

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**INVESTIGATION OF microRNAs ROLES DURING MAIZE LEAF GROWTH
RESPONSE TO PSEUDOMONAS PUTIDA**

ÖMER İLTAŞ
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

**PSEUDOMONAS PUTIDA İLE İNOKÜLE EDİLEN
MISIR BİTKİSİNİN YAPRAK BÜYÜME CEVABINDA
mikroRNA GENLERİNİN ROLÜNÜN
ARAŞTIRILMASI**

ÖMER İLTAŞ
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
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GEBZE

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SUMMARY

The aim of this study was to investigate the putative role of microRNA (miRNA) genes on the maize leaf growth response to plant growth promoting izobacteria *Pseudomonas putida* KT2440. For this context, 9 different maize (*Zea mays* L.) hybrids from Maize Research Institute (Sakarya/Turkey) were subjected to *P. putida* and their leaf growth response were evaluated at morphological, cellular and physiological at the seedling stage. As a results, maize hybrids were displayed different growth response to *P. putida*. In order to understand the leaf growth regulation response to *P. putida*, Sasa139 hybrid was selected for further enzymatic and transcriptional analyses as its leaf elongation rate were increased 15% in *P. putida* treated seedlings. It was observed that antioxidant enzymes activity difference between growth zones. Glutathione reductase (GR) and ascorbate peroxidase (APX) activity were significantly higher in the growth zones of inoculated leaves. Cell cycle genes that control switch between cell cycle checkpoints were highly up-regulated at meristem zone in both conditions. Growth-related miRNAs and their predicted targets were differentially expressed at growth zones. MiR160 and miR169 were significantly up-regulated at maturity in inoculated leaves. Mir319 and miR396 were significantly up-regulated at meristem and mature zones in response to *P. putida*.

As a result, tripartite interactions of maize-miRNA-KT2440 during leaf growth have been shown for the first time in this study. Transcriptional analyzes of cell cycle genes, growth-related miRNAs and their predicted target genes during PGPR interaction have been shown for the first time in the developmental stages of maize leaves.

Key Words: Maize, *Pseudomonas*, miRNAs, growth, PGPR, qRT-PCR.

ÖZET

Bu çalışmanın amacı, bitki büyümesini teşvik eden *Pseudomonas putida* KT2440 rizobakterisinin mısır bitkisi ile etkileşimi sırasında mikroRNA (miRNA) genlerinin yaprak büyümesi üzerindeki olası rolünü araştırmaktır. Bu bağlamda, Mısır Araştırma Enstitüsü'nden (Sakarya / Türkiye) alınan 9 farklı mısır (*Zea mays* L.) hibriti *P. putida* ile inoküle edilerek, fide aşamasındaki yaprak büyüme tepkileri morfolojik, hücresel ve fizyolojik olarak değerlendirilmiştir. Sonuç olarak, mısır hibritlerinin *P. putida*'ya farklı büyüme tepkileri gösterdiği gözlenmiştir. *P. putida*'nın yaprak büyüme regülasyonundaki rolünü daha ayrıntılı araştırmak için yaprak uzama oranında 15%'lik artış saptanan Sasa139 hibridi, enzimatik ve transkripsiyonel çalışmalar için seçilmiştir. Bu bağlamda yapılan çalışmalarda, antioksidan enzimlerin aktivitesinin büyüme bölgeleri arasında farklılık gösterdiği tespit edilmiştir. İnoküle edilen bitkinin büyüme bölgelerinde glutatyon redüktaz (GR) ve askorbat peroksidaz (APX) enzim aktivitelerinin istatistiksel olarak önemli derecede arttığı saptanmıştır. Hücre döngüsünün kontrol noktaları arasındaki geçişi kontrol eden hücre döngüsü genleri, her iki koşulda da meristem bölgesinde yüksek oranda ifade olduğu gözlenmiştir. Büyüme düzenleyici miRNA'lar ve tahmini hedef genlerinin ifadelerinin, büyüme bölgeleri arasında fark gösterdiği saptanmıştır. MiR160 ve miR169'un inoküle edilen bitkilerin olgunluk bölgesinde önemli derecede ifade oldukları tespit edilmiştir. Mir319 ve miR396'nın ifadelerinde, *P. putida* ile etkileşimi sırasında meristem ve olgunluk bölgelerinde önemli derecede artış saptanmıştır.

Sonuç olarak, bu çalışma ile yaprak büyümesi sırasındaki mısır-miRNA-KT2440 üçlü etkileşimi ilk kez gösterilmiştir. PGPR etkileşimi sırasında hücre döngüsü genlerinin, büyümeyle ilgili miRNA'ların ve bunların tahmini hedef genlerinin transkripsiyonel analizleri, mısır yaprağının gelişim evrelerinde ilk kez gösterilmiştir.

Anahtar Kelimeler: Mısır, *Pseudomonas*, miRNA, büyüme, PGPR, qRT-PCR.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
°C	: Celcius
µg	: Micro gram
g	: Gram
µl	: Microliter
Ca ⁺²	: Calcium
°S	: South
°N	: North
gr	: Gram
mg	: Miligram
nt	: Nucleotide
ddH ₂ O	: Double distilled water
H ₂ O ₂	: Hidrojen peroksit
ml	: Mililiter
cm	: Centimeter
mM	: Milimolar
kcal	: Kilocalories
M	: Molar
O ₂	: Oxygen
lt	: Liter
lx	: Lux
<i>AN3/GIF1</i>	: <i>ANGUSTIFOLIA 3/ GROWTH-INRERACTING FACTOR 1</i>
ANT	: <i>AINTEGUMENTA</i>
APX	: Ascorbate peroxidase
ARF	: Auxin response facto
BBP	: Basic blue proein

BSA	:	Bovine serum albumine
bZIP	:	Basic leucine zipper domain
CAT	:	Catalase
CC	:	Chlorophyll content
CDK	:	Cyclin dependent kinase
CDKA	:	A type cyclin dependent kinase
CDKB	:	B type cyclin dependent kinase
cDNA	:	Complimentary DNA
CDS	:	Coding sequence
CFU	:	Colony forming units
cm	:	Centimeter
CP	:	Cell production
CycD	:	D type cyclin
DAPI	:	4'-6-diamino-2-phenylindole
DCL	:	Dicer benzeri enzim
DIC	:	Diffrential interference microscope
DNA	:	Deoksiribonükleik asit
dNTP	:	Deoxynucleotide
dNTP	:	Deoksinükleotit
EDTA	:	Ethylene Diamine Tetra Acetic Acid
EtOH	:	Ethyl alcohol
FLL	:	Final leaf length
GFP	:	Green fluorecent protein
GR	:	Glutathione Rreductase
GRF	:	Growth regulating factor
GSSG	:	Glutathione Disulfide
IAA	:	Indol acetic acid
LA	:	Leaf area
LAC	:	Laccase
LB	:	Luria-Bertani
LER	:	Leaf elongation rate

LL	:	Leaf length
MCI	:	Mature cell length
miRNA	:	Micro RNA
mRNA	:	Messenger RNA
MYB	:	Metiloblastosist
NADPH	:	Nicotinamide Adenine Dinucleotide Phosphate
NBT	:	Nitroblue tetrazolium
NF_YA	:	Nuclear Transcription Factor Y Subunit Alpha
PGPR	:	Plant growth promoting rhizobacteria
POX	:	Peroxidase
PVP	:	Polyvinylpyrrolidone
q-RT-PCR	:	Quantitative real time polymerase chain reaction
RISC	:	RNA-induced silencing complex
RNA	:	Ribonucleic acid
rpm	:	<i>Revolutions per minute</i>
SDW	:	Shoot dry weight
SFW	:	Shoot fresh weight
SL	:	Shoot length
SOD	:	Süperoxide Dismutase
SPL	:	Sporocyteless protein
TCP	:	Transcription factor protein

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1. INTRODUCTION

Zea mays L. is a single annual plant that belongs to *Gramineae* family, which can reach a height of 1-2 m. and originated from South America. It is the third most consumed grain across the world after wheat and rice. Maize is mostly consumed as a second stage product in the form of dairy products in developed countries. In developing countries, maize serves as an essential nutrient for approximately 200 million people. In addition to that, processed form of maize can be turned into biofuel (ethanol) and starch as well. The processed form of starch used as household items (such as syrups, glue, cosmetics, aspirin, etc.) and enzymatic conversion (such as; sorbitol, lactic acid and dextrin). As it is seen in, maize plant, which we use in many areas of life, has actually evolved from a wild grass plant that called Teosinte. As a result of the domestication and several breeding efforts since ancient times teosinte gained an edible fruit and biomass. However, due to the rapid reduction of arable agricultural areas as a result of the wrong soil management and the loss of the crop sown due to various biotic and abiotic stresses, classical breeding activities have become unable to challenge with rapidly rising population. Considering all these beside the classical breeding activities, the need for using biotechnological methods is clearly seen in order to obtain higher yields from the cultivated unit area (promoting growth) and resistant plants against various stresses by unraveling the genetic mechanisms of stress tolerance.

Plant growth is defined as the increase in volume, which is generally not recyclable. Growth at the molecular level is a set of functions controlled by cell division and cell expansion genes spatially and temporarily. Plants reach final size with increasing the cell number by cell division and cell size by cell expansion. Thus, the size of a plant organ depends on the number of cells and cell size [Horiguchi et al., 2006]. In this respect, scientists are investigating cellular and genetic regulation that promote cell production and expansion for elevating biomass [Ferjani et al., 2007]. Cell division and cell expansion is a mechanism which is organized with the participation of many genes at the molecular level. Although the basic mechanism

of these two growth functions at the molecular level is known, information about their regulation during the interaction of various microorganisms is limited.

The relationship between grain crops and rhizospheric bacteria has evolved for more than 60 million years ago when the first definitive evidence for grass pollen existed [Kellogg et al., 2001]. This co-evolution period resulted in an extensive variety of grain-biotic interactions from beneficial to detrimental. Interaction between grain-rhizospheric bacteria has attracted great attention because it ensures opportunities for production of sustainable food from grains. *Pseudomonas* species; specifically: *P. fluorescens* and *P. putida*, have been widely studied for their capacity and ability to support plant growth mechanism [Gravel et al., 2007]. Plant growth promoting rhizospheric bacteria (PGPR) support growth via inoculation on seed and colonization that were first identified by Kloepper and Schroth (1978).

