouped and analyzed by their repeat family. Repeat element types of Zavitan TE-MIRs were compared with wheat whole genome (Kurtoglu et al., 2014) TE-MIRs.

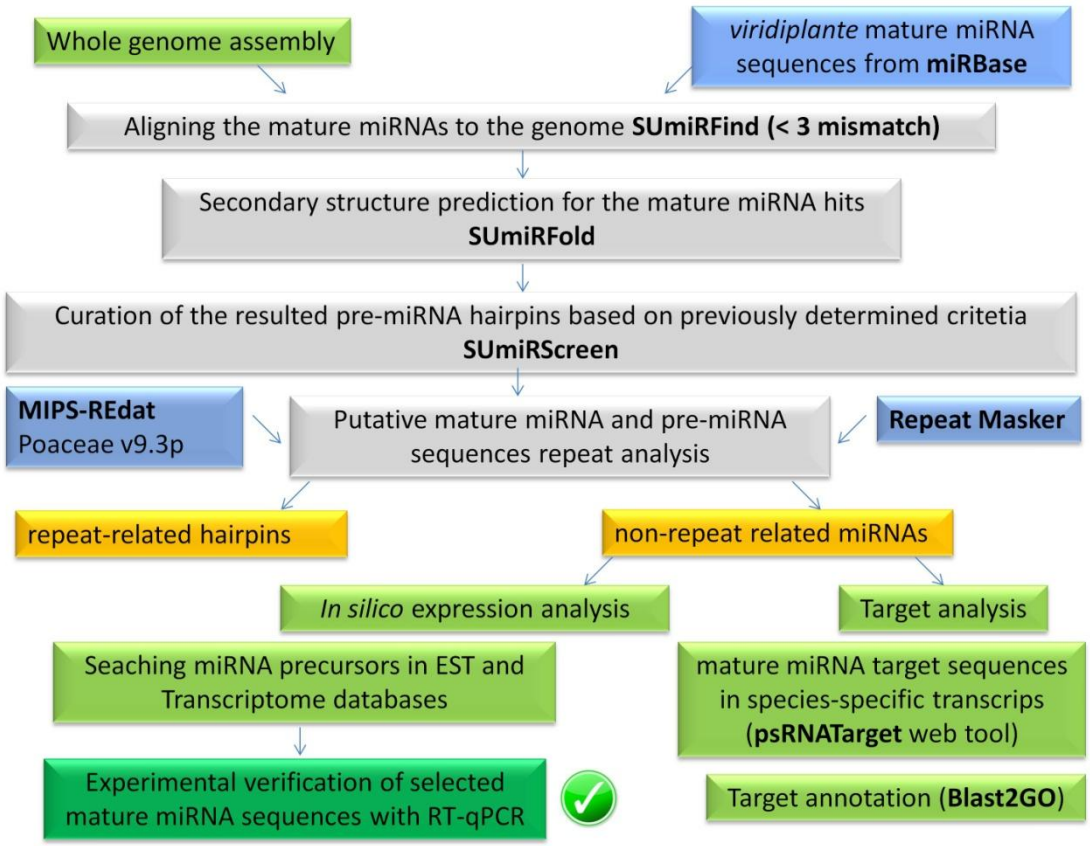

Figure 4: Flowchart representing our *in silico* microRNA prediction, microRNA target prediction and expression analysis strategy.

2.2.3 Computational Identification and Functional Annotation of non-repeat related *Triticum trugidum* spp. *dicoccoides* microRNA Targets

Targets of the non-repeat related microRNAs which gave 99% similarity to an expressed sequence were predicted by online plant small RNA target analysis server psRNATarget (Dai & Zhao, 2011) using "user-submitted small RNAs/ user-submitted transcripts" option and default parameters. Target transcript candidates were searched in

1) a custom file that include *Triticum dicoccoides* expressed sequence tag (EST) (NCBI: 9,343 entries) and transcriptome assembly sequences (Bala Ani Akpinar, Kantar, et al., 2015).

2) *Triticum durum* ESTs (NCBI: 19,721 entries), assembled unique transcripts (8,513 PUT entries) from PlantGDB and RNAseq assemblies (Bala Ani Akpinar, Kantar, et al., 2015).

For the annotation of the durum and emmer wheat transcripts that are targeted by Zavitan miRNAs, they were *BLASTX* searched against NCBI viridiplantae protein database (January 2016) and corresponding protein sequences were found. Functional annotation of the targets was carried out with Blast2GO software (https://www.blast2go.com/) (Götz et al., 2008). Multilevel pie charts representing three sub-groups; molecular function, cellular process and biological function was generated.

2.2.4 *In silico* Expression Analysis

2.2.4.1 *In silico* Expression evidence for microRNA Precursors

In silico expression evidence for the putative miRNAs was performed through searching their unique precursor sequences in hexaploid and tetraploid wheat expression datasets. A separate BLAST (BLASTN 2.2.31) database was constructed for each dataset and BLASTN hits that cover 98% or more of the total pre-miRNA length with 98% or higher identity were kept as *in silico* expression evidence. Utilized datasets are listed in the Table 3.

Table 3: Datasets that are used for providing *in silico* expression evidence

Expression database	**source**	**Description**
(Krasileva et al., 2013)	Separating homeologs by phasing in the tetraploid wheat transcriptome	Supplemental File 15.Published wheat transcripts (non-redundant)
TriFL	http://trifldb.psc.riken.jp/v3/download.pl	Newly sequenced wheat FLcDNA (2.7 MB) http://trifldb.psc.riken.jp/download/ver.3.0/TaRFL4905.fas.gz
TSA_ncbi	http://www.ncbi.nlm.nih.gov/nuccore	search term: txid4565[Organism:exp] AND tsa[prop]
NCBİ_unigene	ftp://ftp.ncbi.nlm.nih.gov/repository/UniGene/Triticum_aestivum/	
NCBİ_ESTs	http://www.ncbi.nlm.nih.gov/nucest	search term: (triticum aestivum) AND "Triticum

		aestivum"[porgn:__txid4565]
plantGDB_ESTs	ftp://ftp.plantgdb.org/pub/Genomes/TaGDB/	TAest175 file
(Pfeifer et al., 2014)	Genome interplay in the grain transcriptome of hexaploid bread wheat	Chinese Spring wheat Illumina HiSeq. 2000 RNA seq assembly of 5 tissues
(Bala Ani Akpinar, Kantar, et al., 2015)	Triticum durum cv. Kızıltan	9 days slow drought stress
	Triticum durum cv. Kızıltan	Control
	Triticum dicoccoides acc. TR39477 (Drought tolerant)	9 days slow drought stress
	Triticum dicoccoides acc. TR39477 (Drought tolerant)	Control
	Triticum dicoccoides acc. TTD-22 (sensitive to drought)	9 days slow drought stress
	Triticum dicoccoides acc. TTD-22 (sensitive to drought)	Control
Pozniak et al. Unpublished	Triticum durum RNA seq assembly	3 solid, 1 hollow stemmed cultivar

2.2.4.2 Aligning wheat microRNAs to Zavitan precursory miRNAs

The reads that are identified as microRNA from the *Triticum aestivum* small RNA sequencing libraries in the literature and miRBase registry (Kozomara & Griffiths-Jones, 2014) were combined and a new and non-redundant mature-miRNA expression list was prepared. Since the shortest reported plant microRNA was 17 nucleotides in the miRBase, the sRNA reads which are shorter than 17 nucleotides were eliminated from the list. These mature miRNA sequences are aligned to the non-redundant pre-miRNA list generated from the Zavitan non-repeat related microRNA sequences. The publications, which the small RNA reads obtained, are listed in Table 4. After creating a BLAST database from the non-repeat related pre-miRNA sequences, the ungapped alignment was performed by using the following criteria: "-evalue 10 –dust no –pect_identity 100 –word_size 17".

Table 4: List of publications that are used to construct mature miRNA expression data-set.

(Kozomara & Griffiths-Jones, 2014)	**miRBase microRNA Registry**
(F. Sun et al., 2014)	Whole-genome discovery of miRNAs and their targets in wheat (Triticum aestivum L.)
(Yao & Sun, 2012)	Exploration of small non coding RNAs in wheat (Triticum aestivum L.)
(Yao et al., 2007)	Cloning and characterization of microRNAs from wheat (Triticum aestivum L.)
(Wei et al., 2009)	Novel microRNAs uncovered by deep sequencing of small RNA transcriptomes in bread wheat (Triticum aestivum L.) and Brachypodium distachyon (L.) Beauv.
(Han et al., 2014)	Identification and characterization of microRNAs in the flag leaf and developing seed of wheat (Triticum aestivum L.)
(Xin et al., 2010)	Diverse set of microRNAs are responsive to powdery mildew infection and heat stress in wheat (Triticum aestivum L.)
(Y.-F. Li, Zheng, Jagadeeswaran, & Sunkar, 2013)	Characterization of small RNAs and their target genes in wheat seedlings using sequencing-based approaches
(Meng et al., 2013)	Development-associated microRNAs in grains of wheat (Triticum aestivum L.)
(Tang et al., 2012)	Uncovering Small RNA-Mediated Responses to Cold Stress in a Wheat Thermosensitive Genic Male-Sterile Line by Deep Sequencing
(B Wang et al., 2013)	Identification of UV-B-induced microRNAs in wheat
(Bing Wang et al., 2014)	MicroRNAs involving in cold, wounding and salt stresses in Triticum aestivum L.

(F. Sun et al., 2014)	Identification and characterization of a subset of microRNAs in wheat (Triticum aestivum L.)
(Pandey, Joshi, Bhardwaj, Agarwal, & Katiyar-Agarwal, 2014)	A Comprehensive Genome-Wide Study on Tissue-Specific and Abiotic Stress-Specific miRNAs in Triticum aestivum
(Ma et al., 2015)	Identification and comparative analysis of differentially expressed miRNAs in leaves of two wheat (Triticum aestivum L.) genotypes during dehydration stress
(Kumar et al., 2015)	Novel and conserved heat-responsive microRNAs in wheat (Triticum aestivum L.)
(A. Li et al., 2014)	mRNA and Small RNA Transcriptomes Reveal Insights into Dynamic Homoeolog Regulation of Allopolyploid Heterosis in Nascent Hexaploid WheatW

2.2.5 Identification of the putative tRNA repertoire of Zavitan Genome

Putative tRNA genes of the non-masked Zavitan genome were predicted by the tRNAscan-SE 1.3.1 software. Prediction was made in default mode that utilizes eukaryotic tRNA models. "-N" parameter was used as the output option for obtaining the codon information instead of tRNA anticodon. Abundance of each tRNA species was count and visualized as bar graphs.

2.2.6 Experimental Verification of Selected Mature microRNAs

Experimental verification of randomly selected mature microRNAs was carried out with RT-qPCR.

2.2.6.1 Plant Materials and Growth Conditions

Seeds of *Triticum turgidum* spp. *dicoccoides* Zavitan accession, were surface sterilized with 10% commercial bleach for 10 min and rinsed with distilled water. Seeds were grown in solid MS media under a cycle of 16 h light and 8 h dark at 25 ^{0}C. Plants were transferred to the pots containing soil supplemented with 200 ppm N, 150 ppm P, 30 S ppm, 5 Zn ppm, 10 Fe ppm,

50 Mg ppm at the seedling stage. Leaf samples were collected from the two month old plants growing in the vegetative mode and frozen in liquid nitrogen immediately. Tissue samples were stored in -80 ^{0}C until their use.

2.2.6.2 Plant RNA material

Total RNA was extracted from 0.2 g frozen leaf tissues by using TRI Reagent (Sigma-Aldrich) following the manufacturer's instructions. Integrity of the RNA was checked via visualizing the major rRNA bands by running 500 ng of RNA on 3% agaroz gel. Quality and quantity of the intact total RNA were measured with Nanodrop ND-100 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Five µg of total RNA was trated with 5 units of DNase I (RNase free) (Fermentas) in a 50 µL volume reaction mixture accoridng to manufacturer's protocol. Reaction was terminated by incubation at 65 ^{0}C for 10 minutes following the addition of 5 µL 50 mM EDTA.

2.2.6.3 RT-qPCR

RT-qPCR experiments were carried out in two steps which employ a special reverse transcription reaction designed for small sized RNA molecules' amplification; stem-loop RT-qPCR (Figure 5). Stem-loop RT and universal Reverse primers were designed as described by Varkonyi-Gasic et al. (Varkonyi-Gasic et al., 2007) (Appendix-I C: List of microRNA primers used through this study). Reverse transcription reaction was performed using RevertAid Reverse Transcriptase kit (Thermo Fisher Scientific) with 100 ng (1µL) DNase treated total RNA in 20 uL total reaction volume. First, the mixture of 100 ng RNA, 1 uL 1uM stem-loop RT primer and 9 uL DEPC-treated H_2O mixed and incubated at 70 ^{0}C for 5 min and chilled on ice. Reaction tubes were incubated at 37 ^{0}C for 5 min after the addition of 4 µL 5x Reaction Buffer, 2µL (10 mM) dNTP mix, 0.5 µL (20 u) RiboLock RNase Inhibitor (Thermo Fisher Scientific). Pulsed reverse transcription reaction was initiated with the addition of 1 µL RevertAid Reverse transcriptase according to following program: 30 min at 16 ^{0}C, 30 s at 30 ^{0}C for 60 cycles, 30 s at 42 ^{0}C and 1s at 50 ^{0}C. No-RT and No-RNA control reactions were performed with the addition of DEPC-Treated H_2O instead of reverse transcriptase and RNA respectively. Reaction was terminated by a heating stage at 85 ^{0}C for 5 min.

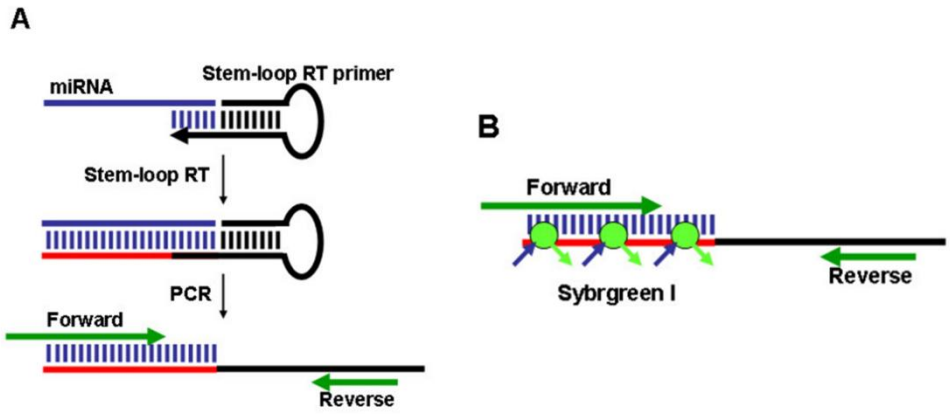

Figure 5: Stem-loop RT-qPCR assay schematic representation (Varkonyi-Gasic et al., 2007)

Quantitative PCR reactions were performed by using PerfeCTa SYBR® Green SuperMix kit (Quanta Biosciences) and Roche LightCycler 480 (Roche Life Sciences) platform. 9.2 µL (1.5 ng) of cDNA which produced separately by using miRNA specific stem-loop primers were used for quantitative PCR. Reactions were carried out by mixing cDNA with 0,4 µL miRNA specific forward primer (10 mM), 0.4 µL universal reverse primer (10 mM) that is specific to the loop region of the RT primers, and 10 µL SYBR Green Supermix. Quantification and melting curve programs were set up as following: 95°C for 10 minutes, 40 cycles at 95°C for 30 seconds, 58°C for 1 minute, 72°C for 30 seconds and by 72°C for 10 minutes. Melting curve generation program was as following: denaturing at 95°C, cooling: 65°C. Products of the RT-qPCR were run on 3% agaroz gel to visualize specific and non-specific amplicons.

2.2.7 Assessment of miR167 as a candidate housekeeping miRNA gene

To be able to utilize in the miRNA relative quantification experiments, expression of a recently proposed wheat microRNA reference gene; miR167 was assessed across five different tissues. Total RNAs extraction and DNAse treatment of samples from *T. dicoccoides* acc. TR39477 and *Aegilops tauschii* acc. AL8/78 leaf and root tissues were carried out as described in 2.2.6.2. Quantitative real time PCR reactions were performed by using these samples and Zavitan leaf tissue RNA as described in 2.2.6.3. Products of the RT-qPCR were run on 3% agaroz gel to visualize specific and non-specific amplicons.

2.2.8 Identification of differential expression of selected mature miRNAs upon shock drought treatment

To be able to see the effect of the drought stress on the selected miRNAs expression levels, we have set another experiment and quantify their expression with RT-qPCR after shock drought treatment.

2.2.8.1 Growth condition and shock treatment of the plants

Zavitan seeds were surface sterilized with 10% commercial bleach for 10 min and rinsed with distilled water and placed on solid MS media supplemented with 2,4-Dichlorophenoxyacetic acid. Wild emmer wheat seedlings were grown under a cycle of 16 h light and 8 h dark at 25 ^{0}C for ten days and transferred to Hoagland's solution inside continuously aerated 2.7 L hydroponics pots. The plants were grown under controlled conditions (24/22°C temperature, 60% relative humidity, 16 h photo- period, and 600–700 µmol m^{-2} s^{-1} photon flux density) and the nutrient solution was renewed every three days. Shock drought stress treatment was applied at the five-leaf stage, at the 24th day after the transfer. Plants were taken out of the hydroponics pots and placed on paper towels under same conditions for i) 6 and ii) 8 hours while control plants were kept inside the pots (Figure 6). At the end of the stress treatment, root and leaf tissue samples from control and stress subjects were frozen inside liquid nitrogen and preserved at -80 ^{0}C.

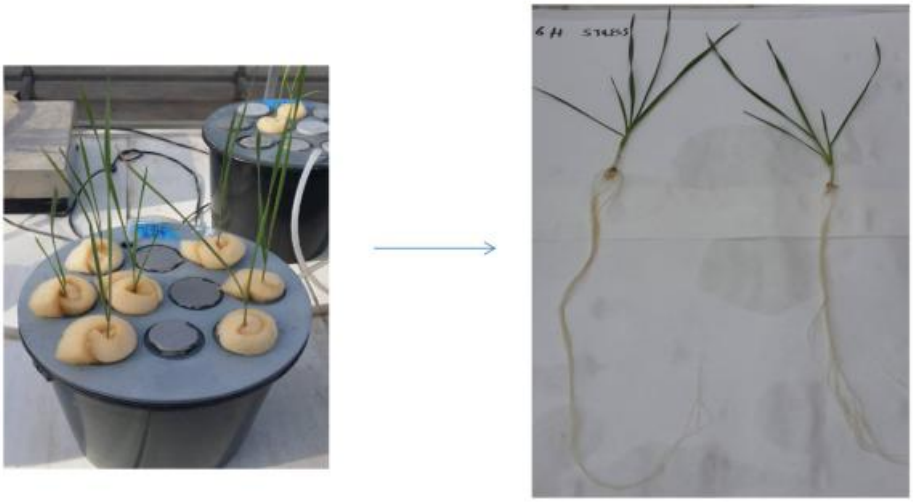

Figure 6: Hydroponics setup and shock drought treatment of the Zavitan seedlings at five leaf stage.

2.2.8.2 Plant RNA material

RNA extraction and DNase treatment of the root and leaf tissues from 6 hour and 8 hour shock drought treated plants and their control samples were carried out as described in section 2.2.6.2.

2.2.8.3 Differential Expression of Selected microRNAs upon Shock Drought Stress

Stem-loop reverse transcription and quantification of mature microRNAs from stress and control tissues were carried out according to Varkonyi-Gasic et al. as described in the 1.2.7.3. Quantification of each miRNA was performed with three technical replicates in addition to no-RNA and no-RT control reactions. The raw fluorescence data from the real time quantification experiments were analyzed for their relative quantification with LinRegPCR software (Ruijter et al., 2009). LinRegPCR calculates the PCR efficiency for each reaction after the baseline correction. Briefly, base-line corrected data is used for the identification of a window-of-linearity for each microRNA amplification reaction, and the values inside this linearity window are used to plot a linear regression line. The slope of the regression lines is used to determine the PCR efficiency. The mean threshold cycles (C_T) and PCR efficiencies of each amplicon are used for the determination of starting amount per sample according to Formula 1.

$$\text{Ratio}_{(\text{test}/\text{calibrator})} = E^{\,C_T(\text{calibrator})/C_T(\text{test})}$$

Formula 1: Calculation of the relative quantification of the RT-qPCR product

Test: The treatment sample

Calibrator: The experimental control

E: individual efficiency of each reaction. $E = 10^{-1/\text{slope}}$

Ratio: relative quantification of the specific amplicon in the test ample according to the calibrator.

3. RESULTS AND DISCUSSION-I

3.1. Zavitan Genome Putative microRNAs

Implementation of homology based *in silico* miRNA prediction pipeline yielded a total of 23,257 fold-back structures in the 10.8 Gb Zavitan genome assembly. Among these 16,553

fold-back structures have passed the precursory miRNA secondary structure criteria implemented by our in house python script (mirScreen.py).

3.2. Repeat masking the fold-back structures

After identification of the fold-back structures which obey the pre-miRNA secondary structure criteria, we have sorted out the ones that are originating from the known repeat elements. This step eliminated 99.96% of the fold-backs as TE-miRs. Final list of Zavitan microRNAs contained 644 miRNAs belonging to 53 different miRNA families. microRNA families and their representation in the Zavitan genome are given in Table 5.

Table 5: microRNA families and their corresponding numbers in the Zavitan genome.

miR-fam.	**#**	**miR-fam.**	**#**	**miR-fam.**	**#**
miR1130	1	miR9671	1	miR399	17
miR1133	2	miR9673	2	miR5049	5
miR1432	7	miR9674	23	miR5062	5
miR156	12	miR9676	2	miR5079	1
miR160	18	miR9677	4	miR5084	84
miR164	4	miR9678	3	miR5168	4
miR166	11	miR9772	5	miR5174	1
miR167	16	miR9776	3	miR5200	9
miR169	25	miR9778	1	miR5205	1
miR171	12	miR9863	2	miR528	2
miR172	5	miR5522	3	miR530	7
miR1878	2	miR6246	1	miR396	12
miR2118	146	miR9652	4	miR397	1
miR2275	17	miR9659	1	miR398	4
miR319	2	miR9661	1	miR9666	50
miR393	7	miR9663	1	miR9667	1
miR394	2	miR9664	2	miR9670	1
miR395	71	miR9665	20		

The average minimum free energy index (MFEI) of these miRNAs was 1.006, where this value was 1.288 for the TE-miRs. Average MFEI values are important indicators of miRNA authenticity as they differ from the values belonging to the other non-coding RNA species. Putative miRNAs of Zavitan had an MFEI average of 1.01 and this value was higher than the average MFEI values of mRNAs (0.64), rRNAs (0.59) and tRNAs (0.64), as expected (Melda Kantar et al., 2012; Schwab et al., 2005). However, this average was lower than the average MFEI values of the pre-miRNAs predicted from transcriptome assemblies belonging to *T. urartu, T. monococcum, T. aegilopoides, Ae. sharonensis, Ae. tauschii,* and *T. aestivum* in a recent study (Alptekin & Budak, 2016). Furthermore, GC percentage of putative Zavitan pre-miRNAs was higher than all averages in the mentioned study.

As the assembled contigs were anchored to the chromosomes, we checked the chromosomal distribution of miRNAs and TE-miRs (Figure 7).

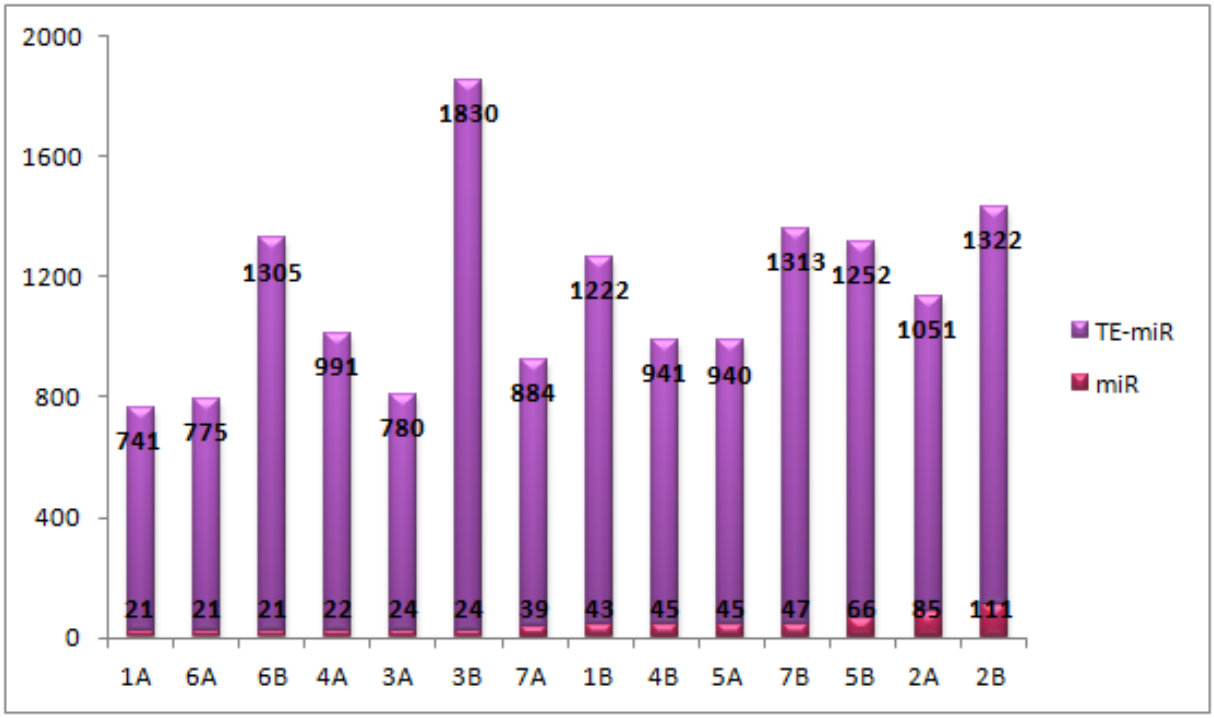

Figure 7: Distribution of miRNAs and TE-miRs in Zavitan chromosomes.

According to this, chromosome 2B was the richest and 1A, 6A and 6B were the poorest chromosomes in miRNA content by coding 111 and 21 miRNAs respectively. However, TE-miRs; fold-back structures with repeat origins, did not resembled the actual miRNA numbers in the chromosomes. With 1,830 TE-miRs, chromosome 3B was the most repeat-rich chromosome. As a general trend, B genome chromosomes had a higher repeat content in

comparison to their A genome counterparts, except for chromosome 4. Increases in the repeat content of the chromosomes are expressed as percentages in Table 6.

Table 6: Percent increases (and decrease for chromosome 4) of the B genome chromosome repeat contents in comparison to A genome chromosome.

Chr_1	Chr_2	Chr_3	Chr_4	Chr_5	Chr_6	Chr_7
64.91	25.78	134.62	-5.05	33.19	68.39	48.53

Mature miRNA and precursor miRNA sequence lengths were counted. 42 % of the mature microRNA sequences were 21 nucleotides and this was followed by 22 nucleotide miRNAs by 34.5 % (Figure 8). Average mature miRNA length was 21.78. Hairpin positions of the mature miRNAs revealed a slight preference for the 3' arm; 39.6 % of the mature miRNAs positioned on the 5' arm while 60.4 % was on 3'.

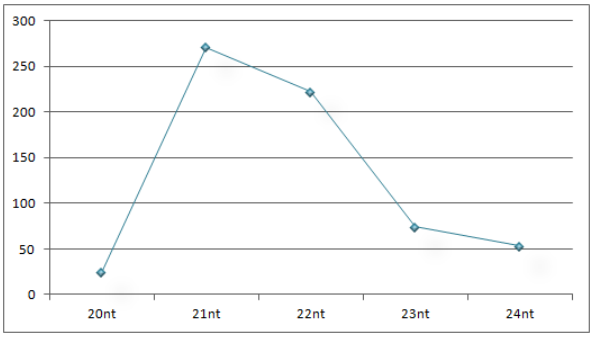

Figure 8: Number of putative microRNAs according to their mature sequence length

Putative precursor miRNA sequence lengths have varied from 89 to 236 nucleotides with the average of 141.96 (Figure 9).

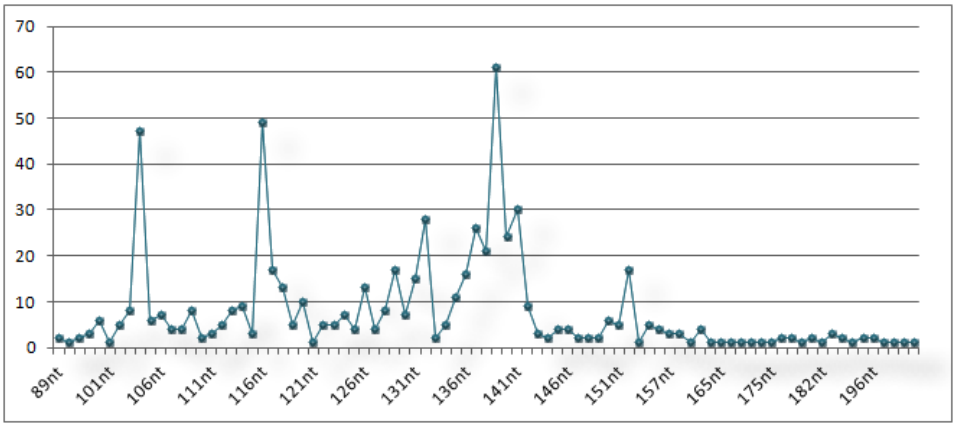

Figure 9: Putative pre-miRNA length distribution.

Characteristics of the precursory microRNAs such as length, GC content and MFEI may vary between species. Statistics of these pre-miRNA characteristics for Zavitan miRNAs are given in Table 7. The diverse distribution of the pre-miRNAs was expected as plant pre-miRNA lengths known to vary between 55 to 930, with the mean length of 146 nucleotides (Thakur et al., 2011). Our predicted miRNAs had an average of 130 nucleotides (Table 7).

Table 7: Statistics related to pre-miRNA characteristics.

	Pre-miRNA length	**GC content**	**MFEI**
Average	130.35	48.05	1.01
Min.	89.00	28.45	0.70
Max.	236.00	69.77	1.60
Median	132.00	47.29	1.00
Std. Deviation	19.63	6.21	0.14

3.2.1. Analyzing the repeat content

Repeat elements are the dynamic actors of genome evolution which are related to gene and pseudo-gene formation over the evolutionary course (Wicker et al., 2011). microRNA gene formation is also closely linked with transposable element activity because of the sequence similarity and closeness of their genomic location with TEs (Y. Li et al., 2011; Piriyapongsa & Jordan, 2008). As previous studies revealed microRNAs which are completely identical to the transposable elements sequences (Y. Li et al., 2011), we have performed a similarity search for the miRNA-like fold-backs against a *Poaceae* repeat element database and select out these repeat-related miRNAs: TE-miRs. As mentioned before 99.96% of the fold-backs were TE-miRs. This high percentage is conceivable considering the highly repetitive contents of *Triticeae* genomes (Bala Ani Akpinar, Yuce, Lucas, Vrána, & Burešová, 2015).

The percentages of the repeat element types were compared with recently published *Triticum aestivum* TE-miRNAs (Kurtoglu et al., 2014) predicted from whole genome shotgun assembly (Brenchley et al., 2013). Repeat element content of the TE-miRs was strikingly similar to that of Zavitan TE-miRs (Figure 10).

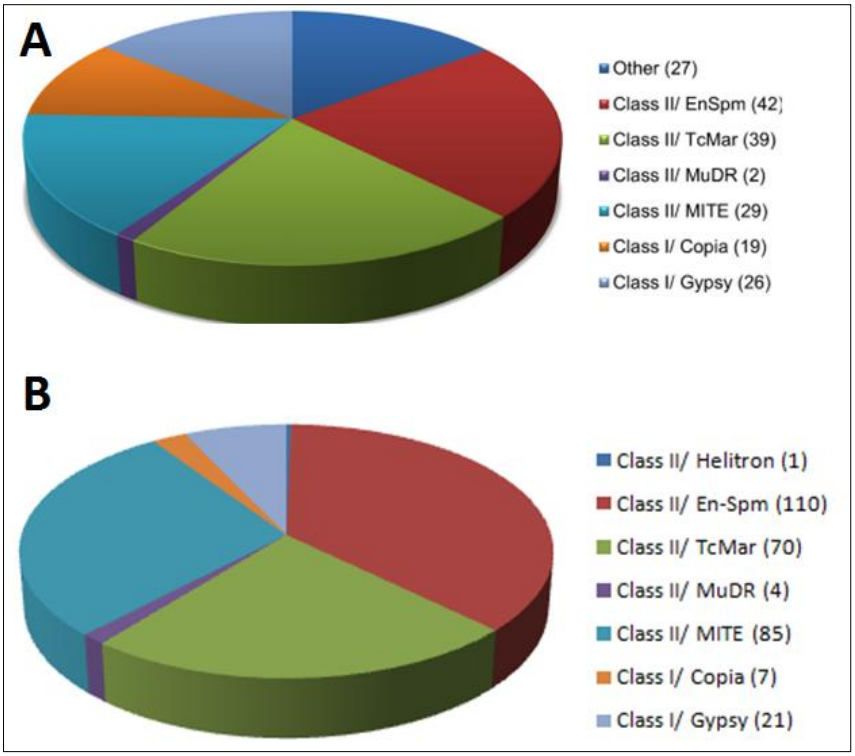

Figure 10: Repeat element distribution of the TE-miRs from A: wheat whole genome miRNAs (Kurtoglu et al., 2014) and B: Zavitan miRNAs.

By 90.6 %, Class II DNA transposons were the most prevailing type while Class I retrotransposons were constituted 9.4% of the fold-back structures. Among Class II repeats, En/Spm subfamily was the most common one being detected inside 110 fold-backs followed by miniature inverted repeat elements (MITEs) (within 85 fold-backs) and TcMar (within 70 fold-backs) sub-families respectively. As repeat elements with palindromic structures resembling the microRNA precursors, MITEs were previously presented as a source of miRNA gene origin and their high percentage in Zavitan genome is expected (Mao, 2000; Tikhonov et al., 1999; Q. Zhang et al., 2000). Other Class II repeats with low representation in Zavitan genome were MuDR (4) and Helitron (1) subfamilies. Class I retroelement content of the TE-miRs were consisting of 21 Gypsy and 7 Copia elements.

In addition to wheat whole genome miRNAs, the prevalence of the En/Spm elements was also seen in the TE-miRs from chromosome 5D of *Triticum aestivum* and *Aegilops tauschii* (Bala A. Akpinar & Budak, 2016). However when considering the repeat elements in the whole chromosome sequence, En/Spm representation has fallen to 16.5 % in *T. dicoccoides* chromosome 5B, and 21 % and 17 % in *T. aestivum* 5D and 5A respectively (Bala Ani

Akpinar, Yuce, et al., 2015). These results are putting this repeat sub-family under the spotlight as an important source of miRNA origin.

3.3. Alignment of wheat mature miRNAs to Zavitan miRNA precursors.

Our wheat mature miRNA list which was compiled from the wheat small RNA sequencing experiments in the literature had a total of 3399 non-redundant sequence. The non-gapped BLAST alignment resulted in the perfect alignment of 239 of these miRNAs against Zavitan pre-microRNAs. This number was increased to 271 when the sequence coverage was decreased to 95 % from 100 %. Precursory sequence of miR5084 and miR395 had the most alignments by the alignment of 64 and 57 unique mature miRNAs followed by pre-miR9666 (50) and pre-miR2118 (36).