Plant growth promoting rhizospheric bacteria show their effects by increasing their metabolism (phosphating, hormone production or nitrogen supply) or by directly using plant metabolism (water and mineral intake), root development, enzyme activity or the interaction of plant with other beneficial microorganisms [Gupta et al., 2000]. Interaction between plants and bacteria is largely facilitated by the rhizospheric soil that supports plant growth [Kloepper et al., 1980]. The growth mechanism promoted by rhizospheric bacteria are quite complex and involves in the host plant physiology with the microbial balance in the rhizosphere. In a study conducted by Kloepper et al., 1980, it was reported that PGPR inoculated cultivated potato increased in yield and growth in field conditions. Most of the research focused on increasing yield and promoting growth by inoculation of beneficial symbiotic rhizobacteria. In addition, it has been discovered that free living rhizospheric bacteria such as *Pseudomonas* and various *Azospirillum* can also be used to support plant growth.

P. putida KT2440 rhizobacteria have been extensively studied to colonize a wide range of plants, to adhere to corn seeds and to arrange colonization through cell to cell communication [Fernández et al., 2012]. *Pseudomonas putida* KT2440 has two auxin carrier genes. The genome of *P. putida* W619 also encodes an enzyme of salicylate 1-monoxygenase (PputW619_2140) that functions the

conversion of salicylate to catechase. This enzyme generally functions in plant growth, development and defense against pathogens. Homologues of this gene are also found in *P. putida* KT2440 genome. It is also assumed that KT2440 has indole acetic acid (IAA) that helps plant growth which is derivate from auxin hormone [Wu et al., 2010]. IAA-producing PGPRs stimulate growth and length of root, further enhancing root area, allowing the plant to reach and get more nutrients from soil [Gilbertson et al., 2007]. In addition to the growth-promoting microorganisms, it has been discovered that some other transcriptional regulation mechanisms are also involved and serve significant functions in growth and development in plants.

Other transcriptional players of these mechanisms are the small RNAs. Small RNAs that function in post-transcriptional gene regulation in plants and animals are known as microRNAs (miRNAs). miRNAs are a class of non-coding small RNAs (sRNAs) which are 19-25 nucleotides in length and adversely affect gene expression by causing mRNA degradation or translational inhibition [Bartel, 2004]. MiRNAs, which are highly conserved among species, were first discovered in studies on *Caenorhabditis elegans* in 1993 [Lee et al., 1993]. Studies on the role of growth and development of plant miRNA genes has attracted a lot of attention in recent years. In this context, in the literature, it has been shown that miR396 in *Arabidopsis* affects leaf growth and plays a role in controlling cell number [Liu et al., 2013]. In another study, miR319, has been shown to play a role in leaf development by regulating the *LANCEOLATE* gene that is target of transcription factor in tomato [Ori et al., 2007].

In this context, the main goal of this study is to clarify molecular mechanism of growth-related microRNAs and their putative targets in maize seedlings in response to *Pseudomonas putida* KT2440.

1.1. Aim, Contribution and Content of the Thesis

Plant growth and PGPR interaction are a complex mechanism which includes numerous genes to regulate growth and development. Although plant-microbe interactions responses of several plants have been studied from the point of some enzymatic activities, enzymatic regulation pathways and some post-translational regulations, miRNA-mediated gene regulation during maize growth mechanisms have not been studied yet. Thus, the main goal of this study was to gain a clear understanding of the regulations of these complex genetic mechanisms. It is important to elucidate the roles of miRNAs and their predicted target genes under the bacterial interactions. Another important point was to reveal out which growth mechanisms (cell division and expansion) were affected from those miRNAs and their target genes. In accordance with this purpose; in this study 9 different maize (*Zea mays* L.) hybrids were treated with *P. putida* KT2440 (10^6 CFU/ml) and as well as non-treated ones will be grown in sterile soil. Then, they were screened at morphological, physiological and cellular levels. The best growing one was selected and used in transcriptional and enzymatic studies. Tissue samples from meristem, elongation and mature zones from control and inoculated plants were harvested after 48 hours after the appearance of the third leaf and then statistical analyzes were performed. Enzyme activity of inoculated and non-inoculated plant were measured by spectrophotometry. The bacteria-plant interaction related microRNAs and their targets were identified *in silico* using bioinformatical tools. For determining transcript levels and analyzing data, quantitative-Real-Time-PCR (qRT-PCR) method was used.

In this study, *P. putida* KT2440 and *Zea mays* interaction were investigated at morphological, physiological, cellular, enzymatical and transcriptional levels. Functions of miRNA genes in maize leaf growth response to PGPR treatment were analyzed. Maize-miRNA-KT2440 tripartite interactions were investigated for the first time in this study.

2. GENERAL INFORMATION

2.1. Maize (*Zea mays* L.) Biology

Maize (*Zea mays* L.) is one of the most consumed cereals belonging to the Gramineae family (Figure 2.1) [Gaut et al., 2000]. It is an annual diploid plant and has 10 chromosomes in its genome ($x = 10$, $2n = 20$). Maize is thought to have emerged 55-70 million years ago in Central or Southern Mexico. It has a wide range of cultivated geographical area from Chile 40 °S to Canada 50 °N at altitudes of 3400 meter above sea level [Tenaillon and Charcosset, 2011].

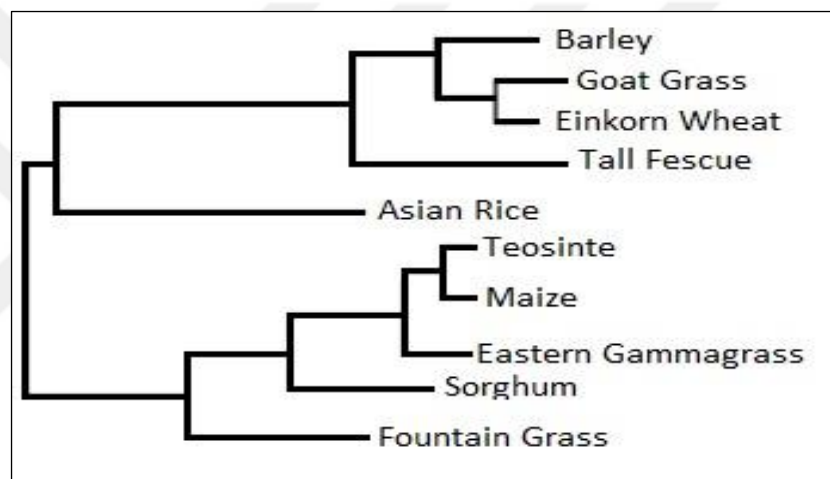


Figure 2.1: A phylogeny of diploid grass species.

The plant has abundant branched and thin root system and in optimal conditions length of roots can reach up to 1500 meters. Maize stem has approximately twenty leaves on which is arranged spirally and in opposite rows. Blade of leaf is narrow, long and wavy towards the tip and hairless. The leaves are braced by a pronounced middle rib along its overall length. Along the whole leaf surface stoma formed in rows. Abaxial surface of leaf has more stomata than on the adaxial surface. Motor cells are occurs on the upper-side of leaf surface (Figure 2.2).

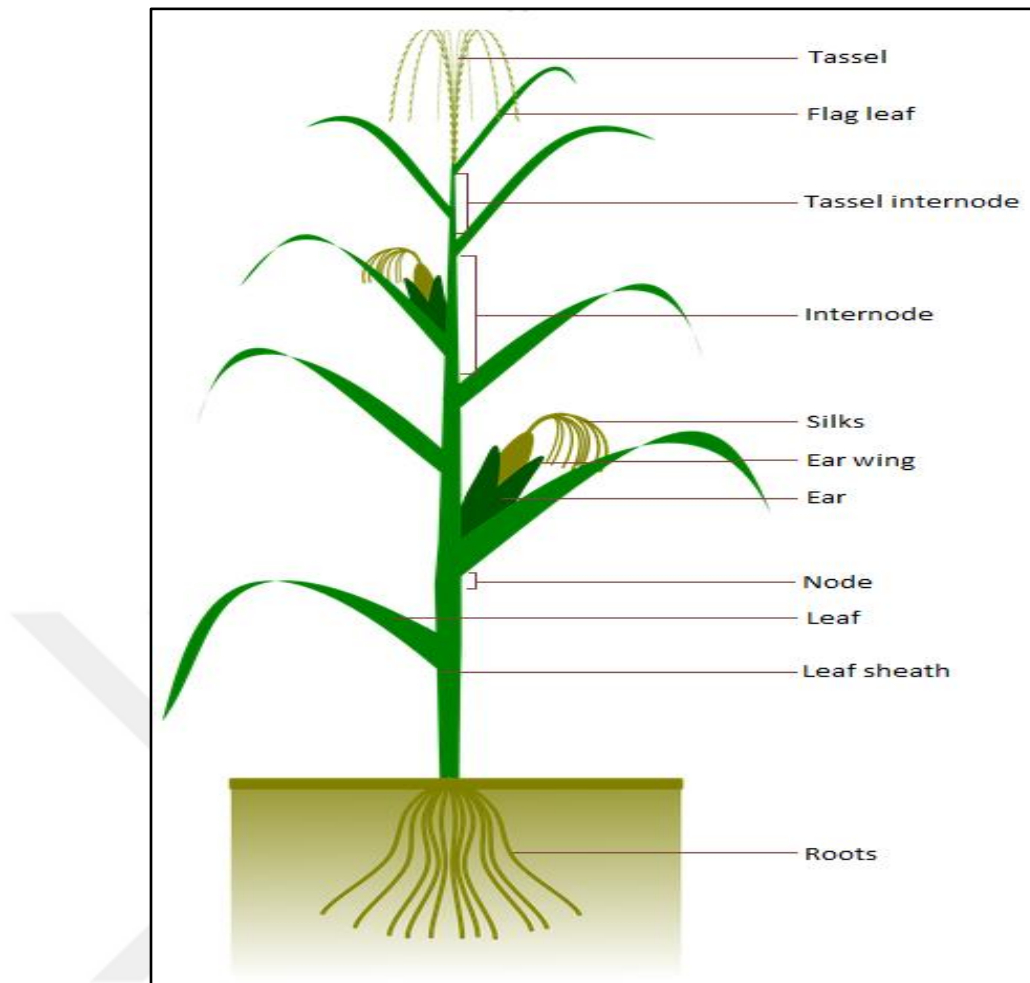


Figure 2.2: Schematic representation of parts of the corn plant.

Maize is monoecious plant, which means that the reproductive organs (male and female) are found on the same plant. The silk of the flowers which is located at the bottom emerge first. The flowers ripen after 60-70 days of plant growth. Male staminate flowers transform into tassels, located at the top end of the main stem. Female pistillate flowers are located in the nodes along the stem and found in one or more ears [Du Plessis, J. 2003].

Maize (*Zea mays L.*) has the largest production of approximately 800 million tons of product types produced in 2013 and constitutes 32% of the total grain production all around the world. According to the statistical data, 90% percent of this product used as human nutrition and animal feed (approximately 65-70% of produced maize) and 20% of this produced maize from 90% consumed directly by humans. Remaining part (8-10%) is for industrial use. Besides being an economically important cereal, for more than 100 years, maize (*Zea mays L.*) has been used as a

model organism for genetic and molecular studies. Genetic mapping, cytogenetics and epigenetics studies have successfully been done in this organism [Freeling and Walbot, 1994]. Because of these extensive characteristics of maize, it attracts great attention for future uses and studies.

2.2. Plant Growth

The irreversible persistent increase in the size of an organ or in a single cell is described as growth. In general, this irreversible process is accompanied with by anabolic and catabolic mechanisms and energy expenditure. Unlimited growth capacity of plants throughout their life make them unique organisms. This unique ability of plants is by virtue of the presence of meristems in certain places in the body. The meristem cell has capable of division and self-sustaining. The meristem cell loses its capacity by the time and divide and turned to the form the plant stem. This type of growth, in which new daughter cells are added to total body with the activity of meristem, is defined as open growth [Gonzalez et al., 2012]. The growth period of plant usually occurs in three stage; the meristematic, elongation and maturation (Figure 2.3) (Web 1). Meristematic stage include continuously dividing cells, both at the root apex and the shoot apex. The cells near the meristematic zone represent the elongation zone. In this phase, enlargement of cell and new cell wall deposition occurs. Further away from elongation zone, lies the part of the axis which undergoes the maturation phase. The cells in this zone reach their maximum dimensions in terms of wall thickening and protoplasmic modifications.

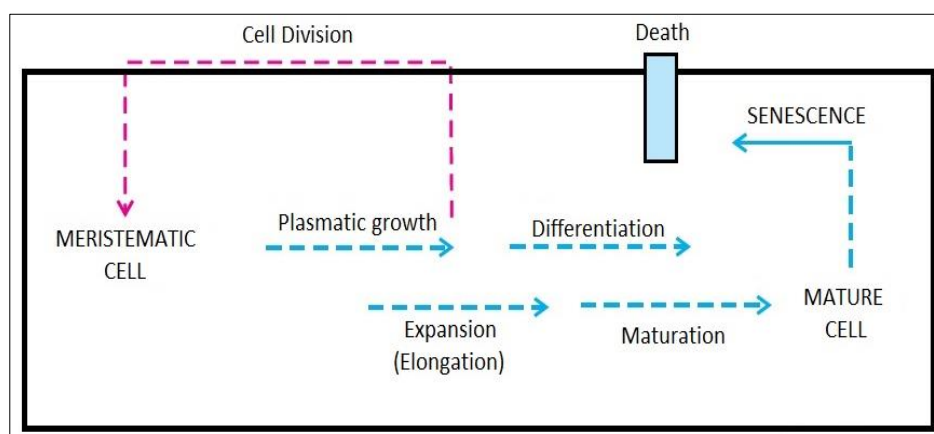


Figure 2.3: The growth and developmental processes of a plant cell.

Cellular growth is mainly a result of an increase in the quantity of protoplasm. Since it is difficult to measure the increase in protoplasm directly, one measures some amounts, which more or less proportional. Thus, growth is evaluated by various parameters which include; fresh and dry weights, length, area, volume and increase in cell number. Each plant has its own growth rate, which is highly variable among plants. For example; apical meristem of maize root can lead to more than 17,500 new cells per hour, while watermelon can increase up to 35,000 times. Growth is mainly expressed as an increase in the number of cells; the second refers to as an increase in the cell size [Taiz and Zeiger, 2006].

Concisely, meristematic cells lead to increase in plant organs and allowed to the plant to grow throughout its life cycle. During development, cell enlargement and cell wall changes, loosening and *de novo* synthesis occurs in expansion phase. Cell maturation phase is the final differentiation process and cells become fully functional. All these phases are under the control of complex gene regulations. In previous studies, by analyzing mutants and transgenic lines, it has been suggested that coordination of developmental stages under the genetic regulations is significant for final organ size. In this context, scientists have tried to identify genes involved in growth by making targeted mutations to observe variation in cell number and size.

Studies have shown that the number and size of small-leaf mutants decreased significantly but this could be the result of mutation of photosynthetic genes [Horiguchi et al., 2006]. In the same study, results indicate that the mutants were larger than the wild type and the cells number were increased. However, there were no cases where the cell size and number was increased at the same time. Namely, overexpressed mutants transcription factors such as *AINTEGUMENTA (ANT)*, *Arabidopsis thaliana GROWTH REGULATING FACTOR 5 (AtGRF5)* and *ANGUSTIFOLIA 3/ GROWTH-INTERACTING FACTOR 1 (AN3/GIF1)* which increased the leaf size in wild types is linked to give rise in cell number. However the size of cell in these plants remained the same [Mizukami and Fischer, 2000]. In contrast, the increase in leaf size was found to be proportional to cell size in mutants that overexpress AtGRF1 [Kim et al., 2003].

Besides, studies carried out to date has shown that some post-transcriptional regulators, called miRNAs, play versatile roles in plant growth and development as well. In consequence of these studies, it has been revealed that several growth-related miRNAs were preserved among several plant species. For instance, miR159; target the MYB and *TCP* family transcription factors (TFs) and play a role in floral asymmetry and leaf development [Jones et al., 2004], mir166; encode ATHB-15, MYB and bZIP family TFs, and play role in axillary initiation of meristem, leaf and vascular development [Qian and Huan, 2015], mir396 target gene is GRF family, TFs and CP29 and play role in leaf and cotyledon development, mir164; encode *TCP* and *CUC2* gene family and play role in leaf development [Liu et al., 2009; Rodriguez et al., 2010]. miR319 and its predicted target *TCP* (*TEOSINTE BRANCHED1/CYCLOIDEA/PCF*) affect growth through direct regulation of cell cycle genes or modifications of the signalling of hormones such as cytokinin and gibberellin. *TCP* genes promote the transition from proliferation to differentiation, and overexpression of miR319 leads to an extended proliferation phase leading to larger and more complex leaves [Ori et al., 2007].

2.3. Plant Cell Cycle

The cell cycle is an operation for growth of the plant including a series of biochemical activities and morphological modifications starting with the perception of the division signals of the cells. Plant cell cycle including 4 essential component phases; post-mitotic inter-phase (G1), DNA synthesis phase (S-phase), pre-mitotic inter-phase (G2) and mitosis/cytokinesis. Division of cells in plants is under the control complex mechanism which controlled by cyclin-dependent kinases (CDKs) and cyclins (Figure 2.4) [De Veylder et al., 2007].

Plant genomes are codify many different cyclin-dependent kinases (CDKs) and cyclins. Model organism *Arabidopsis thaliana* has 12 separate well-intentioned CDKs and more than 49 different types of cyclin [Vandepoele et al, 2002 and Wang et al., 2004]. CDKs and cyclins are classed in different groups according to their sequence, which is a classification correspond to their functional variety. The main drivers of the plant cell cycles are the A, B and D type CDKs. A type CDKs (CDKA) are

mainly concerned with mammalian CDK1 and CDK2 and during cell cycle control the G1-S and G2-M transition [Inzé and De Veylder, 2006]. B-type CDKs (CDKBs) are mainly specific to plant and accumulate depend on the phase of the cell cycle, attainment to the maximum level of G2-M transition where their activity is required [Boudolf et al., 2006]. D-type cyclins (CYCD) regulator of the G1-S transition, but there is accumulating proof that CycD's may play a role in G2-M transition as well [Vandepoele et al, 2002].

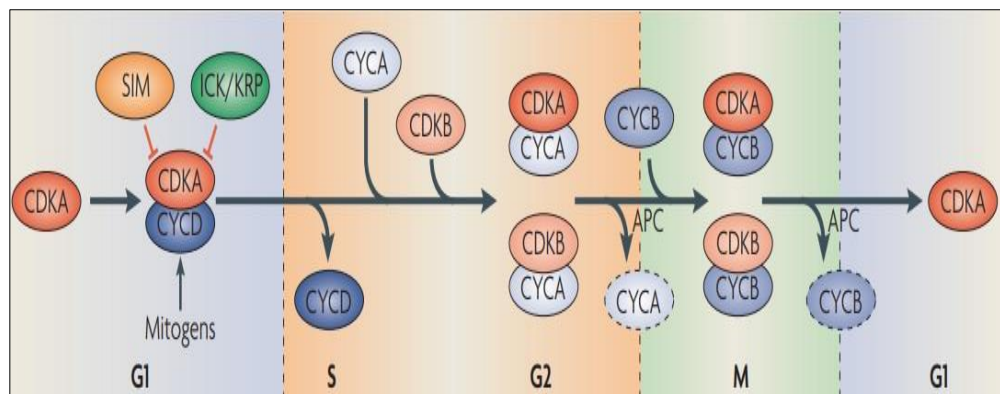


Figure 2.4: The major cell-cycle machinery during cell division processes in plants.

Cell cycle processes requires the successive unity of CDKs with different cyclin types. According to past studies a number of deviations from this general functional assignment have been reported. For example, *CYCA2* showed that it has promoted to specific cell cycle kinase activity during G2-M transition in *Medicago sativa* [Roudier et. al., 2000]. The two different CDKB sub-groups were found in both monocots and dicots; this finding indicate their unique role in the cell cycle regulation for each of the CDKB subgroups. During the S, G2 and M phases *CDKB1* transcripts actively accumulate while the expression of *CDKB2* is specific to the G2 and M phases. [Breyne et. al., 2002; Corellou et al., 2005 and Fobert et al., 1996].

2.4. Maize Leaf Growth and Development

The leaves of monocotyledons are usually strip-shaped and have narrow footing and the leaf base surrounds the body. They grow from the basal meristem. Leaf development in monocot varies considerably. In some families, the leaf

primordium has a distinct abaxial and adaxial surface. Development occurs as a result of continuous meristematic divisions towards the apical growth remains in its original state and the growth center is established in an abaxial manner. Vertical growth on the leaves begins from the end of the primordium. The vegetation point at the end stops after a while, intercalations that occur in the basal part are provided by the tissues formed at the base with the meristem activity and growth. The transverse growth takes place in the lamina. Sub-epidermal cells add new cells to the top and bottom of the leaf, while the middle vein and the lamina differentiates. Factors determining the specific shape of the leaf; shape of leaf primordium, number, location and orientation of cell division, amount and distribution of cell expansion. The growth zone of maize leaf covers about 10 cm (Figure 2.5). This growth zone is separated like; first 3 centimeters of the growth zone is part of meristem, 4-9 centimeters is part of elongation and 9-10 centimeters is part of maturity zones [Avromava et al., 2015].