3.4. In silico Target Prediction and Annotation Analysis

Plant miRNAs exert their actions by binding on their corresponding target mRNAs by near perfect complementarity (Pasquinelli, 2012). They mostly act through target mRNA degradation but can also cause translational repression (Gu & Kay, 2010). As it has excelled among the other plant miRNA target prediction tools in terms of performace, target analysis has been carried out by using psRNATarget online tool (http://plantgrn.noble.org/) (Srivastava, Moturu, Pandey, Baldwin, & Pandey, 2014). Targets of the putative Zavitan miRNAs which have expression evidence, were searched inside *T. dicoccoides* and *T. durum* expressed sequences (Transcriptome assemblies or EST sequences). Annotation of the putative target sequences revealed stress metabolism related proteins and transcription factors that can give information about the corresponding miRNAs significance in terms of drought stress responses. The annotated miRNA target gene ontologies have been group under; related cellular component, biological function and molecular function categories and represented as pie-charts Figure 11, 12 and 13. Annotated putative targets are presenting a rich resource to be utilized for crop improvement studies in the future.

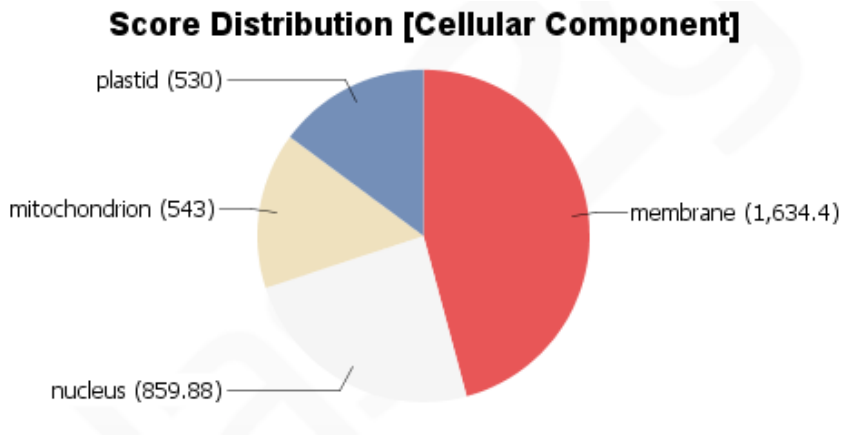

Figure 11: Putative microRNA targets' distribution in terms of related cellular components according to the gene ontology analysis

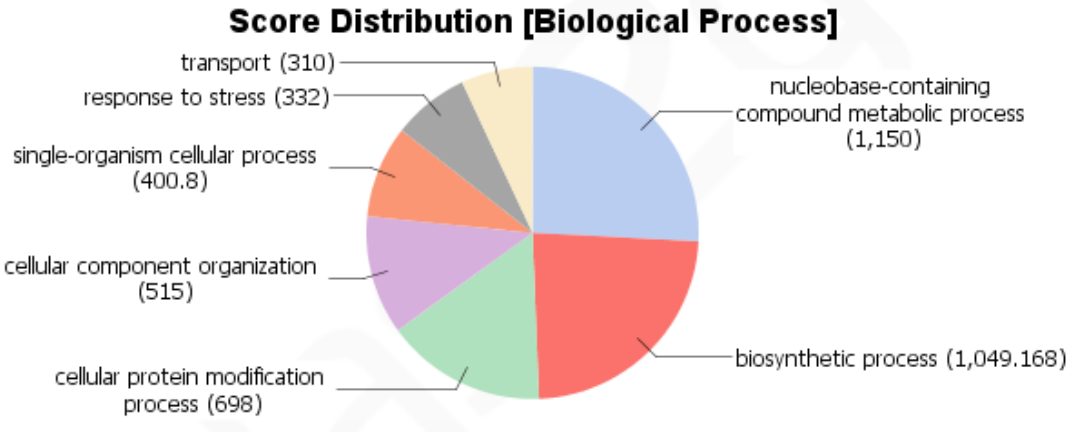

Figure 12: Putative microRNA targets' distribution in terms of their biological process GO terms according to the gene ontology analysis

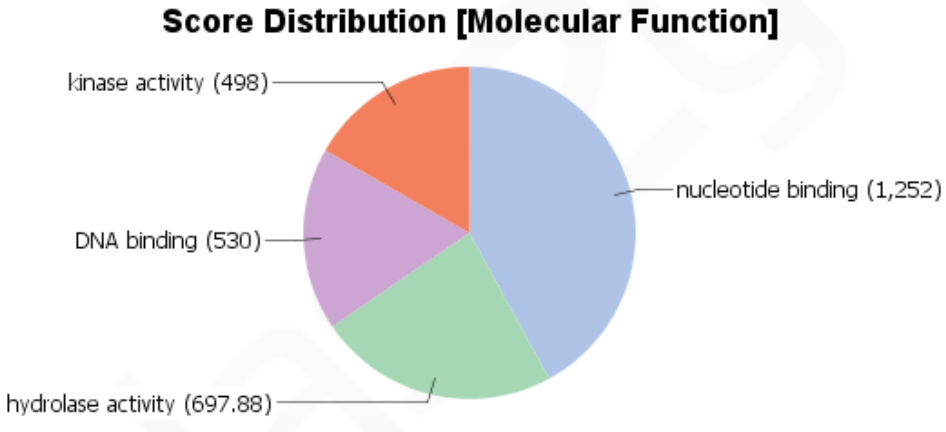

Figure 13: Putative microRNA targets' distribution in terms of their molecular function GO terms according to the gene ontology analysis

3.5. In silico Expression Evidence for Zavitan miRNA Precursors

Expression evidence for the putative Zavitan microRNA precursors were searched inside expression databases consisting of tetraploid and hexaploid wheat transcriptomes and EST sequences. In total, 400 sequences from the expression libraries gave hit to predicted precursor sequences belonging to 133 different miRNA and 31 miRNA families (Appendix-I D). As microRNAs, therefore their precursor sequences are expressed in spatiotemporally diverse conditions, many microRNA sequences might be overlooked when using small RNA sequencing or RNA sequence for the microRNA prediction (Sunkar, 2012). Our genome sequence based approach overcomes this issue. By using many different expression sources, and extending our transcription libraries with both hexaploid and tetraploid wheat sequences we were able to provide expression evidence for 20 % of the miRNAs. Pre-miRNA sequences with *in silico* expression evidence are listed in Appendix D.

3.6. Prediction of Putative tRNA Genes

tRNA gene search on the non-masked genome assembly yielded a total of 9401 putative tRNA (Figure 14). Among them, 12.86 % (1210) was denoted as pseudo-tRNA gene and 225 predictions had intron bounds (Table 8). 110 of the sequences were undetermined/unknown isotypes. 17 of the tRNAs were grouped under possible suppressor tRNA sequences (Sup) and 5 of them were selenocystein (SeC).

Table 8: tRNA species that were predicted as intronic and pseudo-gene.

Chr. #	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Intronic							
A	22	12	20	5	10	9	4
B	29	10	30	6	11	43	4
Pseudo							
A	64	81	101	81	82	60	78
B	94	88	102	69	84	75	103

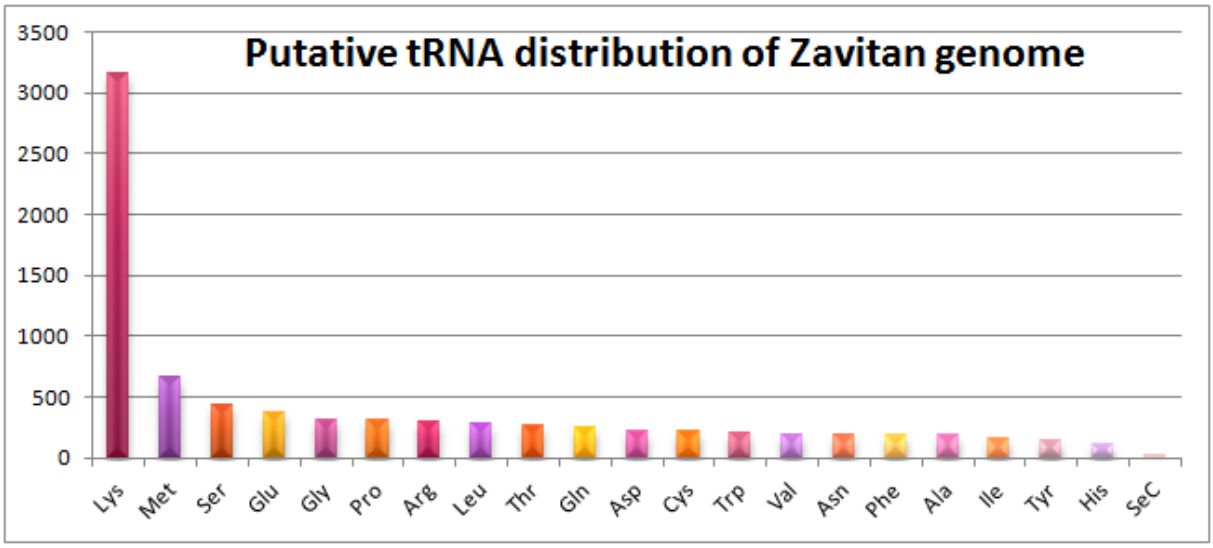

Figure 14: Number of each putative tRNA species.

As can be seen from Figure 14 and Figure 15, $tRNA^{Lys}$ species was the most prevalent tRNA species followed by $tRNA^{Met}$ and $tRNA^{Ser}$. High abundance of $tRNA^{Lys}$ species has been attributed to their transposable element originated formation and therefore high numbers in genomes which have high repeat-content (Bala Ani Akpinar, Yuce, et al., 2015). This high $tRNA^{Lys}$ content was also observed in the previous studies that have predicted the putative tRNA repertoire of flow-sorted chromosome 5D of *T. dicoccoides* (Bala Ani Akpinar, Yuce, et al., 2015), *T. aestivum* chromosome 5D (Lucas et al., 2014) and 6B (Tanaka et al., 2014), and Aegilops tauschii chromosome 5D (B. a Akpinar, Lucas, Vrána, Doležel, & Budak, 2014).

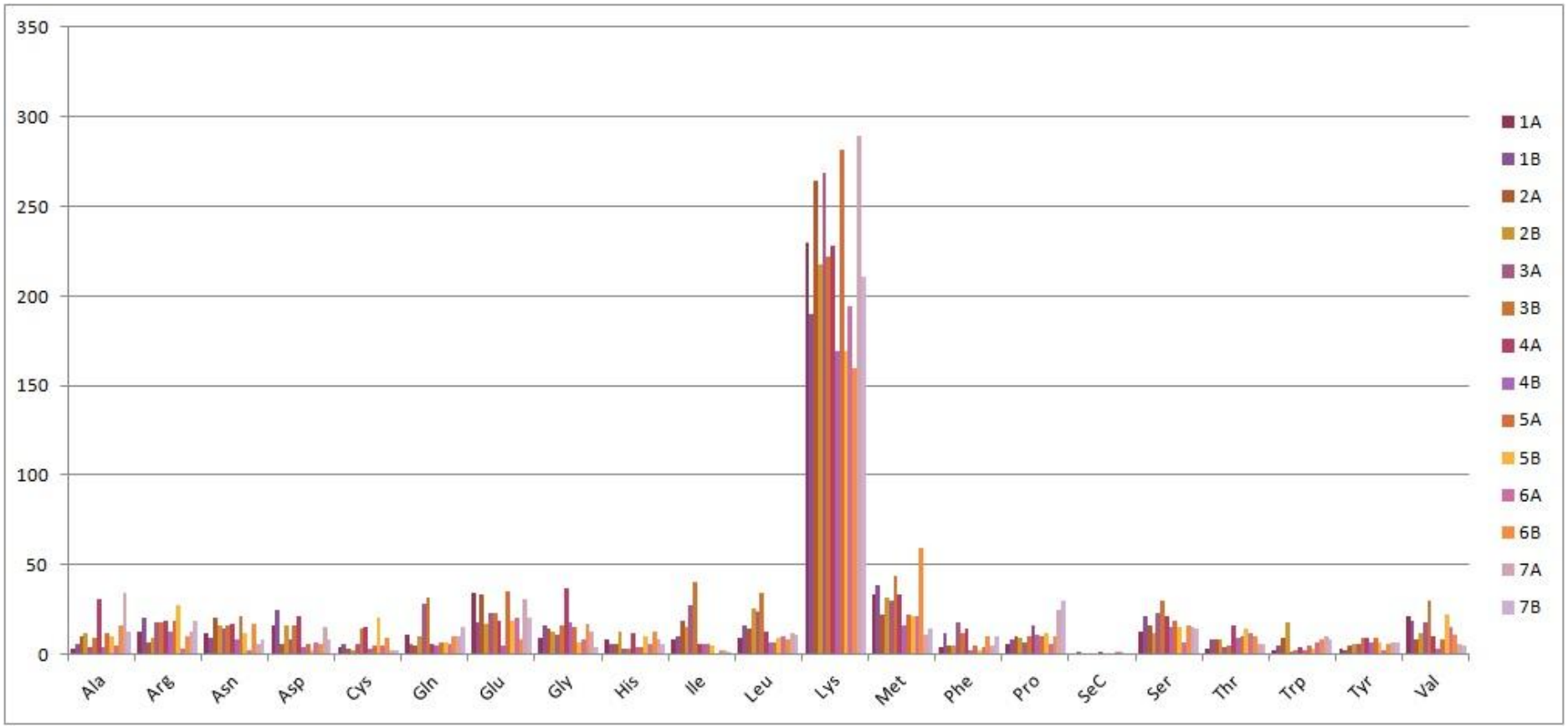

Figure 15: tRNA species distribution according to chromosomes

3.7. Experimental Verification of Selected miRNAs

After the computational identification, we selected some of the microRNAs for experimentally verifying their authenticity. Mature microRNAs' expression was checked in the Zavitan leaf tissue, sampled from two mounts old plants which were watered in every other day. RT-qPCR reactions were performed with no-RT and no-RNA controls. Reactions with non-specific amplifications and potential primer-dimers were eliminated by both comparing the fluorescence signals and agaroz gel visualizations of the no-RNA and no-RT controls with experimental reactions. Sixteen of the mature microRNAs' expression was demonstrated via RT-qPCR with stem-loop cDNA synthesis approach. The melting curves and agaroz gel images belonging to those 16 mature miRNA are given in Figures 16-20.

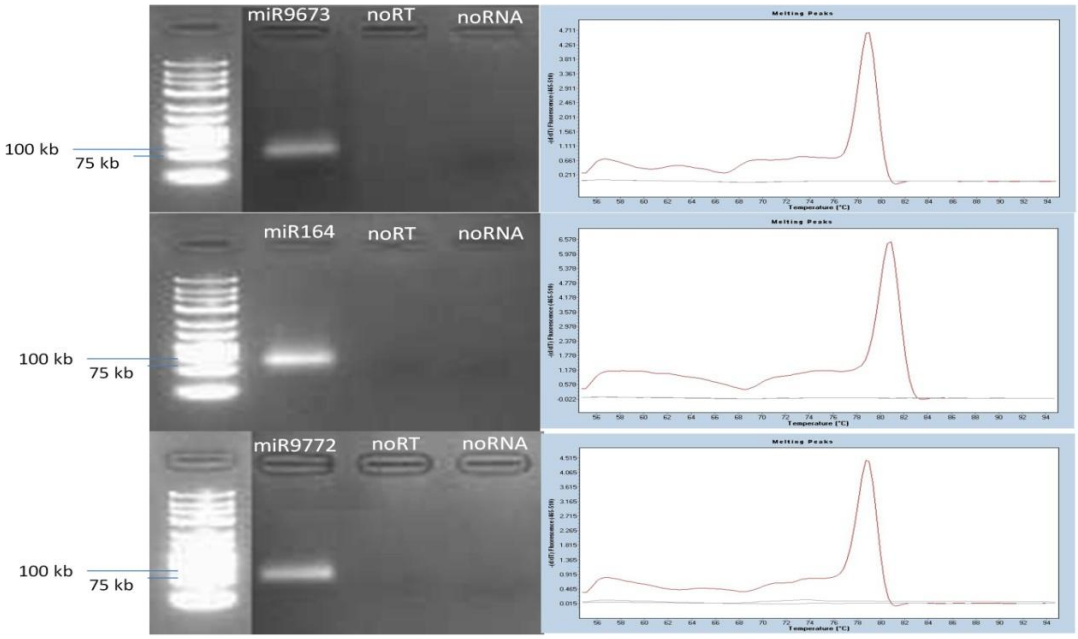

Figure 16: Gel electrophoresis and Melting peak images of miR9673, miR164 and miR9772

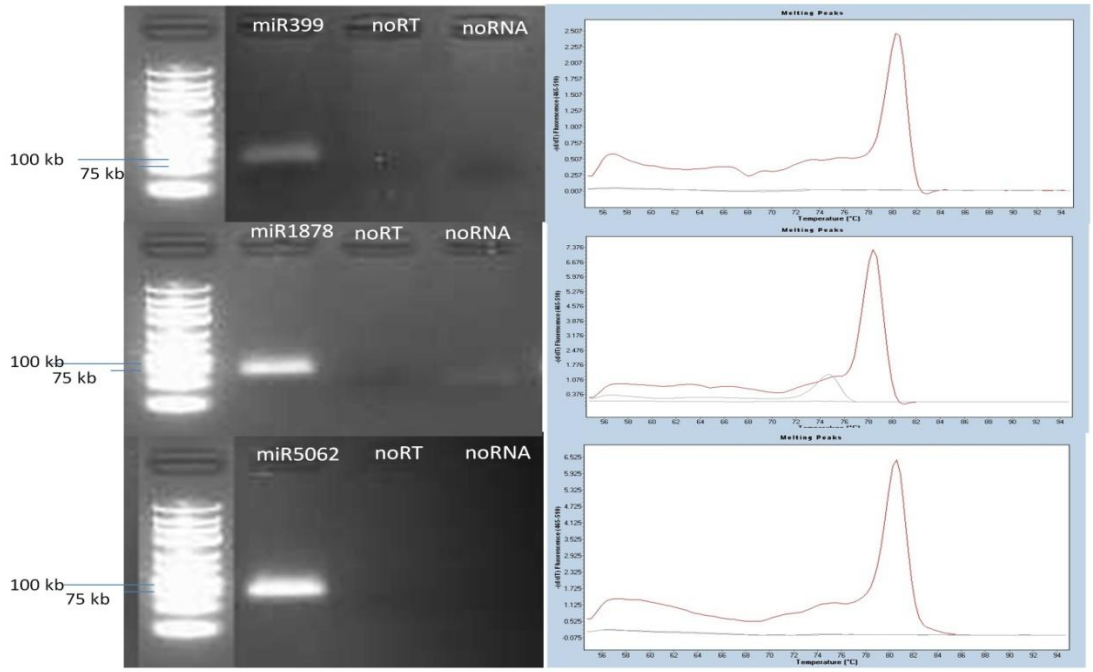

Figure 17: Gel electrophoresis and Melting peak images of miR399, miR1878 and miR5062.

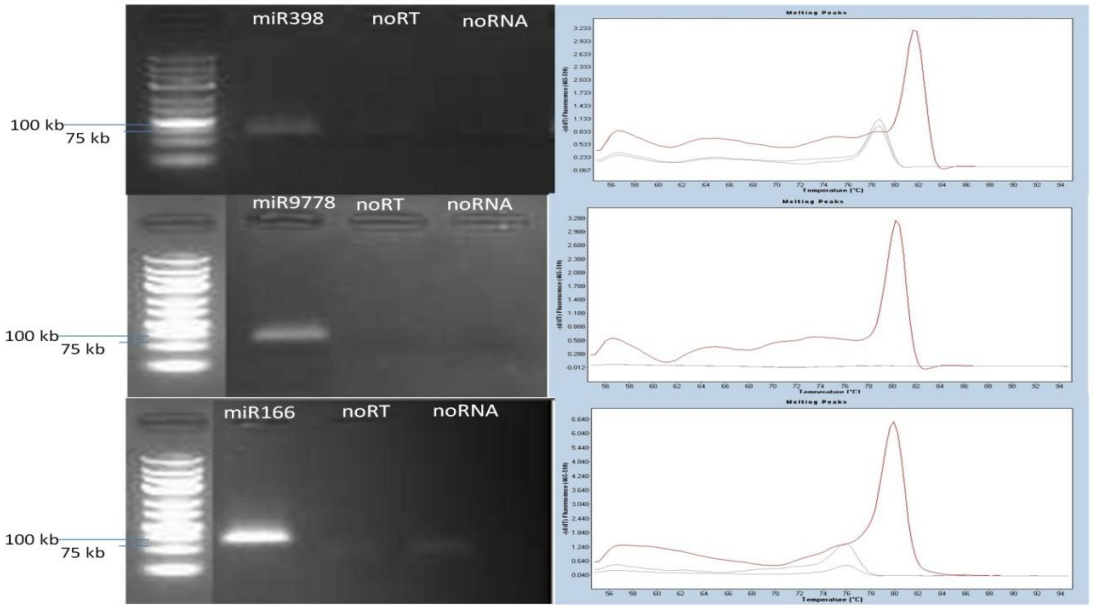

Figure 18: Gel electrophoresis and Melting peak images of miR398, miR9778 and miR166.

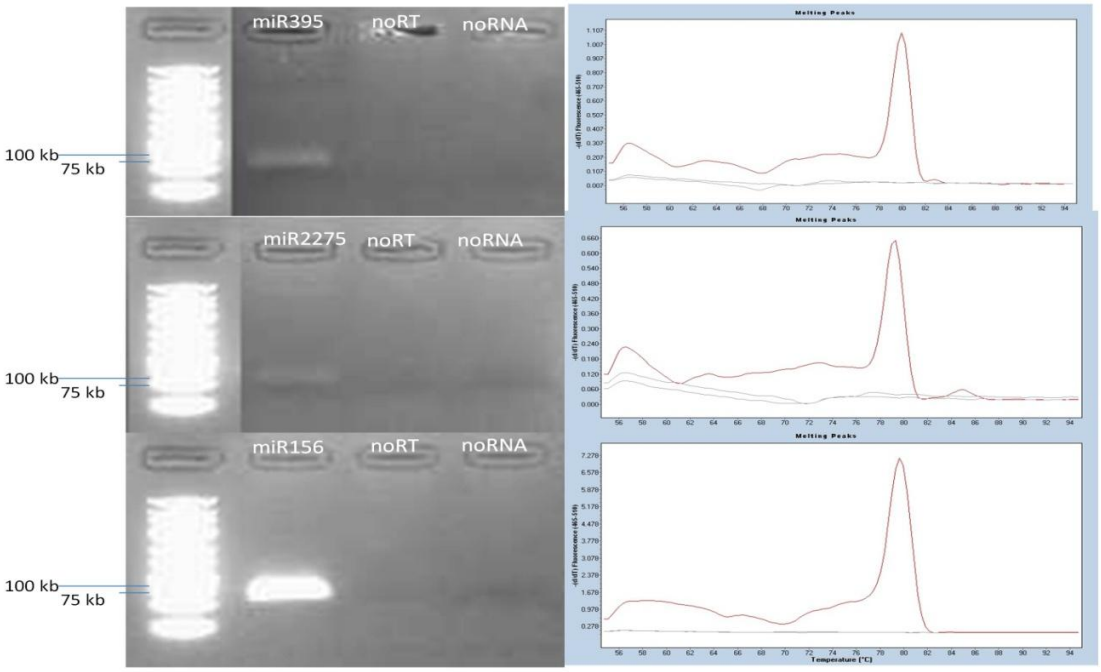

Figure 19: Gel electrophoresis and Melting peak images of miR395, miR2275 and miR156.

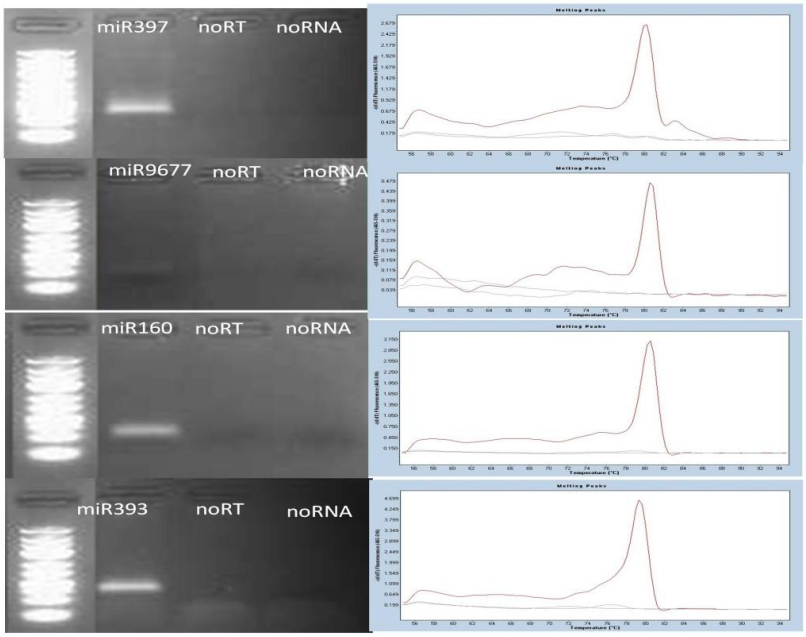

Figure 20: Gel electrophoresis and Melting peak images of miR397, miR9677, miR160 and miR393.

According to the resulted amplification curves, miR5062 was the microRNA with the highest expression level in the leaf tissue among the microRNA families that we have tested as the fluorescent signal belonging to its amplification was detected from the beginning of the 22nd cycle. This cycle is known as the quantification cycle (Cq), and it is advised to have a Cq cut-off at 35 to eliminate the non-specific amplifications and background signals in the conventional qPCR experiments (Mestdagh et al., 2008). However, miRNA expression levels may require higher cut offs for more sensitive detection (Gallo, Tandon, Alevizos, & Illei, 2012). This is because of the very low expression levels of some of the microRNAs in diverse spatio-temporal conditions (Budak & Kantar, 2015). Therefore, we designed our experiment with 40 cycles and cautiously examined the amplification signals. The Cq values of miR395, miR2275 and miR9677 were 36, 38 and 38 respectively. However their melting curves and amplification products showed their specificity and therefore, we kept these microRNAs in our experimentally verified miRNA list as the microRNAs with subtle expression level in the mature leaf tissue. The Cq values and amplification curves of the mature miRNAs are given in Figure 21.

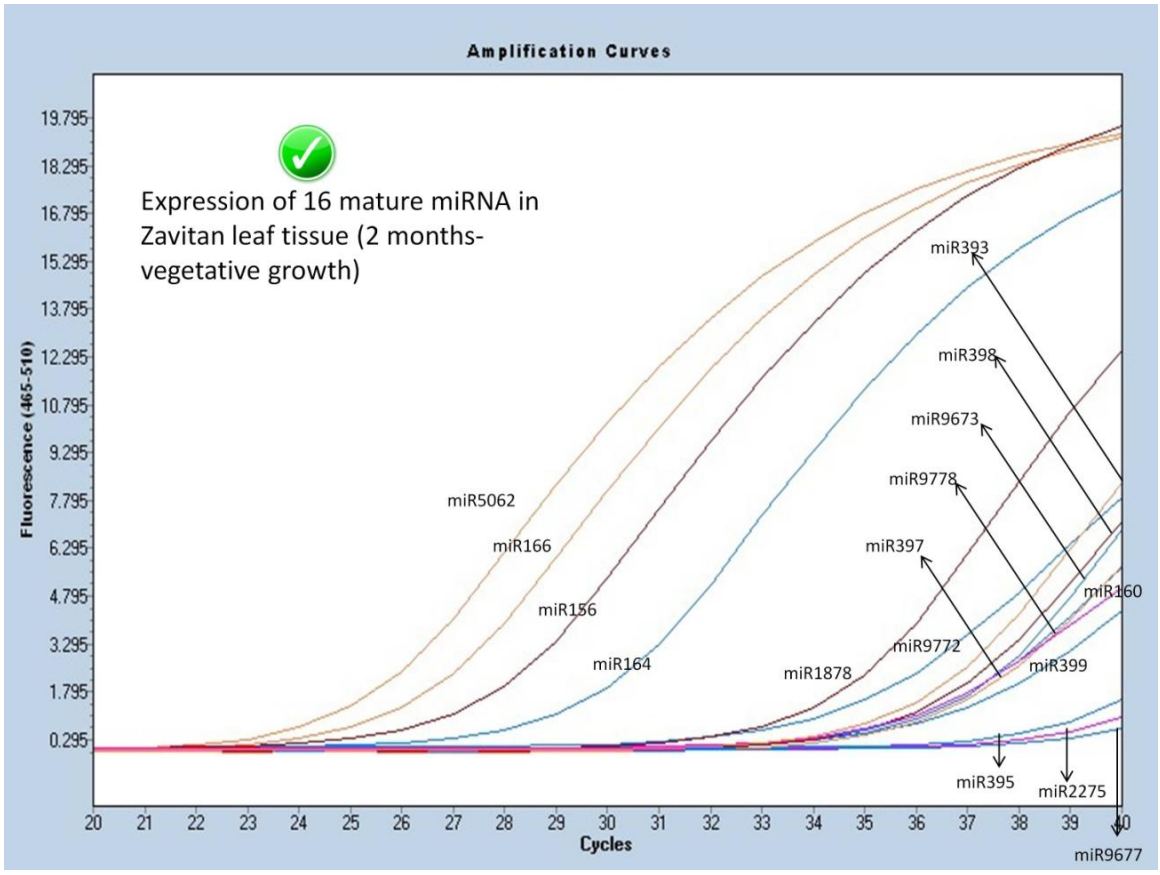

Figure 21: Amplification curves of the mature microRNAs that are expressed in the mature leaf tissue of Zavitan plants.

To be able to use in the quantification reactions, a recently proposed housekeeping wheat microRNA; miR167, expression was assessed in both the adult leaf tissue of Zavitan and *T. dicoccoides* acc. TR39477 and *Aegilops tauschii* acc. AL8/78 leaf and root tissues. However, miR167 expression was only detected in the root tissue of *T. dicoccoides* acc. TR39477. Therefore it could not be used as a reference gene in the quantification experiments. MicroRNA levels were only compared according to the control experiment levels and described as fold changes. Original gel images belonging to the mature microRNA RT-qPCR experimet are given in Appendix-I F.

3.8. Expression Levels of microRNAs upon 6 and 8 Hours of Shock Drought Stress

microRNAs selected from the ones that have both computational and experimental validation were used for drought treatment experiments. For this, Zavitan seedlings were grown in MS agar media supplemented with 2,4-Dichlorophenoxyacetic acid and then transferred to continuously aired hydroponics pots supplemented with Hoagland's solution. Hydroponics system was chosen to be able to involve root tissues in our experiments as the precursory (Bala Ani Akpinar, Kantar, et al., 2015) and mature microRNA (Melda Kantar et al., 2011) expression levels in the root tissue are substantially affected in *Triticum durum* and *Triticum dicoccoides* plants upon drought stress.

Shock stress treatment was applied as previously described by Ergen et al. (Ergen, Thimmapuram, Bohnert, & Budak, 2009b). The integrity of the RNA samples extracted from the leaf and root tissues were checked by visualizing the major rRNA bands in agaroz gel (Figure 22).

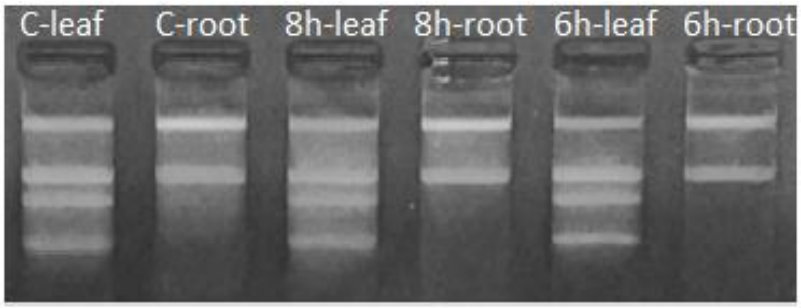

Figure 22: Major rRNA bands from the leaf and root tissue samples belonging to 8 and 6 hours of shock drought treatment along with the control samples.

PCR efficiencies were calculated by using LinRegPCR program and expression levels of mature miRNAs were presented as their fold changes according to the control samples. Our relative expression analysis highlighted the drought responsive expression profiles of the microRNAs.

miR156, miR393 and miR395 were down regulated in the root tissues upon shock drought while miR166 and miR397 had increased expression levels. In the leaf tissues, miR156, miR164, miR9673, miR9772 and miR9778 were up-regulated while the only miRNA with decreased expression was miR160. microRNA expression levels in the other were either up-regulated or down-regulated according to the stress durations. This type of fluctuating relative expression profiles are commonly seen in small RNA sequencing or qPCR based analysis of miRNA expression under diverse biotic and abiotic stress conditions (Pandey et al., 2014; Wu, Shu, & Jin, 2014) and may refer to their changing duties in early and late stages of the response.

According to the relative fold changes, miR156 was detected as the "most responsive" miRNA during drought treatment. miR156 is a known regulator of the *SQUAMOSA PROMOTER PROTEIN-LIKE 9* gene, controlling the flower development. It is known that crop yield and quality is highly affected by the trade-off between the development and drought response (Peleg, Fahima, & Saranga, 2008). Under stress conditions, delay in the development is provided by the miR156 dependent regulation of the SPL transcription factors, for executing a better stress response (Cui, Shan, Shi, Gao, & Lin, 2014; Stief et al., 2014).

While being up-regulated in the leaf samples, miR156 expression significantly decreased in the root tissue. The differential regulation of miR156 upon drought stress was previously reported in different plant species and it's a widely known drought responsive miRNA (Bala Ani Akpinar, Kantar, et al., 2015; Ferdous, Hussain, & Shi, 2015; Melda Kantar et al., 2011; Liu, Tian, Li, Wu, & Zheng, 2008; Shen, Xie, & Xiong, 2010).

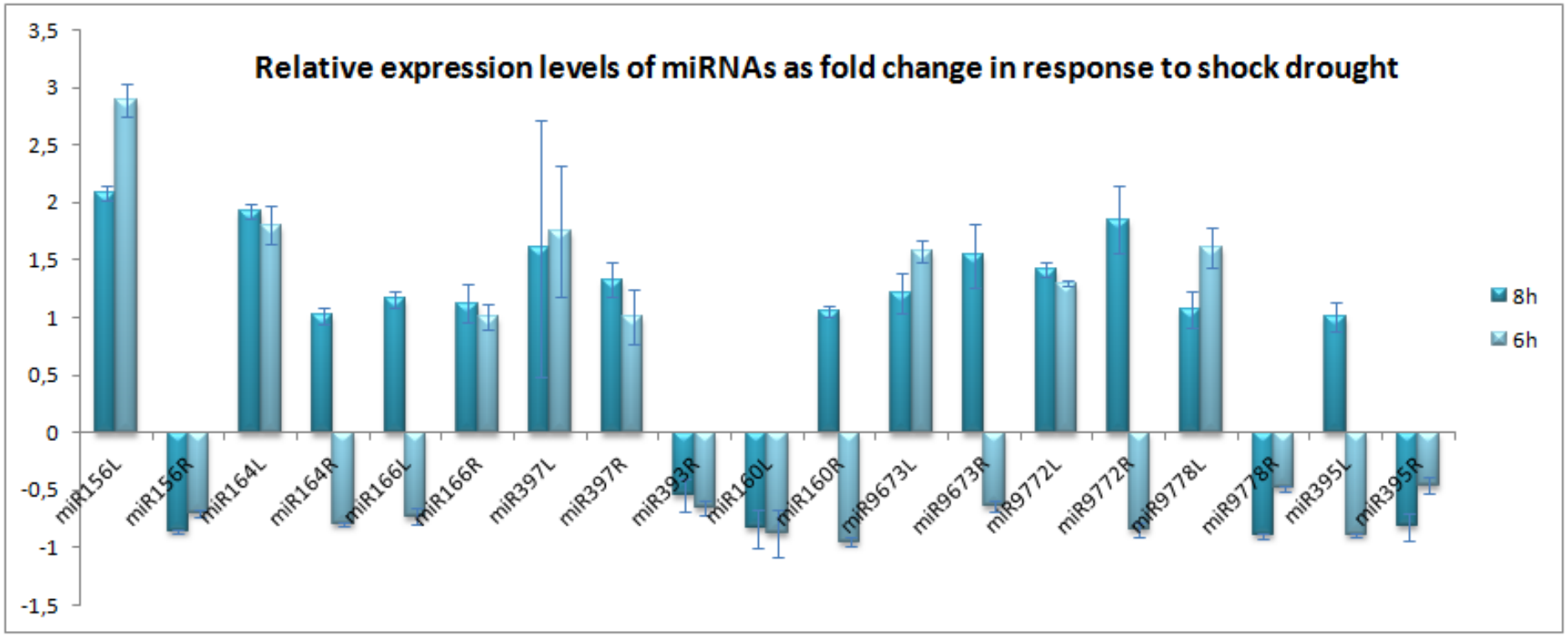

Figure 23: Differential expression of selected miRNAs in root and leaf tissues treated with 8h and 6h shock drought.