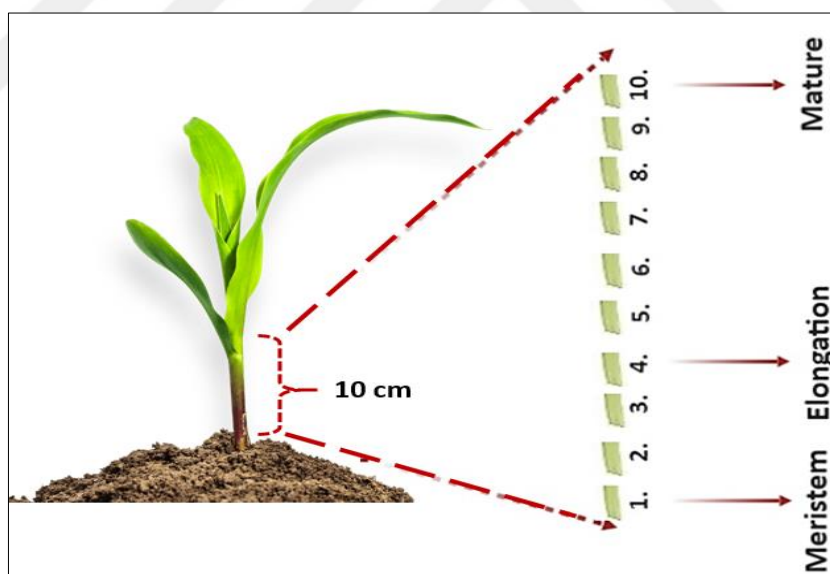


Figure 2.5: Growth zone of maize leaf.

In general, maize plant has particular growth stages are numbered from 0 to 10 (Figure 2.6) [Web 2]. The growth stage is the zero stage which involves seed sowing until the seedling can be seen just above the soil surface. Growth stage 10 cover when the biological maturity occurred. The stage of 0 includes the part from sowing to seed formation. Germination part start with of whole stem with growth

point which take part under 25 to 40 mm of soil surface. Under warm and humid conditions seedlings became visible between six or ten days. The temperature conditions for germination is ranged between 20 °C and 30 °C with together 60% humidity. At stage one, maximum leaves and the lateral shoots are preformed and newly occurred leaves are opens up and down every three days. At this stage, growth zone is still under the surface of the soil.

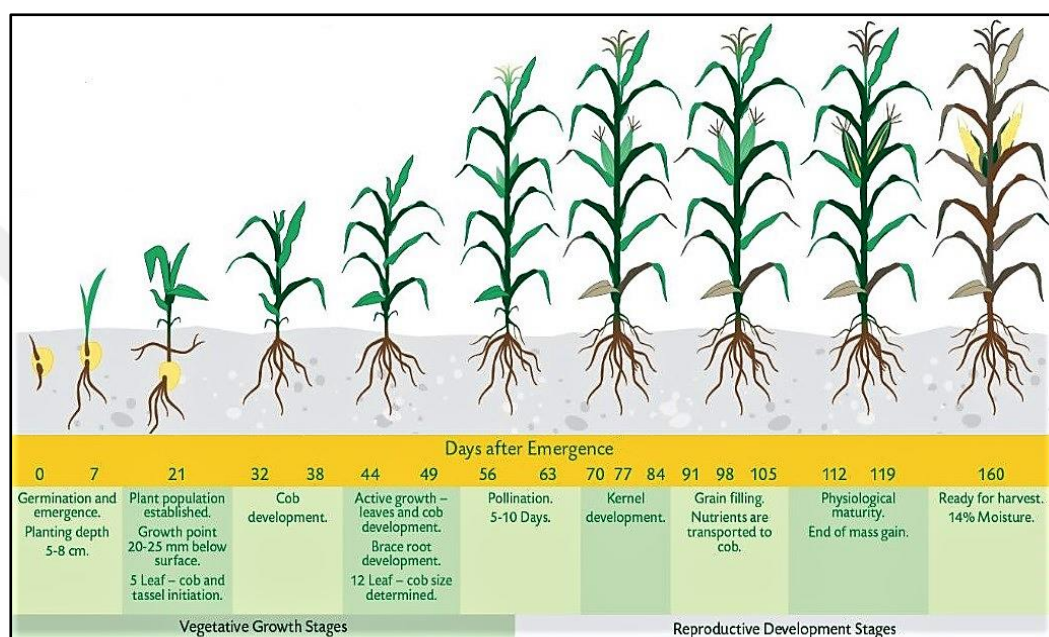


Figure 2.6: Growth stages of Maize.

Growth stage 1 followed next step by completely opening of eight leaves. In this stage, the leaf area of plant increases as 5-fold, while the stem mass increases 50-folds. The initiation of the ear has already begun at stage 1. Tillers are start to forming from the nodes under the soil surface. In stage 2, the growth zone is about 5.0 to 7.5 cm higher than the soil surface. Stage 3, in growth result as comprise of twelve leaves on stem. The tassels were start to develop swiftly. Lateral shoots with cobs increase swiftly from the sixth to eighth nodes. Growth stage 4; the stem extends very fast and the tassel develops almost completely.

Stage 5 in growth; all leaves are fully opened and the tassels are became visible. The stage 6; the ear, lateral shoot and bracts developed completely and starch start to accumulate in the endosperm. Grain mass of maize plant hold to increase and sugars are converted into starch at the stage 7. In stage of 8, sugars in

the kernel start to disappear swiftly. In stage of 9, kernels are reaches its maximum dry mass, a black cell layer develops at the base of the kernel.

Lastly maize passed to through growth stage 10; while grains have reached physiological maturity, they have to reduce moisture before reaching biological maturity. Drying of grains occurs about 5% up to 20% per week, followed by deceleration. Biological maturity occurs and the kernels are became completely dried.

2.5. Plant Microbe Interactions in the Rhizosphere: Plant Growth Promoting Rhizobacteria (PGPR)

The root is an organ which is a chlorophyll-free section that grows downwards and sideways below the ground, connects the crops to the soil by forming arms, and helps them absorb nutrients from surrounding soil. This interaction creating a root-soil interface known as rhizosphere .The term rhizosphere, formed by the Greek words rhiza (root) and sphere (influence zone), and firstly defined by the plant-physiologist Hiltner in 1904. Nutrients rich this zone is parallel to the bulk soil, by virtue of accumulation of various organic compounds and flows freely by exudates, secretion and deposition from the roots [Ligaba et al., 2004]. Root exudates are nutrient rich and contain high and low molecular weight compounds like polysaccharides, proteins, organic acids, vitamins, fatty acids and other compounds like carbon dioxide, protons, hydroxides, etc. [Ahmad et al., 2011]. Microbial growth and activity in the rhizosphere is intense while organic compounds are used as carbon and energy sources by microorganisms from this zone. Plant and the bacterial population living in the rhizosphere bring several kinds of connections together such as being mutualistic, symbiotic or harmful to the host if the microbes are pathogenic. However, the bacterial realization of root colonization is subject to complex procedures involving survivability, latitude and competition with native rhizospheric microorganisms [Somers and Vanderleyden, 2004]. The role of bacteria in the rhizosphere identified by studying the genes and proteins involved in colonization of root is understood in the case of *Pseudomonas*.

Recent studies showed features of proteins joined in adherence to biotic surfaces and biofilm formation [Yousef et al., 2008; Nielsen et al., 2011; Nilsson et al., 2011], proteins that playing role in flagella formation [Duque et al., 2013], proteins takes place in chemotaxis processes [Matilla et al., 2007], proteins related to stress adaptation mechanisms [Reva et al., 2006], proteins involved in transport processes, protein secretion [Barret et al., 2011], and metabolism related proteins [Ramos-González et al., 2005; Matilla et al., 2007; Cheng et al., 2009] pointed out that strong selective pressures are acting in the vicinity of roots (Figure 2.7) [Matilla et al., 2007].

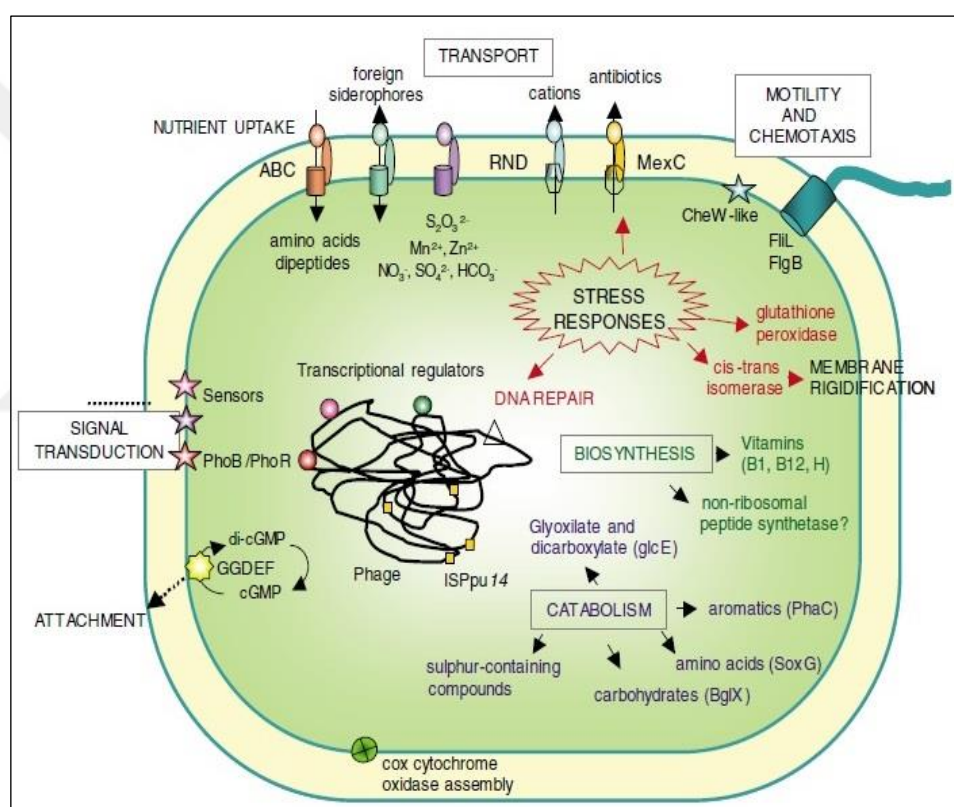


Figure 2.7: Scheme showing relevant bacterial functions induced in rhizosphere-pseudomonas interaction.