A microarray based survey on the drought response of *T. dicoccoides* miRNAs showed the up-regulation of miR156 upon 8h drought stress in the four-leaf stage root tissue and this pattern was further validated with qPCR analysis (Melda Kantar et al., 2011). In contrast, miR156 levels were decreased in comparison to the control samples in 8h shock drought root tissues in our results (Figure). In another study, precursors of this miRNA were found in both stress treated and control roots of slow drought stressed *T. durum* and stress treated roots of two *T. dicoccoides* accessions while their control samples did not express miR156 (Bala Ani Akpinar, Kantar, et al., 2015).

miR156 was up-regulated in Arabidopsis thaliana, *T. dicoccoides*, *Hordeum vulgare*, *Populus tomentosa* and *Prunus persica*; while being down-regulated in *Oryza sativa* (Ferdous et al., 2015). It should be noted that all of the mentioned studies were carried out on different stress treatment techniques which were applied on diverse developmental stages. Therefore, our results have provided a detailed expression pattern for miR156 in *T. dicoccoides*

miR164 is another drought responsive conserved miRNA in *Poaceae*, which were only found in the control root samples of the drought tolerant *T. dicoccoides* accession TR39477 but not in the stress samples, therefore it was down-regulated upon slow drought stress (Bala Ani Akpinar, Kantar, et al., 2015). In our analysis, miR164 was up-regulated in the leaf tissues and increased its expression level from 6h to 8h. However, in the root samples, its expression was lower than the control at 6h stress and higher at 8h. Delineation of the miR164 expression characteristics are significant as miR164 is known to target the NAM, ATAF1/2, and CUC2 (NAC) transcription factor family that are shown to negatively regulate drought response when they are over-expressed in rice (Fang, Xie, & Xiong, 2014).

Drought induced up regulation of miR166 in the root tissues were in line with the previous studies (Melda Kantar et al., 2011) which targets the HD-ZipIII transcription factors (Juarez, Kui, Thomas, Heller, & Timmermans, 2004) which have important roles in development and drought-responsive stress adaption (Agalou et al., 2008).

We presented a second evidence for the presence of the mature miR397 and 395 in *T. dicoccoides* with the results of Akpınar et al. (Bala Ani Akpinar, Kantar, et al., 2015), and first computational and experimental evidence for miRNAs; miR9673, miR9672 and miR9778.

4. CONCLUSION-I

Exploiting the 10.8 Gb whole genome sequence assembly, we have identified genome-wide putative microRNAs of *Triticum turgidum* spp. *dicoccoides* subpopulation *judaicum*, accession Zavitan. Our *in silico* approach was able to detect 644 putative microRNAs belonging to 53 families. In addition to these putative microRNAs, microRNA-like fold-back structures; TE-miRs, which are originating from repetitive elements were identified to be pervading the Zavitan genome. In line with the previous bread wheat whole genome, and flow sorted chromosome miRNA studies in both tetraploid and hexaploid wheat, En-Spm sub-family was the most prominent repeat type, emphasizing its role in the miRNA gene evolution. Genome-wide tRNA prediction analysis also underscored the repeat rich content of the Zavitan genome.

Precursory sequences of the putative Zavitan microRNAs have been searched in the available tetraploid and hexaploid wheat expression sources and this analysis has computationally validate 20% of the pre-miRNAs expression with 98% sequence identity and coverage. To further legitimize the authenticity of our predicted miRNAs, we performed RT-qPCR experiments for the selected mature microRNA sequences and successfully established 16 mature microRNAs expression. Since microRNA expression is spatio-temporally, we investigated the drought responsive profiles of 10 microRNAs in root and leaf tissues of Zavitan seedlings under two different shock-drought stress durations. Our results have provided detailed information on the stress response related expression fluctuations of the miRNAs. These results, along with the computational target identification analysis, have highlighted some microRNA and microRNA targets as new targets for the future crop improvement studies.

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CHAPTER II

1. INTRODUCTION-II

1.1 Food packaging and Shelf Life

Food packaging is essential for the healthy consumption and this practice serves as i) a protection layer for the food contaminants and spoilage microbes ii) an accommodation for the transportation and storage processes iii) an ease for the equal division and measurement of the food and iv) communicating the food-related information to the consumer. Among these, the protection of the food content from the environment is the most essential purpose as it greatly affects the shelf life. Dust, gases, water contact or water evaporation, vibration and microorganisms are the environmental factors that imperil the food safety. Selection of the right packaging system greatly affects the microbial burden on the stored material and can prevent food-borne illnesses (Robertson, 2010).

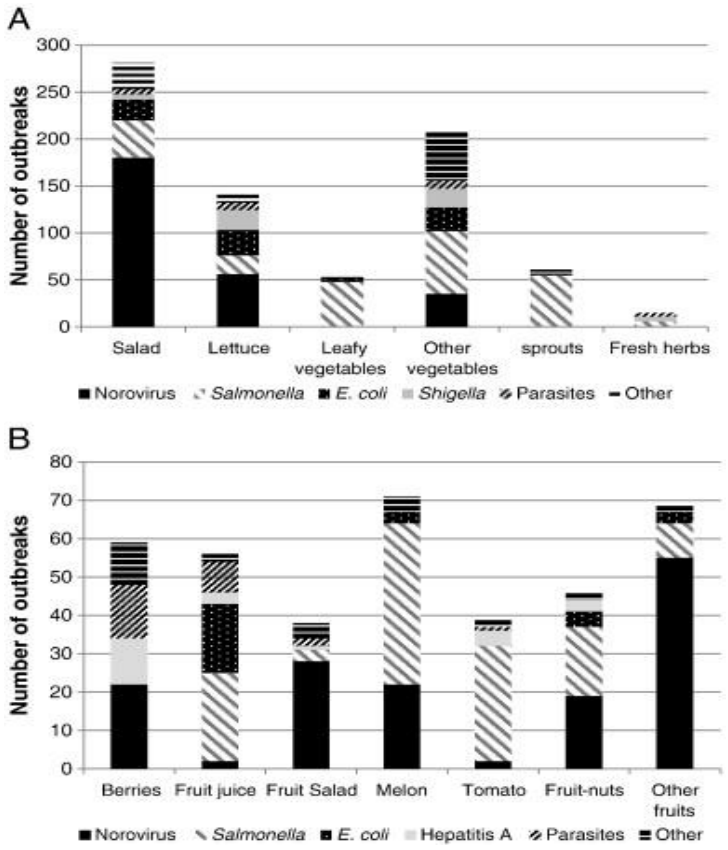


Figure 1: Reported food borne outbreaks in vegetables (A) and fruits (B) (Ramos et al., 2013).

The selection of the right strategy for the packaging maintains the beneficial effects of the food processing efforts until its consumption. Packaging strategy should be created based on

the needs of the food material and benefit both transport and protection while being cost-effective. The *Total product concept* must be applied when designing the packaging system, in which the package and product are considered as a whole. This approach requires preliminary assessment and characterization of the both components such as detailed knowledge of the foods deterioration process and it's biophysical and biochemical features, and adverse outcomes of the interactions between the package material and food (compatibility) (Coles, McDowell, & Kirwan, 2003).

1.2 Biodeterioration of the Food

Biodeterioration is defined as the spoilage and lesion formations on the food caused by the microbes or the toxic compounds that are produced by the food-borne microorganism. The introduction of the food pathogen to the packaged food can be either pre-harvest/pre-processing or during the packaging and processing practices through the contacted surfaces. The introduced microorganism number and variety dictates the shelf-life of the product. While physical effects also facilitate the biodeterioration process, the main actors are mostly the enzymes produced from the pathogenic fungi and bacteria to metabolize and consume the food composite in their environment. In addition, enzymes which are produced by the food itself comprise another source of biodeterioration. While there are a number of bacteria which secrete enzymes to the food surface, this is a more general trend for moulds. Considering the spore producing life cycle of the moulds, their reduction and decontamination from the food is a tough challenge. The biodeteriorating effects of the enzymes are less likely to be seen in the canned products as their preparation involves long heat treatment steps in high temperatures, however fresh fruits and vegetables are not suitable for this and the lack of effective management of the enzyme producing microorganisms and enzymes shortens the shelf life (Coles et al., 2003).

Water existence in the environment generally promotes food borne bacterial growth by facilitating the transportation and availability of the small molecules and nutrients that are crucial for their metabolism. While the 20 % level of available water furnishes their growth, bacterial growth does not detected at 5% (Coles et al., 2003). Bacteria is the most widely investigated and studied food pathogen and its managements efforts have borne fruit in some of the EU countries in terms of the decline in the related disease rates. However complete management of the bacterial pathogens from the food is a challenging task.

Physiology and growth requirements of bacteria greatly vary towards different extremities in terms of heat, pH or aerobic conditions, blocking the application of holistic approaches for their control. Every food's preservation needs the consideration of characteristics of the microorganisms which play a role in their spoilage. For example, *Clostridium botulinum* is one of the most lethal food-borne pathogen bacteria which can form heat resistant spores and neurotoxins. Applying very high temperature is required for its elimination from the food. If the food's nature is not convenient for the heat treatment, other means of control must be employed. Other important food pathogens *Listeria monocytogenes, E. coli and Salmonella* can be reduced by 6-log via heat treatment at 70 ^{0}C for 2 minutes (Coles et al., 2003).

Salmonella is a well recognized food pathogen that generally colonizes livestock animals and mostly contaminates meat products in addition to a wide range of other foods. Due to its ability to inhabit various environments it is prone to evolve resistance against many undesirable conditions. While *Salmonella* species are mainly isolated from the meat and egg, recent studies showed its contaminations in fresh fruit and vegetables (Abadias, Usall, Anguera, Solsona, & Viñas, 2008; Newell et al., 2010). Another well known food pathogen which is of primary concern for the public health is *E. coli* (Figure 1). There are a number of well recognized *E. coli* strains which are associated with different diarrheal diseases and related outbreaks all over the world. Just as *Salmonella*, the high rate of the evolution of resistant strains is the major drawback for the management of *E. coli* pathogenicity and sprout. Other major food borne pathogen bacteria can be named as *Listeria monocytogenes, Staphylococcus aureus, Bacillus* and *Camplylobacter* species.

1.3 Food preservation and Human Health

Numbers in the deaths caused by diarrheal illnesses that are resulted from the consumption of contaminated food and water have a decreasing trend. Predictions foresee the continuity of the trend in the future in the developed world as a result of the implementation of international standards and legislations. However the predictions for the developing countries do not follow. Therefore microbiologic safety is continuing to be a critical issue in both developed and underdeveloped countries. Moreover, the practice of the latest standards by itself also do not result in the precise elimination of the pathogens (Newell et al., 2010). It was reported that the annual number of illness cases related to food-borne pathogens are 76 million in the United States (Mead et al., 1999). Many food-borne pathogens and microbes are known, and instead of being eliminated with the new antimicrobial packaging and processing applications,

they continue to pose serious challenges for the microbiological food safety by evolving resistant strains (Newell et al., 2010). The lack of effective monitoring methods through the food chain jeopardizes their detection and management. Studies on the infectious intestinal disease records state that more than 50 % of the infectious agents are undefined (Tompkins et al., 1999) and this leads to the underestimation of the importance of the bacterial and fungal originated toxins. As a result of the tardiness in detection techniques' development, the numbers of microorganisms that are recognized as food borne pathogens are increasing day by day. The increase in the number of recognized pathogens potentiates new means of processing and packaging for diverse food and packaging system combinations for the effective elimination of diseases caused by the consumption the contaminated food.

1.4 Food Preservation and Packaging

The first practices on the food preservation was the physical methods such as heat treatment (Pasteurization, blanching, canning, sterilization, cooling, refrigeration and freezing), drying, salting and sugaring, pH modifications, smoking, irradiation and the use of controlled atmosphere conditions during packaging, storage and transportation. However, with the large scale manufacturing and industrialization, chemical management strategies have been developed and employed. Chemical preservatives of the foods are generally the food additives such as benzoate, sorbate, sulphur dioxide, nitrite, propionate and parabens. As these chemicals are added to the food directly, their use is strictly controlled and they need to fulfill some requirements to be considered as a potential food additive. A food preservative should act on only the microorganisms that are known to be contained in the related food product and should not cause the formation of resistance. The food preservative substance must kill (microbiocidal action) the food-borne microorganisms rather that inhibiting their reproducibility (microbiostatic action). Chemical compounds which may pose any toxicity danger and without proper detection assays are also not permitted for the chemical preservation. While they are widely used in the food industry there are several adverse effects of the currently used chemical food additives such as causing allergic reactions and gastric irritations when consumed in large amounts. Among them, nitrite and proptonate are recognized as potential carcinogens. These concerns have brought up the request for the food products that are free of chemical preservatives. However, commercial production of the majority of the foods is not suitable for chemical-free practice (Coles et al., 2003).

1.4.1 Physical Methods for Fresh Fruit and Vegetable Preservation

Modified packaging strategy is the most commonly used physical packaging method for extending the packaged products' shelf life. Simple modified packaging refers to vacuuming the oxygen during packaging and sealing operation. On the other hand, modified atmosphere packaging (MAS) is concerned about the atmosphere composition inside the package and based on the modifications on the gas composite by introducing nitrogen and carbon dioxide gases instead of oxygen for the minimization of the metabolic activity of the fresh fruit or vegetable (Ramos et al., 2013).

Use of cold gas plasma for decontaminating the surfaces is another newly introduced technique in the food industry. Cold gas plasma consists of non-thermal ionized gases with the ability to reduce bacterial growth. The technique allows the applications at low temperatures and therefore is desirable for packaging and pre-processing of various foods. Studies have shown the effectiveness of the method on common food borne pathogens *E. coli* and *Salmonella* (A. Fernandez, Noriega, & Thompson, 2013; Niemira & Sites, 2008).

When the food texture or nutrients is not negatively affected by the use of high pressures during or before the packaging, High Pressure Processing (HPP) is preferred. In this, food is exposed to high pressure in between 100 to 1000 MPa with the aim of disrupting the food pathogens' metabolism and enzymatic activities. As the method allows low or high processing temperatures, it has a wide application area in the food industry (Ramos et al., 2013).

Another method utilizes the gamma-ray, X-ray or ionizing radiation for the reduction of the microorganisms and the level of water. Use of ionizing radiation in low doses has been shown to be reducing the protozoan and bacteria effectively (Lu, Yu, Gao, Lu, & Zhang, 2005). The maximum allowed level of radiation was dictated as 1.0 kGy by The United States Food and Drug Administration (FDA) (Parish et al., 2003). Ultraviolet radiation is also used for the fresh fruits and vegetables packaging. Ranging from 280 nanometers to 315, UV-C is by far the mostly used dose. However one drawback for the use of radiation in packaging is the potential for introducing resistant pathogen strains as it may cause DNA damage in the microorganism (Ramos et al., 2013).

Use of ultrasound waves and pulsed light for inhibiting microbial growth are among other less common approaches for protecting the packaged food by physical means. Though physical methods are indispensable for food packaging and processing, they must be combined with

the other chemical or nanotechnology based applications for progressively improving the food quality inside the package and its shelf life.

1.4.2 Chemical Methods for Fresh Fruit and Vegetable Preservation

Use of chemical agents for the control of post harvest diseases and extending the shelf life of the fresh fruits and vegetables is an extensively adopted and effective approach. Hypochlorite, chlorine dioxide, sodium chloride, trisodium phosphate (TSP), hydrogen peroxide (H_2O_2), organic acids, peroxyacetic acid, calcium based solutions and electrolyzed water are some of the chemicals that are being widely utilized.

As most of these chemicals have very strong bactericidal and bacteriaostatic activity, their effect on human health is a matter of concern. Therefore their use and dosage is strictly controlled. Hydrogen peroxide exerts its detrimental effect by producing cytotoxic agents and used by adding to the wash water of the fruits and vegetables. While low concentrations fail to work, high concentrations may affect the overall quality and flavor of the food (Ölmez & Kretzschmar, 2009). Lactic acid, citric acid and acetic acid are the organic sanitizers which introduce an acidic environment in the food surface and interfere with the biochemical function of the pathogens cell membrane. In addition to their inclusion in the wash water, there are practices where organic acids added to the fruit juices (Velázquez, Barbini, Escudero, Estrada, & Guzmán, 2009). The mostly used chemical sanitizer in the food industry is chlorine. However, its activity can be inhibited by the organic materials and they may produce chlorine by-products (CBPs) which are hazardous for human. Its dioxide form is also another effective alternative which does not produce CBPs, but also known to interact with amino acids and RNA inside the cell and therefore requires detailed research (Keskinen, Burke, & Annous, 2009; Ramos et al., 2013).

1.5 Antimicrobial Agents and Active Packaging

Increasing the food safety during the processes of global food trade and distribution is a critical issue. The large scale outbreaks caused by the food pathogens indicates the urgency of the need for easily established protection techniques for the fresh and minimally processed food products. The introduction of the antimicrobial food packaging strategies has been suggested as novel and effective solution. Use of antimicrobial agents in the food packaging applications will serve to increase the shelf life and play an important role in maintaining the quality.

As the used antimicrobial agents have contact with the food, antimicrobial packaging is an active packaging system. There can be a number of different forms of antimicrobial food packaging. Antimicrobial agents could be encapsulated in polymers or they can be linked to the polymer based surface coatings. In the volatile form, they can be as absorbed in sachets that are placed inside food packages. These applications mostly utilize nanotechnology and nanomaterial based applications.

1.5.1 Use of Nanomaterials in Food Packaging

Nanomaterial based packaging is an active and intelligent packaging (A&I packaging) system which preserves the product through incorporating different types of antimicrobial or sanitizing agents to the packaging system. As the food processing and related operations with "natural" and "organic" keywords are preferred by the general public, nanotechnology has took its place in the food industry relatively late in comparison to the other areas, notwithstanding its broad potential applications. Though it is not the only application area in food industry, nanotechnology has its greatest share in the packaging step. In 2008, global nanotechnology based food packaging market was 4.13 billion dollars with 11.65 % annual growth rate (http://www.innoresearch.net/).

Designing active nanomaterial based packaging systems requires a thorough understanding of the pathogen and food interactions and the spoilage mechanisms. Using nanotechnology based materials provides several advantages over other conventionally used materials in the packaging systems. For example; nanocomposites; the materials which have at least one phase with nano-scale dimensions, offer environment-friendly solutions while preserving or even promoting the desirable mechanical properties of the food packaging materials (Ramos et al., 2013; Roy, Saha, Kitano, & Saha, 2012). Also nanotechnology based antimicrobials yield higher efficiencies in comparison to the conventional antimicrobials as they have greater surface area to volume ratio, facilitating the contact with microorganism (Malhotra, Keshwani, & Kharkwal, 2015). Thermal stability, chemical resistance, conductivity, preserving optical properties and low gas permeability are among other advantages of the nanotechnology based applications, but it should be noted that inadequacy in the information on their effect on human health and food contact properties is a drawback for their applications in the market (De Azeredo, 2013).

Use of the volatile antimicrobials absorbed to the sachets and placed inside the packages is the most widely used and successful technique so far. They exhibit their discouraging effect on

the microbial growth by absorbing different gases inside the packaging system like oxygen or generating ethanol vapor (Appendini & Hotchkiss, 2002; Ozdemir & Floros, 2004). Ethanol vapor generators inhibit the mold growth. Their gas relies rates must be in very low levels and strictly controlled (Day, 2008). Even though these systems has many commercial applications and have been useful for the food preservation, a major drawback is its undesirable effect on the taste of the food (Appendini & Hotchkiss, 2002).

Another promising active packaging strategy is the incorporation of antimicrobial agents on the polymer surfaces. Although this method has not been widely applied in the industrial practices as the absorbents and evaporators, there has been extensive research related to its use and effects on food-borne bacteria and fungi. They have been tested in both paper and plastic packaging materials. Incorporation of polymers such as polypropylene and polyethylene, with zeolites which are substituted with silver in their Na residues is the most popular form. This system relies on the antimicrobial effect of silver molecule on the cellular processes of the microorganisms (Fernandez, Soriano, Hernandez-Munoz, & Gavara, 2010). Spraying the antimicrobials on the food surface is a common practice. But antimicrobials that have been introduce to the food surface through spraying lost their activity in the long term and require more than one operation over time. Polymer embedded antimicrobials provides a controlled mean of long term release. As it allows for the incorporation of many different types of molecules, and even the incorporation of the combinations of different types of molecules, this approach has a great potential to be implemented in many food packaging units and non-food related areas (Appendini & Hotchkiss, 2002).

Methods for the incorporation of antimicrobial agent into the polymers may differ according to their physical and chemical characteristics. The antimicrobial effect of the agent should not be affected by the production process. For example use extruder technology is only suitable for the agents which are resistant to the heat treatments therefore cannot be employed in case of using heat-sensitive enzymes as antimicrobial agents (Del Nobile et al., 2009). Despite the great variety in the theoretical applications of the surface bound antimicrobials, their incorporation and even distribution on the polymer surface is a daunting task and requires a strictly controlled environment in the production process (Torres-pacheco, 2006). Also the migration and diffusion of the surface bound agent to the outside of the package needs control.

As mentioned above, heat sensitive profile of many antimicrobial agents hinder their incorporation into polymers. However, they can be coated on the surface after polymer production. Adsorption of nisin on multiple different surfaces like ethylene-vinyl acetate, poly ethylene, polyamide and polyethylene terephthalate are some of the early examples of antimicrobial coating (Appendini & Hotchkiss, 2002).

Immobilization of the antimicrobial agent on the surface via ionic and covalent bonds is another method of choice when the antimicrobial agent and preferred packaging material's surface have suitable functional groups in their structure. Polystrene, polyvinyl chloride and polyethylene are some of the materials with functional groups in their surface which are commonly used food packaging industry. However the lack of functional groups on the surface of the polymer could be overcame by surface functionalization before antimicrobial coating (Pasquier, Keul, Heine, & Moeller, 2007). An example for the physical functionalization of the polymer surface has been done on polyamide films by UV treatment. This application has resulted in the elevated antimicrobial activity as a consequence of increased number of amine groups (Appendini & Hotchkiss, 2002). Other means of functionalization of inert surfaces can be named as ion beam, electron beam, laser and plasma treatments (Ozdemir, Yurteri, & Sadikoglu, 1999).

Another alternative application for surface coating is the use of spacer molecules to generate an easier movement and increased contact for the antimicrobials as a result of conformational freedom (Appendini & Hotchkiss, 2002). Covalent bonding between the antimicrobial agent and the packaging material should be preferred when the diffusion of the antimicrobial agent is non-desirable and the potential catalytic reactions and their byproducts should not be overlooked.

Chemical structure of some of the polymers resembles antimicrobial characteristics without the adsorption of extraneous antimicrobial agents. This is mostly due to the interaction between the charged groups of the polymer surface with the microorganism's cell walls. Bacterial cells outer surface has a net negative charge; presented by the lipoteichoic acids in Gram positive bacteria cell wall, phospholipids and lipopolysaccharides in the Gram negative bacteria outer membrane and inner cell membrane. The negatively charged nature of the bacteria cell envelope aided the use of positively charged functional groups in the polymer surface to both attract and disrupt the integrity of the cell envelope. This type of polymers could be hydrophilic macromolecular tails that are bound to cationic groups, or functional

groups on randomly polymerized blocks consisting of hydrophobic monomers and hydrophilic co-monomers (Timofeeva & Kleshcheva, 2011). Such polymers were shown to disrupt the bacterial cell wall and outer membrane in the contact areas and this mode of the antimicrobial activity was associated with the length of the polymer macromolecule (Broxton, Woodcock, & Gilbert, 1983; Broxton, Woodcock, Heatley, & Gilbert, 1984). One of the commonly used cationic polymers, polyhexamethylene biguanide chloride (PHMB) was tested in terms of its mode of action as antimicrobial. The proposed action starts with the rapid recruitment of the negatively charged bacterial cells to the coated surface followed by the disassembly of the outer membrane as a consequence of physical contact. After passing over the outer membrane, PHMB interacts with the phospholipid content of the inner membrane, causing a rapid increase in the membrane permeability. This sequence of events finally leads to the leakage of the intracellular constituents and bacteriostasis caused by total loss of inner membrane integrity (Gilbert & Moore, 2005; J. Y. Maillard, 2002).

1.5.2 Quaternary Ammonium Compounds

Quaternary ammonium compounds (Figure 2) are a group of surfactants or surface-active agents. Same as all the other surfactants, their molecular structure consists of one hydrophobic and one hydrophilic/polar region. Since their hydrophobic group is positively charged, quaternary ammonium compounds (QACs) are grouped under cationic surfactants. QACs are also membrane-active, and function through the same proposed mechanism as for PHMB by binding to the membrane phospholipids. However in high concentrations QACs may coagulate the membrane proteins and restrict the leakage of the cytoplasmic content (Davies, Bentley, & Field, 1968). Also the length of the alkyl moieties linked to the nitrogen in hydrophobic part effects the antimicrobial activity and alkyl chains with 16-18 carbon atoms are defined as the optimal (Ahlstrom, Chelminska-Bertilsson, Thompson, & Edebo, 1995; Ahlström, Thompson, & Edebo, 1999; Calas et al., 2000; Lindstedt, Allenmark, Thompson, & Edebo, 1990). Their proposed action mechanism is supported with the experiments which utilize methods like Atomic Force Microscopy (AFM) and Transmission Electron Microscopy (TEM) (Timofeeva & Kleshcheva, 2011). Polymerization based increase in the antimicrobial effect was observed for two quaternary ammonium compounds; polyvinylbenzyl ammonium chloride and polymethacrylate with pendant biguanide groups. When tested on Gram-positive and Gram-negative bacteria, poly(methacrylates) with ethyl or methyl quaternary ammonium chloride side groups showed strong antimicrobial activity on the Gram-negative bacteria, while inhibiting effect was mild in the Gram-positive (Gottenbos, Grijpma, van der Mei,

Feijen, & Busscher, 2001; Kenawy, Abdel-Hay, El-Shanshoury, & El-Newehy, 1998). Quaternary ammonium compound are also active against lipophilic viruses by putatively interacting with the proteins in the viral envelope (Fraise et al., 2012; Shirai, Kanno, Tsuchiya, Mitsubayashi, & Seki, 2000). Studies showed that while disrupting the capsid, QACs did not alter the viral genome (J. Y. Maillard, Beggs, Day, Hudson, & Russell, 1996; J.-Y. Maillard, Beggs, Day, Hudson, & Russell, 1995)

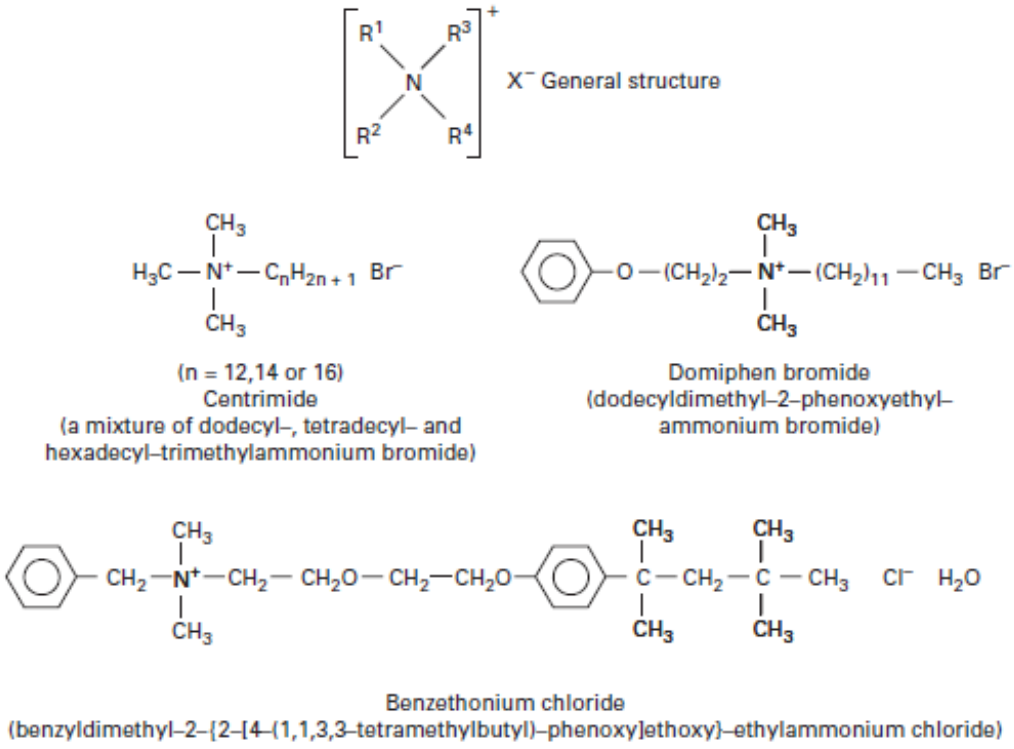


Figure 2: General structure and some of the examples of quaternary ammonium compounds (Fraise et al., 2012).

Low levels of QAC resistance was reported for *S. aureus*, *P. aeruginosa, P. Stutzeri*, coagulase-negative staphyloccoci, *Achromobacter xylosoxidans* and *Burkholderia cepacia* related with the hydrophobicity, charge, and protein composite of their cell surface (Fraise et al., 2012). Also, ineffectiveness of QACs against bacterial spores was stated in the past, however a recent study that assessed the activity of a commercial QAC product on the *Mycobacterium bovis* reported high effectiveness of the product (Elkholy, Hegab, Ismail, & Hassan, 2016). In line with the observations on mycobacteria, investigations on the antifungal efficacy of QACs showed variation according to the QAC type, working concentration, target fungi and exposure time (Fraise et al., 2012). Therefore the antimicrobial activity of QACs; either effective or ineffective, cannot be ruled out from individual studies.

Quaternary ammonium compounds also interact with DNA. The interaction results in the precipitation of the nucleic acid (Del Sal, Manfioletti, & Schneider, 1989). While they are generally recognized as safe and environmentally friendly, recent reports drawn attention on potential mutagenicity of quaternary ammonium salts (Dmochowska et al., 2011; Grabińska-Sota, 2011). Furthermore, high levels of toxicity of benzalkonium and some other QACs have been reported on the cornea cells (Olson & White, 1990; Sasaki, Nagano, Yamamura, Nishida, & Nakamura, 1995). Therefore their common use requires detailed mutagenicity and toxicity tests.

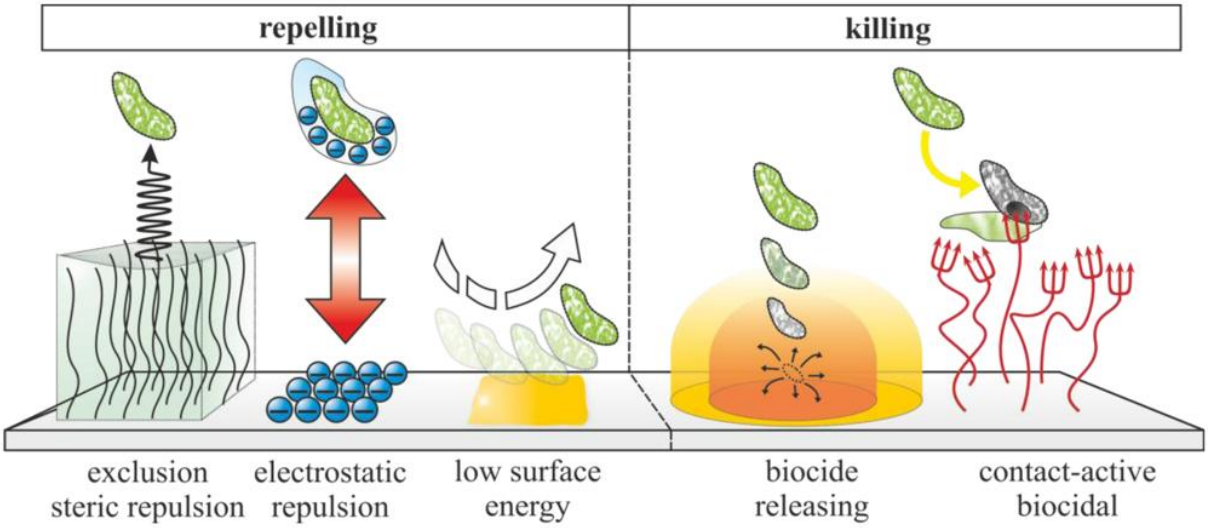


Figure 3: Action mechanisms of antimicrobial surfaces (Siedenbiedel & Tiller, 2012).

Other examples for cationic antimicrobial polymers are chitosan and poly-L-lysine (Goldberg, Doyle, & Rosenberg, 1990). As an alternative to the currently used synthetic fungicides, chitosan have been used to coat the surface of the fresh fruits and vegetables. It showed promising performance for the food preservation both pro- and post-harvest by executing its action on plant as well as on pathogens. Besides its antifungal activity, chitosan decreases the respiration rate of the fruit by forming a semi-permeable film on the surface, which also serves as a base for the incorporation of other possible food additive molecules (Romanazzi, Feliziani, Baños, & Sivakumar, 2016).

Use of antimicrobial polymers in food packaging needs the consideration of the action mechanism of different polymers, their chemical composition, and the biophysical

characteristics of the microorganisms within the target spectrum. For example, diffusion kinetics is the point of concern when the antimicrobial agent acts on the microorganisms by being released from the surface and enter the cell cytoplasm. Their position and flexibility on the package surface is not as crucial as the surface active polymers. Polymer kinetics can change depending on the tested environment. The activity tests conducted in the growth media or other mediums may not resemble the "real-life" activity on the foods surface or inside the package (Appendini & Hotchkiss, 2002). Furthermore, nutrient rich components of some food materials could hinder the use of some antimicrobials like zeolites substituted with silver, as the antimicrobial activity is reduced in the presence of sulfur containing amino acids (Ohlsson & Bengtsson, 2002).

Additives that are used to strengthen or increase the quality of the food packaging polymers may also have an altering effect on the polymerization and therefore the antimicrobial activity. On the other hand, the antimicrobial polymers and coatings sometimes results in the formation of undesirable properties. Thickness, transparency, seal strength, opacity, tensile and other physical and mechanical characteristics are typically affected especially in the thin films (Appendini & Hotchkiss, 2002). Incorporation of natural extracts; propolis, chitosan and clove extract in the production process and the fabrication of the films by blowing extrusion had an inhibitory effect on some of the food pathogens such as *Fusarium oxyporum*, *Lactobacillus plantarium*, *S. cerevisiae* and *E. coli*, but among them, chitosan addition significantly altered the mechanical and physical characteristics of the films (Hong, Park, & Kim, 2000). Antimicrobial additives may alter the packaging materials physical properties either by impeding or facilitating, therefore needs individual consideration.