Plant interacted bacteria that can colonize roots surfaces are called rhizobacteria and can be divided into useful, harmful and neutral groups based on their effect on growth. Useful rhizobacteria stimulating the growth of plants are firstly described by Kloepper and Schroth (1978) and commonly mentioned to as plant growth-promoting rhizobacteria (PGPR). PGPRs colonize the roots of several plants by following inoculation onto seeds, and promote growth by direct or

indirect mechanism in plants. Those microorganisms have common traits which comprise the colonization process include the (1) ability for surviving after inoculation onto seed; (2) attaching to the surface of roots; and (3) start colonizing for promote root system [Kloepper, 1994]. It has been reported that several isolated PGPR has been successfully in promoting the growth of crops such as maize, canola, soybean and wheat [Glick et al., 1997; Timmusk et al., 1999]. Increasing of plant growth by PGPR demonstrates its capacity as use biofertilizers in agriculture. Bertrand et al. (2001) described that genus of *Pseudomonas*, *Varivorax*, *Agrobacterium* and *Phyllobacterium* are the most productive PGPRs that interact with plants.

2.5.1. The Pseudomonas Genus

Genus of *Pseudomonas* are considered to be ubiquitous in the environment, since they can be found in a variety of milieus all along the globe. The name of *Pseudomonas* were first identified and defined in rather vague terms by Migula in 1894. Genome sequence of *Pseudomonas* species were completed and published in 2000. More recently, other strains has been sequenced and published which is include; *P. aeruginosa* strains PAO1 (2000), *P. putida* KT2440 (2002), *P. protegens* Pf-5 (2005) and *P. syringae* pathovar tomato DC3000 (2003). Among the described species *Pseudomonas putida*, *Pseudomas fluorescens*, *Pseudomonas aeruginosa*, etc. being up to 200 species conforming the group [Palleroni and Moore, 2004]; the G+C content is 58% to 69% of genome. Taxonomic classification of *Pseudomonas* is as showed in Table 2.1

Table 2.1: Taxonomic classification of *Pseudomonas* species.

Domain: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Pseudomonadales
Family: Pseudomonadaceae
Genus: <i>Pseudomonas</i>

Pseudomonas genus are oxidase positive and metabolise glucose via the Entner-Doudoroff pathway [Castillo et al., 2007]. This genus includes water and soil saprophyte bacteria with a high metabolic versatility, can colonize a wide variety of media and assimilate a variety of carbon sources, including fractionous compounds. There are strains that can synthesize iron chelators with iron chelators in their iron-limiting conditions. The characteristic trait between species is the presence of plasmids that provide the ability to cope with antibiotics, other antibacterial agents and catabolize toxic compounds; 13 plasmids groups have been identified to be compatible with *Pseudomonas* species [Greated et al., 2002]. There are members with potential uses and interests in environmental and agricultural sectors, such as pollutants degraders and plant growth promoting bacteria as *Pseudomonas putida* and *Pseudomonas fluorescens* [Lugtenberg and Dekkers, 1999]. *Pseudomonas* is easily cultured with a wide variety of nutrition under laboratory conditions, with versatile carbon sources, neutral pH and mesophyll temperature range [Palleroni, 1986].

Among the sequenced *Pseudomonas* there innocuous strains like *Pseudomonas putida* KT2440 [Nelson et al., 2002] and *Pseudomonas fluorescens* Pf-5 [Paulsen et al., 2005]. Genomic analyses of these innocuous strains revealed that they were not carry genetic machinery that encoding exo-toxins, hydrolytic enzymes and type-III secretion systems. However, both types of strains shares feature like stress responsive proteins, adhesins and type IV pili [Nelson et al., 2002]. Some *Pseudomonas* strains can synthesize phytohormones, iron chelators, to solubilize soil-less compounds, toxic substances to other microorganisms, which make them impressive candidates for agriculture as PGPR and/or biocontrol agents

[Haas and Keel, 2003]. Other members of the genus are able to cope with and / or catabolize environmental pollutants, this successfully managing make them suitable for use in environmental processes such as rhizoremediation and bioremediation treatments [Segura et al., 2009].

Therefore, the genus of *Pseudomonas* has great interest as its use in biotechnology, improvement of polluted areas, control of biological agents and promoting the plant growth in agriculture.

2.5.2. *Pseudomonas putida*

In 1995, scientists at the Genomics Research Institute in Germany sequenced the full genome of *Pseudomonas putida*. By this sequenced genome, thirty microbial strains were completed and sorted completely [Kowalski, 2002]. Genomic analysis of *Pseudomonas putida* showed that it has approximately 6.2 million base pair.

Pseudomonas putida is a rod-shaped, non-spore-forming, gram-negative, utilizes aerobic metabolism and grows optimally at 25-30 °C. This bacteria contains several polar flagella for mobility. *Pseudomonas putida* is environmentally sensitive and suppresses changes in the rotation of flagella after detecting chemo-attractants. This feature is very useful for guiding *Pseudomonas putida* towards the plant seeds that feed the bacterial cells [Harwood et al., 1989].

They have been isolated from agricultural, forest and industrial soils, and from continental waters. These species can be colonized in the rhizosphere and has a potential use in the biological treatment of contaminated soils [Segura et al., 2009]. *Pseudomonas putida* species are found to be non-pathogenic for both animals and plant. This has been taken as positive for the development of biotechnological techniques, like the design of catabolic pathways aimed for pollutants degradation [Ramos et al., 1986] production of intermediates in the chemical synthesis of complex molecules [Wubbolts and Timmis, 1990]. Further, several strains have been proven being good colonizers of a variety of plants rhizosphere, like *Pseudomonas putida* KT2440 in maize rhizosphere (Figure 2.8) [Espinosa et al., 2002].

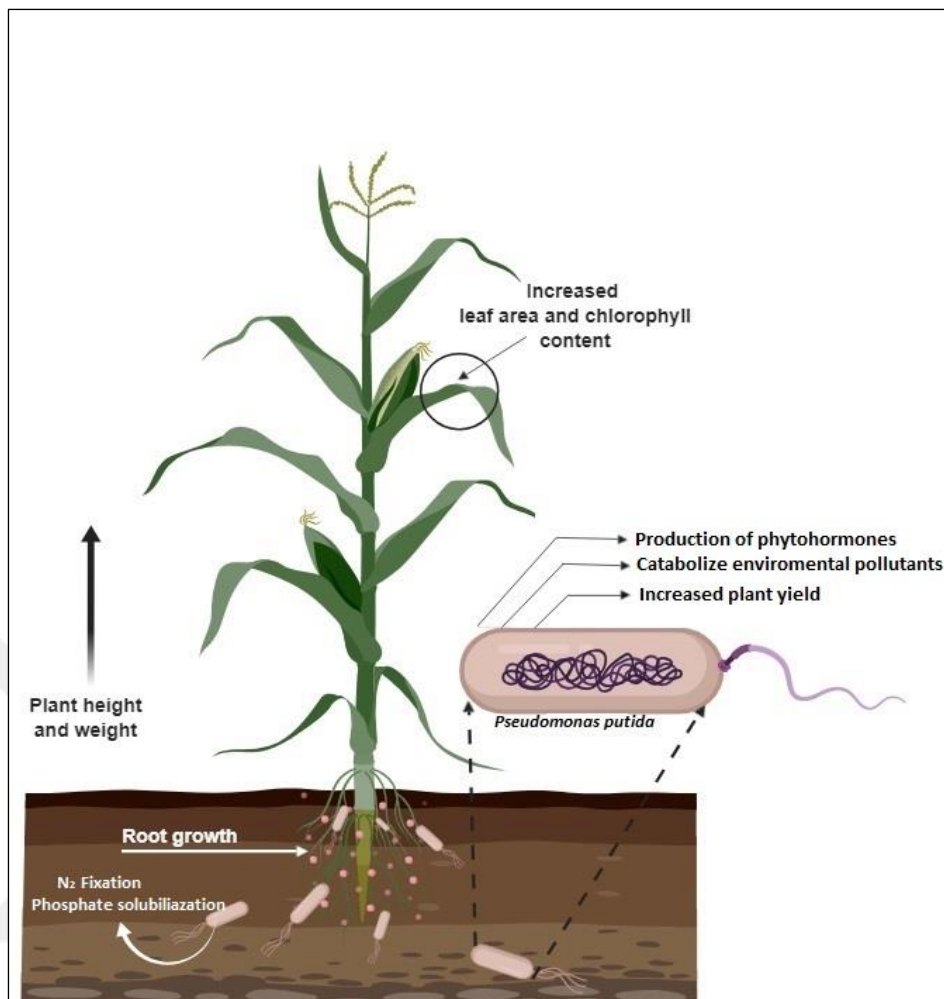


Figure 2.8: Beneficial effect and interacton of *Pseudomonas putida* in rhizosphere of maize plant.

2.5.3. *Pseudomonas putida* KT2440

The plasmid-free *P. putida* KT2440 (Figure 2.9) was firstly isolated in Japan from a garden soil in 1963 by Hosakawa. Detailed analysis of the genome of KT2440 strain showed that it has 6,181,863 nucleotides with 61.6 % G+C content. This detailed analysis found out that KT2440 has important genes with high capacity different responses [Nelson et al., 2002]. For example 10 genes encode for universal stress proteins_(10: PFAM00582; *Halobacter*: 11; PAO1: 8), 6 genes about cold shock_proteins (PFAM 00313; PAO1: 6; *M. loti*: 7), 5 genes about small heat shock proteins (PF00011; *M. loti*:7; PAO1 and *Caulobacter* both have two) and 15

genes for stringent response, starvation related proteins_(PFAM00043, PAO1: 18/15; *M. loti*: 10/12; *Caulobacter*: 19/19).

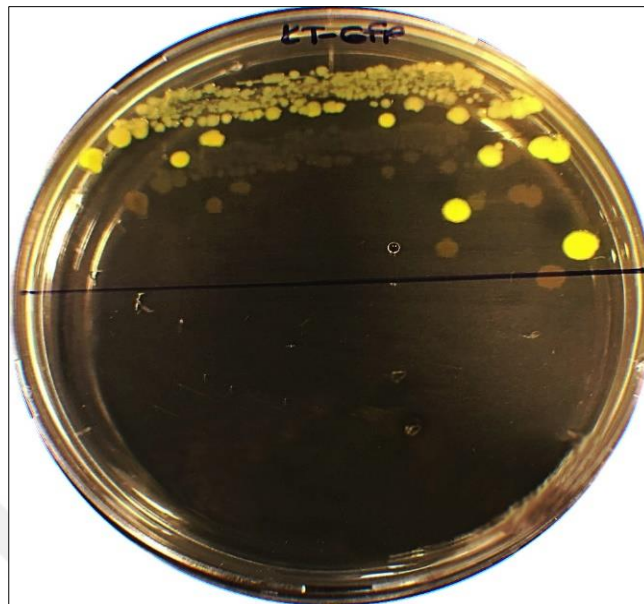


Figure 2.9: *Pseudomonas putida* KT2440 carrying pSEVA237-G plasmid (<http://seva.cnb.csic.es>) on LB agar.

It has been also studied for their high capacity to colonize wide range of plant by adhering the seeds and regulate the colonization by cellular interactions [Fernandez et al., 2012]. Genome analysis of the KT2440 revealed that the presence of some genes involved in pathways has growth promoting properties. These genes are facilities plant growth by production of phytohormones [Wu et al., 2010]. For example the genome of *P. putida* W619 has gene that encode a salicylate 1-monooxygenase (mahG, PputW619_2140) which is involved in the conversion of salicylate into catechol. Homologs of this gene found in *P. putida* KT2440 genomes as well. Salicylate is a phytohormone that functioning in plant growth and development. Also, it has important role in resistance to pathogens by inducing the production of pathogenesis-related proteins [Huijsduijnen et al., 1986]. These wide beneficial effects of KT2440 made this bacteria interesting for promoting growth in agriculture.

2.6. Uses and Applications of PGPR Microorganisms in the Environment

The increased knowledge about rhizosphere management and the potential benefits of microorganisms gives biotechnology the capacity of reducing fertilizers and agrochemicals and contribute to elimination of pollutants in agriculture. PGPR microorganisms become a common component of bio-fertilizer, bioremediation and rhizoremediation processes in agricultural applications and environmental applications [Vessey et al., 2003; Nakkeeran et al., 2005].

To improve the yield of agricultural products, PGPRs are become commercial formulations in agriculture. Various PGPR strains have been characterized and identified as suitable for use in modern sustainable agriculture [Bhattacharyya and Jha, 2011]. There are several categories of PGPR based on action mechanisms, including; i) biofertilizers, ii) phyto stimulators, and iii) biopesticide, (Table 2.2) [Bhattacharyya and Jha, 2011]. Moreover, PGPR may enhance plant development through the combination of more than one of the cited properties such as phosphorous solubilization, production of hormones, nutrient solubility, induction of systemic disease resistance, nitrogen fixation, resistance to water stress, and rhizoremediation.

Table 2.2. PGPR forms and their mechanism of action stimulating plant growth.

PGPR forms	Definition	Mechanism of action
Biofertilizer	A substance that contains live microorganisms which, when applied on the seed, plant surface or soil, colonizes the rhizosphere and promote plant growth through increased supply of primary nutrients for the host plant	Biological nitrogen fixation Utilization of insoluble phosphorus
Phyto stimulator	Microorganism, with the ability to produce phytohormones such as indole acetic acid, gibberellic acid, cytokinins and ethylene	Production of phytohormones
Biopesticide	Microorganisms that promote plant growth by controlling phytopathogenic agents	Acquired and Induced systemic resistance. Production of antibiotics, siderophores, HCN. Production of hydrolytic enzymes

PGPRs can perform various useful interactions that lead to strong and eco-friendly solutions for sustainable farming. The application of agricultural crops with the desired bacterial populations of rhizosphere soil has provided important promises in both laboratory and greenhouse experiments. Specifically engineered

rhizobacteria can target growth mechanism, clean-up various pollutants and resistance biotic/abiotic stresses. Recent advances in molecular biology and biotechnology in clarifying the rhizobacterial interactions with roots will promote an appropriate field of research in PGPR mechanisms for colonization.

2.7. Plant MicroRNAs; Biogenesis and Functions

MicroRNAs (miRNAs) are small, endogenous, non-coding RNA that has a major impact on nearly all biological processes. miRNAs mediate post-transcriptional gene silencing that play a key role in regulation of main processes such as growth, development and stress responses [Budak et al. 2015].

These regulatory small RNAs were first discovered by scientists two decades ago [Lee et al., 1993; Wightman et al., 1993] but were not recognized until 2001. In comparison with other organisms, plant miRNAs were identified over two decades in 2002, several groups cloned small RNAs and found plant miRNAs [Llave et al., 2002; Reinhart et al., 2002]. Since then, 872 miRNAs from 42 families have been identified in 71 plant species.

Biogenesis of miRNA is a complex mechanism that needed many steps to create mature miRNAs from miRNA genes (Figure 2.10) [Zhang et al., 2006]. Firstly, primary miRNA (pri-miRNA) transcribed by a miRNA gene. Pol II enzymes control this step during pri-miRNA transcription. Secondly, the pre-miRNA occurs from pri-miRNA with cleaved to a stem loop intermediate. Dicer-like 1 enzyme (DCL1) controlled this step in plants [Tang et al., 2003]. miRNA duplex occurred as a result of miRNA dividing by dicer-like enzyme 1 (DCL1) takes place in the nucleus or place called D-bodies in the nucleus rather than in the cytoplasm. Then HASTY (the plant orthologue of exportin) translocated the duplex into the cytoplasm [Park et al., 2005]. In the cytoplasm, helicase resolved plant miRNAs as single stranded mature miRNAs. Lastly, RNA-induced silencing complex (RISC) occurs by stepping of mature miRNAs in a ribonucleoprotein complex [Hammond et al., 2000] where they regulate targeted gene expression. This suggests that miRNA biogenesis is complex; several enzymes are required to process mature miRNAs from long pri-miRNA to 20–24 nt [Bartel, 2004; Kurihara and Watanabe, 2004].

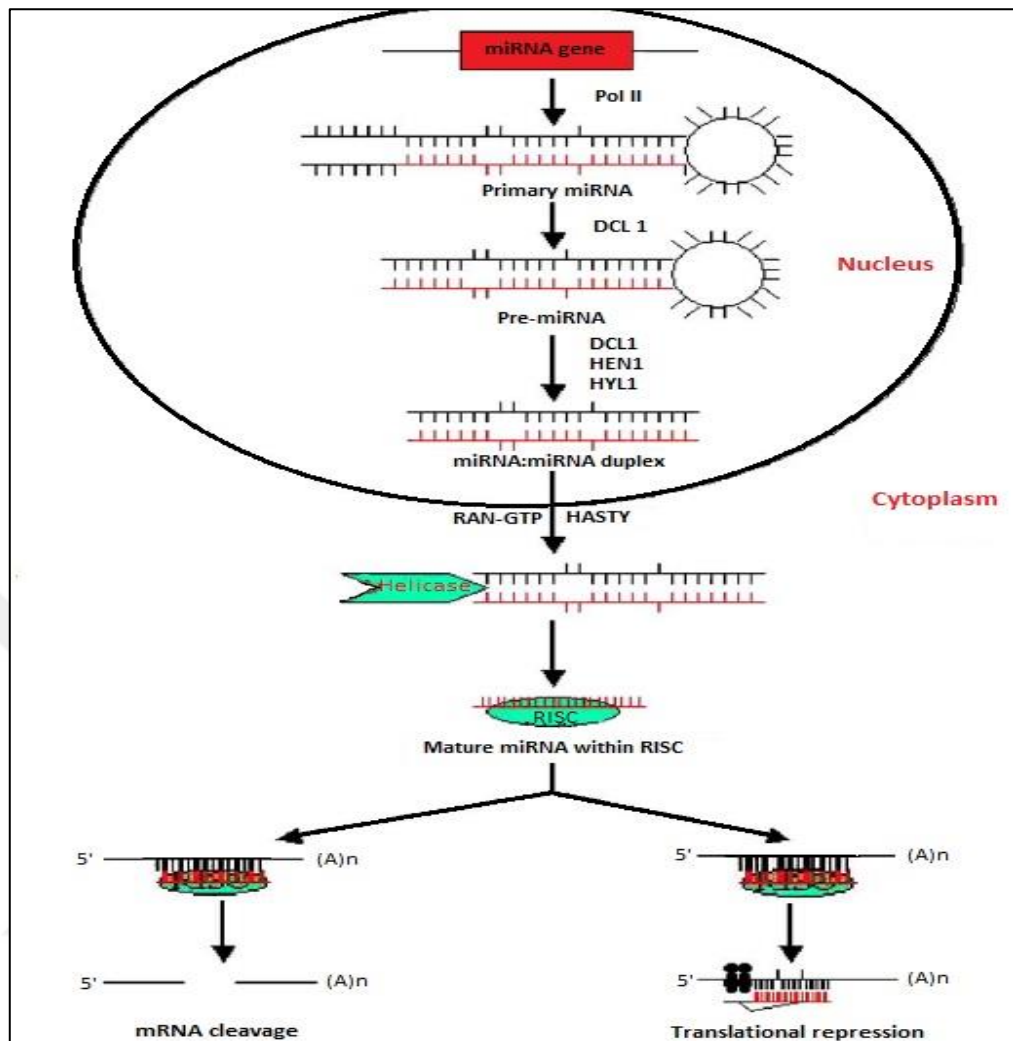


Figure 2.10: Biogenesis plant miRNAs; a simplified model.

2.8. MicroRNAs in Plant Microbe Interaction

Recent studies have done in plant-microbe interactions indicate that microRNAs (miRNAs) play significant role in the interaction between plants and microorganism [Simon et al., 2009]. They have important roles in many plant processes such as development, differentiation and response to biotic and abiotic stresses [Guleria et al., 2011; Hajdarpasic et al., 2012]. The study conducted in soybean roots, showed that some miRNAs were highly up-regulated and down-regulated after inoculation by bacterium *Brodyrnia japonicum* [Subramanian et al., 2008]. In tomato leaves, the expression of miR393 challenged with flg-22 (*Pseudomonas syringae* pv. tomato DC3000 flagellin derived peptide) indicated

important role of miRNA in plant disease resistance [Navarro et al., 2006]. In another study established that miR397 and miR408 were played significant roles in the metabolism of cell wall during growth and development response to *P. indica* [Wei et al., 2014].

2.9. Growth Related MicroRNAs

The studies have done in animals showed that roughly approximately 60% of the protein encoding genes appeared by regulation of miRNAs [Friedman et al. 2009]. In spite of that, plant microRNAs solely target a small number of mRNA targets [Addo-Quaye et al., 2008]. Despite the number of miRNAs is small, the overall effect of plant miRNAs cannot be underestimated due to most of their target are transcription factors [Jones et al., 2006]. These transcription factors may potentially contribute to most developmental processes from seed germination to maturation in plants. These evidence point out role miRNAs are in a range of developmental processes in plants (Table 2.3) [Marco et al., 2017].