1.5.3 Antimic

The proposed product patented by Sabancı University and licenced to Nanotego Co. and named as Antimic® in the market is colorless and oderless material that forms nanolayers on the surfaces and its long-term conservation on surfaces inhibits the growth of microorganisms. Antimic has a partially quaternized ammonium organosilane composition (Taralp, Menceloglu, Simsek, & Acatay, 2011). Antimic is reported to have a wide spectrum of target microorganisms. Antimic is resistance to high temperatures and reported to be resistant up to 100 ^{0}C.

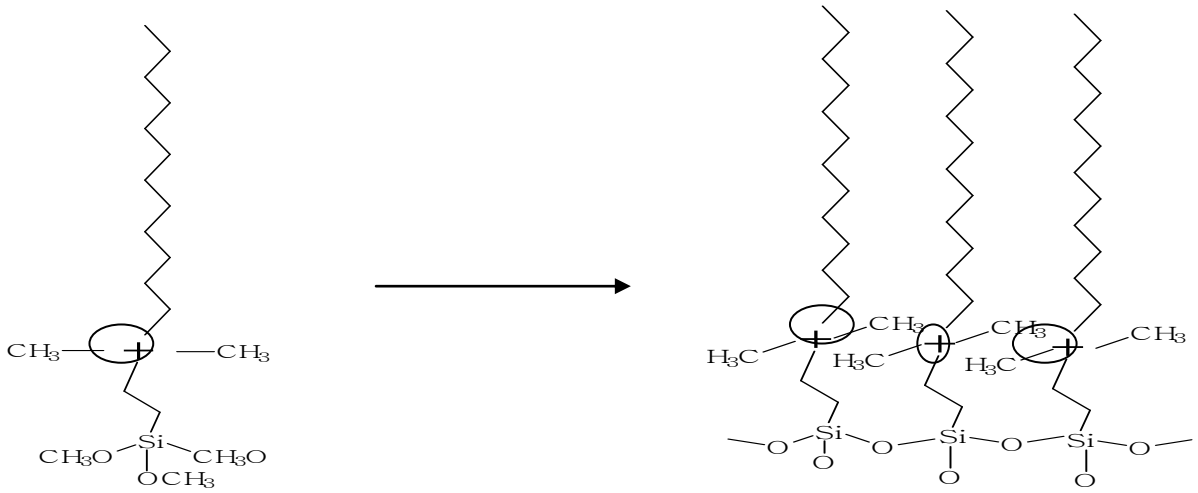


Figure 4: Sol-gel polymerization reaction of Antimic.

It is a water-based compound which is stated to be environmentally friendly. It is also possible to prepare alcohol based Antimic solutions regarding the characteristics of the defined surface. As a result of the sol-gel polymerization reaction, it covalently binds to the applied surfaces with the oxygen atoms in its silane groups (Figure 4). Sol-gel polymerization is a highly advantageous processing technique which is used to entrap compounds with diverse functions onto different surfaces. It requires shorter processing time and lower temperatures in comparison to the other solution and solid-state based techniques (Danks, Hall, & Schnepp, 2016). Sol-gel based inorganic oxides are commonly used for the formation of antimicrobial coatings (Xing, Yang, & Dai, 2007).

Based on the proposed mechanism for quaternary ammonium compounds, negatively charged microorganisms are attracted by the positive charge of the ammonium groups of Antimic layer and they are killed by the physical action of the repeating units in its polymer chain.

1.6 Regulations

Regulations and international standards for testing the antimicrobial effects of the polymers used for the food packaging are still in their infancy. Safety of the food contact substances are regulated and monitored by The Office of Food Additive Safety (OFAS) at the U.S. Food and Drug Administration's (FDA) Center for Food Safety and Applied Nutrition (CFSAN) in the Unites States. The food contact substances are broadly define as "any substance intended for use as a component of materials used in manufacturing, packing, packaging, transporting , or

holding food if such use is not intended to have any technical effect in such food". Some of the food contact substances were listed as; polymers, materials used in the production of paperboard and antimicrobial agents. In 1996, the legitimate definitions of the food contact substances "food additives" and "pesticide chemicals" have been updated and handed over the regulatory authority of the food contact antimicrobials to the EPA under "pesticide chemicals" group. However the antimicrobial agents, those are included in the packaging materials, or applied to them are not considered as "chemical pesticides" and there is no specific regulation for active packaging; therefore they are regulated by FDA as food additives. The safety of the food contact substances are stated as "reasonable certainty in the minds of competent scientists that a substance is not harmful under the intended conditions of use" by FDA. According to the legislation, a number of pre-requisite must be fulfilled for a new agent to be approved as a food contact substance. Detailed toxicology information, data related to the environmental effects and chemical properties of the new compound should be reported in Food Contact Notification form. Mutagenicity and carcinogenicity related information is expected to be documented in detail in the toxicology report. As the antimicrobial residues in raw agricultural commodities are under the jurisdiction of EPA under "pesticide chemicals" category, antimicrobial food contact substances needs to be registered in EPA in addition to FDA (http://www.fda.gov). While there are a number of official test methods for assessing the effects of the microorganisms on materials like plastics, the examination of the antimicrobial activity of antimicrobial packaging systems lacks a set of legitimate techniques. Since they are considered as food additives, current regulations on the control and assessment of the food additives and their activity are apply for antimicrobial packaging systems (Vernaud & Rosca, 2006).

The legislation is more detailed in Europe, under European Food Safety Authority (EFSA) (https://www.efsa.europa.eu). In EU regulations, antimicrobial substances are categorized as either; active, additive or polymer production aid substance and regulated accordingly. Food contact materials and active and intelligent packaging systems are in the scope of REGULATION 1935/2004/EC and REGULATION 450/2009/EC respectively. Regulation of the food contact materials states that the material shall not transfer its constituents to the food in the quantities which may pose a hazardous risk for the human health. If the contact affects the organoleptic characteristics of the food, the changes should not mask the spoilage and need to be in the limits of the legislations. In case of market use, these compounds should be adequately labeled. Also, if the active packaging system contains more than one

active/intelligent components, both of them must full fill legislation requirements. Most importantly, before their authorization, substances must undergo a safety assessment by EFSA. For this, overall migration of the active substance and degradation products resulted by its activity and the toxicology properties should be reported (Restuccia et al., 2016).

1.7 Efficiency Measurement of the Antimicrobial Packaging Materials

Antimicrobial properties of the antimicrobial containing products have been tested with 'Film Contact Method' in Japan as a standard method. This technique applies to the antimicrobial products which involve inorganic antimicrobial agents and produced as sheets and films. Method is based on the inoculation of the packaging product with the selected bacteria and determining the bacterial growth by colony counting. Agar plate, dynamic shake flask and minimal inhibitory concentration (MIC) are other assays which have been employed for the direct assessment of the antimicrobial effect on the food borne pathogens (Zweifel, Maier, & Schiller, 2009).

Co-incubation of the different concentrations of antimicrobial packaging polymers inside test tubes with the selected microorganism for differing time periods and the subsequent spectrophotometric measurement of the microbial concentration lets the determination of the minimum inhibitory concentration of an antimicrobial compound. Therefore this technique facilitates the comparison of the strength of different concentrations and even different antimicrobials. Reporting the MIC results require the information about polymer composition, polymer dimensions and changing characteristics according to used sample types.

A second test method is the agar plate method in which an antimicrobial product specimen is placed on an agar plate that has been inoculated with the selected microorganism. After the incubation time that is required for the visual detection of the microbial growth, the area without microbial growth in the samples' surrounding is measured and indicates the diffusion.

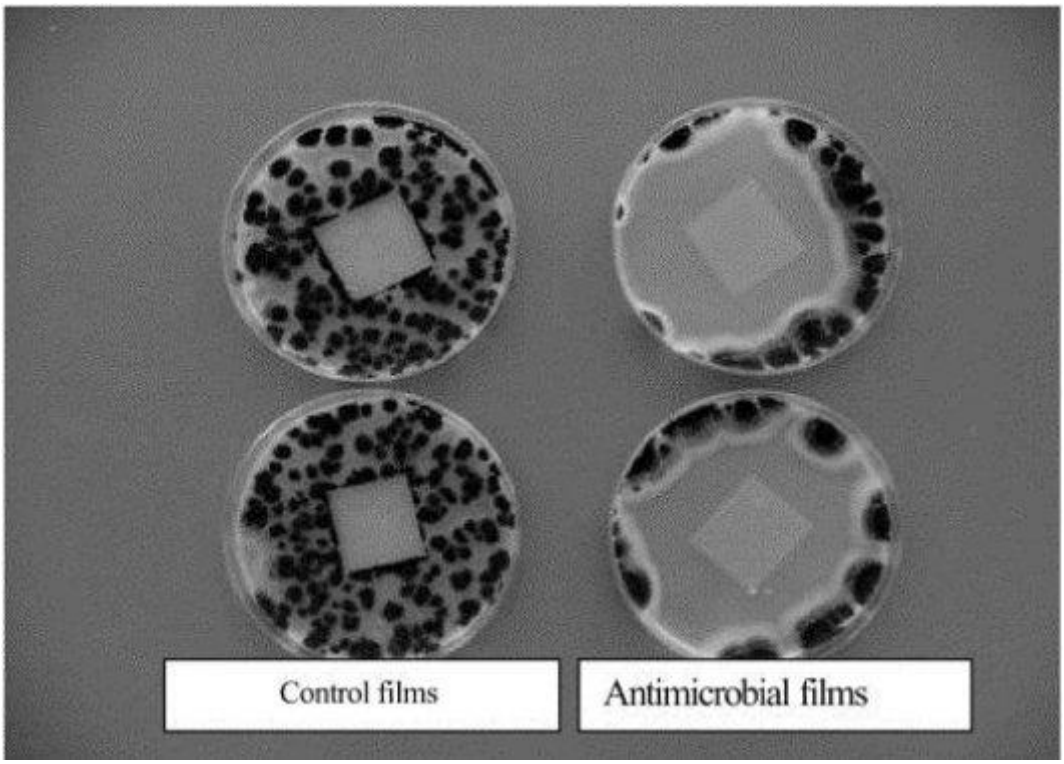


Figure 5: Antimicrobial coated plastic products effect on *Aspergillus niger*.

The third commonly used method is the shake flask method which benefits the identification of the kinetic characteristics of the antimicrobial agent. In this method, antimicrobial polymers are placed into the flasks which have a selected buffer inoculated with the target microorganism. The flasks are mildly agitated to enable the maximum contact between polymer and the microorganisms. Growth rates or the inhibition of the growth is monitored through the sample collection from the samples in multiple time points. Liquid phase of the test can be both broth media and different buffer solutions. Microbiocidal effect of the polymer can be assessed by using buffer solutions and kinetic properties of microbial growth can be obtained from the tests that have been carried out in broth media. When reporting the results of shake flask tests, surface are of the polymer must be specified along with the microbial inoculums concentration and buffer solution volume; since the polymer surface are and its ration to the buffer volume greatly effects the antimicrobial activity levels and should resemble real packaging system's conditions (Appendini & Hotchkiss, 2002).

1.8 Food Microbiology Testing

Tests which conducted on the food materials are in the scope of food microbiology studies. Even though latest improvements in the clinical instrumentation have introduced molecular and real time applications like biosensors and ELISA assays, issues such as cost effectiveness and reliability keeps the food microbiology laboratories relying on the conventional techniques (Gracias & McKillip, 2004; Jaykus, 2003). These methods assess the number and/or recovery of the microorganisms in the food matrix. Selective or differential media or standard plate count methods are the long-lasting and reliable methods that do not require complex instrumentation.

Standard plate count method utilizes a plethora of commonly used non-selective or semi-selective media like Tryptic Soy Agar (TSA), Potato Dextrose Agar (PDA), Standard Plate Count agar (SPC) and Aerobic Plate Count agar (APC). These plates could gain selectivity by the addition of antibiotics or different types of selective layers on top of the standard media. Selective layers aim to recover the bacteria after the inhibitory effects of the treatments during food processing and allowing realistic assessment of the number microorganisms (Harrigan & McCance, 1966; Roberts & Greenwood, 2003). The application relies on pouring the inoculum obtained from the food on the non-selective media and incubation for the recovery, prior to layering the selective medium (Gracias & McKillip, 2004). Pre-enrichment of the samples in broth media before testing is another technique for the resuscitation of the microorganisms (T. Zhao & Doyle, 2001).

Another commonly used method in the food microbiology is the separation or concentration of the targeted microorganism from the food slurry. For example, separation of the specific bacteria can be achieved by immunologic magnetic separation (IMS) through antibody coated magnetic beads and use of magnetic field. Subsequent detection can utilize Polymerase Chain Reaction (PCR) or agar plate based techniques (Cudjoe, 1995).

Color-formation based agar plates; named as fluorogenic and chromogenic media, are also available for the detection of specific food borne pathogens. This type of detection is based on the specific activity of the enzymes that are incorporated on the surface of the plate, on different microorganisms. In this system, enzymatic cleavages are designed so as to leaf fluorescence or color-forming reactions. Various forms of these media are available for the distinction of Gram-negative and Gram-positive bacteria as well. More specifically, some of

the kit based methods are used for the detection of pathogen related chemicals or toxins (Gracias & McKillip, 2004).

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

The chemicals used in this research are listed in Appendix-II A.

2.1.2 Equipment

The equipments used through this study are listed in Appendix-II B.

2.2 Methods

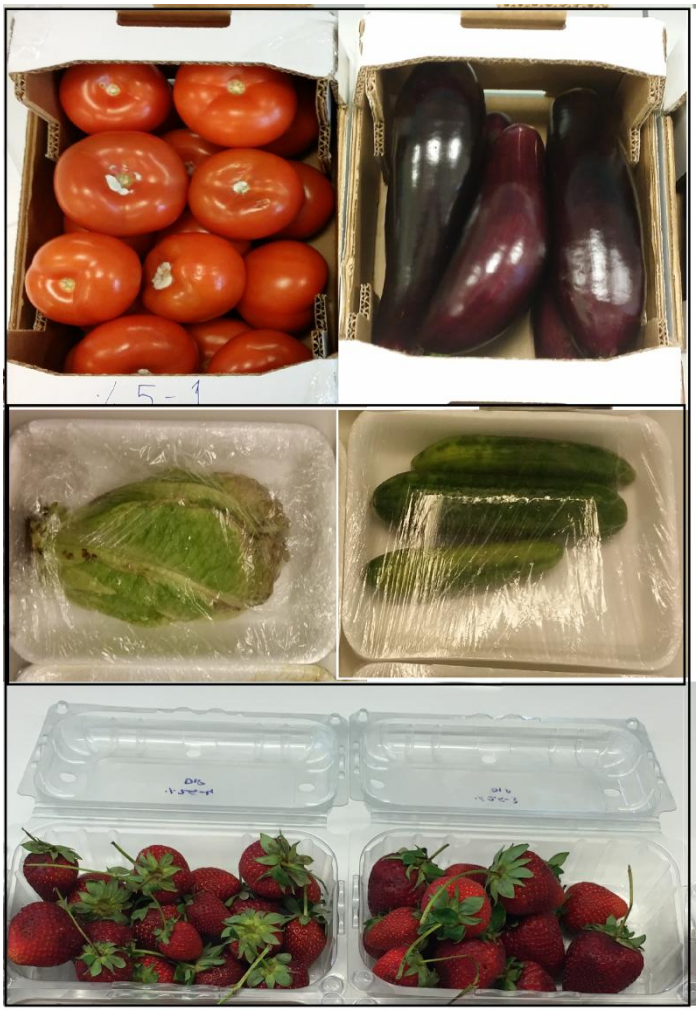
2.2.1 Production of the Antimicrobial Packaging material

To be able to analyze the effect of Antimic-6000 surface-active agent on the shelf life, various packaging material units were used for the storage of selected fruits and vegetables in their suggested storage conditions. The packaging units and the stored material couples are presented in the Table 1. Representative packaging unit and stored fruit/vegetable combinations are shown in Figure 6.

Table 1: Storage material and food combinations

Packaging Unit	Stored fruit/vegetable
Polystrene plates + Stretch film	Apple, Banana, Lettuce, Cucumber
Corrugated cardboard box	Egg plant, Cucumber, Tomato, Zucchini
Polyethylene Terephthalate container	Strawberry

Figure 6: Representative packaging unit and stored fruit/vegetable combinations.



2.2.1.1 Stretch film

Polyvinyl chloride (PVC) stretch film materials used through this study was produced by Rotopaş and Antimic was dispersed inside the film structure during compounding process with stretch film extruder machine. The test samples contained 0.05 % Antimic-6000 whereas control samples were produced without the addition of the Antimic. The quality and properties of the samples were examined by physical and microbiological tests.

2.2.1.2 Polystyrene plates and Polyethylene Terephthalate container

Commercially available polystyrene plates and polyethylene terephthalate containers were supplied from the market. Antimic-6000 solutions were prepared by dissolving Antimic-6000 in isopropyl alcohol. The antimicrobial agent coating of the material was carried out by i) spraying the Antimic-6000 solution with various concentrations via high pressure nozzle spraying system or ii) dip coating of the packaging material into the Antimic-6000 solutions.

Polystyrene plates coated with the Antimic-6000 were kept at room temperature on blotting papers for two days prior to their use.

2.2.1.3 Corrugated cardboard boxes

Corrugated cardboard boxes with 25 cm height, 45 cm width and 35 cm length were used through this study. Cardboard boxes were selected as they will allow respiration wilt an open top and ventilation holes and suitable for the cold chain to avid deterioration. Cardboard boxes were prepared by spraying the Antimic-6000/ IPA solutions with different concentrations through high pressure nozzle spraying system. Sprayed boxed were dried at room temperature for two days on blotting papers and folded prior to their use.

All Antimic-6000/IPA solution concentration and packaging material with the stored fruit/vegetable combinations used throughout this study were given in the Table 2.

Table 2: Antimic-6000/IPA concentrations used for the production of the packaging materials.

Packaging material	**Antimic-6000 concentration**	**Stored material**
Stretch film	0.05 %	Apple Cucumber Lettuce Banana
Corrugated cardboard box	50 ppm/m^2 140 ppm/m^2 230 ppm/m^2 320 ppm/m^2 410 ppm/m^2 500 ppm/m^2 1 % (0.25 L/m^2) 2.5 %(0.25 L/m^2) 5 % (0.25 L/m^2) 10 %(0.25 L/m^2)	Tomato Egg plant Zucchini
Polyethylene Terephthalate container	50 ppm/m^2 140 ppm/m^2	Strawberry

	230 ppm/m^2 320 ppm/m^2 410 ppm/m^2 500 ppm/m^2 1 % (0.25 L/m^2) 2.5 %(0.25 L/m^2) 5 % (0.25 L/m^2) 10 %(0.25 L/m^2)	
Polystyrene plate	1 % (0.25 L/m2) 2,5 %(0.25 L/m2) 5 % (0.25 L/m2) 1% - dip-coating 2.5 % - dip-coating 5 % - dip-coating	Banana Lettuce Cucumber

2.2.2 Mechanical and Physical Properties of the Packaging Material

2.2.2.1 Tensile, Thickness and Optical Characterization of the Packaging Material

For analyzing the physical characteristics of the produced stretch film material; tensile strength and elongation, thickness, luminous transmittance, haze and clarity properties were measured. Tensile strength and elongation characteristics of the material were tested by using universal testing machine type Zwick/Roell Z100. Thickness test was performed on the cross sections of Polyethylene Terephthalate container packaging units by using KLA-Tencor P6 Surface Profilometer. For thickness tests, half of the crossections of samples were coated with Antimic-6000/IPA solution with 5% and 10% concentrations by applying two different coating methods which are dip-coating and spraying. Profilometer scanned through the border between coated and non-coated area. This area was also visualized by the optical microscopy during profilometer measurements. Luminous transmittance, haze and clarity characteristics were tested with BYK-Gardner Haze-Gard equipment. The sample surface was illuminated with a 90^0 angle and the haze was measured with integrated measurement sphere. Haze was expressed with transmittance and transparency percents. Transmittance is the ratio of the transmitted light to the intensity of the incident light while passing through a sample.

2.2.2.2 Fourier Transform Infrared Spectroscopy (FTIR) Analysis of the Antimic-6000 coated Packaging Material

Assessment of the sol-gel polymerization reaction of Antimic-6000 on the coated packaging material surface was carried out using Nicolet iS10 Fourier Transform Infrared Spectrometer (FTIR). Measurement was performed on the polyethylene terephthalate containers and corrugated cardboard box with varying that are prepared either through spraying or dip-coating method by using multiple Antimic-6000/IPA concentrations. Assessment of the Antimicrobial Activity of Antimic-6000 on Packaging Material via Microbiologic Tests

After the physical characterization, microbiological tests were carried out to examine the antimicrobial activity of the Antimic-6000 coated storage materials. First, international standard tests were performed for the assessment of the antimicrobial activity. Second, the effect of Antimic-6000 on the shelf life and potential inhibiting effect on the microbial burden was examined.

2.2.2.3 Assessment of the Antimicrobial Activity of Antimic-6000 Agent by Using ISO 22196 Standard Test

Assessment of the antimicrobial activity of the Antimic-6000/IPA coated samples was performed by using a modified version of ISO 22196 standard test "*Measurement of antibacterial activity on plastics surfaces*". Though the standard was prepared to test plastic specimens, corrugated cardboard box samples were also included in the experiment. *E. coli* cells were used as testing bacteria.

2.2.2.3.1 Preparation of the test specimen and coverage of films by Antimic solutions

Test specimens from 1) stretch film that was produced with 0.5 % Antimic-6000 2) corrugated cardboard boxes that are coated with 5%, 2.5% and 1% Antimic-6000/IPA solution through spraying, and 3) polystyrene plates and polyethylene terephthalate containers that are coated with 5%, 2.5% and 1% Antimic-6000/IPA solution through both spraying and dip-coating were cut as flat surfaces with 5cm x 5cm square dimensions by using a sterile scalpels. Coverage of films were performed with the same method from commercial stretch film layers in 4cm x 4cm square form. Three and six specimens were prepared from each treated and non-treated control samples, respectively.

2.2.2.3.2 Preparation of the inoculum

One loop full *E. coli* stock culture was inoculated on nutrient agar slant media and incubated at 37 ^{0}C for 18 hours. A colony/pure strain from the agar slant was sub-cultured to fresh nutrient broth media and incubated inside the orbital shaker at 37 ^{0}C and 150 rpm for 24 hours. Nutrient Broth media was suspended by 500 fold with distilled water and sterilized and chilled at 10 ^{0}C before its use. 10^{-1}, 10^{-1} and 10^{-1} fold suspensions of this culture media were prepared with 500 fold diluted nutrient broth. The number of *E. coli* cells inside this suspension series was estimated spectrophotometrically through their absorbance values at 600 nm wavelength. Targeted solution with $6x10^{5}$ cells in a milliliter concentration was prepared with the assumption of the OD_{600} value of 1 of 24 hours incubated E. coli cells are corresponding to $8x10^{8}$ cells. This dilution was used as the testing inoculum.

Figure 7: Inoculation of the test specimen with E. coli according to ISO 22196 test procedure

2.2.2.3.3 Testing procedure

Test specimens were placed in sterile Petri dishes as their Antimic-6000 coated surface face on top.

Cross sections of stretch film and polyethylene terephthalate containers were inoculated with 100 µL of test inoculums while 200 µL inoculums was used for the corrugated cardboard and polystyrene plate cross sections to prevent the leakage of the inoculums under the cover films (Figure 7). Inoculated specimens were immediately covered with cover films. Petri dishes were covered with their lids and test samples excluding the "zero contact" samples were incubated at 37 ^{0}C for 24 hours.

Three replicates of the non-treated control samples were used as "zero contact" samples and the remaining steps of the test procedure was carried out without the 24 hour incubation step. Inoculums were washed from the zero contact samples, control samples and test samples by pouring 10 mL of tryptic soy broth media onto the test specimens and cover films. Washing was completed by pipetting the broth media onto the test material for 5 to 6 times. Hundred and thousand fold dilutions of the wash media containing the inoculums from each sample was prepared by using phosphate-buffered physiological saline solution. One mL volume from this dilutions and 15 mL of plate count agar media (60 ^{0}C) were transferred into sterile Petri dishes. Petri dishes were gently shaken to distribute the bacteria equally and kept at room temperature until they are fully solidified. Agar plates were incubated at 37 ^{0}C for 48 hours. Formed colonies were counted and recorded after the two day incubation. Colony counts of the zero contact, control and test specimens were compared for the antimicrobial activity assessment.

2.2.2.4 Assessment of the Antimicrobial Activity of Antimic-6000 Agent by Using AATCC 147-2004: Parallel Streak Method

For the further validation of the antimicrobial activity of the Antimic-6000, and to be able to test the activity on the corrugated cardboard material, we also employed the AATCC 147-2004 method. The main principle of the AATCC 147-2004 technique is to directly expose the antimicrobial surface to the relevant bacteria colonies which were inoculated on an agar media in the form of parallel streaks. The antimicrobial effect of the material is measured according to the inhibition of the bacterial growth surrounding the borders of the antimicrobial product sample. The method allows the usage of both gram negative and positive bacteria. *Escherichia coli* (gram negative) and *Staphylococcus aureus* (gram positive) cell cultures were used as the gram negative and gram positive bacteria which are involved in the spoilage process of many fruits and vegetables.

2.2.2.4.1 Preparation of the test specimen

The test specimens of polystyrene plates, polyethylene terephthalate containers, corrugated cardboard boxes that are coated with 5%, 2.5% and 1% Antimic-6000/IPA solution through either dip-coating and spraying methods; and stretch film produced with 0.5 % Antimic-6000 were cut in the 25 mm x 50 mm rectangular form along with the non-coated control samples from each material. Antimicrobial activity assessment through AATCC 147-2004 test was

conducted on the following materials: 1) stretch film that was produced with 0.5 % Antimic-6000 2) corrugated cardboard boxes and polystyrene plates that are coated with 5%, 2.5% and 1% Antimic-6000/IPA solution through spraying, and 3) polystyrene plates, polyethylene terephthalate containers that are coated with 5%, 2.5% and 1% Antimic-6000/IPA solution through dip-coating.

2.2.2.4.2 Preparation of the inoculums and incubating the samples on inoculated plates

Glycerol stocks of *E. coli* and *S. aureus* bacteria were first revived by streaking a loop full of bacteria on tryptic soy agar plates. Agar plates were incubated at 37 ^{0}C for 48 hours and pure strains of each bacterium were inoculated in tryptic soy broth media. Culture mediums were incubated in the orbital shaker at 37 ^{0}C and 150 rpm for 24 hours. Test inoculums were prepared by transferring 1 mL of 24 hour culture to 9 mL of sterile distilled water in test tubes and vortexing the resulted culture media dilution. A loop full of diluted cultures were inoculated on tryptic soy agar plates by making five streaks that are approximately 10 mm apart from each other across the Petri dish. Streaks were made without refilling the inoculating loop. The test specimens were placed on the inoculated plates as they will be positioned perpendicular to the culture media streaks, and gently pressed onto the agar by using a sterile spreader without disrupting the streaks. Inoculated plates and test specimens inside the sealed Petri dishes were incubated at 37 ^{0}C for 24 hours.

2.2.2.4.3 Assessment of the antimicrobial activity

The clear zone lengths that were lacking the microbial growth surrounding the borders of the test specimen were measured. The clear zones from each side of the specimen were measured and average zone of inhibition (W) was calculated according to the Formula 1 where D stands for the test specimen diameter, T stands for the total clear zone across the specimen in millimeters.

$$W = \frac{T - D}{2}$$

Formula 1: Calculation of the zone of inhibition according to the interrupted regions of bacterial growth

The sample was not included to the analysis if there is bacterial growth under the test specimen. Assessment of the antimicrobial activity was performed by comparing the average zone of inhibition lengths of Antimic-6000 coated samples with non-coated control samples.

2.2.3 Testing the effect of Antimic-6000 on the Shelf life of Fruits and Vegetables in various packaging units

Effectiveness of the Antimic-6000 coating in terms of preserving the stored fruit and vegetables and prolonging the shelf life was surveyed by the microbiological tests and physiological examinations carried out on the stored material. Storage times of the packages were determined according to the suggested storage life of the fruits/vegetables. The number of the fruit/vegetabel inside a packaging unit was adjusted as it will allow the food and package contact optimally. All of the tested packaging units and the stored fruits/vegetables are given in the Table 3.

Table 3: All packaging units and stored material combinations along with their Antimic-6000 dose used througt the study.

Packaging unit	Stored fruit/vegetable	Number of replicates	Test days and storege time	Antimic-6000 concentration
Corrugated cardboard box	Egg plant 10 ^{0}C	2	6, 15, 23, 34 - mic	500ppm, 230ppm, 50ppm
Corrugated cardboard box	Tomato 5 ^{0}C	2	Day 20 , 34-mic 10, 34 - phys	1%, 2.5% , 5%
Corrugated cardboard box	Zucchini 5 ^{0}C	3	28	5%, 10%
Polystyrene plate + stretch film	Banana 12 ^{0}C RT	12 6	28 14	1%, 2.5% , 5% 1%, 2.5% , 5%
Polystyrene plate + stretch film	Cucumber 12 ^{0}C RT	6 6	15 8-mic,30-phys	1%, 2.5% , 5% 1%, 2.5% , 5%

Polystyrene plate + stretch film	Lettuce 0 ^{0}C RT	6 6	25-mic 14-mic, 25-phy	1%, 2.5% , 5% 1%, 2.5% , 5%
Polystyrene plate + stretch film	Apple 0 ^{0}C	10	75 days	0.05% stretch No-Antimic600-stretch
Polyethylene Terephthalate container	Strawberry 2 ^{0}C	6	1-mic 5-mic-phys 10-mic-phys	Spray:1%, 2.5% , 5% Dip-coating: Spray:1%, 2.5% , 5%

Fruits and vegetables were provided from local shops and only the ones which do not have any noticeable bruises and rotten were included in the experiments. Previously prepared and characterized packaging material was used for the storage as replicated experiments along with the control sets. Fruits and vegetables were stored at their optimum storage temperature inside a fridge and microbiological test were performed on either the selected whole fruit/vegetable or the cross-sections from them. Microbial burden on these samples were rinsed off inside sampling bags with distilled sterile water or peptone water by agitating the bags in orbital shakers.

Three different types of agar plates were used for the detection of fungal and bacterial growth. Potato dextrose agar (PDA) growth media was selected as it allows the growth of a wide range of bacteria and fungi. A selective media for inhibiting the growth of the bacteria while allowing fungi, was prepared by the addition of streptomycin sulphade antibiotic on potato dextrose agar media (PDA-ss) as the antibiotic will block the prokaryotic protein synthesis by binding to the 30S subunit of the ribosome (Leclerc, Melancon, & Brakier-Gingras, 1991). 0.2 grams of streptomycin sulphade was dissolved in 10 mL of sterile distilled water and added to one liter of autoclaved PDA media through 0.22 μm syringe filters. Second selective media was prepared for detecting only the bacteria and inhibit saprophytic fungal growth by adding cycloheximide antibiotic to tryptic soy agar media as it hinders the eukaryotic protein synthesis (Cundliffe, Cannon, & Davies, 1974). 0.1 grams of cycloheximide was dissolved in 10 mL of sterile distilled water and added to one liter of autoclaved tryptic soy agar media through 0.22 μm syringe filters. All agar media were kept at +4 ^{0}C prior to their use.

Serial dilutions of the washing liquid were prepared and spread on PDA, PDA-ss and TSA-cyc plates. TSA plates were incubated at 37 ^{0}C for two days where PDA-ss plates were kept at room temperature for the fungal growth. As they will facilitate the growth of both bacteria and fungi, PDA-ss plates were kept at room temperature following the two days incubation at 37 ^{0}C. At the end of their incubation, colony formations on the agar plates were counted and recorded for the further analysis and comparison.

2.2.3.1 Statistical analysis of the test results

Statistical significance of the Antimic-6000 antimicrobial activity was tested on the results of microbiologic tests after fruit and vegetable storage. One way ANOVA test was performed in Microsoft Excel, for PDA, PDA-SS and TSA-cyc plate counts separately to test whether the sample groups have an overall significant difference. Least Significant Difference method (LSD) was performed as a post-hoc test when the p-value from the one way ANOVA test is lower than 0.05, to determine the groups that are significantly differed. t critical values were calculated by using Formula 2 and t-distribution table, where $t_{0.05}$ is the t value where α level is 0.05 and DFW is the degrees of freedom within groups in the one way ANOVA test result.

$$\text{t critical value} = \frac{t_{0.05}}{2\text{DFW}}$$

Formula 2: Calculation of the t critical value

After the calculation of the t critical values, LSD values between groups were calculated according to Formula 3, where; $LSD_{\text{A,B}}$= Least Significant Difference value, MSW= Mean Square Error Within Groups, n_a= number of items in the group A.

$$LSD_{\text{A,B}} = \frac{t_{0.05}}{2\text{DFW}} \sqrt{MSW(1/n_a + 1/n_b)}$$

Formula 3: Calculation of the LSD value

Absolute value of the difference between groups were calculated and compared with LSD values. Null hypothesis of our work is defined as “Antimic-6000 coating on the packaging material does not create a significance difference in terms of inhibiting the microbial growth on the stored fruits and vegetables”. The null hypothesis was rejected where the absolute value of the difference between groups is greater than the LSD value.

3. RESULTS AND DISCUSSION

3.1 Mechanical and Physical characteristics of the produced packaging material

Antimic-6000 sol-gel polymerization and the quality of the packaging material were assessed with mechanical and physical tests.

3.1.1 Tensile, Thickness and Optical Characterization of the Packaging Material

The stretch film material which was produced by the Rotopaş Corporation was subjected to tensile and optical transmittance tests for the comparison of the Antimic-6000 containing product with the commercial stretch films. Appearance of the stretch film material containing 0.05 % Antimic-6000 and standard commercial product is shown in the Figure 8. The addition of the Antimic resulted in the red-orange color formation in the stretch film.

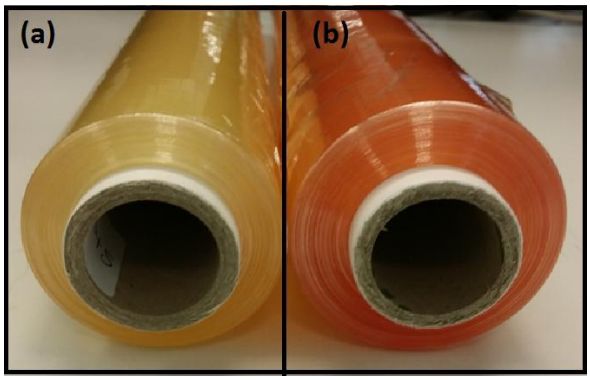


Figure 8: a: Standard commercial PVC stretch film **b**: PVC stretch film that contains 0.05 % Antimic.

3.1.1.1 Tensile and Elongation

Tensile test results (Table 4) of the standard and 0.05 % Antimic containing stretch films. According to the results, the addition of Antimic increased the tensile elongation 3.67 % and 25 % longitudinally and transversely respectively as a result of its reinforcing effect on the polymer matrix. However, elastic modulus of the stretch film was decreased by 25 % and 22.15 %. Longitudinal tensile strength of the material has decreased by 15.13 % while transverse tensile strength had increased by 56.69%.