For example; it has been shown that miR396 affects leaf growth and especially plays a role in controlling cell number in *Arabidopsis thaliana* [Liu et al., 2013] and miR319 encoding a transcription factor and played a role in development of leaf in tomato [Ori et al., 2007]. Another study suggested that miR156 specifically targeted *SPL* genes and *CYP78A5/KLUH* and playing a role in plastochron length and developed size specific organs in *Arabidopsis thaliana* [Wang et al. 2008].

Table 2.3: Plant miRNAs and their targets in development processes.

miRNA	Target	Target function	Species
miR156	<i>SPL family</i>	Plastochron length, promoting flowering and corn development in <i>Zea mays</i>	<i>Arabidopsis</i> <i>Zea mays</i>
miR159	<i>MYB33</i>	Anther, silique, and seed development	<i>Arabidopsis</i>
miR164	<i>CUC</i>	Meristem boundary identity, auxiliary meristem formation, and leaf serration	<i>Arabidopsis</i> <i>Solanum</i>
miR165/166	<i>HD – ZIP II</i>	Maintaining meristematic cells, adaxial identity of leaves, lateral root growth	<i>Arabidopsis</i>
miR167	<i>ARF6 and AR</i>	Male organ development	<i>Arabidopsis</i>
miR169	<i>CBF</i> and <i>AtNF-Y</i> family	Enhancer of C homeotic gene transcription, Flowering in response to abiotic stress	<i>Antirrhinum majus</i> <i>Arabidopsis thaliana</i>
miR172	<i>AP2 family</i>	Represses flowering, flower meristem identity and patterning; carpel and stamen development in <i>Z. mays</i>	<i>Arabidopsis</i> , <i>Z. mays</i> ,
miR319	<i>TCP family</i>	Control of cell growth and proliferation during development	<i>Arabidopsis</i> and <i>Solanum lycopersicum</i>
miR390	<i>TAS3</i>	tasiRNA biogenesis for ARF repression and indirect miR165/166 regulation	<i>Arabidopsis</i>
miR390	<i>TIR1 and AFB</i>	Auxin homeostasis	<i>Arabidopsis</i>
miR393	<i>LCR</i>	Meristematic identity suppression via WUS downregulation	<i>Arabidopsis</i>
miR396	<i>GRF</i>	Cell proliferation in leaves, determining morphology	<i>Arabidopsis</i> , <i>Medicago</i> , and <i>Oryza</i>
miR857	<i>LACCASE7</i>	Secondary growth	<i>Arabidopsis</i> and <i>Citrus sinensis</i>

2.10. Reactive Oxygen Species (ROS) and Enzymatic Antioxidants in Plants

Plants are continuously exposed to several abiotic and biotic stress factors in field, which affect their production. These environmental adversities generally induce the accumulation of reactive oxygen species (ROS), which can cause severe oxidative damage to plants. ROS are involved in processes such as growth, development, response to biotic and abiotic environmental stimuli programmed cells and can act as signal transducers. Stress factors, hormones, development and several other metabolic pathways can stimulate the production of ROS; that can trigger other pathways or act directly as defense compounds [Bailey et al., 2006]. Knowledge of oxidative mechanisms in plants can contribute to the development of plants that are most adapted to the environment and resistant to pathogens. Plants have antioxidant defense systems to clean ROS. Plant cells have systems that produce and eliminate specific ROS in different organelles, such as chloroplasts, mitochondria, and peroxisomes. Removal or detoxification of excess ROS is carried out by enzymatic and non-enzymatic antioxidant systems. Enzymatic antioxidants include enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) [Sharma et al., 2012].

Superoxide dismutase is enzyme that catalyze the exchange of O_2 to H_2O_2 ; therefore, its form a frontline defense against ROS. Catalases are responsible for the removal of H_2O_2 by reducing H_2O_2 to $2H_2O$ [Scandalios, 2002]. It is known that increased catalase activity helps the plant adapt to difficult conditions and protects metabolic processes by minimizing the toxic level of H_2O_2 [Ashraf et al., 2010]. Ascorbate peroxidases are enzymes that play a key role in catalyzing the conversion of H_2O_2 into H_2O and use ascorbate as a specific electron donor. Plants have different APX isoforms that are distributed in distinct subcellular compartments, such as chloroplasts, mitochondria, peroxisomes, and the cytosol. The APX genes are differentially modulated by several abiotic stresses in plants [Rosa et al., 2010 and Caverzan et al., 2012]. Glutathione reductase is an NAD(P)H-dependent enzyme that catalyze the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH). It is a key enzyme of the AsA-GSH cycle; it protects cells against oxidative

damage. A high GSH/GSSG ratio is essential for protection against oxidative stress [Foyer and Noctor, 2005]. Peroxidases are glycoproteins that involved in defense against pathogens and injury, lignin, ethylene biosynthesis, and stress response. It is recognized as antioxidant enzymes that protect cells from the destructive effects of H_2O_2 and derived oxygen species [Radić et al., 2006]. Plants are activating antioxidant defense mechanisms under stress conditions which helps to preserve the structural integrity of cell components and mitigates oxidative damage. In general, tolerant species have good capacity to protect themselves from oxidative stress by providing an increase in antioxidant enzyme activity under stress conditions [Hediye et al, 2007].



3. MATERIAL AND METHODS

3.1. Plant Material

In this study, 9 maize (*Zea mays* L.) hybrids (Table 3.1) provided by Maize Research Institute (Sakarya) were used as plant material. First of all, comparative secreening analysis was performed between 9 maize hybrids according to growth response to *P.putida* KT2440. At the end of screening analysis, a maize hybrid among 9, which was evaluated with positive growth response, were selected for further detail analysis to uncover interaction between the maize leaf growth and bacteria interaction.

Table 3.1: The name of maize (*Zea mays* L.) hybrids were used in this study.

No:	Name	Description
1	Sasa-99	Single Cross Hybrid- For Silage- FAO-700 Maturity Group
2	Sasa-152	Single Cross Hybrid- For Silage- FAO-700 Maturity Group
3	Sasa-5	Single Cross Hybrid-For Silage- FAO-720 Maturity Group
4	Sasa-166	Single Cross Hybrid-For Grain- FAO-650 Maturity Group
5	Sasa-139	Single Cross Hybrid-For Grain- FAO-650 Maturity Group
6	Sasa-137	Single Cross Hybrid-For Grain- FAO-650 Maturity Group
7	Sasa-186	Single Cross Hybrid-For Grain- FAO-650 Maturity Group
8	Ada-1650	Single Cross Hybrid-For Grain- FAO-700 Maturity Group
9	Adasa-16	Single Cross Hybrid-For Grain- FAO-650 Maturity Group

The seeds of maize were firstly rinsed in 70% ethanol for 3 times and then sterilized in a 10% bleach solution by stirring in a shaker for 5 minutes. Then, the seeds were washed with the ddH₂O to remove the remaining bleach solution.

3.2. Growth of Bacteria

Pseudomonas putida KT2440 carrying pSEVA237-G plasmid were kindly provided by Prof. Dr. Victor de Lorenzo (Systems and Synthetic Biology Program, National Biotechnology Center, Madrid, Spain) (<http://seva.cnb.csic.es>). In this study, *Pseudomonas putida* KT2440 carrying pSEVA237-G plasmid were used as a plant growth promoter rhizobacteria.

Pseudomonas putida KT2440 was took from the stock at -80 °C and transferred to LB (Luria-Bertani) growth medium. The sample that transferred to LB

medium was allowed to grow overnight at 200 rpm at 30 °C. After a night, proliferating cells were harvested by centrifuging at 12,000 rpm. The harvested cells washed 2 times in 10 mM MgCl₂ solution. Finally, according to our previous study the washed culture was diluted to 10⁶ CFU/ml and became ready for inoculation.

3.3. Inoculation of Seeds with *Pseudomonas putida* KT2440

For comparative analysis mock and bacterial inoculated groups were grown in optimum conditions. For the mock group, sterilized maize seeds planted in the pots and allowed to germinate under control conditions in the growth cabinet. For the inoculated group; the sterilized seeds were inoculated in prepared bacteria solution (10⁶ CFU/ml) by shaking at 45 rpm for 3 hours at room temperature. After inoculation, the seeds were planted into the pots and allowed to germinate under normal conditions in the growth chamber. Sterilized seeds were transferred and grown under condition following 16-hour at 25 °C/day and 8-hour at 20 °C/night with 15,000 lx light intensity and 70% humidity. After germination, the seedlings were planted in 1.5 liters pots of peat and transferred to the climate cabinet. Plants were grown in the control conditions until fourth leaves became fully matured.

3.4. Detection of the KT2440 from Root Surface

15 days old inoculated maize roots (purified from soil) washed with 10 mM MgCl₂ solution to extract bacteria from surface of root. After that, the washed solution was planted on agar supplemented Luria-Bertani (LB) Broth for visualizing the presence of green fluorescence protein (GFP) labeled KT2440 via imaging system (Azure Biosystem c600). In addition to that, GFP labelled KT2440 were also visualized under DIC (Differential Interference Microscope, Leica DM2500) microscope. In this context, 15 days old inoculated maize roots (purified from soil) were washed with of 4',6-diamidino-2-phenylindole (DAPI) buffer (50 mM NaCl, 5 mM EDTA, 10 mM TRIS-HCL (pH 7) 2 times and kept on ice and in dark. Then root was quickly mounting on a microscopy glass and covering it with a cover glass for observing the presence of bacteria on root surfaces.

3.5. Determination of the Effect of Bacteria on Chlorophyll Content by SPAD

The effect of the bacterium on the content of photosynthetic pigments were determined by using the Chlorophyll meter SPAD-502 Plus (Konica Minolta) instrument (Figure 3.1). Chlorophyll content of maize seedlings were measured after 15 days until 3th leaves were fully matured.



Figure 3.1: Chlorophyll measurement from maize leaf with SPAD-520Plus.