Table 4: The results of the tensile and optical tests of the PVC stretch films

	Longitudinal			Transverse		
	Elastic-modulus	**Tensile strength**	**Tensile elongation**	**Elastic-modulus**	**Tensile strength**	**Tensile elongation**
Standard	0.084	2.7	122.5	0.0591	1.27	154
0.05% Antimic	0.0599	2.29	127	0.0460	1.99	192.5
improvement			3.67 %			25 %

3.1.1.2 Luminous transmittance, Haze and Clarity

PVC stretch films were also characterized for their optical properties. The effect of the Antimic addition on the haziness of the PVC film was measured. Haziness has been expressed as percent luminous transmittance and percent clarity as given in the Table 5.

Table 5: Haziness test results of the control and Antimic containing PVC film in terms of luminous transmittance and clarity.

	Haze	
	Transmittance	**Clarity**
Standard	88.7	99.5
0.05% Antimic	88.3	99.2

According to the values on the Table 5, Antimic addition in the PVC stretch film production has a slightly retarding effect on transmittance and clarity. Luminous transmittance has decreased by 0.45 % while clarity decreased by 0.3 %.

3.1.1.3 Thickness

The polyethylene terephthalate packaging material was used for the investigation of the effect of the Antimic coating and coating technique on the thickness. Surface profiles of the Antimic coated polyethylene terephthalate cross-sections prepared either through dip-coating and spraying method was measured with profilometer and also visualized with optical microscopy. Two of the highest Antimic-6000/IPA concentrations were chosen for the

measurements. Optical microscopy images of the samples produced via dip-coating and spraying are shown in the Figure 9 and Figure 10 respectively.

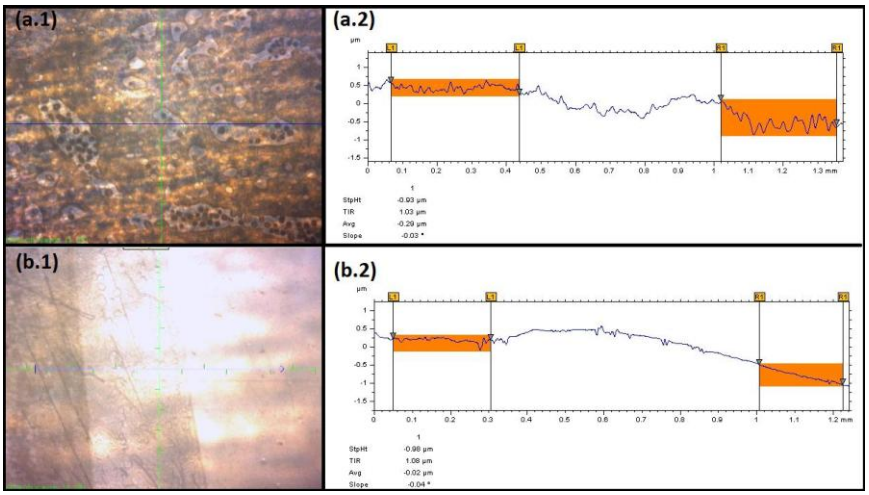


Figure 9: Optical microscopy images and surface height profiles of the polyethylene terephthalate cross-sections which are prepared with dip-coating method. a: 5% b: 10% Antimic-6000/IPA concentration.

Surface height profile of the samples which are produced by dip-coating method were measured by scanning from the coated surface to the non-coated area. The decrease in the height profile of the dip-coating samples of the both concentrations can be seen in the Figure 8.

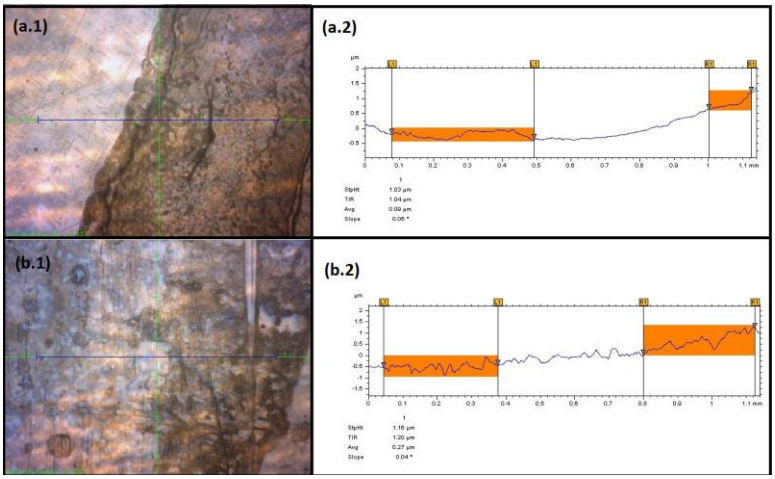


Figure 10: Optical microscopy images and surface height profiles of the polyethylene terephthalate cross-sections which are prepared by spraing method. a: 5% b: 10% Antimic-6000/IPA concentration.

Surface height profile of the samples which are produced by spraying method were measured by scanning from the non-coated area to the coated surface. The increase in the height profile of the dip-coating samples of the both concentrations can be seen in the Figure 9.
Tickness of the samples are shown in the Table 6.

Tablo 6: The thickness of the polyethylene terephthalate container cross-sections which are coated with Antimic-6000/IPA solution either through dip-coating or spraying.

	The thickness of the Antimic-6000 layer on the surface of the dip-coating samples (µm)	**The thickness of the Antimic-6000 layer on the surface of the spraying samples (µm)**
5%	0.93	1.03
10%	0.98	1.16

According to the surface thickness profile measurement results, the thickness of the polyethylene terephthalate has increased by 5.38 % in the dip-coating method and 12.6 % in the spraying method when the Antimic-6000/IPA concentration has doubled from 5% to 10%. While the concentration change did not make a drastic change in the surface thickness, coating method had a greater effect as samples which are prepared by spraying method were thicker. When the working concentration was 5%, spraying resulted in a 10.75 % greater thickness level than the dip-coating technique and this level was 18.37 % when the concentration has doubled. Therefore, profilometer results confirmed the presence of Antimic on packaging surface and as the concentration of Antimic increases, the thickness increases gradually.

3.1.1.4 FTIR

The Antimic-6000 sol-gel polymerization on the surface of the packaging materials was screened with the Fourier Transform Infrared Spectroscopy. The FTIR spectra of the polyethylene terephthalate container cross-section coated with 500 ppm Antimic-6000/IPA solution, the control sample and the Antimic-6000 itself are given in the Figure 11.

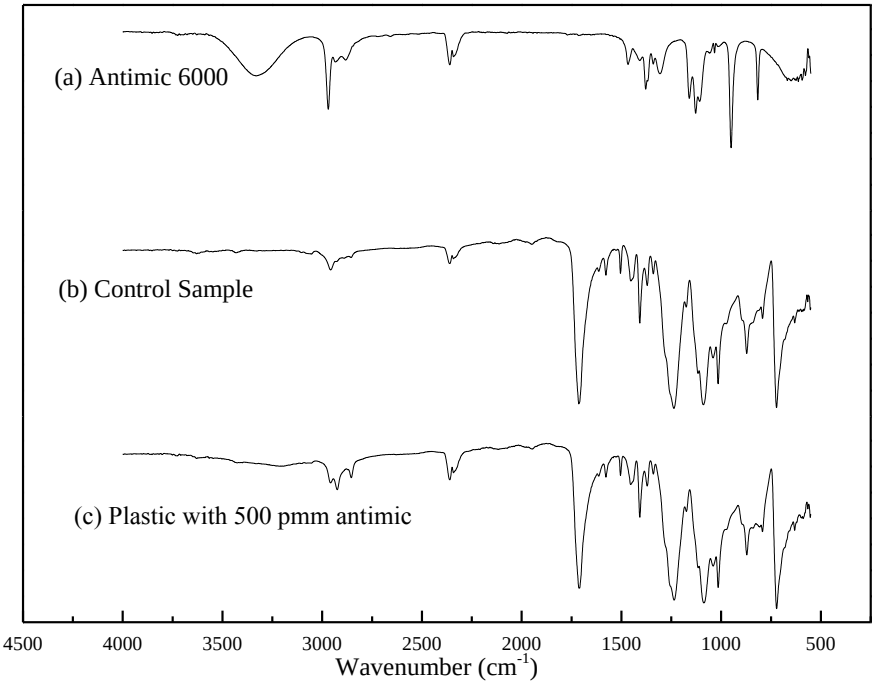


Figure 11: FTIR spectra of a: Antimic-6000, b: non-coated polyethylene terephthalate and c: polyethylene terephthalate coated with 500 ppm Antimic-6000/IPA solution.

Infrared absorptions of the functional groups in the Antimic-6000 sol-gel polymer were detected. Figure 10 a exhibits the FTIR spectra of Antimic-6000 with the characteristic vibrations of SiOC–H methoxy groups at 2970 cm^{-1} (Cetin, Ozmen, Tingaut, & Sebe, 2005) Si–O stretching at 955 cm^{-1} (Jang, Bae, & Kang, 2001) as well as strong absorption bands at 1160 and 1032 cm^{-1} for Si–O–Si stretching vibrations and the peak at 1463 cm^{-1} is attributed to C–H deformations of the methoxy groups at SiOC–H (Cetin et al., 2005) . Figure 11 b and c show the FTIR spectra of polymeric substrate before and after applying the antimic solution, respectively. As it is seen in Figure 11 c most of the antimic related spectra are overlapped with polymeric substrate but 2970 cm^{-1} peak clearly confirms the presence of Antimic on the substrate.

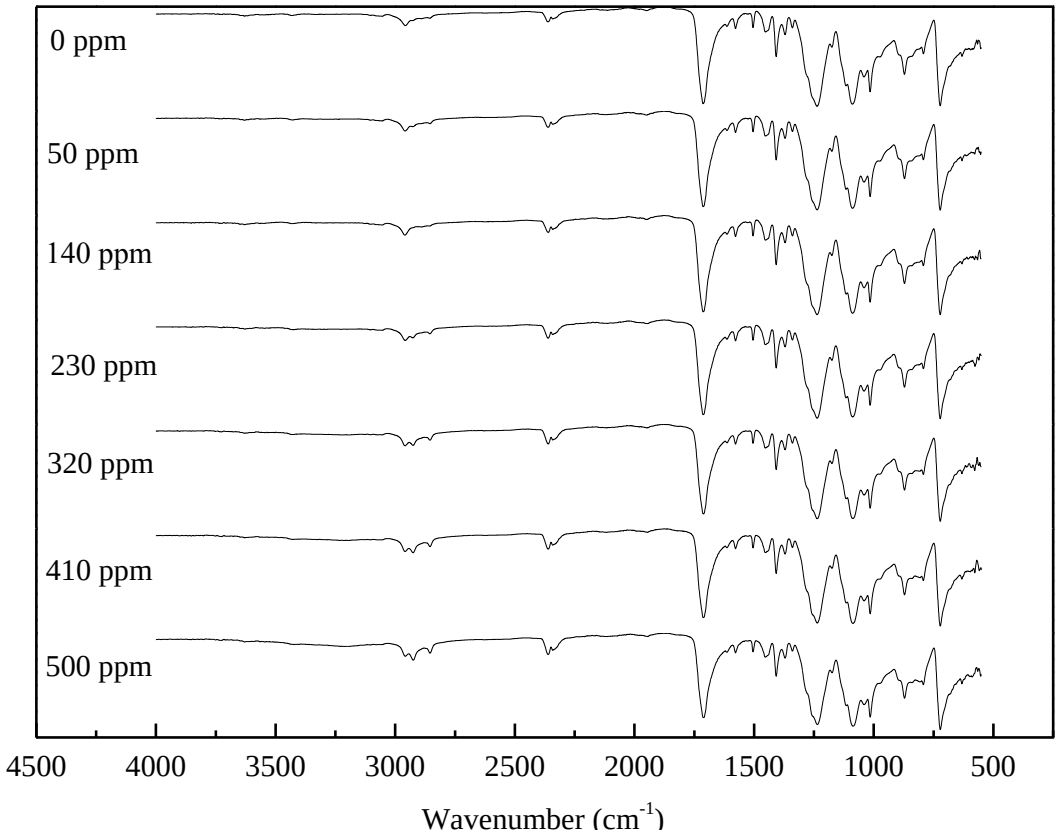


Figure 12: Polyethylene terephthalate with different Antimic-6000/IPA solution concentrations

For the investigation of the minimum Antimic-6000 polymerization formation, FTIR spectra on the polyethylene terephthalate cross-sections that are coated with the small incrementations of the Antimic-6000/IPA concentration were used. Figure 12 exhibits the polymeric substrate FTIR spectra at different antimic solution concentration on polymeric substrate and it is obviously seen that intensity of peak at 2970 cm^{-1} increased by increasing the concentration while other peaks are remain constant starting from the lowest concentration.

FTIR spectra of the corrugated cardboard box samples coated with 500 ppm Antimic-6000/IPA solution with spraying method along with Antimic-6000 and non-coated control samples are given in the Figure 12. Again, the FTIR spectra for the corrugated cardboard box samples that are coated with four different concentrations between 50 and 500 ppm along with their control sample are given in the Figure 13.

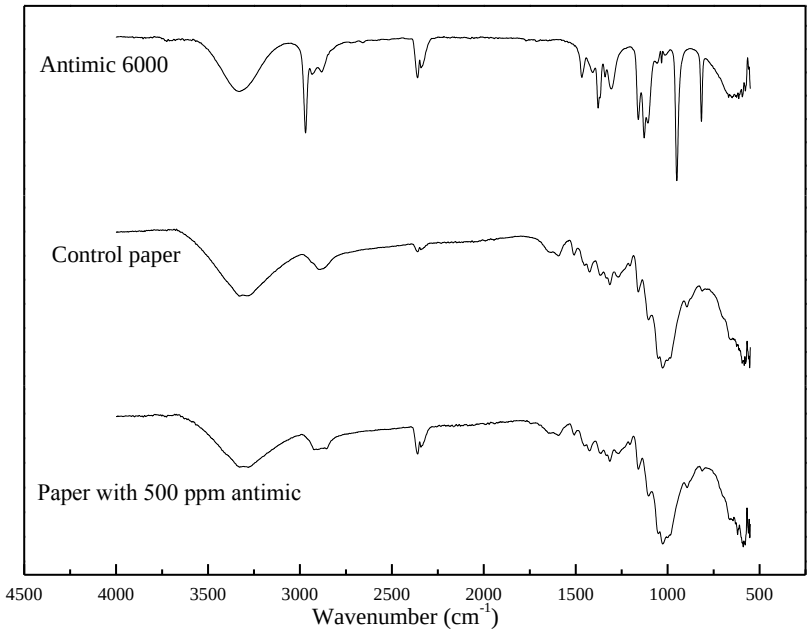


Figure 13: FTIR spectra of a: Antimic-6000, b: non-coated corrugated cardboard box and c: corrugated cardboard box coated with 500 ppm Antimic-6000/IPA solution.

Similar to previous section, peak at 2970 cm^{-1} identifies the presence of Antimic-6000 sol-gel polymerization on the substrate surface. It can be seen that intensity of peak at 2970 cm^{-1} increased by increasing the concentration while other peaks are remain constant (Figure 14).

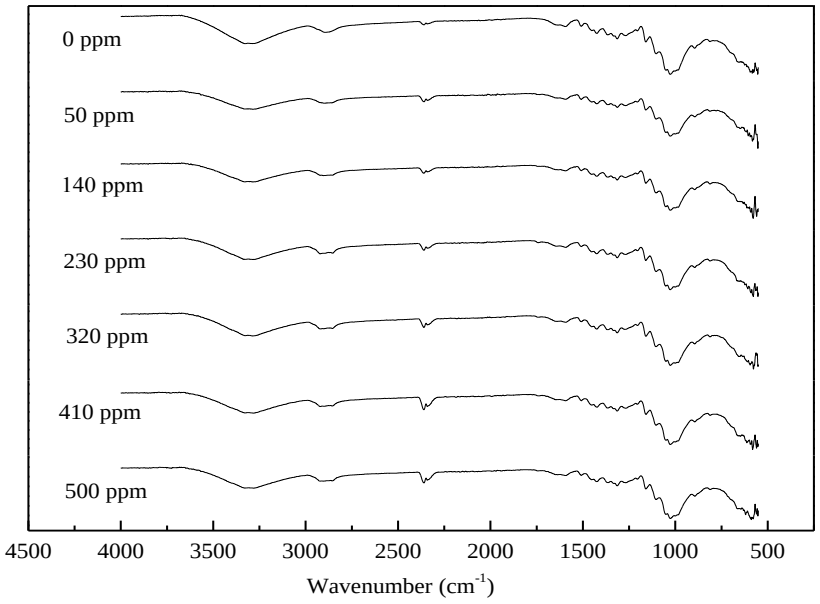


Figure 14: FTIR spectra of corrugated cardboard boxes with different Antimic-6000/IPA solution concentrations prepared by spraying.

The sol-gel polymerization was also demonstrated in the two highest concentrations; 5% and 10%, that have been used throughout the study by using corrugated cardboard boxes and spraying method Figure 15. This measuremenst also confirms the Antimic-6000 sol-gel polymerization on the surface of the material as can be seen from the 2970 cm^{-1} peak and the increasing intensity of it proportionally to the increased concentration.

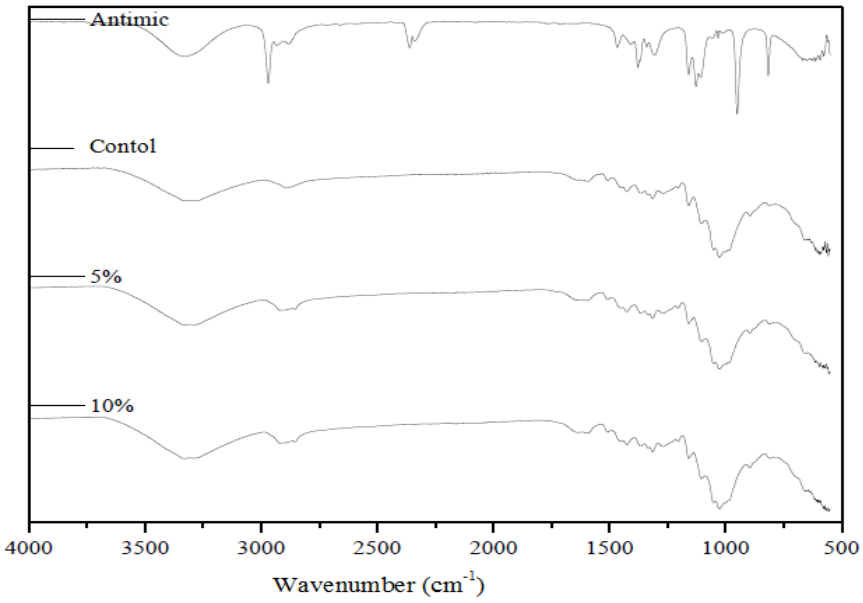


Figure 15: FTIR spectra of corrugated cardboard boxes with 5 and 10 % Antimic-600/IPA solution along with Antimic-6000 and control samples.

3.2 Assessment of the Antimicrobial Activity of Antimic-6000 on Packaging Material via Microbiologic Tests

3.2.1 Assessment of the Antimicrobial Activity of Antimic-6000 Agent by Using AATCC 147-2004: Parallel Streak Method

The parallel steak method was first described for the assessment of the antimicrobial activity of textiles with antimicrobial finishes. However utilization of this method to assess the antimicrobial activity of materials such as polyvinyl chloride (PVC) and polyurethane (PU) is also applicable and reported (Nichols, 2004). In addition to the plastic materials, we applied this technique on the cardboard material. The method relies on the measurement of the area surrounding the test material which the microbial growth is reduced if the microbial agent is migrating from the sample. However, the effectiveness is estimated by the inhibition of the bacterial growth only the surface of the sample which is in contact with the media with the bacterial incubation as a streak, when the agent is not released from the material.

This method had allowed the determination of whether Antimic-6000 is effective in terms of bacterial growth inhibition and gave the information about its adherence on the applied surface. Results of the parallel streak method applied on the Polyethylene terephthalate, polystyrene plate, corrugated cardboard box and PVC Stretch film are summarized in Table 7.

Table 7: The summary of the parallel streak test results.

	Polyethylene terephthalate			
	Dip-coating			
	Control	1%	2.5 %	5%
E. coli	0	0.5	0.15	0.1
S. aureus	0	0.7	0.9	1.15
	Polystyrene plate			
	Dip-coating			
	Control	1%	2.5 %	5%
E. coli	0	0.45	0.2	0.2
S. aureus	0	0.85	0.75	0.25
	Polystyrene plate			
	Spraying			
	Control	1%	2.5 %	5%
E. coli	0	0.65	0.1	0.4
S. aureus	0	0	0.65	0.05
	Corrugated cardboard box			
	Spraying			
	Control	1%	2.5 %	5%
E. coli	0	0.4	0.15	0.45
S. aureus	0	0	0.3	0.25
	PVC Stretch film			
	0.05% Antimic		Control	
E. coli	0		0.5	
S. aureus	0		0.15	

According to the results, non-of the control sample type has inhibited the bacterial growth except PVC stretch film. This result suggested that the used materials did not have an autogenous antimicrobial effect. In case of the polyethylene terephthalate container material, all sprayed concentrations of the Antimic-600/IPA solution was effective in terms of inhibiting the growth. However, the degree of inhibition did not followed by the increasing concentration of the antimicrobial agent when the test organism was *E. coli* and the 1% was identified as the most effective dose. However, when the testing bacteria was Gram-positive *S. aureus*, the increase in the antimicrobial agent concentration, had enhanced the effectiveness.

To see the effect of the coating method on the antimicrobial activity, the tests on the polystyrene plate material have been carried out on the specimens which were produced by i) dip-coating and ii) spraying method. Again, the test results of the dip-coating samples have highlighted 1% as the most effective concentration as the zone of inhibition has decreased by the increase in the dosage. However, the results have fluctuated when the coating method was spraying. Also the effectiveness was abolished in the 1% coating. The difference in the dosage based antimicrobial activity trends was thought to be caused by the potential uneven distribution of the antimicrobial agent on the surface in the spraying method. Comparison of the results of these two sub-groups favored the dip-coating method in terms of producing reliable results. In the cardboard experiments, all doses provided the inhibition of bacterial growth except 1% when *S. aureus* was used as the test organism. The concentration and antimicrobial effectiveness did not overlap. When stretch film material was tested, 0.05 % Antimic addition did not provide antimicrobial effect.

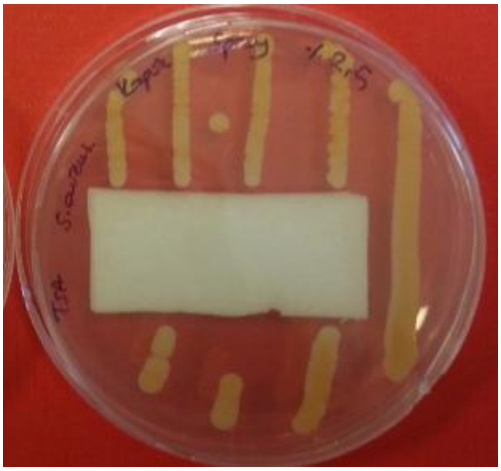


Figure 16: Agar plate showing the inhibition of the bacterial growth in the antimicrobial specimens' surrounding area.

Overall, parallel streak tests suggested the antimicrobial agent migration from the surface. The migration of the antimicrobial agent from the package surface is crucial for the effective preservation of the food by the antimicrobial agent (Lacoste, Schaich, Zumbrunnen, & Yam, 2005). While the antimicrobial agent is being released from the surface, the conservation of the antimicrobial agents' concentration over the minimum inhibitory concentration (MIC) is crucial for sustaining the activity in the long term (Corrales & Fernández, 2014). However, this mode of action applies to the antimicrobial agents which are designed as they will exert their biocidal activity on the microorganisms as they get released from the surface (Figure 3). According to Antimic-6000's proposed activity mechanism, it creates an antimicrobial

coating on the surface and inhibits the bacterial growth. This mechanism puts Antimic-6000 under § 201(q)(1)(B)(ii) of FFDCA according to FDA. However the test results suggested a second and effective mode of action for the Antimic agent as acting on the microorganisms by migrating from the surface.

3.2.2 Assessment of the Antimicrobial Activity of Antimic-6000 Agent by Using ISO 22196 Standard Test

ISO 22196 is a standard testing method for measuring the antibacterial activity of antimicrobial agents added to, or applied on the plastic materials. Again, because of the lack of test standards for the assessment of the antimicrobial activity on the paper material, we included the corrugated cardboard box material produced by the Antimic-6000 coating to ISO 22196 tests. Test results are presented in Table 8, 9 and 10 as bacterial colony counts.

Table 8: ISO 22196 test method colony count results summary of the polyethylene terephthalate container samples

Polyethylene terephthalate															
	Spraying														
	zc			tc			1%			2.5%			5%		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
10^{-2}	165	208	143	364	168	280	108	115	104	77	92	61	63	62	78
10^{-3}	12	25	13	28	17	12	14	34	16	10	24	5	12	5	9
10^{-2}-$\bar{x}$	172			271			109			77			68		
10^{-3}-$\bar{x}$	17			19			21			13			9		
	Dip-coating														
	zc			tc			1%			2.5%			5%		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
10^{-2}	165	208	143	364	168	280	123	23	132	124	112	135	71	94	107
10^{-3}	12	25	13	28	17	12	4	5	11	3	9	27	6	5	8
10^{-2}-$\bar{x}$	172			271			93			124			91		
10^{-3}-$\bar{x}$	17			19			7			13			6		

Table 9: ISO 22196 test method colony count results summary of the polystyrene plate samples

Polystyrene plate															
	Spraying														
	zc			tc			1%			2.5%			5%		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
10^{-2}	Over growth														
10^{-3}	245	289	197	372	224	312	252	172	178	128	264	184	100	266	184
10^{3}-$\bar{x}$	244			303			201			192			183		
	Dip-coating														
	zc			tc			1%			2.5%			5%		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
10^{-2}	Over growth														
10^{-3}	245	289	197	372	224	312	264	269	280	280	276	264	104	236	200
10^{-3}-$\bar{x}$	244			303			280			273			180		

Table 10: ISO 22196 test method colony count results summary of the corrugated cardboard box samples

Corrugated cardboard box															
	Spraying														
	zc			tc			1%			2.5%			5%		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
10^{-2}	Over growth						228	148	364	252	212	188	208	156	292
10^{-3}	105	201	142	53	144	280	45	35	52	43	39	46	27	37	44
10^{3}-$\bar{x}$	149			159			44			43			36		

Table 11: ISO 22196 test method colony count results summary of the PVC stretch film samples

PVC stretch film									
	zc			tc			0.05 % Antimic-6000		
	1	2	3	1	2	3	1	2	3
10^{-2}	289	301	252	384	304	300	332	204	176
10^{-3}	46	15	28	65	32	37	42	30	19
10^{-2}-$\bar{x}$	282			329			237		
10^{-3}-$\bar{x}$	30			45			30		

According to the number of colonies counted from the zero contact time control samples and the control samples that were incubated with the experimental samples, none of the four packaging material showed autogenous antibacterial activity. The bacterial growth continued through the incubation time in the "tc" control samples. Both spraying and dip-coating methods provided antimicrobial protection for the samples from the polyethylene terephthalate container material. The results of the spraying samples were more stable according to the increase in the concentration. Likewise, results of the polystyrene plate samples also showed antimicrobial effectiveness of the Antimic-6000 and exhibited the better performance yield when the spraying method is used. The number of colonies in the zero contact time controls were higher than the "tc" controls in the corrugated cardboard material, and experimental samples which were coated with Antimic-6000 had a 25 % decrease in bacterial growth in comparison to the controls. The effectiveness of the stretch film material was much lower than the other materials.

3.2.3 Statistical analysis of the microbiology tests on raw fruits and vegetables

After the physical characterizations and antimicrobial activity assessments, Antimic-6000 activity has been examined for its impact on the preservation of raw fruits and vegetables. Microbiological tests have been carried out on the fruits/vegetables that were stored inside different packaging materials with varying Antimic-6000/IPA concentrations along with the control samples (Table 2). Statistical analyses could not be carried out on some of the test groups' results either because of their infufficient number of replicates or the improper plate

count results. The test groups, which we were able to analyze statistically, and their corresponding p-values are given in the Table 12.

Table 12: The p-values of the test groups according to the one way ANOVA test.

	PDA	PDA-ss	TSA-cyc
Lettuce	0.006786	0.000104	0.003439
Cucumber	1.82E-05	5.3E-05	0.00074871
Banana	0.563783	0.00485	0.296451
Apple	0.95359	0.89898	1
Lettuce	0.71191	0.55827	0.39506
Cucumber	0.83975	0.5208	0.8141
Apple	0.53415	0.95813	0.86265
Strawberry-day1	0.738169	0.884135	0.843813
Strawberry-day4	0.000293	0.000352	0.000226
Strawberry-day8	0.998834	0.985211	0.046707

According to the selected alpha level (0.05), p-values of the test groups that have a statistically significant variation are represented as underlined in the Table 12. In Lettuce, Cucumber and Strawberry day-4 tests, the colony counts of each agar media yielded significant variation whileonly the PDA-ss counts and TSA-cyc counts resembled variation in Banana and Strawberry-day8 test respectively. In these groups, the pre-defined null-hypothesis: "Antimic-6000 coating on the packaging material does not create a significance difference in terms of inhibiting the microbial growth on the stored fruits and vegetables" was rejected. For the interpretation of the variation, Fishers Least Significant Difference test have been carried out. Calculated LSD values are represented in the Table 13.

Table 13: LSD values of the test groups with the sitatistically significant variation between groups

	PDA	PDA-ss	TSA-cyc
Lettuce	**55.415**	**39.609**	**51.469**
Cucumber	**49.298**	**51.268**	**51.481**
Banana	**-----**	**15.226**	**-----**
Strawberry-day4	**0.091**	**0.082**	**0.107**
Strawberry-day8	**-----**	**-----**	**0.324**

To be able to determine the antimicrobial effectiveness of Animic and its best working dosage in different fruit/vegetable-packaging systems, the absolute values of the difference between groups have been compared with the LSD number.

All of the Antimic dosages have provided protecting effect in comparison to the control packages for Lettuce. Antimic have acted on both the bacterial and fungal microorganisms in this group as the PDS-ss and TSA-cyc plate counts have concluded the inhibition in the microbial growth. However, the increasing Antimic dosage did not create a difference in the protective effect; suggesting that the lowest (1%) test concentration or lower doses are sufficient for the effectiveness.

Curiously, cucumber test results have ascribed a negative effect to the Antimic coating for the protection of the ftuit. Packages with the highest antimic dosage were the ones that have the most microbial growth. This could be originated by the high microbial burden or bruises on the starting material which cannot be detected by human eye and causing unwqual starting conditions for the different packages. Another possible reason could be an undefined negative effect of Antimic on the outher surface of the fruit, which facilitate the biodeterioration process.

The Banana results have suggested the protective effect of the Antimic on the fungal microorganisms on the fruit surface. In this group, 1% and 2.5 % Antimic coating have provided better protection than the control samples. However increasing the dosage to 5% did not make a significant difference.

The PDA counts in the Strawberry-day4 tests revealed the 1% Antimic concentration is the most effective dose for strawberry preservation as the 2.5% and 5% packages did not enhaced

the antimcirobial activity. However all of the doses resulted in the better protection in comparison to the control samples. According to the PDS plate counts of 2.5% packages, spraying and dip-coating methods did not differ in the resulted degree of protection. The same trand was seen in the TSA-cyc counts of 2.5% and 1% packages. Furthermore the 5% packages had a decreased protection in comparison to 2.5 and 1%. Strawberry-day8 results also did not favoured dosage increment in increasing antimicrobial protection according to PDA-ss and PDA plate counts. This suggested that Antimic has failed to act on the fungal pathogens on the fruit surface. Althought, in both Strawberry-day4 and –day8 tests, bacterial growth have been inhibited in 5% and 2.5% dosages while 1% coating did not provide protection.

4. CONCLUSION

Inefficient preservation of fresh fruits and vegetables after their harvest results in a high percentage of spoilage and consequently: economic loss. In addition, consumption of these poorly preserved foods causes a considerable amount of diarrheal and digestive system diseases in addition to food poisoning. Even though current antimicrobial packaging systems and strict regulations are providing a degree of improvement; the need for novel, environment-friendly and effective food packaging systems still continues. Quaternary ammonium compounds are being used as one of the promising nanotechnology based solutions in the food packaging systems for the reduction of microbial growth. In this study, we have assessed the antimicrobial effectiveness of a novel quaternary ammonium compound: Antimic, when used as a coating material in post-harvest food packaging systems. We have confirmed its unique polymerization that is required for the antimicrobial activity, on the surface of the applied packaging materials. Furthermore, the effect of the Antimic addition on the physical and mechanical characteristics of the material was investigated. After the physical characterization, materials were subjected to standard tests for the assessment of antimicrobial activity. We demonstrated the inhibitory effect of Antimic on selected bacteria. Finally, Antimic coated packaging units were tested for their effect on the shelf life of different raw fruit and vegetable types. The microbial growth on the stored fruits and vegetables was comparatively assessed for the Antimic-coated and control packages. The results have proven the protective effect of Anitmic in several fruit/vegetable-package units. However, the diversity in the optimum protective dosage of Antimic in different packaging units suggested that each food type must be tested before being stored with Antimic or other

quarternery ammonium compounds since their action and target microorganism spectrum may resemble different profiles in different food material.

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APPENDIX-I A

Enzymes, Chemicals and Molecular Biology Kits

6X DNA Loading dye	Thermo Scientific	R0611
Absolute ethanol	Riedel de Haen	32221
Agarose	PRONA	8016
Boric acid	Sigma	B6768
Calcium chloride ($CaCl_2$)	Sigma	B6768
Calcium nitrate ($Ca(NO_3)_2$)	Sigma-Aldrich	237124
Chloroform	Merck	102.445
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich	40718
Dioxyribonuclease I (DNase I)	Thermo Scientific	EN0525
dNTP Mix	Thermo Scientific	R0193
EDTA iron (III) sodium salt	Sigma-Aldrich	E6760
Ethidium bromide	Applichem	A1151
Ethylenediaminetatraaceticacid (EDTA)	Calbiochem	324503
GeneRuler 100 bp DNA Ladder	Thermo Scientific	SM0241
GeneRuler DNA Ladder Mix	Thermo Scientific	SM0332
Isopropanol	Merck	1.09634
Magnesium chloride ($MgCl_2$)	Fluka	63063
Nuclease free water	Qiagen	129114
Potassium phosphate monobasic (KH_2PO_4)	Sigma-Aldrich	P0662
Potassium sulfate (K_2SO_4)	Sigma-Aldrich	P0772

RevertAid Reverse Transcriptase	Thermo Scientific	EP0441
RiboLock RNase Inhibitor	Thermo Scientific	EO0381
TRI Reagent	Sigma	T9424
Trisbase	Sigma	T1503
Zinc sulfate ($ZnSO_4$)	Sigma-Aldrich	96495
Murashige and Skoog with vitamins	Cassion Labs	A00240
Potassium chloride (KCl)	Sigma	12636
Magnesium sulfate (MgSO4)	PhytoTech.Laboratories	m150
Sodiun hydroxide	Merck	106462
Manganese (II) sulfate monohydrate	Merck	1.05941.0250
Copper (II) sulfate pentahydrate	Merck	1.02790.0250
2,4-Dichlorophenoxyacetic acid	Sigma-Aldrich	D7299
RNase-ExitusPlus	AppliChem	A7153.0250
PerfeCTa SYBR® Green SuperMix	Quanta Biosciences	95053-100
Plant agar	Duchefa Biochemie	P1001.1000
Sucrose	Cassion Labs	S011-1KG
MES monohydrate	PhytoTech. Laboratories	A00233

APPENDIX-I 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 ^{o}C Bosch, TURKEY
	-80 ^{o}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
Heating block:	HDV Life Sciences, AUSTRIA
	Thermostat Bio TDB-100, LATVIA
Hydroponic tanks:	GroWell, UK
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:	Bosh, 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
	Roche Life Sciences, USA
Refrigerator:	+4 ^{o}C 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:	Nanodrop, ND-1000, USA
Sterilizer:	Steri 350, Simon Keller Ltd., SWITZERLAND
Thermocyler:	Eppendorf, Mastercycler Gradient, GERMANY
Tissue Lyser:	Qiagen Retsch, USA
Vortex Mixer:	VELP Scientifica 2X3, ITALY
Water bath:	Memmert, GERMANY

APPENDIX-I C

List of mature miRNA Primers

universal_R(60.6)	5' CAG TGC AGG GTC CGA GGT A 3'
tdic_zavi_miR156*909_F	5' CTC GGC TCA CTG CTC TTC CT 3'
tdic_zavi_miR156*909_RT	5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC GATGAC
tdic_zavi_miR160*189_F	5' TCA GCG TGC AAG GAG CCA 3'
tdic_zavi_miR160*189_RT	5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC CATGCT
tdic_zavi_miR164*47_F	5' CGG GAT GGA GAA GCA GGG 3'
tdic_zavi_miR164*47_RT	5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC TGCACG
tdic_zavi_miR166*773_F	5' GAC CTC TCG GAC CAG GCT T 3'
tdic_zavi_miR166*773_RT	5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC GGAATG
tdic_zavi_miR1878*924_F	5' GCG GGA TTT GTA GTG TTC GGA T 3'
tdic_zavi_miR1878*924_RT	5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC AAACTC
tdic_zavi_miR2275*348_F	5' GCC GGG TTT GGT TTC CTC C 3'
tdic_zavi_miR2275*348_F	5' GCC GGG TTT GGT TTC CTC C 3'
tdic_zavi_miR2275*348_RT	5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC TAAGAT
tdic_zavi_miR2275*348_RT	5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC TAAGAT
tdic_zavi_miR393*90_F	5' CGG ACT CCA AAG GGA TCG CAT 3'
tdic_zavi_miR393*90_RT	5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC GGATCA
tdic_zavi_miR395*106_F	5' GGC GAG TGA AGT GTT TGG GG 3'
tdic_zavi_miR395*106_RT	5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC GAGTTC
tdic_zavi_miR397*20228_F	5' GAT AAT CAC CGG CGC TGC AC 3'
tdic_zavi_miR397*20228_RT	5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA

	TTC GCA CTG GAT ACG AC CATTGT
tdic_zavi_miR398*403_F	5' GCG TGG TGT GTT CTC AGG TC 3'
tdic_zavi_miR398*403_RT	5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC CGGGGG
tdic_zavi_miR399*160_F	5' GCG AGT GCC AAA GGA GAG TT 3'
tdic_zavi_miR399*160_RT	5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC CAGGGC
tdic_zavi_miR5062*441_F	5' GGG CGT GAA CCT TAG GGA AC 3'
tdic_zavi_miR5062*441_RT	5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC ATGCGG
tdic_zavi_miR9673*22370_F	5' GGG AGC GGT AAG AAG CAA ATA G 3'
tdic_zavi_miR9673*22370_RT	5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC CATGTG
tdic_zavi_miR9772*673_F	5' GGG AGC GTG AGA TGA GAT TAC C 3'
tdic_zavi_miR9772*673_RT	5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC GTATGG
tdic_zavi_miR9778*22436_F	5' CCC CGT GCA TCA TCT CGA AC 3'
tdic_zavi_miR9778*22436_RT	5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC CGACGA

APPENDIX-I D

List of pre-miRNAs with in silico expression evidence

miRNA name	Subject ID	Mature miRNA seq.	Pre-miRNA seq
miR5062*440	Ku_c80400	UGAACCUUGGGGAAAAGCCGCAU	AAAAGGTTCACATAGCTTCTTGAACCTTGGGGAAAAGCCGCATAACACCATTAACTTGAAAACCATGTATTTCCTATCCCAAAACTGCTCCTAGCACAAGGGGTAGAAGATGGTTTTTAGTTGTGTGGTCATGCGGATTTTTCACCAAGATTCAAGTGGTTATCTGGATCTTTTGA
miR5062*442	Ku_c85469	UGAACCUUAGGGAACAGCCGCAU	GTAAGGTTCACGTAGCTTCTTGAACCTTAGGGAACAGCCGCATAACACCATTAACTTGAAAACCACTTACCTTATCCCAAAACTGCTCCTACCACAATATAAGGGGTAAGGAATGGTTTTTGGTTTGTGAGGTCATGCGGATTTTTCACCAAGGTTCAAGTGGTTATCTGGATCTTTTGA
miR9674*634	Ku_c66069	AUAGCAUCAUCCAUCCUACCA	TCCTCTGGTTGAATTTGTCCATAGCATCATCCATCCTACCATTCAATGGTGGCTGATGCTGCCCAACGGTGGGTAGGTGGCTGGTGCTATGGATAGCTTCAACCCGATGTTG
miR2118*309	Ra_c19387	UUCCUGAUGCCUCCCAUUCCUA	GAGTGAGCTCAAGAGCACTGGGAATGGGAACATGGAGGAAAGCCCTGCTTGTTTGTTTGCACCTGCAGTGTTGGAGGTGCAAATATTGGGCATGGATCCTTTCCTGATGCCTCCCATTCCTACCGTTCTTGTGCACTTCTTC
miR5079*7350	Ra_c218	UUUGGAUUUGUUAUUUUGGUAU	TATTCGCAATTTATTTACATACTAGAACAAATCCAAATTGGAAGGGTACGAATTCTGCACTTATAGCTTCGATAGGATTTCTTATAATTTGGATTTGTTATTTTGGTATAAATCTATTAGGAATAGGCT
miR167*24	Ku_c81432	UGAAGCUGCCAGCAUGAUCUA	GTCATGCCCATGTGAATGAGTGAAGCTGCCAGCATGATCTATCTTTGATTCGCTTCTGCGTGTGAATCCTTGTTAGATCATGACTGACAGCCTCATTTCTTCCCAGCAATGGGCATA
miR2118*244	Ra_c19387	GGGAAUGGGAACAUGGAGGAA	ATAGAGGGCCTTAGAGTAGTGGGAATGGGAACATGGAGGAAAGCCTAACTTGCTCGGTTTTCACCTCCAGTGTTGGAGGTGAGAATTTAGTTTGGTGTTTCCCAATGCCTCCCATTCCTATTGTTCTAAAGCACTCCTTCC
miR2118*263	Ra_c39275	GGGAAUGGGAACAUGGAGGAA	AAAGAGGGCATAAGAGCAGTGGGAATGGGAACATGGAGGAAAGCCTAAGTTGCTTTGTTTTCACCTCTAGTGTTGGAGGTGAGAACTTGCTTTGGTGTTTCCCGATGCCTCCCATTCCTATTGTTCTAAAGCACTCCTTCC
miR167*25	Ra_c98389	UGAAGCUGCCAGCAUGAUCUG	CAAGGTGCACCACGAGCTGGTGAAGCTGCCAGCATGATCTGATGACCTAACTCATGGATCAGAGTCCATGTCAATCAGGTCATGCTGGAGTTTCATCTGCTGGTCGGAGCACCACGA
miR9674*661	Ku_c66069	GGUGCUAUGGAUAGCUUCAAC	AGAACTGAAAATCCTCTGGTTGAATTTGTCCATAGCATCATCCATCCTACCATTCAATGGTGGCTGATGCTGCCCAACGGTGGGTAGGTGGCTGGTGCTATGGATAGCTTCAACCCGATGTTGGTGACATACCC
miR530*7377	JD_c14615	GCUGCAUUUGCACCUGCACCU	AAGAAGAATGGCGTGATATGGCTGCATTTGCACCTGCACCTGCGACGAAGAACAAAGGAAACGCTTTTTGTCCTCCTCAAGTTGCTAGGTGCAGTGGCACATGCAGCCGTATCACATGAATTTTCTGAA
miR2118*310	Ra_c19387	UUCCCAAUGCCUCCCAUUCCUA	TAGAGGGCCTTAGAGTAGTGGGAATGGGAACATGGAGGAAAGCCTAACTTGCTCGGTTTTCACCTCCAGTGTTGGAGGTGAGAATTTAGTTTGGTGTTTCCCAATGCCTCCCATTCCTATTGTTCTAAAGCACTCCTTC
miR160*186	Ex_rep_c87015	GCGUGCACGGAUCCAAGCAUA	GGATATGATATGATATGATGTGCCTGGCTCCCTGTATGCCACTCATCCAGAGCAACACCTTTTGCAATAAGGTTGCCTGCGATGGATGGCGTGCACGGATCCAAGCATATCGAACCCTCTCCCTCTCCC

miR530*1760	JD_c3045	UGCAUUUGCACCUGCACCUAC	AGAATTTGGCGTGATATGGCTGCATTTGCACCTGCACCTACGACGAAGAACAAAGGAAACGCCTTTTGTCATCCTCAAGTTGCTAGGTGCAGTGGCACATGCAGCCGTATCACATGAATTCTCTG
miR530*7376	JD_c3045	GCUGCAUUUGCACCUGCACCU	GAAGAATTTGGCGTGATATGGCTGCATTTGCACCTGCACCTACGACGAAGAACAAAGGAAACGCCTTTTGTCATCCTCAAGTTGCTAGGTGCAGTGGCACATGCAGCCGTATCACATGAATTCTCTGAA
miR160*189	Ra_c94450	GCGUGCAAGGAGCCAAGCAUG	ATCAGCTCGTCGTGCGTGCGTGCCTGGCTCCCTGTATGCCACTCATGTAGCCCAACCCGCGGCGTGATTGGATGCTGTGGGTGGCGTGCAAGGAGCCAAGCATGCGTACATACATGCCTCCCTC
miR2118*294	Ra_c39275	UUCCCGAUGCCUCCCAUUCCUA	AAGAGGGCATAAGAGCAGTGGGAATGGGAACATGGAGGAAAGCCTAAGTTGCTTTGTTTTCACCTCTAGTGTTGGAGGTGAGAACTTGCTTTGGTGTTTCCCGATGCCTCCCATTCCTATTGTTCTAAAGCACTCCTTC
miR398*403	Ku_c29821	UGUGUUCUCAGGUCGCCCCCG	TACGCGAGGAAATTCCTGCGGGGTCGAACTGGGAACACATGGGATACGAACCGCTTGATTGACGAGCGACTCTTATCAACTCATGTGTTCTCAGGTCGCCCCCGCTGGAGCCTCCTCGCCGCAG
miR530*7375	JD_c3045	GCUGCAUUUGCACCUGCACCU	AGCAAGGGTTATGTGATAGAGCTGCATTTGCACCTGCACCTAGAGAGGAAGAAGAAAGGTCGAAGGCTTGGAACTTGGCCATGCATGCGATTGCGATGCATGCAACGTTCTCCTTGACCCCTCGCTCTTCCTGTAGGTGCAGTGGCATATGCAACTCTATCACAGGCATCCTAGGTA
miR530*21091	JD_c14615	UGCAGUGGCAUAUGCAACUCU	AGCAAAGGGTTATGGGATAGAGCTGCATTTGCACCTGCACCTAGAGAGGAAGAAAGGTCGAGGGCTTGGAACTTGGCCATGCATGCGATTGCGATGCATGCAACGTTCTCCTTGACCCTCTCGCTCTTCCTGTAGGTGCAGTGGCATATGCAACTCTATCACAGGCATCCTAGGTAC
miR530*7378	JD_c14615	GCUGCAUUUGCACCUGCACCU	GCAAAGGGTTATGGGATAGAGCTGCATTTGCACCTGCACCTAGAGAGGAAGAAAGGTCGAGGGCTTGGAACTTGGCCATGCATGCGATTGCGATGCATGCAACGTTCTCCTTGACCCTCTCGCTCTTCCTGTAGGTGCAGTGGCATATGCAACTCTATCACAGGCATCCTAGGTA
miR530*21090	JD_c3045	UGCAGUGGCAUAUGCAACUCU	CAGCAAGGGTTATGTGATAGAGCTGCATTTGCACCTGCACCTAGAGAGGAAGAAGAAAGGTCGAAGGCTTGGAACTTGGCCATGCATGCGATTGCGATGCATGCAACGTTCTCCTTGACCCCTCGCTCTTCCTGTAGGTGCAGTGGCATATGCAACTCTATCACAGGCATCCTAGGTAC
miR9674*663	Ku_c100499	GGUGCUAUGGAUAAAUUCAAC	GAGGGAGCCAGTTATCCGGTTTAATTTGTCCATAGCATCCGCGATCCCGCCATTCAGCTGTGATAGCTGCTGCCCTATTGCACGTTGCGGAAGATAGCTGGTGCTATGGATAAATTCAACCCGATGGTTGTGCATGCCCG
miR2118*243	Ra_c19387	GGGAAUGGGAACAUGGAGGAA	AGAGTGAGCTCAAGAGCACTGGGAATGGGAACATGGAGGAAAGCCCTGCTTGTTTGTTTGCACCTGCAGTGTTGGAGGTGCAAATATTGGGCATGGATCCTTTCCTGATGCCTCCCATTCCTACCGTTCTTGTGCACTTC
miR395*106	contig69372	GUGAAGUGUUUGGGGGAACUC	GCATTCTTTCAAATGCCATTGTGAAGTGTTTGGGGGAACTCTTGCTGATGCCAAACTTCATTCAATGCCAGTCAAGAAACAAAGGAGACGGTCATCACAAAGCATGTTTGGTCTTACCATGGGTTCGCTGCAAGCACTTCACGAGGCATTATTAGAAATGTCAT
miR169*80	contig79327	UAGCCAAGGAUGACUUGCCUA	TGCAAGGGTCTTACCTCTGATAGCCAAGGATGACTTGCCTATGTCTTCATCCATGTTCAATGCTTCATTAGCCTTGGCGTGGGTTCTCATGGGCAGTCGCCTTGGCTAGCCTGAGTGGCTCTTGCT
miR169*75	contig74010	UAGCCAAGGAUGACUUGCCUA	TGCAAGGGCCTTATCTCTGATAGCCAAGGATGACTTGCCTATGTCTTGTTCCTCCCTCAAGGCTTAATTGGCCTTGGGGTGTGGTTTCATGGGCAGTCTCCTTGGCTAGCCTGAGTGGCTCTTGCC
miR169*72	contig79327	UAGCCAAGGAUGACUUGCCUA	TGCAAGGGCCTTACCTCTGATAGCCAAGGATGACTTGCCTATGTCTTCATCCATGTTCAATGCTTCATTAGCCTTGGCGTGGGTTCTCATGGGCAGTCACCTTGGCTAGCCTGAGTGGCTCTTGCT
miR169*7269	contig74010	UAGCCAAGGAGACUGCCCAUG	GCGGCAAGAGCCACTCAGGCTAGCCAAGGAGACTGCCCATGAAACCACACCCCAAGGCCAATTAAGCCTTGAGGGAGGAACAAGACATAGGCAAGTCATCCTTGGCTATCAGAGATAAGGCCCTTGCAG

miR1878*924	contig00397	AUUUGUAGUGUUCGGAUUGAGUUU	CTTACAAACCACTTTTGCAAACTTAGTCTTGGCACTATAAATTTAAAATGTGCATGTGAAAATATGCTCATTATAAATTTGTAGTGTTCGGATTGAGTTTATAATAGTCGTTCTAAGATT
miR6246*7369	CL36112Contig1	GUGGGGAUUUCCUGCCGGAGGGA	CGGCATTGGAGACCCTCGGCGTGGGGATTTCCTGCCGGAGGGAGCTTTGCGGACGAAAAAGCTGCCTTCGGGAGGAACTCTTCCAGCCCCGAGGGTTTGGTCTTGTGTG
miR5200*606	gi|22547791|gb|BU099992.1|BU099992	UGUAGAUACUCCCUAAGGCUU	AAGCTTTAAGCACTAATTAAGCCTTAGTGAATATCTACACTAGTAAGTTTTTCTTCATGTTTGAAATGCTTAGCATTGTAGATACTCCCTAAGGCTTGGGTGGTATTGCGTTGTGTG
miR395*114	gi|39556435|gb|CK194045.1|CK194045	AUGAAGUGUUUGGGGGAACUC	CATGTTTGGTCTTACCATGGGTTCCTTGCAAGCACTTCATGAGGCATTATTTGAAATGCCACTATGAAGTGTTTGGGGGAACTCTTGGTGATGCCAAGCTTTAT
miR169*73	gi|383556880|gb|HX098125.1|HX098125	UAGCCAAGGAUGACUUGCCUG	GCGACGAGGGCTTCTTCTGGTAGCCAAGGATGACTTGCCTGTGGATATATAGCTTGATCGATTGCAGTTACATCTCTGCATGTATATATGTGATCTCTCACTATGTACAACAGGCAGTCTCCTTGGCTAGCCCGAGTGGCCCTCATCCTTCA
miR399*160	gi|93057089|gb|CJ667854.1|CJ667854	UGCCAAAGGAGAGUUGCCCUG	AATTAAGCAATCCAGTTTCAGGGCTCCTCTTTATTGGCAGGGAGCGTGTGAGGCCATGTAGCTCCATTCAGCGCTCTGCCAAAGGAGAGTTGCCCTGTAACTGGAAATAGCTTAAGG
miR2275*348	gi|25149453|gb|CA597098.1|CA597098	UUUGGUUUCCUCCAAUAUCUUA	AATTTTGGCAGATGAATTTGAGTGTTGGATGGGACCAAATCTTACCGCCGGGTGTGGCAAGATTTGGTTTCCTCCAATATCTTATGTTCATATGTCAGACACTG
miR5200*609	gi|22547791|gb|BU099992.1|BU099992	AAGCCUUAGUGAAUAUCUACA	CAAAGCTTTAAGCACTAATTAAGCCTTAGTGAATATCTACACTAGTAAGTTTTTCTTCATGTTTGAAATGCTTAGCATTGTAGATACTCCCTAAGGCTTGGGTGGTATTGCGTTGTG
miR2275*349	gi|25149453|gb|CA597098.1|CA597098	UUUGGUUUCCUCCAAUAUCUCG	AACCTCGCAAGCTGAATGTGAGATTTGGATGGAACCAAATCTTCAGCTATAGTGGCACAAGATTTGGTTTCCTCCAATATCTCGTCTTCAACTGTCCCTGATGA
miR9678*22376	gi|20312117|gb|BQ167596.1|BQ167596	UCUGGCGAGGGACAUACACUGU	ATTGATTCGTCGTAGGATCATCTGGCGAGGGACATACACTGTATAAGTGAAAAGCGGCGCGCCGTGCGGCGTGTACAGTTTATGTCCCCGGCAGACGATCTACGACGGCTCTGCGAC
miR9772*674	gi|25237721|gb|CA659209.1|CA659209	UGAGAUGAGAUUACCCCAUAC	TTTCTCTCTGTAGCGGCCGTTGAGATGAGATTACCCCATACCCTATAGCGGCAGCTCGACTCCGCACACGCGTAGGGGGCAATCTCACCTCAACAGCTGCTCTCAGTGAGCTTT
miR9678*22374	gi|93247093|gb|CJ652184.1|CJ652184	UCUGGCGAGGGACAUACACUGU	ATTGATTCGCCCTAGGATCATCTGGCGAGGGACATACACTGTACAAGTGAATCGCGGCGCGCCGTGCGGCCTGTACAGTTTATGTCCCCGGCAGACGATCTACGACGGCTCTGCGAC
miR2118*341	gi|141708292|gb|CJ796550.1|CJ796550	UUCCUGAUGCCUCUCAUGCCUA	AAGAGGGCCTAAGAGCGGTGGGCATGGGACATGATGGAAAGCTAAAGACCTCAGTTCCTCGTCTAACACTGTAAGATGTTTTCTGTCAAAAGAAAAAAAAACACTGTAAGATGCTTTGTTTTGAGTTTCCTGATGCCTCTCATGCCTATTGTTCTTTGGTGTTCCTTC
miR9778*22436	gi|39569001|gb|CK206611.1|CK206611	UGCAUCAUCUCGAACUCGUCG	TGGCCTCCTTGTGGAAGCCCTGCATCATCTCGAACTCGTCGGTGCGATAGCGTGCAGCGTGACATGCTGTTCATCACCAACGAGTTCCAGACGATGCAGGCCTTCATCAAGGCGGAGCGC
miR2118*7327	gi|93731993|gb|CJ497229.1|CJ497229	UUCCCAAUGCCUCCCAUGACUA	AAGAGGGTCTAAGAGCGGTGGACATGGGAACATGGAGGAAAGAAAAAAAAGGTCATCCCTCTAGAAATAGAGGTTGTGATTTGTTTCGCATTTTCCCAATGCCTCCCATGACTATTGTTCTCAGATGCTCCTTC

miR160*45	gi|93162853|gb|CJ641547.1|CJ641547	UGCCUGGCUCCCUGUAUGCCA	GAGGTGAAAACAATGGGATATGCCTGGCTCCCTGTATGCCACTCGCGTAGCTGCCAACTCCCCAAAGCTTGCGTTGGCTCTACCGCGGATGGCGTGCGAGGAGCCAAGCATGACCGTCTCTCTCTCTCTCTC
miR2118*7332	gi|391026464|gb|JV844579.1|	UUCCCGAUGCCUCUCAUGCCUA	ATGAGCATCTAAGAGCATTAGGCATGGGAACATTGAGGAAGGCCTAAACTAGTGTGTCCCTGTCCAACTAGGTTCCAACATTTGTTTTGGAATTTTCCCGATGCCTCTCATGCCTAATGTTGTTAGGTGTTCCTTC
miR2118*334	gi|391033584|gb|JV851699.1|	UUCCUGAUGCCUCCCAUGCCUA	ATGAGTATCTTAGAGCGGTGGGCATGGGAACATGGAGGAAAGCCTAGATTTCTTGGCCTCTTAATTTAGGGGCAAAGATTGTTTTGTGTTTTCCTGATGCCTCCCATGCCTATTGTTCTTAGTTATTCCTTC
miR2275*342	gi|391173497|gb|JV991609.1|	UUUGUUUUUCUCCAAUAUCUCA	TTGTGCAGGCACTGAAAGTGAGTGTTGGAGGAAAGCAAACTGGGAAATCCGTCTCAACAGTGCTGATGTTTCCAGTTTGTTTTTCTCCAATATCTCATTTTCGGTAGATTTACCTGA
miR2118*935	gi|391112807|gb|JV930921.1|	UUUCUGAUGCCUCCCAUUCCUA	AAGTGGGTCTAAGAGGTGTAGGAATGGGAACATGAAGAAAATCAACTGTAATGCCTCCTCTAGTATTGGAGGGGAGAAATGGTCTGATGTTTCTGATGCCTCCCATTCCTATGGATCTTAGGTTCTCCTTT
miR2118*952	gi|391099696|gb|JV917810.1|	UUCCUGAUGCCUCCUAUUCCUA	GAGAATCTCTAAGAGCGGTGGGGATAGGATCATGGAGGAAAGCCTAGAGCTCAAATTCTCCTCCTCTGTCAGAGCCGAGGTAGAGATTTGCTTTGTGTTTTCCTGATGCCTCCTATTCCTATCGATTTTAAGTACTCCTTC
miR2118*322	gi|391033584|gb|JV851699.1|	UUCUUGAUGCCUCCCAUGCCUA	ATGAGTATCTCAGAGCGGTGGGCATGGGAACATGGAGGAAAGCCTAGATTTCTTGGCCTCTTAATTTAGGGGCAAAGATTATTTTGTGTTTTCTTGATGCCTCCCATGCCTATTGTTCTTAGGTATTCCTTT
miR2118*324	gi|391123601|gb|JV941714.1|	UUCCUGAUGCCUCCCAUGCCUA	ATGAGTATCTCAGAGCGGTGGGTATGGGAACATGGAGGAAAGACTAGACCTCTTGGCCTCTTAATTTAGGGGCGAAGATTGTTTCGTGTTTTCCTGATGCCTCCCATGCCTATTGTTCTTAGGTATTCCTTA
miR167*23	gi|391092574|gb|JV910688.1|	UGAAGCUGCCAGCAUGAUCUG	CAAGGTGCACCACAAGCTGGTGAAGCTGCCAGCATGATCTGATGACCTAACTCATGGATCAGAATCCATGTCAATCAGGTCATGCTGGAGTTTCATCTGCTGGTCGGAGCACAACGA
miR2118*339	gi|391061419|gb|JV879534.1|	UUCCUCAUGCCUCCCAUGCCUA	ATGACTATCTTAGAGCGGTGGGCATGGAAACATGGAGGAAAGCATAGATTTCTTGGCCTCTTAATTTAGGGGCAAACATTGTTTTGTGTTTTCCTCATGCCTCCCATGCCTATTGTTCTTAGGTATTCCTTC
miR393*373	gi|509298353|gb|GAJL01178934.1|	UUCCAAAGGGAUCGCAUUGAU	TTCAGGAAGCTAGTGGAGGATTCCAAAGGGATCGCATTGATCCATCTCTCCGTAACCGGCGCTCAAGATCGATGGATCAGTGCAATCCCTCTGGAATTCTCCGCTTGCCTCCGCCTCC
miR2118*320	gi|391033584|gb|JV851699.1|	UUCCUGAUGCCUCCCAUGCCUA	ATGAGTATCTTAGAGCGGTGGGCATGGGAACATGGAGGAAAGCCTAGATTTCTTGGCCTCTTAATTTAGGGGCAAAGATTGTTTTGTGTTTTCCTGATGCCTCCCATGCCTATTGTTCTTAGGTATTCCTTC
miR2118*7336	gi|391025262|gb|JV843377.1|	UUCCCGAUGCCUCUCAUGCCUA	ATGAGTATCTTAGAGCAATGGGCATGGGAACATGGAGGAAAGCCTAGACTTCTTGGCCTCTAAATTTAGGGGCCAAGACTGTTTTGTATTTTCCCGATGCCTCTCATGCCTATTATTCTTAGATATTCCTTC
miR2275*347	gi|391137755|gb|JV955867.1|	UUUGGUUUCCUCCAAUAUCUCA	AACCTTGCAAGCTGAATGTGAGATTTGGATGGAACCAAATCTTCAGCTATAATGGCACAAGATTTGGTTTCCTCCAATATCTCATCTTCAACTGTCCCTGATGA
miR2118*7335	gi|391197049|gb|JW015162.1|	UUCCCGAUGCCUCUCAUGCCUA	ATGAGCATCTAAGAGCATTAGGCATGGGAACATTGAGGAAGGCCTAAATTAATGTGTCCCTGTCCAACTAGGTTCCAACATTTGTTTTGGAATTTTCCCGATGCCTCTCATGCCTAATGTTCTCAGGTGTTCCTTC
miR2118*270	gi|391153804|gb|JV971916.1|	UUCCUAAUGUCUCCCAUUCCUA	AAGATAGCATAAGAGCACTAGGAATGGGAACATGGTGGAAAGTGTAACTTGTTTGTTTACACCCGTCATGTTGGAGGTGCAAATAATTAGTTTTGTGCTTTCCTAATGTCTCCCATTCCTATGGTTCTTGAGCACTCCTCC
miR166*193	gi|391208740|gb|JW026853.1|	GGAAUGUUGUCUGGUUCAAGG	AAGCTATTTTGCTTCTGGGTGGAATGTTGTCTGGTTCAAGGTCTCGCTCTGGTTTGAGGATGGAAAACTTTCATTTCTCTCAGCTTCCTGCGATCTCGGACCAGGCTTCATTCCCCTCAGAGATAGCTTCAACCAT
miR9673*2237	gi|509215499|gb|GAJL01259080.1|	UAAGAAGCAAAUAGCACAUG	TGCGGGGAATCACCTCGAGGTAAGAAGCAAATAGCACATGCAGGAGGTAAGAAGCAAACGAATGTGTGTGCTCTTTCCTTCTTACCCTCGAGCCGACTCCCGGAACC

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miR9666*21745	gi|391018508|gb|JV836623.1|	CGGUAGGGCUGUAUGAUGGCGA	AATACAAATCATTCGGATTCGCCATCATACGCCCAACCGTGCATTTGATATGCATATATATGCATCACGAGCCACGGTAGGGCTGTATGATGGCGATACCGATTGGTTTGTTCTAG
miR2118*946	gi|391160185|gb|JV978297.1|	UUCCUCAUGCCUCCCAUUCCUA	GAGATAACTCGAGAGCACTAGGAATGGGAACATAGAGGAAACCCCTGCTTGTTTGTTTGCACTTCTAGTGTTTGAGGTGCAAATATTTTCACAGTTCATTTCCTCATGCCTCCCATTCCTACCATTCTTGTGCACCCCTTC
miR9863*771	gi|391085358|gb|JV903473.1|	UGAGAAGGCAGAUCAUAAUAGC	ATAATAAACAAGTCTTTAGATGAGAAGGCAGATCATAATAGCTGAGCGAAATGTAAGTGATACCGTTGCTCAATTGTTATGATCTGCTTCTCATCTGAAGACTAGTTTATTACAT
miR2118*246	gi|346477762|gb|JP210263.1|	GGGAAUGGGAACAUGGAGGAA	ATAGAGGGCCTTAGAGTAGTGGGAATGGGAACATGGAGGAAAGCCTAAATTGCTCGGTTTTCATCTCTAGTGTTAGAGGTGAAAATCTAGTTTGGTGTTTCCCAATGCCTCCCATTCCTATTGTTCTAAAGCACTCCTTCC
miR2275*959	gi|391173269|gb|JV991381.1|	UUCAGUUUCUUCUAAUAUCUCA	TGCATGCACCGCTCAAAGCGAGGATTAGAGGGAACTGAACCATGAACAATCCATTGGAAACATGAGTGATGGTTCAGTTTCTTCTAATATCTCACCTTGACTGGTCCACTGTGC
miR166*773	gi|391208740|gb|JW026853.1|	UCUCGGACCAGGCUUCAUUCC	GCTATTTTGCTTCTGGGTGGAATGTTGTCTGGTTCAAGGTCTCGCTCTGGTTTGAGGATGGAAAACTTTCATTTCTCTCAGCTTCCTGCGATCTCGGACCAGGCTTCATTCCCCTCAGAGATAGCTTCAACC
miR5062*443	gi|509292474|gb|GAJL01184809.1|	GCAGAUUUUUCACCAAUAUUCAAG	AAAAAGTCTACATAACCTCTTGAATCTTGGGAAAAAGCTGCATAACTCCAATAAATTGGAAACCTCATTTTCTATCCCAAAATTGCTTGCAAACACAATTTCTGGGGTAAAATATATGGTTTGAAGTTATGAGGTTATGCAGATTTTTCACCAATATTCAAGTGGTTATCTAGATTTTTTTA
miR2275*360	gi|391156891|gb|JV975003.1|	UUUGGUUUCCUCCAGUAUCUCA	AACCTTGGAAGTTGAATGTGAGATTTGGAGGGAACCAAATCTTAAGCTATCATGCCACAAGATTTGGTTTCCTCCAGTATCTCATCTTCAACTGTCCCTGATGA
miR2118*7337	gi|391156658|gb|JV974770.1|	UUCCCGAUGCCUCCCUUGCCUA	ATGAGCATCTAAGAGTATTGGGCATGGGAACATGGAGGAAGGCCTAAACTAGTGTGTCCCTGTCGAACTAGGGTCCAATTTTTGTTTGGAATTTTCCCGATGCCTCCCTTGCCTATTGATCTTAGGTGTTCCTTC
miR398*405	gi|391172652|gb|JV990764.1|	GGGUCGAGCUGGGAACACAUG	TACGCGAGGAAATTCCTGCGGGGTCGAGCTGGGAACACATGAGATATGAACCGCTTGATTGAATATCAACTCATGTGTTCTCAGGTCGCCCCCGCTGGAGCCTCCTCGCCGCAG
miR2275*960	gi|391122558|gb|JV940671.1|	UUCAGUUUCUUCUAAUAUCUCA	TGCATGCACCGCTCAAAGCGAGGATTAGAGGGAGCTGAACCATCCATTGGAAACATGGAGTGATGGTTCAGTTTCTTCTAATATCTCACCTTGACTGGTTCATCCGGG
miR9772*673	gi|509293551|gb|GAJL01183734.1|	UGAGAUGAGAUUACCCCAUAC	TTTCTCTCTGCAGCGGCCGTTGAGATGAGATTACCCCATACCCTATAGCGGCAGCTTGACTCCGTGCACGCGTAGGGGGCAATCTCACCTCAACAGCCGCTCCCAGTGAGCTTT
miR2118*7323	gi|391033584|gb|JV851699.1|	UUCCCAAUGCCUCCCAUGCCUA	ATGAGCATCTAAGAGTATTGGGCATGGGAACATGGAGGAAGGCCTAAACTAGTGTGTCCCTGTCCAACTAGGGTCCAATATTTGTTTGGAATTTTCCCAATGCCTCCCATGCCTATTGTTCTTAGGTGTTCCTTC
miR9677*667	gi|391099801|gb|JV917915.1|	UGGCCGUUGGUAGAGUAGGAGA	CAAAGGCGGCCTCGCTTCTCTTCCACTCTACCAACAGCCACGCTGCCACGTCTAGCCCGCGACCATGGCGTGGCCGTTGGTAGAGTAGGAGACGAGCATGGCCACCTGTCCC
miR2118*317	gi|391028619|gb|JV846734.1|	UUCCCGAUGUCUCCUAUUCCUA	AAGATAGCCTGAGAGCAGTGGGGATAGGAACATGGAGGAAAGCCTAACTCGCTTTTTTCACCTCTTATTGGAGGCGAGAATTTAGTTTGGTATTTCCCGATGTCTCCTATTCCTATTGTTCTCAAGCACTCCTTC
miR2118*337	gi|391156658|gb|JV974770.1|	UUCCUGAUGCCUCCCAUGCCUA	ATGAGTATCTCAGAGTGGTGGGCATGGGAACATGGAGGAAAGCCTAGATTTCTTGGCCACTTAATTTAGGGGCAAAGATTGTTTTGTGTTTTCCTGATGCCTCCCATGCCTATTGTTCTTAGGTATTCCTTC
miR166	gi|391208740|gb|JW02	CUCGGACCAGG	AGCTATTTTGCTTCTGGGTGGAATGTTGTCTGGTTCAAGGTCTCGCTCTGGTTTGAGGATGGAAAACTTTCATTTCTCTCAGCTT

*916	6853.1|	CUUCAUUCCC	CCTGCGATCTCGGACCAGGCTTCATTCCCCTCAGAGATAGCTTCAACCA
miR2275*955	gi|509251861|gb|GAJL01223109.1|	UUCAGUUUCUUCUAAUAUCUCA	GACATGTGCCGCTTGAAGTGAGAGTTAGATAGAACTGAACCACCTCCGGTCGACCGCCTGTTGGTTTGGGTGTTGTTCAGTTTCTTCTAATATCTCACTTTGAATGGTCCATCTGTC
miR2118*335	gi|391197049|gb|JW015162.1|	UUCCCGAUGCCUCUCAUGCCUA	ATGAGCATCTAAGAGCATTAGGCATGGGAACATTGAGGAAGGCCTAAATTAATGTGTCCCTGTCCAACTAGGTTCCAACATTTGTTTTGGAATTTTCCCGATGCCTCTCATGCCTAATGTTCTCAGGTGTTCCTTC
miR2118*327	gi|92496469|gb|CJ509470.1|CJ509470	UUCCUGAUGCCUCCCAUGCCUA	ATGAGTATCTCAGAGCGGTGGGCATGGGAACATGGAGGAAAGCCTAGATTTCTTGGCCATTTAATTTAGGAGCAAAGATTGTTTTGTGTTTTCCTGATGCCTCCCATGCCTATTGTTCTTAGGTATTCCTTC
miR2118*936	gi|391172157|gb|JV990269.1|	UUUCUGAUGCCUCCCAUUCCUA	AAGCGGGTCTAAGAGGTCTAGGAATGGGAACATGAAGAAAATCAATTGTAATGCCTCCTCTAGTATTAGAGGGGTGAAATGGTCTGATGTTTCTGATGCCTCCCATTCCTATGGATCTTAGGTTCTCCTTC
miR397*20228	gi|391124107|gb|JV942220.1|	UCACCGGCGCUGCACACAAUG	GAAGAGACGCAAAGGTGCCATTGAGTGCAGCGTTGATGAACCGTCCTACTCCTCCGAGGCCGGAGCGGTTCACCGGCGCTGCACACAATGACGGCTCTGCTTTCTCCGGC
miR393*91	gi|509298353|gb|GAJL01178934.1|	UCCAAAGGGAUCGCAUUGAUCC	TCAGGAAGCTAGTGGAGGATTCCAAAGGGATCGCATTGATCCATCTCTCCGTAACCGGCGCTCAAGATCGATGGATCAGTGCAATCCCTCTGGAATTCTCCGCTTGCCTCCGCCTC
miR167*27	comp4383964_c0_seq1	UGAAGCUGCCAGCAUGAUCUA	CGAGTGCCCAAGAGAAAGCGTGAAGCTGCCAGCATGATCTAACTCTTGAAGCTGGCCGCGTGGTGCTGGTTAGATCGTGCTGTGACAGTTTCACTCTTTCCCTGTTGGGCA
miR167*201	comp4383964_c0_seq1	GAUCGUGCUGUGACAGUUUCACU	CGAGTGCCCAAGAGAAAGCGTGAAGCTGCCAGCATGATCTAACTCTTGAAGCTGGCCGCGTGGTGCTGGTTAGATCGTGCTGTGACAGTTTCACTCTTTCCCTGTTGGGCACATG
miR1878*925	comp375313_c0_seq1	AUUUGUAGUGUUCGGAUUGAGUUU	CTTACAAACCACTTTTGCAAACTTAGTCTTGGCACTATAAATTTAAAATGTGCATGTGAAATATGCTCATTATAAATTTGTAGTGTTCGGATTGAGTTTATAATAGTCGTTCTAAGATT
miR5062*441	c13281_g1_i2	UGAACCUUAGGGAACAGCCGCAU	GTAAGGTTCACGTAGCTTCTTGAACCTTAGGGAACAGCCGCATAACACCATTAACTTGAAAACCACTTACCTTATCCCAAAACTGCTCCTACCACAATATAAGGTTTAAGGAATGGTTTTTAGTTTGTGAGGTCATGCGGATTTTTCACCAAGGTTCAAGTGGTTATCTGGATCTTTTGA
miR156*37	c40546_g1_i1	GCUCACUGCUCUAUCUGUCAGC	TGGGTGCTGTGTGTGGAAGTTGACAGAAGAGAGTGAGCACCCATGGTGTTTCCTTAGCATCAAGGGGCATGCCAGGGAACTATGCGTGCTCACTGCTCTATCTGTCAGCCACTGATCAGACCCCATTTC
miR156*34	c40546_g1_i1	UGACAGAAGAGAGUGAGCAC	TGGGTGCTGTGTGTGGAAGTTGACAGAAGAGAGTGAGCACCCATGGTGTTTCCTTAGCATCAAGGGGCATGCCAGGGAGCTATGCGTGCTCACTGCTCTATCTGTCAGCCACTGATCAGACCCCG
miR156*181	c40546_g1_i1	GCUCACCCUCUCUCUGUCAGC	GGAGCTGGGCTTTGGAGGTCTGACAGAAGAGAGTGAGCACACGCGGTGGTTTCCTAGCATGCGAGCGCCATGTTTGGAGCTGCGCGTGCTCACCCTCTCTCTGTCAGCCATTTACCATGCCCATCTCT
miR393*90	c66520_g1_i1	UCCAAAGGGAUCGCAUUGAUCC	AACAATGCCTTGGGGAAGCATCCAAAGGGATCGCATTGATCCTTCCATCCATGGTGTTGATGGCTGTCATCAATCAACCGGCTCGACGGATCATGCGATCCTTTTGGAAGCTTCCTCTAGCCATTACTCC
miR156*38	c40546_g1_i1	GCUCACUGCUCUAUCUGUCAGC	TGGGTGCTGTGTGTGGAAGTTGACAGAAGAGAGTGAGCACCCATGGTGTTTCCTTAGCATCAAGGGGCATGCCAGGGAGCTATGCGTGCTCACTGCTCTATCTGTCAGCCACTGATCAGACCCCGTTTC
miR156*31	c40546_g1_i1	UGACAGAAGAGAGUGAGCAC	GGAGCTGGGCTTTGGAGGTCTGACAGAAGAGAGTGAGCACACGCGGTGGTTTCCTAGCATGCGAGCGCCATGTTTGGAGCTGCGCGTGCTCACCCTCTCTCTGTCAGCCATTTACCATGCCCAT

miR156*32	c40546_g1_i1	UGACAGAAGAGAGUGAGCAC	TGGGTGCTGTGTGTGGAAGTTGACAGAAGAGAGTGAGCACCCATGGTGTTTCCTTAGCATCAAGGGGCATGCCAGGGAACTATGCGTGCTCACTGCTCTATCTGTCAGCCACTGATCAGACCCCA
miR156*910	c27534_g2_i1	GCUCACUGCUCUUCCUGUCAUC	CCGGTACGGGCGCCGGGCGGTGACAGAAGAGAGTGAGCACACGGCCGGTCGTTACGGCACCGCCCGGGTGTGCCGTCGCGGCCGCGTGCTCACTGCTCTTCCTGTCATCCACTCTCCCGCTTCCCTTCC
miR156*909	c27534_g2_i1	GCUCACUGCUCUUCCUGUCAUC	CCGGTACGGGCGCTGGGCGGTGACAGAAGAGAGTGAGCACACGGCCGGTCGTTACGGCACCGCCCGGGTGTGCCGTCGCGGCCGCGTGCTCACTGCTCTTCCTGTCATCCACTCTCCCGCTTCCCTTCC
miR164*47	c56266_g1_i1	UGGAGAAGCAGGGCACGUGCA	GTTGGTGAGGAGGTCCCTGTTGGAGAAGCAGGGCACGTGCAAATTATGATCGTAGCACGCTTCGTGCTTGTATAGTACGTGCATGCATGGATCGTCGTGCACGCGCTCCCCTTCTCCACCATGGCCTTCTCACCTAGCCT
miR9673*22371	c39934_g1_i1	UAAGAAGCAAAUAGCACAUG	TGCGGGGAATCGCCTCGAGGTAAGAAGCAAATAGCACATGCAGGAGGTAAGAAAGAAGCAAGCGAATGCGTGTGCTCTTTCCTTCTTACCCTCGAGCCGGCTCCCGGAACC
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miR2275*957	c64793_g1_i1	UUCAGUUUCCUCUAAUAUCUCA	TACATGTGCCACTTGAAGTGAGAGTTAGATGGAACTGAACCACCTCCGTTACAGCCGATTGCATGTTGATCTGGGGTTCAGTTTCCTCTAATATCTCACTTTGAATGGTCCATCCGCC
miR396*14	c55124_g1_i1	UUCCACAGCUUUCUUGAACUG	GTGTGGCCCTCTTTGCTGTCTTCCACAGCTTTCTTGAACTGCATCTGCAATTGGTGGATGCTAGCTTCTACCGGCAAGATCTGCAGTTCAATAAAGCTGTGGGAAATTGCAGAGAGAGACCAATTG
miR2275*958	c10697_g2_i2	UUCAGUUUCUUCUAAUAUCUCA	TGTATGCACCGCTCAATGCGAGGATTAGAGGGAACTGAACCATCCATTGGAAACATGGAGTGATGGTTCAGTTTCTTCTAATATCTCACCTTGACTGGTCCATCCAGG
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miR9674*656	CUFF.8427.1	UGAAUUUGUCCAUAGCAUCAU	AGAACAGAAAATCCTCTGGTTGAATTTGTCCATAGCATCATCCATTCTACCATTCAACGGTGGCTGCGCTGCCCGCAGTGGGTAGGATGGCTGGTGCTATGGATAGCCTCAACCCGATGTTGGTGACATACCC
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miR9676*22372	CUFF.48370.1	UGGAUGUCAUCGUGGCCGUACA	ACCGGGAGCTCTTCACACCGTGGATGTCATCGTGGCCGTACATGAATCGTCCCCCACATCCAGCTCTGTTCAATTTCAGGCAAAGTGGACGTTTCATTGTACGGCCTGATGACATCCACGGCGTGGCTCTCCCAGAAACA
miR319*369	CUFF.22037.1	AGCUGCCGAUUCAUUCAUUCA	GATGGTTGTAGGGTTTAATTAGCTGCCGATTCATTCATTCACCTACCAAGAACCAAGGCGGAGCGCATGTCTTGGTAGCTGACTGGATGACGCGGGAGCTAATATTTAGCTCCACGTTGTTT
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miR160*44	CUFF.5578.1	UGCCUGGCUCCCUGUAUGCCA	TGCTGGGATATGATATGATGTGCCTGGCTCCCTGTATGCCACTCATCCAGAGCAACATCTTTTGCAATAAGGTTGCCTGCGATGGATGGCGTGCACGGATCCAAGCATATCGAACCCTCTCCCTCTCGT
miR5062*439	gi|509237224|gb|GAJL01237634.1|; Ku_c80400	GCGGAUUUUUCACCAAGAUUCAAG	AAAAGGTTCACATAGCTTCTTGAACCTTGGGGAAAAGCCGCATAACACCATTAACTTGAAAACCATGTATTTCCTATCCCAAAACTGCTCCTAGCACAAGGGGTAGAAGATGGTTTTTAGTTGTGTGGTCATGCGGATTTTTCACCAAGATTCAAGTGGTTATCTGGATCTTTTGA
miR167*202	Ku_c81432	GAUCAUGACUGACAGCCUCAUU	GTCATGCCCATGTGAATGAGTGAAGCTGCCAGCATGATCTATCTTTGATTCGCTTCTGCGTGTGAATCCTTGTTAGATCATGACTGACAGCCTCATTTCTTCCCAGCAATGGGCATA
miR9674*660	Ku_c66069; Ex_c53441;gi|282836252|gb|FL488864.1|FL488864	UGAAUUUGUCCAUAGCAUCAU	AGAACTGAAAATCCTCTGGTTGAATTTGTCCATAGCATCATCCATCCTACCATTCAATGGTGGCTGATGCTGCCCAACGGTGGGTAGGTGGCTGGTGCTATGGATAGCTTCAACCCGATGTTGGTGACATACCC
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miR2275*361	gnl|UG|Ta#S13123008	AGUGUUGGAUGGGACCAAAUC	AATTTTGGCAGATGAATTTGAGTGTTGGATGGGACCAAATCTTACCGCCGGGTGTGGCAAGATTTGGTTTCCTCCAATATCTTATGTTCATATGTCAGACACTG
miR9772*672	gi|25237721|gb|CA659209.1|CA659209	AGGGGGCAAUCUCACCUCAAC	TTTCTCTCTGTAGCGGCCGTTGAGATGAGATTACCCCATACCCTATAGCGGCAGCTCGACTCCGCACACGCGTAGGGGGCAATCTCACCTCAACAGCTGCTCTCAGTGAGCTTT
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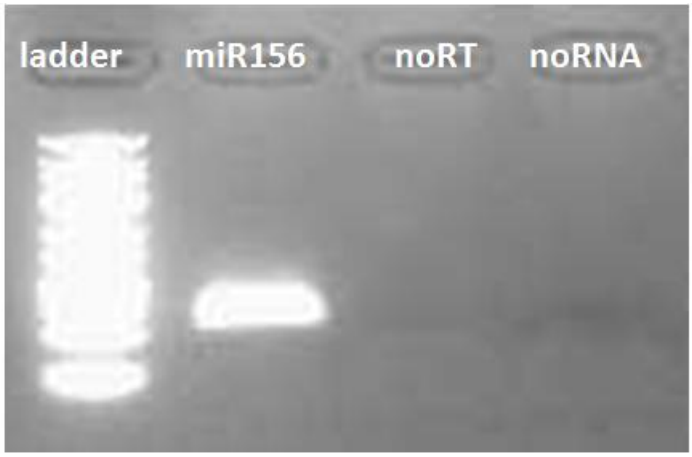
miR166*52	gi|391208740|gb|JW026853.1|	UCGGACCAGGCUUCAUUCCCC	AAGCTATTTTGCTTCTGGGTGGAATGTTGTCTGGTTCAAGGTCTCGCTCTGGTTTGAGGATGGAAAACTTTCATTTCTCTCAGCTTCCTGCGATCTCGGACCAGGCTTCATTCCCCTCAGAGATAGCTTCAACCAT
miR9863*772	gi|391085358|gb|JV903473.1|	UGUUAUGAUCUGCUUCUCAUC	ATAATAAACAAGTCTTTAGATGAGAAGGCAGATCATAATAGCTGAGCGAAATGTAAGTGATACCGTTGCTCAATTGTTATGATCTGCTTCTCATCTGAAGACTAGTTTATTACAT
miR2118*7383	gi|391156658|gb|JV974770.1|	GGCAUGGGAACAUGGAGGAAGG	ATGAGCATCTAAGAGTATTGGGCATGGGAACATGGAGGAAGGCCTAAACTAGTGTGTCCCTGTCGAACTAGGGTCCAATTTTTGTTTGGAATTTTCCCGATGCCTCCCTTGCCTATTGATCTTAGGTGTTCCTTC
miR398*404	gi|391172652|gb|JV990764.1|	UGUGUUCUCAGGUCGCCCCCG	TACGCGAGGAAATTCCTGCGGGGTCGAGCTGGGAACACATGAGATATGAACCGCTTGATTGAATATCAACTCATGTGTTCTCAGGTCGCCCCCGCTGGAGCCTCCTCGCCGCAG
miR9772*671	gi|509293551|gb|GAJL01183734.1|	AGGGGGCAAUCUCACCUCAAC	TTTCTCTCTGCAGCGGCCGTTGAGATGAGATTACCCCATACCCTATAGCGGCAGCTTGACTCCGTGCACGCGTAGGGGGCAATCTCACCTCAACAGCCGCTCCCAGTGAGCTTT
miR2118*7384	gi|391033584|gb|JV851699.1|	GGCAUGGGAACAUGGAGGAAGG	ATGAGCATCTAAGAGTATTGGGCATGGGAACATGGAGGAAGGCCTAAACTAGTGTGTCCCTGTCCAACTAGGGTCCAATATTTGTTTGGAATTTTCCCAATGCCTCCCATGCCTATTGTTCTTAGGTGTTCCTTC
miR9677*669	gi|391099801|gb|JV917915.1|	UUCCACUCUACCAACAGCCACG	CAAAGGCGGCCTCGCTTCTCTTCCACTCTACCAACAGCCACGCTGCCACGTCTAGCCCGCGACCATGGCGTGGCCGTTGGTAGAGTAGGAGACGAGCATGGCCACCTGTCCC
miR393	gi|509298353|gb|GAJL	AUCAGUGCAAU	TCAGGAAGCTAGTGGAGGATTCCAAAGGGATCGCATTGATCCATCTCTCCGTAACCGGCGCTCAAGATCGATGGATCAGTGCA

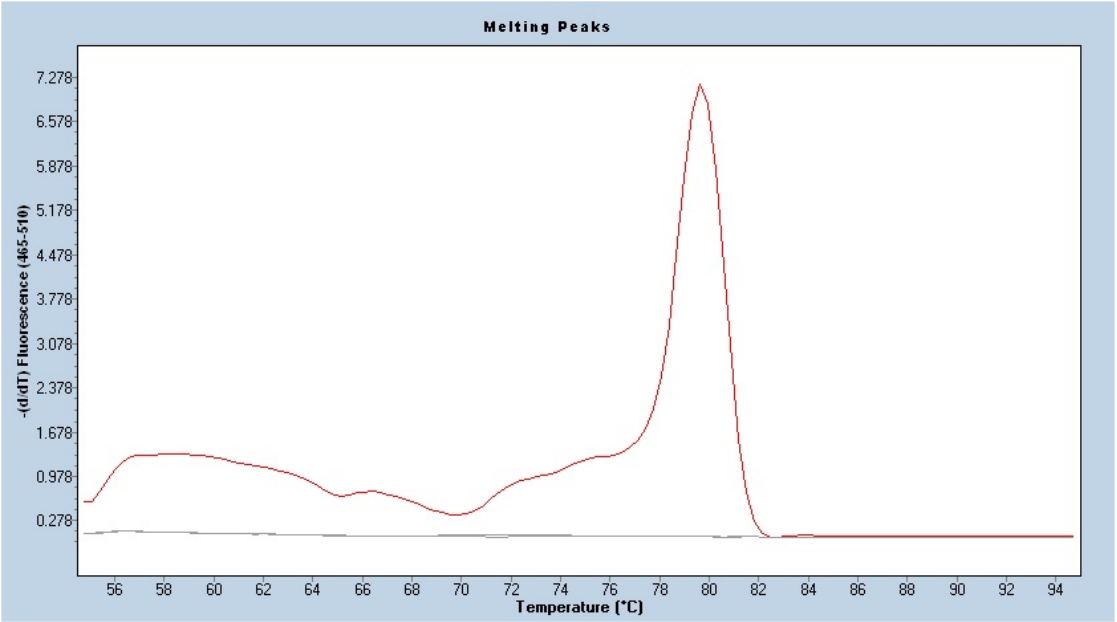
*23241	01178934.1|	CCCUCUGGAAU	ATCCCTCTGGAATTCTCCGCTTGCCTCCGCCTC
miR396 *1881	c55124_g1_i1	UCCCACAGCUU UAUUGAACUG	ATTGGTCTCTCTCTGCAATTTCCCACAGCTTTATTGAACTGCACATCTTGCCGGCAGAAGCTAGCATCAACCAATTGCAGATGCA GTTCAAGAAAGCTGTGGAAGACAGCAAAGAGGGCCACACAC
miR396 *1882	c55124_g1_i1	UCCCACAGCUU UAUUGAACUG	ATTGGTCTCTCTCTGCAATTTCCCACAGCTTTATTGAACTGCAGATCTTGCCGGTAGAAGCTAGCATCCACCAATTGCAGATGCA GTTCAAGAAAGCTGTGGAAGACAGCAAAGAGGGCCACACAC
miR396 *158	c55124_g1_i1	GUUCAAUAAAG CUGUGGGAAA	GTGTGGCCCTCTTTGCTGTCTTCCACAGCTTTCTTGAACTGCATCTGCAATTGGTGGATGCTAGCTTCTACCGGCAAGATCTGCA GTTCAATAAAGCTGTGGGAAATTGCAGAGAGAGACCAATTG
miR319 *368	CUFF.22037.1	ACUGGAUGACG CGGGAGCUAA	GATGGTTGTAGGGTTTAATTAGCTGCCGATTCATTCATTCACCTACCAAGAACCAAGGCGGAGCGCATGTCTTGGTAGCTGAC TGGATGACGCGGGAGCTAATATTTAGCTCCACGTTGTTT
miR160 *187	CUFF.5578.1	GCGUGCACGGA UCCAAGCAUA	TGCTGGGATATGATATGATGTGCCTGGCTCCCTGTATGCCACTCATCCAGAGCAACATCTTTTGCAATAAGGTTGCCTGCGATG GATGGCGTGCACGGATCCAAGCATATCGAACCCTCTCCCTCTCGT

APPENDIX-I E

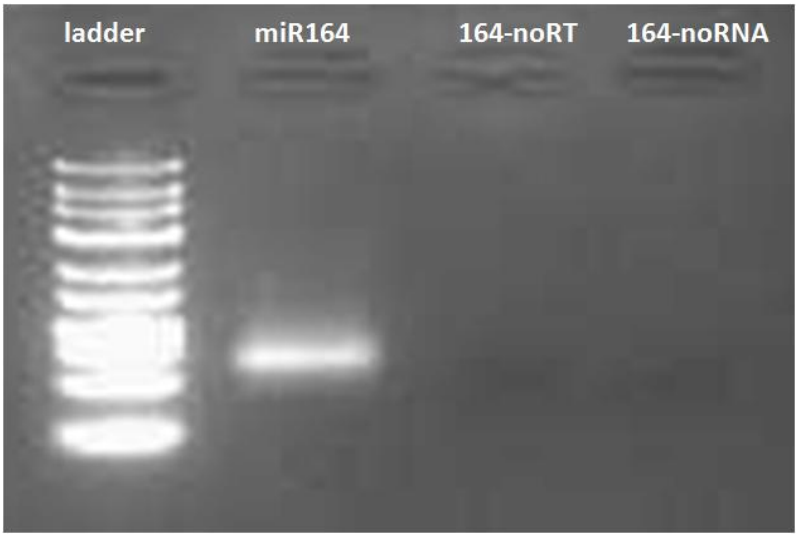
Original gel images belonging to the mature microRNA RT-qPCR experimet.



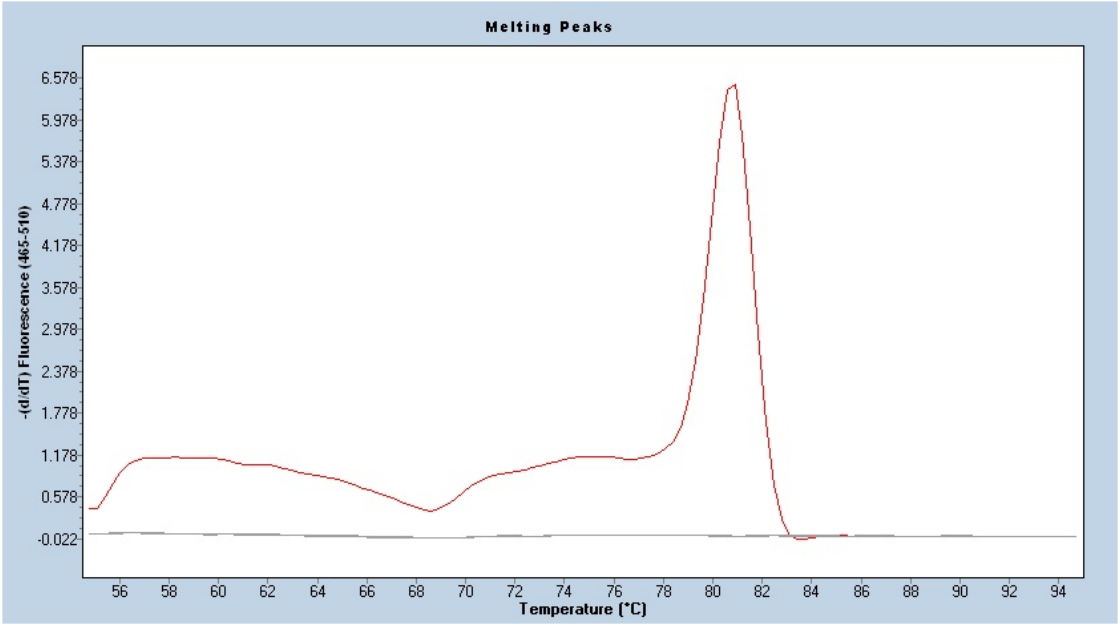
miR156 gel image



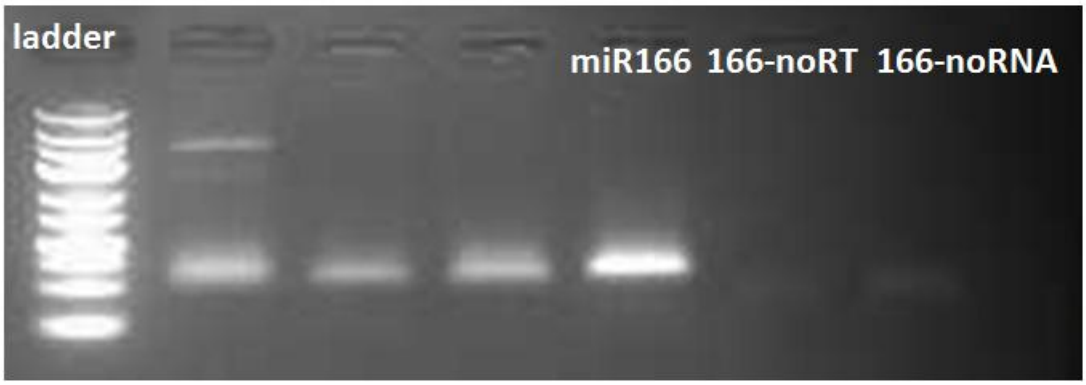
miR156 melting curve



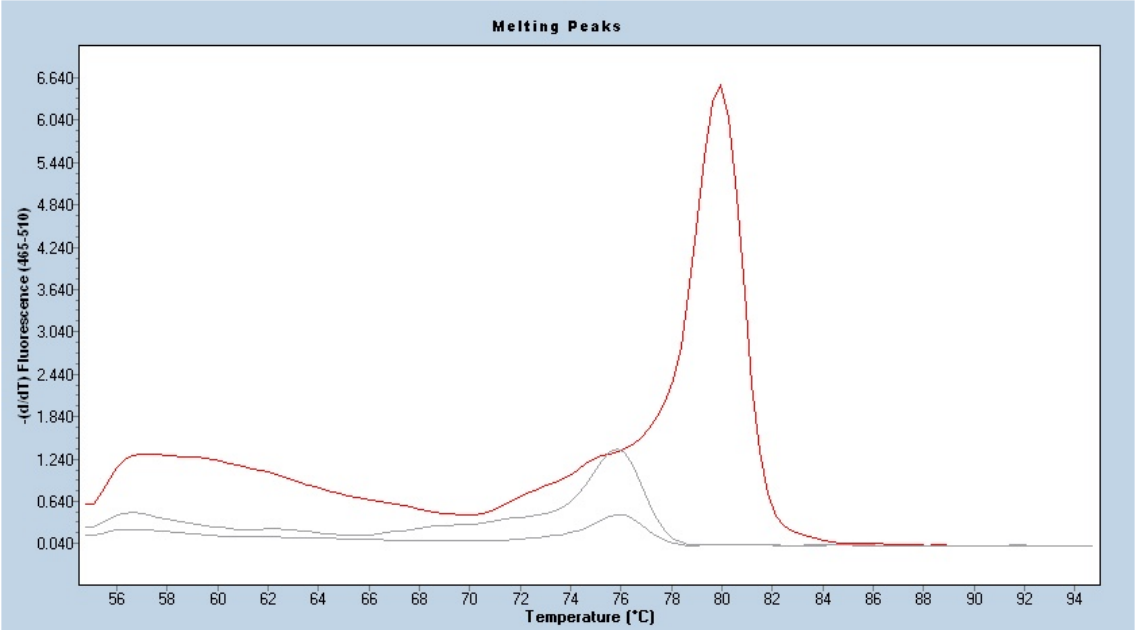
miR164 gel image



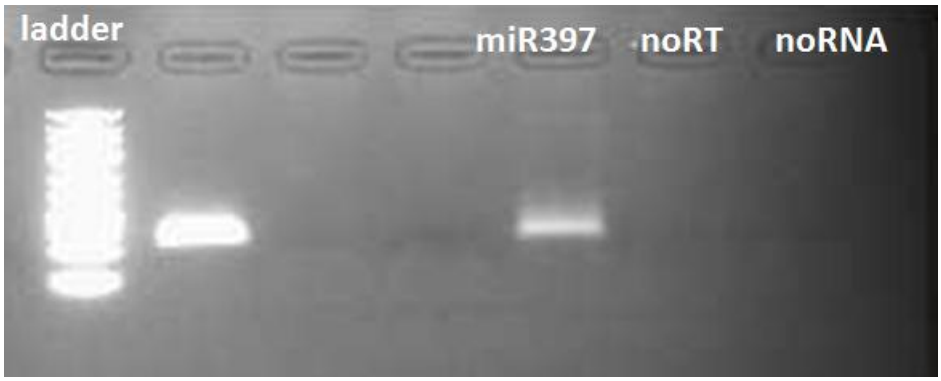
miR164 melting curve



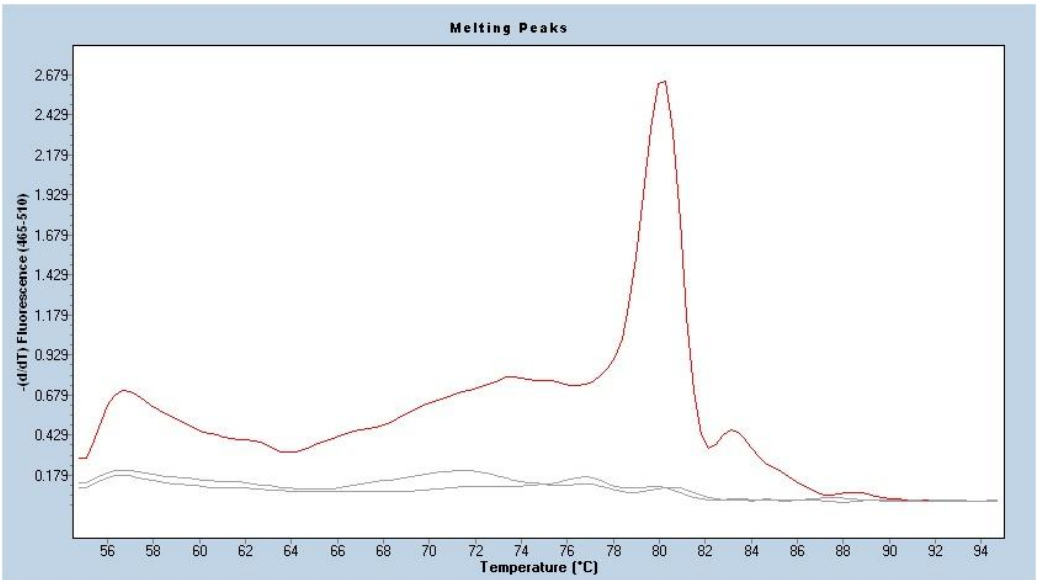
miR166 gel image



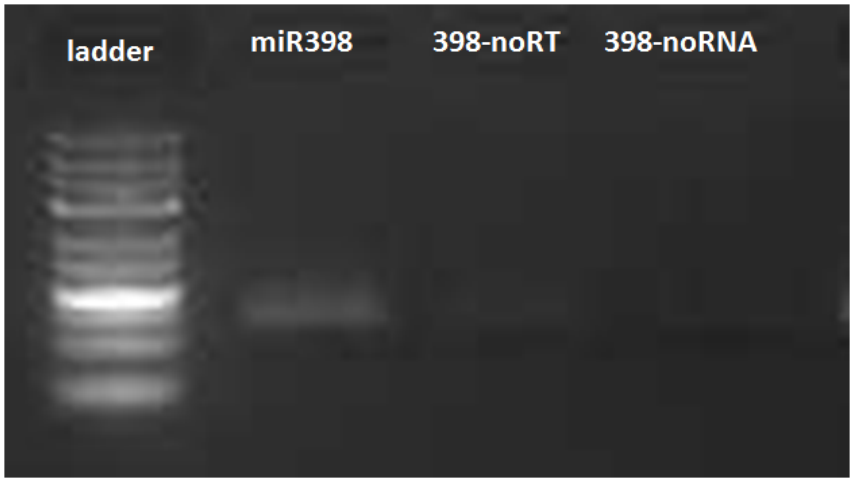
miR166 melting curve



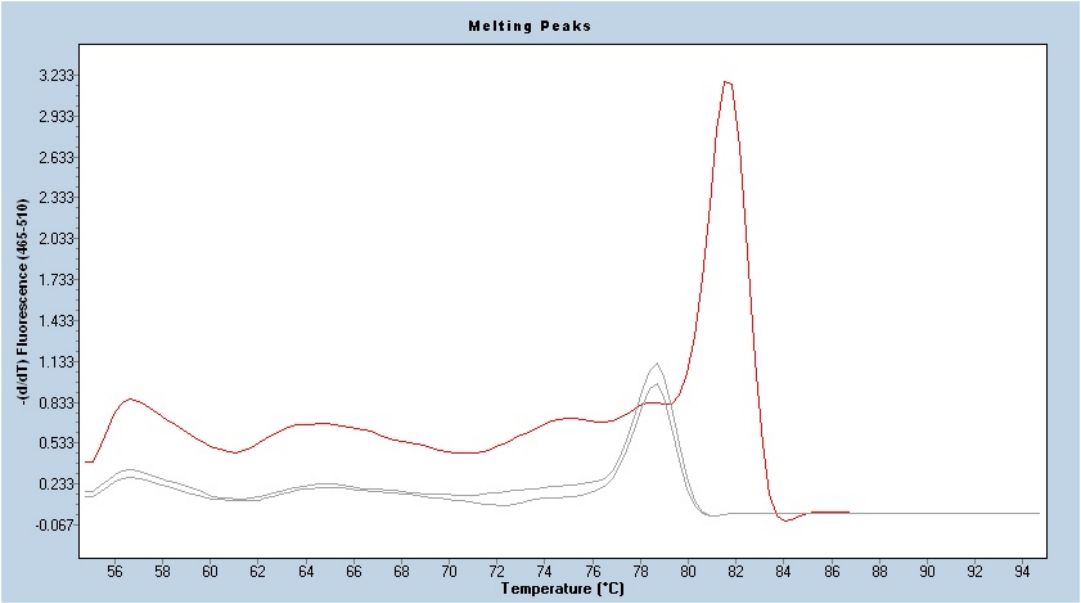
miR397 gel image



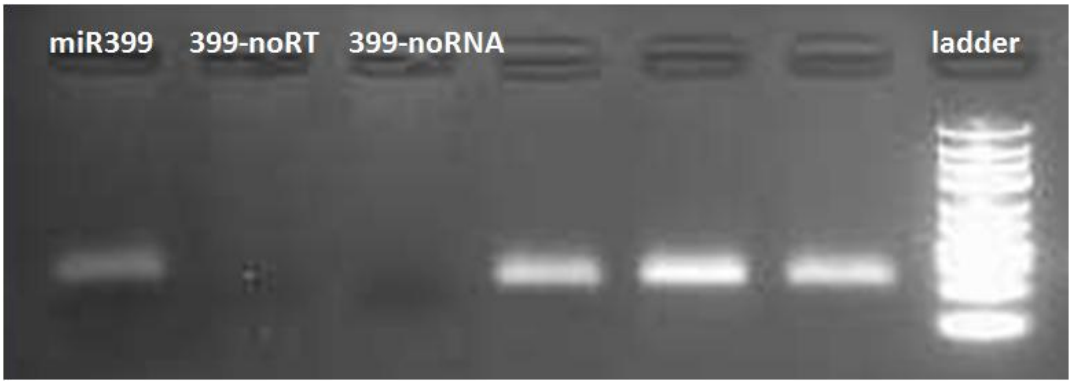
miR397 melting curve



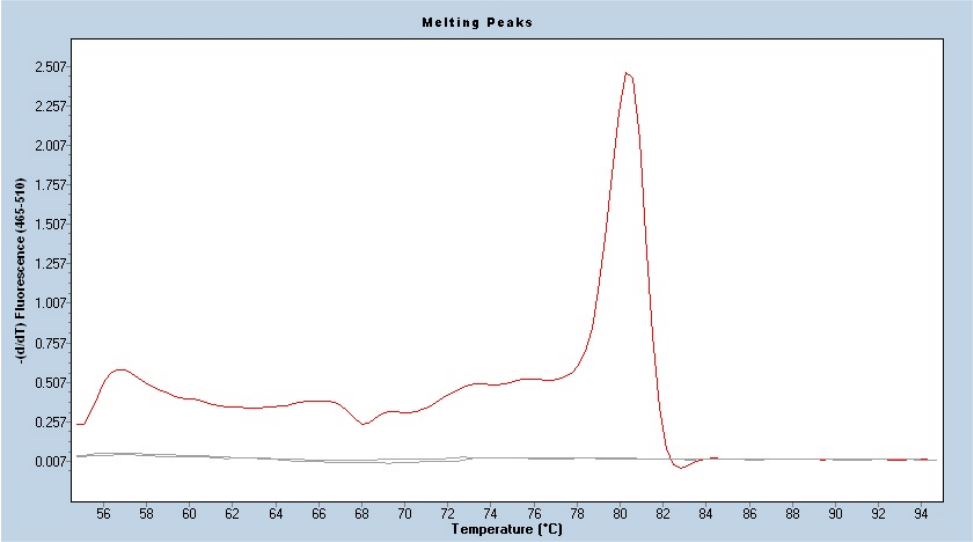
miR398 gel image



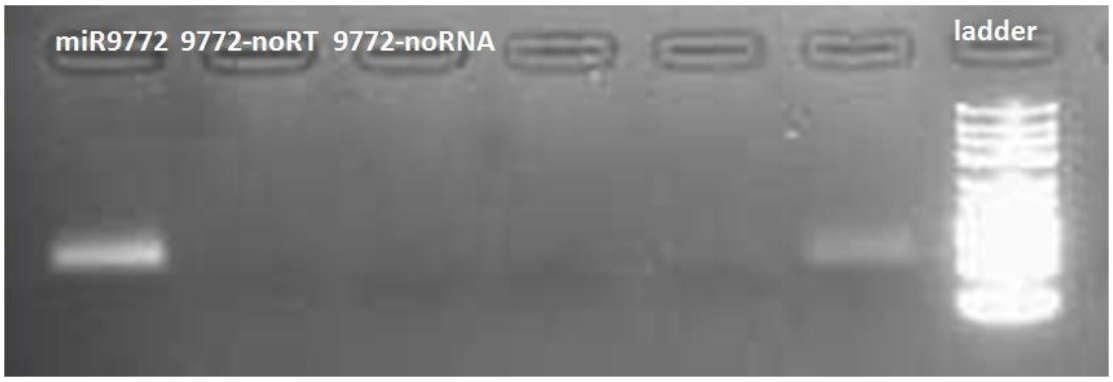
miR398 melting curve



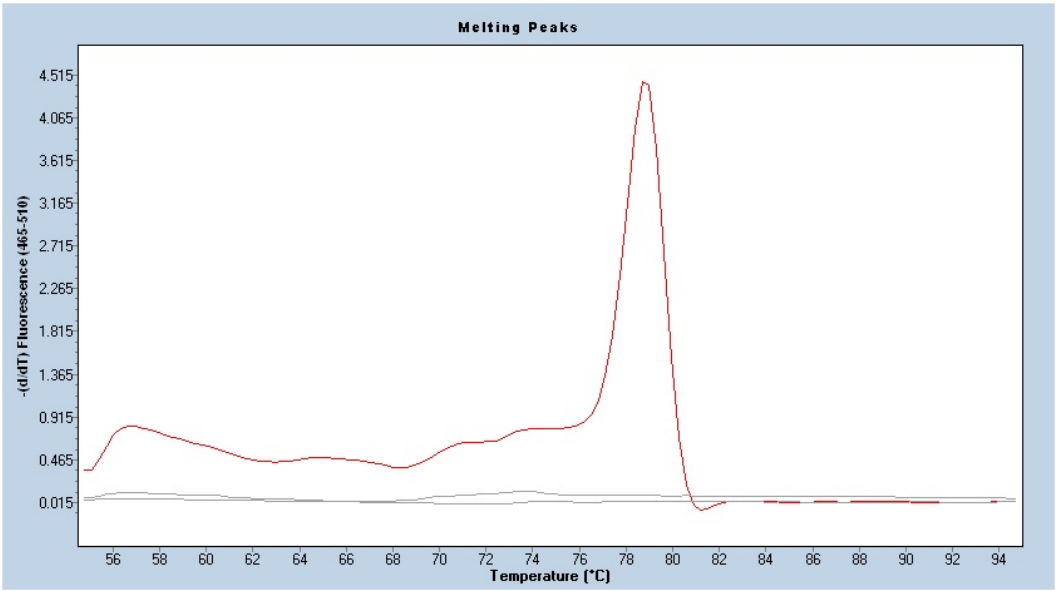
miR399 gel image



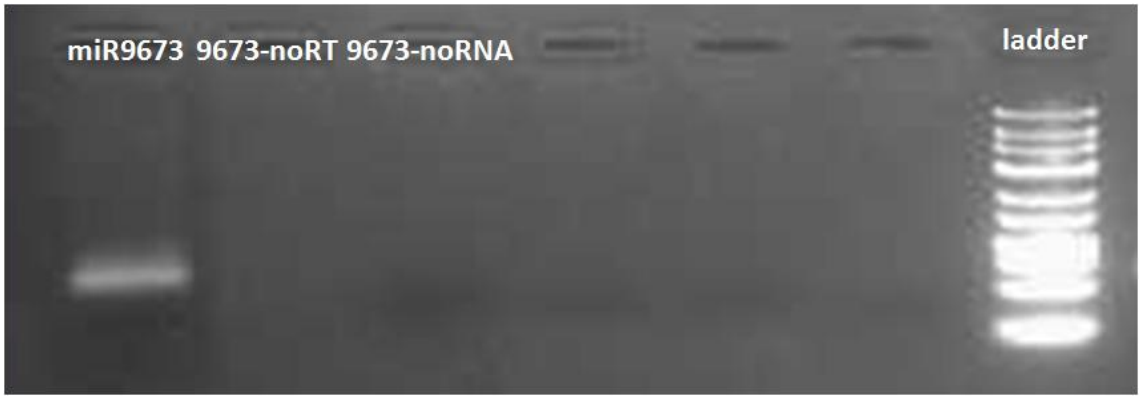
miR399 melting curve



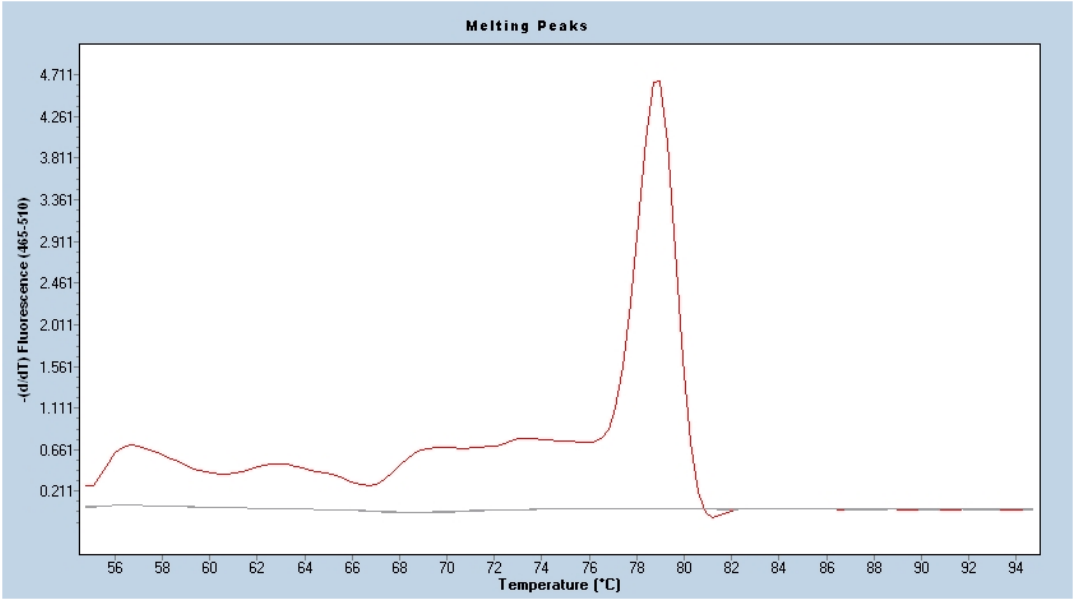
miR9772 gel image



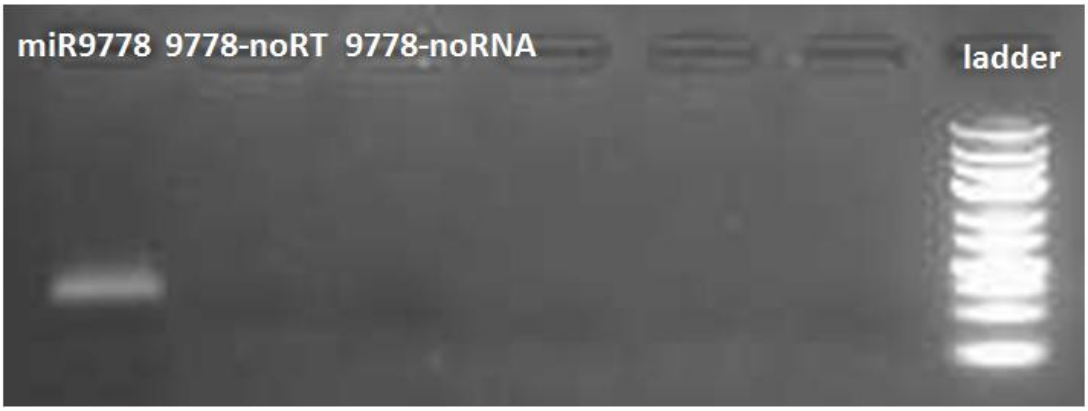
miR9772 melting curve



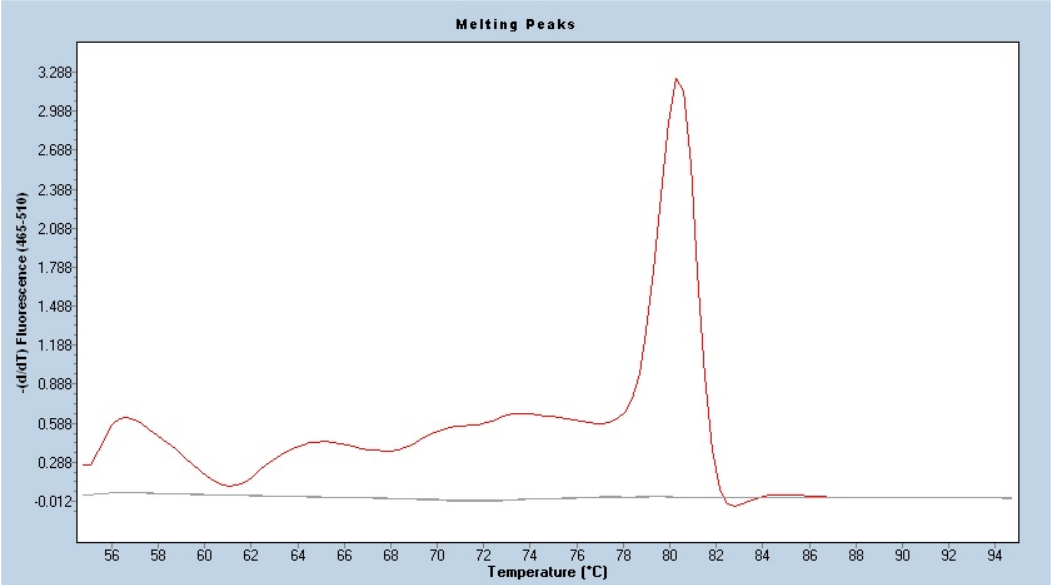
miR9673 gel image



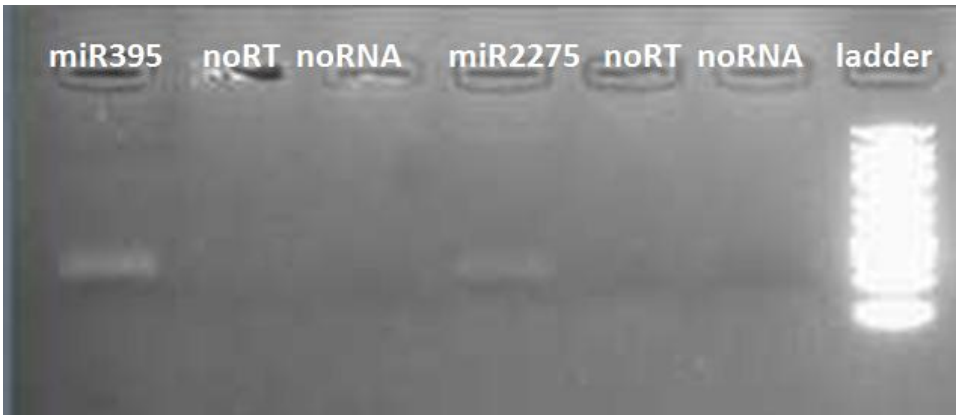
miR9673 melting curve



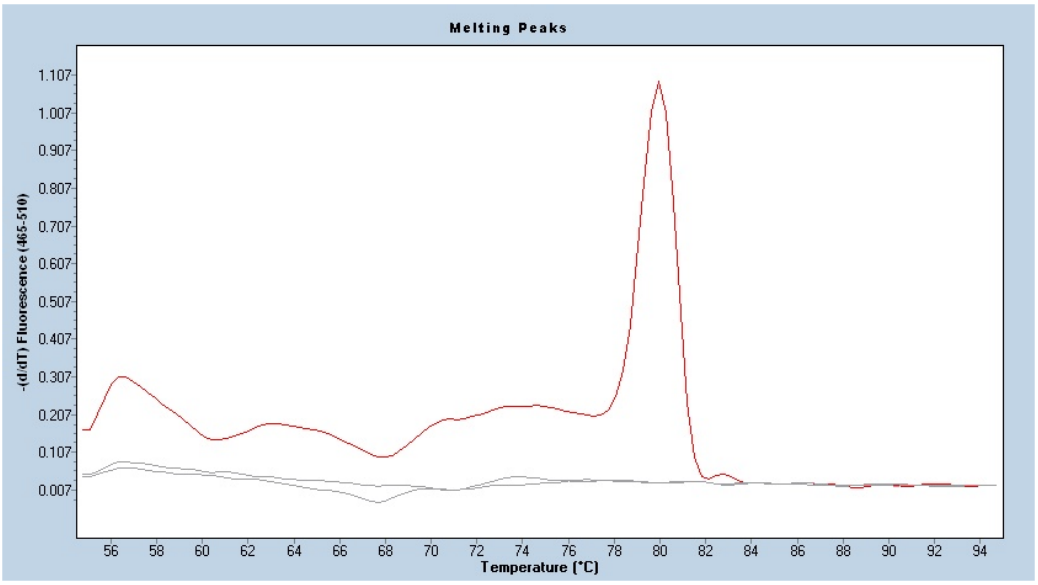
miR9778 gel image



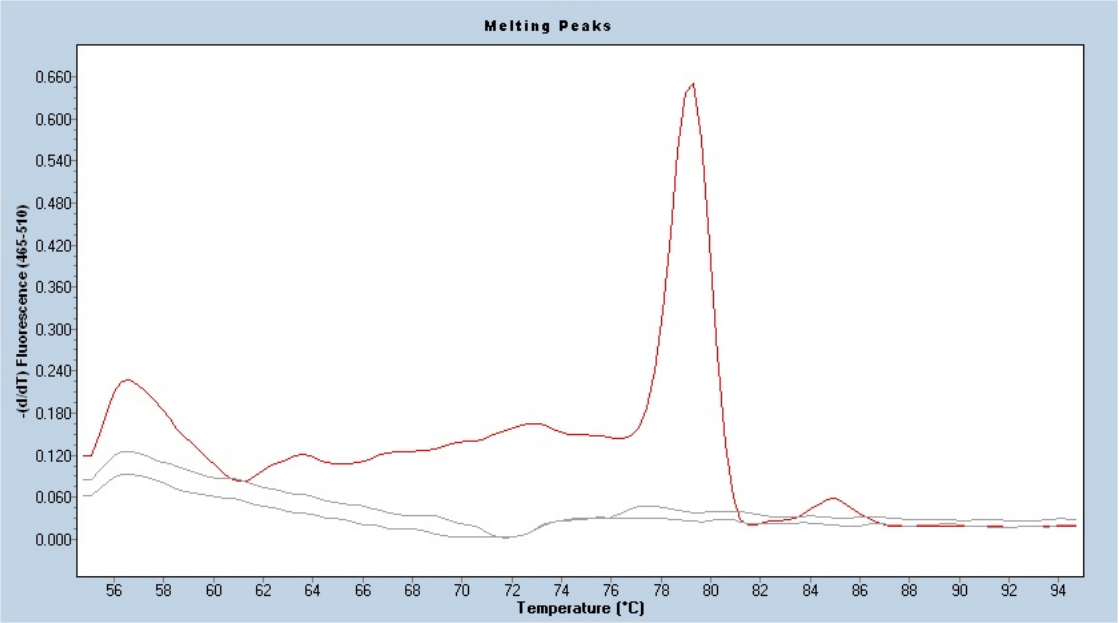
miR9778 meting curve



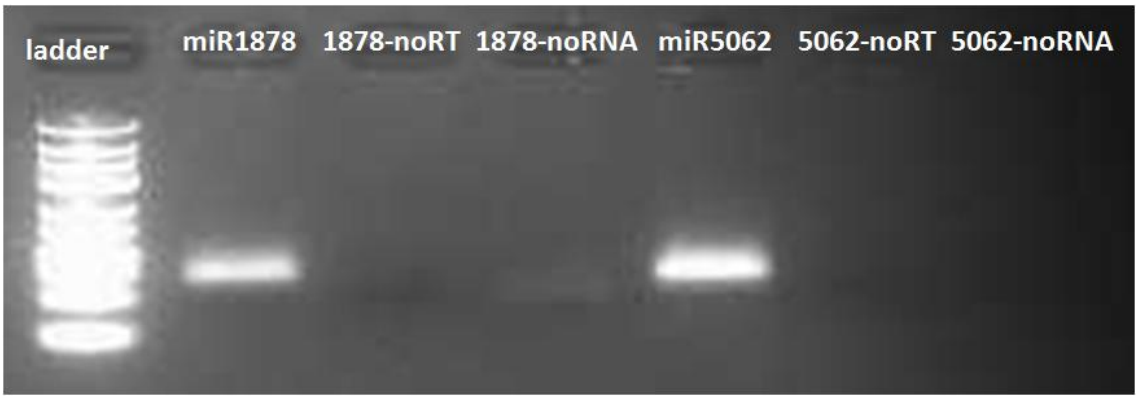
miR395 and miR2275 gel image



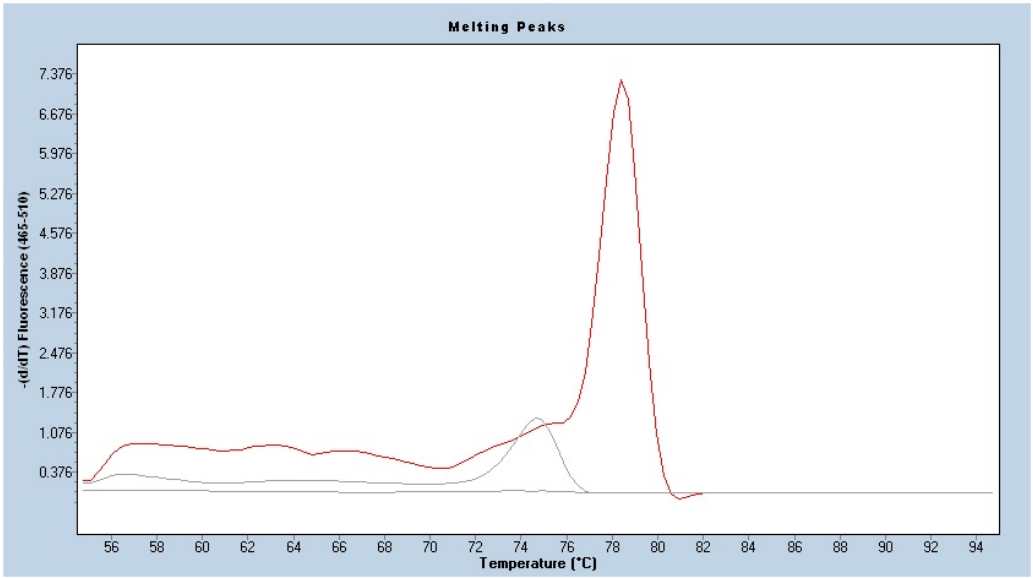
miR395 melting curve



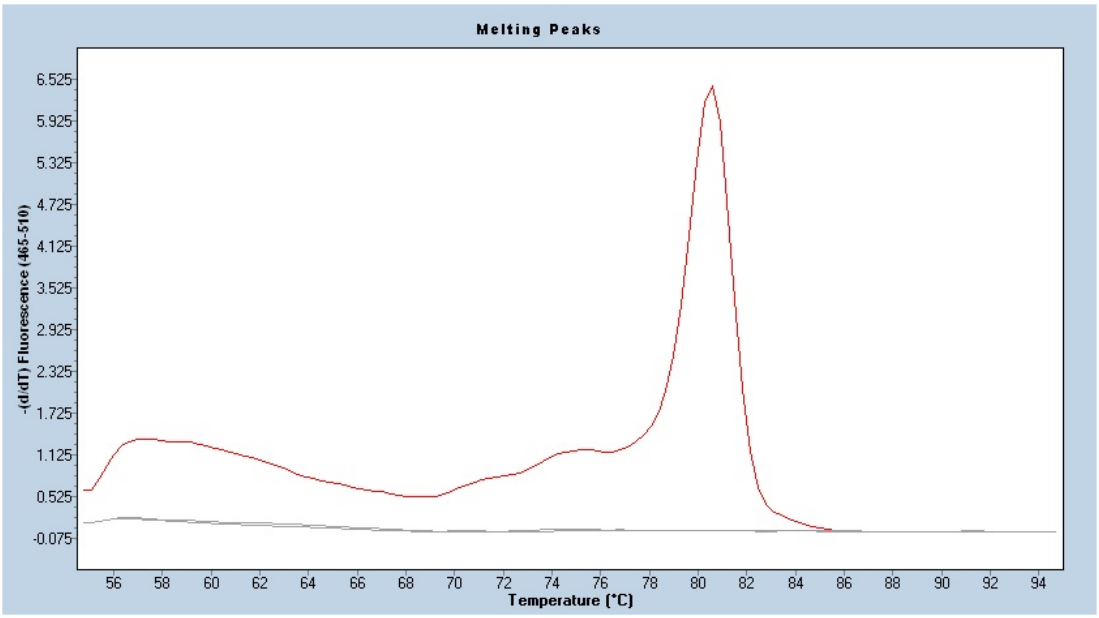
miR2275 melting curve



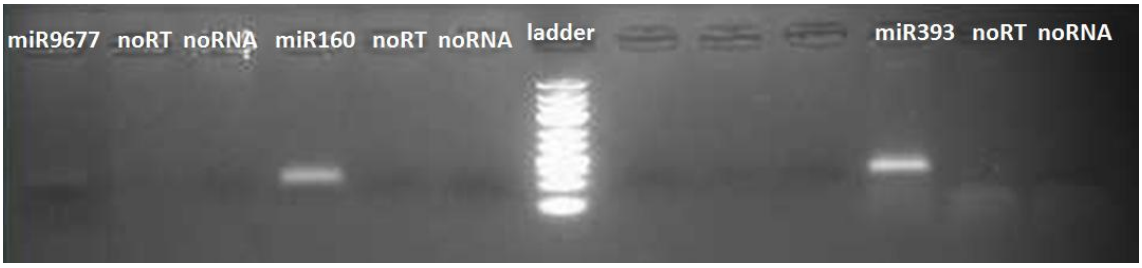
miR1878 and miR5062 gel image



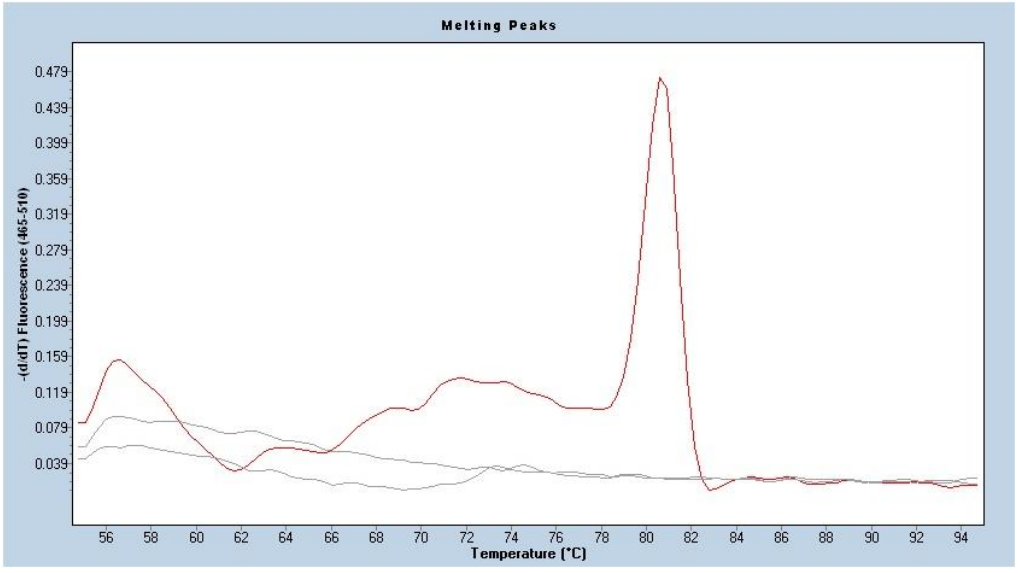
miR1878 melting curve



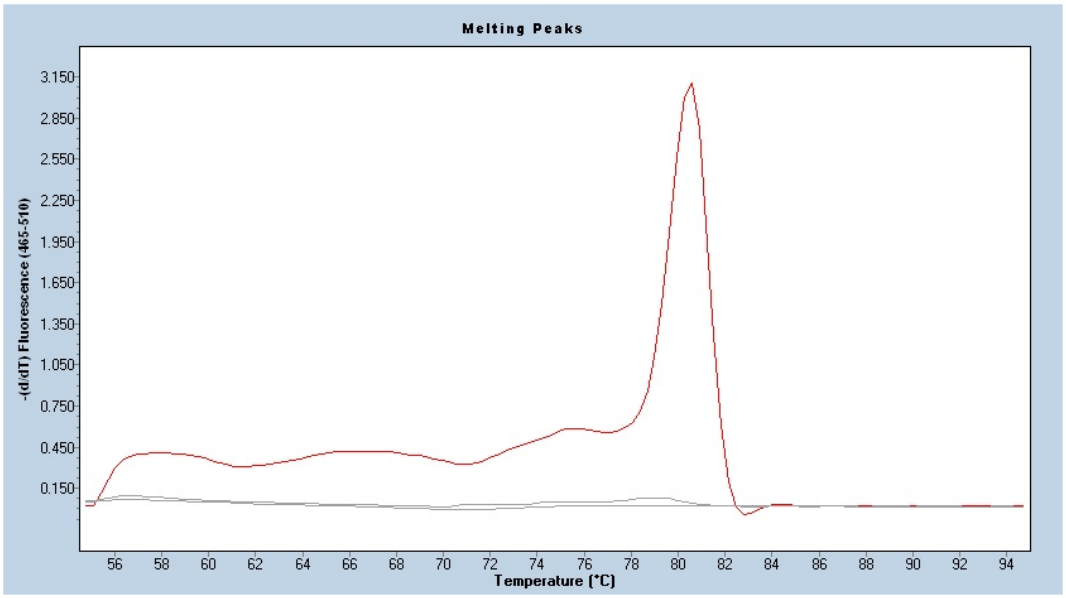
miR5062 melting curve



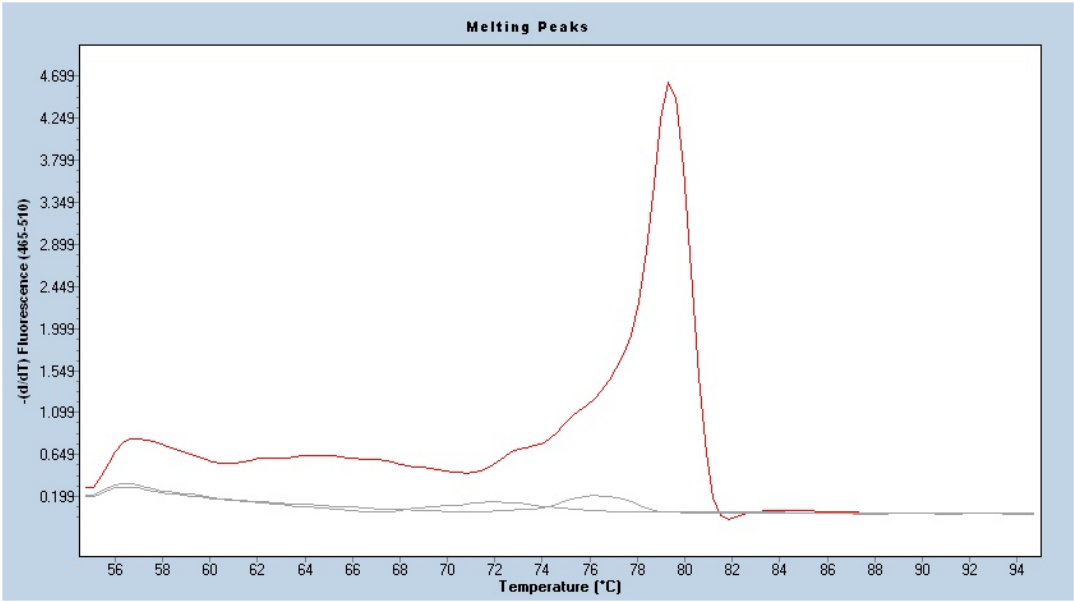
miR9677, miR160 and miR393 gel image



miR9677 melting curve



miR160 melting curve



miR393 melting curve

APPENDIX-II A

Chemicals

Antimic	Nanotego Co.	
Absolute ethanol	Riedel de Haen	32221
Isopropanol	Merck	1.09634
Potato Dextrose Agar	Biolife	4019352
Tryptic Soy Agar	Fluka	22091
Tryptic Soy Broth	Fluka	T8907
Nutrient Agar	Fluka	70148
Nutrient Broth	PhytoTech. Laboratories	n611
Cycloheximide	Applichem	A0879
Streptomycine sulphade	Applichem	A1852
Sodium Chloride	Sigma-Aldrich	S3014
Potassium Chloride	Sigma	12636
Sodium phosphate dibasic (Na_2HPO_4)	Sigma-Aldrich	255793
Potassium Dihydrogen Phosphate (KH_2PO_4)	Sigma	04243
Plate Count Agar	Fluka	70152

APPENDIX-II 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
Distilled Water:	Millipore, Elix-S, FRANCE
	Millipore, MilliQ Academic, FRANCE
Fiter papers:	Whatman General Purpose Filtration Paper WHASE1141,
	Sigma, MO, USA
Heating block:	HDV Life Sciences, AUSTRIA
	Thermostat Bio TDB-100, LATVIA
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:	Bosh, TÜRKİYE
Oven:	Memmert D06062 Modell 600, GERMANY
pH Meter:	WTW, pH540, GLP MultiCal, GERMANY
Refrigerator:	+4 oC Bosh, TÜRKİYE
	Ugur 374 DTK-Y, 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:	Nanodrop, ND-1000, USA
	Fourier Transform Infrared Spectrophotometer, Nicolet iS10
	Thermo Fisher Scientific, USA
Sterilizer:	Steri 350, Simon Keller Ltd., SWITZERLAND
Vortex Mixer:	VELP Scientifica 2X3, ITALY
Universal testing machine	Zwick/Roell Z100
Haze-Gard	BYK-Gardner, USA
Profilometer	P6 Surface Peofilometer, KLA-Tencor,USA
Thermometer	Isolab 059.02.002, Germany
High pressure spray nozzle	
Glass beads	
Tweezers	
Petri dishes	
Sterile scalpels	
Inoculation loop	

APPENDIX-II D

	PDA	PDA-ss	TSA-cyc
Lettuce-1 LSD	**55.4154**	**39.6099**	**51.4691**
Control-1%	61.1667	87.1667	74
Control-2.5%	78.6667	88.5	82,3333
Control-5%	102.333	100.667	99.1667
1%-2.5%	17.5	1.33333	8.33333
1%-5%	41.1667	13.5	25.1667
2.5%-5%	23.6667	12.1667	16.8333
Cucumber-2 LSD	**49.2981**	**51.2682**	**51.481**
Control-1%	13.3333	13.6667	-14.333
Control-2.5%	-3.66667	8.66667	13.6667
Control-5%	-128	-118.833	-99.667
1%-2.5%	-17	-5	28
1%-5%	-141.333	-132.5	-85.3333
2.5%-5%	-124.333	-127.5	-113.333
Banana-1	-----	**15.2261**	-----
Control-1%	-----	22.1667	-----
Control-2.5%	-----	26.9167	-----
Control-5%	-----	20	-----
1%-2.5%	-----	4.75	-----
1%-5%	-----	-2.1667	-----
2.5%-5%	-----	-6.9167	-----
Strawberry-day 4LSD	**0.091**	**0.082**	**0.107**
%5-D vs %5-S	~~-0.066~~	-0.137	-0.268
%5-D vs %2.5-S	-0.214	-0.287	-0.302
%5-D vs %2.5-D	-0.181	-0.241	-0.284
%5-D vs %1-S	-0.390	-0.519	-0.523
%5-D vs %1-D	~~-0.288~~	-0.436	-0.528
%5-D vs Kontrol	-1.078	-1.034	-1.256
%5-S vs %2.5-S	-0.148	-0.150	~~-0.034~~

%5-S vs %2.5-D	-0.115	-0.104	~~-0.016~~
%5-S vs %1-S	-0.323	-0.382	-0.254
%5-S vs %1-D	-0.222	-0.298	-0.260
%5-S vs Kontrol	-1.012	-0.897	-0.987
% 2.5-D vs % 2.5-S	~~-0.033~~	~~-0.046~~	~~-0.018~~
% 2.5-S vs %1-S	-0.176	-0.232	-0.221
% 2.5-S vs %1-D	~~-0.074~~	-0.148	-0.226
% 2.5-S vs Kontrol	-0.864	-0.747	-0.954
% 1-D vs % 1-S	-0.102	-0.084	~~0.006~~
% 1-D vs Kontrol	-0.790	-0.598	-0.727
%1-S vs Kontrol	-0.688	-0.515	-0.733
Strawberry-day8 LSD	-----	-----	**0.324**
%5-D vs %5-S	-----	-----	~~0.158~~
%5-D vs %2.5-S	-----	-----	~~0.002~~
%5-D vs %2.5-D	-----	-----	~~-0.064~~
%5-D vs %1-S	-----	-----	-0.749
%5-D vs %1-D	-----	-----	-0.708
%5-D vs Kontrol	-----	-----	-0.885
%5-S vs %2.5-S	-----	-----	~~-0.156~~
%5-S vs %2.5-D	-----	-----	~~-0.222~~
%5-S vs %1-S	-----	-----	-0.907
%5-S vs %1-D	-----	-----	-0.866
%5-S vs Kontrol	-----	-----	-1.043
% 2.5-D vs % 2.5-S	-----	-----	~~0.066~~
% 2.5-S vs %1-S	-----	-----	-0.752
% 2.5-S vs %1-D	-----	-----	-0.710
% 2.5-S vs Kontrol	-----	-----	-0.888
% 1-D vs % 1-S	-----	-----	~~-0.041~~
% 1-D vs Kontrol	-----	-----	~~-0.177~~
%1-S vs Kontrol	-----	-----	~~-0.136~~