3.6. Measurement of Leaf Elongation Rate

The 3th leaves of the seedlings were measured daily from the day of emergence until fully got matured from the soil as reference point to leaf tip. The mean and standard deviation of all data were determined and a leaf length (LL) profile graph have been formed. Leaf elongation rate (LER) were calculated according to formula 1.1 by taking derivative of leaf length. In formula, x_1 is the length measured on the first day and x_2 is the length measured on the second day. t shows the time between two measurements. The significance of the difference between the groups was determined according to the Students T-test in the Microsoft Excell program.

$$\mathbf{LER} = \frac{x_2 - x_1}{t_2 - t_1}$$

1.1

3.7. Measurement of Mature Cell Length

To measure of mature cell length of the 9 hybrids, 10th cm of the mature third leaf were harvested. To determine cell length profile in the growth zones (10 cm from leaf base to tip) of Sasa139, the 3th leaves of maize seedlings were harvested 48 hours after emergence. The 3th leaf was divided into segments of 1 cm after been harvested from the leaves surrounding the outside itself (Figure 3.3). All segments were left in ethanol for chlorophyll removal. The fixation and solution was changed periodically. The leaf segments were then transferred to glass bottles containing 90% lactic acid for cleaning, storage and preparation of microscope slides. Then, segments were examined by using a DIC microscope (Differential Interference Microscope, Leica DM2500), with microscope-connected camera system (Leica DFC 3000G) under a 20, 40 or 63 X (depending on the cell size) objective magnification (Figure 3.2). Cells in both positions were measured as cell development showed a gradient from tips to the bottom of the leaf. The cells at the bottom of the leaf between the leaf margin and the middle vein were measured. Approximately 50 or more cells were measured to obtain sufficient data. A calibration scale was used for each image. The obtained images were opened in the ImageJ (<https://imagej.nih.gov>) program and the average cell area was determined by dividing the total area by the cell number. The data were represented as mean and standard deviation.

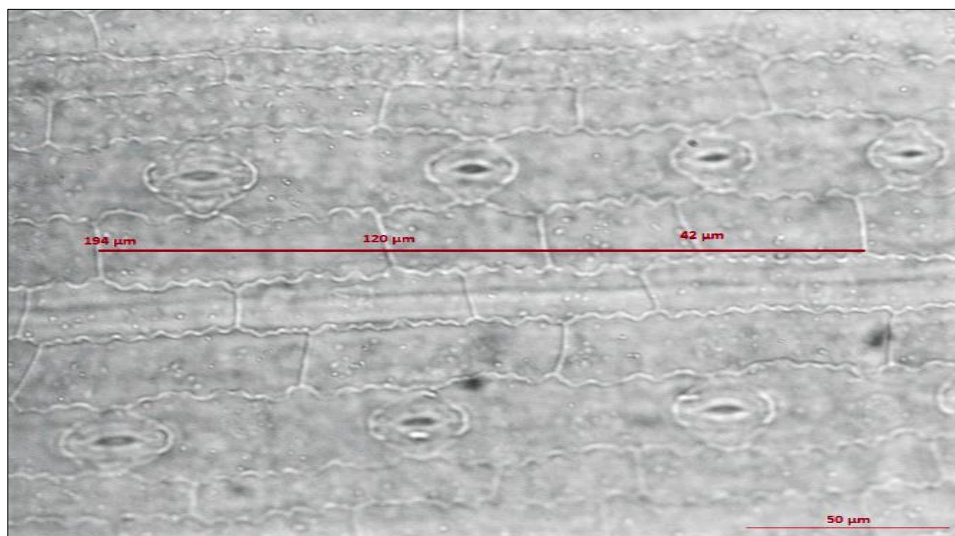


Figure 3.2: The appearance of the mature cells at 20X objective magnification.

3.8. Measurement of Leaf Area

To determine of leaf area (LA) of the 9 hybrids, the 3th leaves were cut from the leaf sheath of the seedlings which were inoculated with bacteria and control conditons. After harvesting, 3th leaves were being scanned with a scanner, the leaf area was calculated by using ImageJ program (<https://imagej.nih.gov>) on the basis of the calibration scale and converted to pixel mm (Figure 3.3). Freehand tool was used to determine the leaf area. The data were represented as mean and standard deviation.

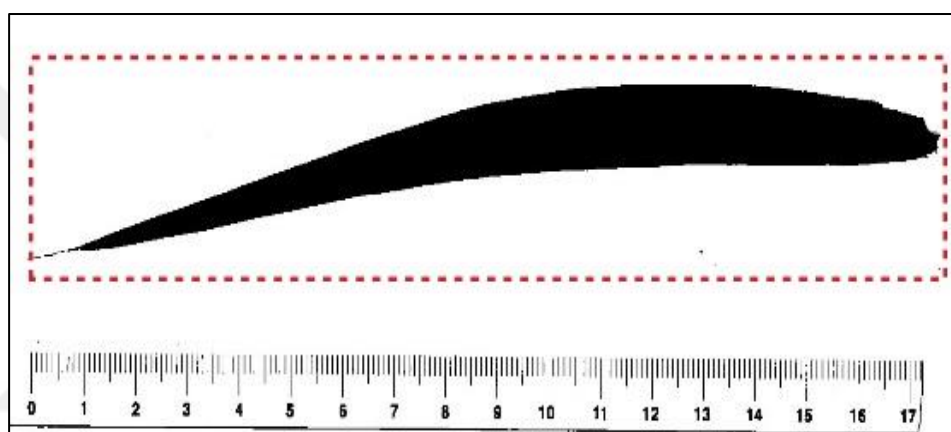


Figure 3.3: A leaf area of maize fourth leaf measured via ImageJ program

3.9. Enzymatic Analysis

3.9.1. Extraction and Determination of Total Soluble Protein in Plant Leaf Tissues

For total protein extraction, the 4th leaves of Sasa139 seedlings grown under control and inoculated conditions were harvested after 48 hours exiting. The growth zones of the 4th leaves were divided into one-centimeter segments representing the meristem (1th cm), elongation (4th cms), maturity (10th cms), respectively. The segments were quickly transferred to 2 ml ependorfs and fixed in liquid nitrogen. Samples were stored at -80 °C until the experiments. In total 30 samples, including three biological repeats of each samples seedlings grown in

inoculated and control conditions were used for perform to comparative and statistical analyses.

Protein extraction of each samples performed according to Bradford, (1976). Leaf segment samples were taken from -80 °C to liquid nitrogen. Three metal beads (4 cms in diameter) were put in the ependorf tubes and were placed in homogenizer blocks. The homogenizer was set to 45 seconds/25 frequencies and operated. After the device was stopped, ependorf tubes were rapidly taken into liquid nitrogen. 1 ml extraction buffer containing 50 mM KH_2PO_4 (pH = 7.8), 1mM EDTA and 2% PVP was added on the samples. Ependorf tubes were thoroughly vortexed. Vortexed samples were centrifuged at + 4 °C for 30 min at 10,000 rpm. The supernatant parts of the centrifuged samples were transfered to the clean ependorf tubes without touching the beads and kept at -20 °C for experiment.

Total amount of protein was determined according to Bradford Assay [Bradford, 1976]. For the test of Bradford from 2 mg/ml stock BSA solution; the concentration amounts were adjusted from 0 mg/ml to 1.2 mg/ml. Dilution ratio of the samples were prepared as 0 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, 1 mg/ml and 1.2 mg/ml. Diluted BSAs were placed in the cell culture plate in the order of 5 μ l BSA, and 250 μ l Bradford reagent. For Blank; 250 μ l Bradford reagent and for protein samples; 5 μ l protein and 250 μ l Bradford reagent were used. The wells were mixed by pipetting. Samples were incubated for 10 minutes. The absorbance value of the Spectral scanning multimode reader (Thermo Scientific Varioskan Flash) was set up to 595 nm. The standard curve was estimated.

3.9.2. Determination of Catalase Enzyme Activity

Catalase enzyme activity was performed according to the method proposed by Chance and Maehly (1955). 1.5 ml quartz cuvette used for experiment. For the reaction in the experiment; 50 mM 850 μ l of potassium phosphate buffer (pH: 7.0) 200 mM of 100 μ l of H_2O_2 and 50 μ l of (4-11 μ g/ml) of protein extract were used. For the blank; (900 μ l of potassium phosphate buffer, 200 mM of 100 μ l of H_2O_2) and for working solution; (850 μ l of potassium phosphate buffer, 100 μ l of H_2O_2 and 50 μ l of protein) were used. Absorbance was set up to 240 nm and samples were

measured for 90 seconds and recorded on spectrophotometer (Shimadzu, UV-1280). The activity of catalase was calculated according to Aebi (1983). Catalase break down H_2O_2 to release water and oxygen. 1 unit of enzyme catalase was introduced as the amount of enzyme needed to break down 1M of H_2O_2 and release water oxygen. The results were calculated by taking the mean of 3 biological and technical replicates and the error bars indicated as standard error on the graph. The significant of difference between the inoculated and control groups was determined according to the Student's T test in the excel program. If P value was less than 0.05, the difference was considered as significant.

3.9.3. Determination of Superoxide Dismutase (SOD) Enzyme Activity

Superoxide dismutase (SOD) enzyme assay was performed according to the method proposed by Banowertz et al., (2004). The method is based on inhibitory effect of SOD on nitroblue tetrazolium (NBT) dye by superoxide radicals produced from auto-oxidation of hydroxylamine hydrochloride. The decrease of NBT was followed by an increase in absorbance at 540 nm. 96 well TPP cell culture plate was used for assay. 40 μl of 250mM potassium phosphate buffer (pH = 7.8), 26 μl of 100 mM methionine, 4 μl of 5mM EDTA, 20 μl of 750 mm of NBT, 90 μl of ddH₂O, 20 μl of protein extract and 0.002 g of riboflavin were added into the wells for reaction. For Blank, 40 μl of 250mM potassium phosphate buffer (pH = 7.8), 26 μl of 100 mM methionine, 4 μl of 5mM EDTA, 20 μl of 750 mm NBT, 90 μl of ddH₂O and 0.002 g of riboflavin were added. The cell culture plate put into Varioskan spectrophotometer (Thermo Scientific Varioskan Flash) and were shaken for 1 min. After shaking samples were measured at 560 nm absorbance. After measurement, cell culture plate was exposed to fluorescent light for 10 min in a dark room. The samples were again shaken on the spectrophotometer for 1 min and the absorbance values at 560 nm were measured.

The activity of the superoxide dismutase enzyme was calculated according to Kono (1978). The 1 unit enzyme is the amount of enzyme required to inhibit the absorbance difference from the chromogen production at 540 nm by 50% at one minute under the experimental conditions. The results were calculated by taking

the mean of 3 biological and technical replicates and the error bars indicated the standard deviation.

3.9.4. Determination of Glutathione Reductase (GR) Enzyme Activity

Glutathione reductase catalyzes the reduction of glutathione disulfide (GSSG) by oxidation of NADPH. Glutathione reductase enzyme activity method performed according to Çakmak et al. (2006). 1.5 ml quartz cuvette used for experiment. For the reaction in the experiment; 50 mM potassium phosphate and 0.1 mM 850 μ l of EDTA buffer solution, 0.5 mM 50 μ l GSSG, 0.12 mM 50 μ l NADPH, 50 μ l protein extract were prepared. For the blank; 850 μ l of 50 mM potassium phosphate buffer, 50 μ l of 0.1 mM EDTA, 0.5 mM 50 μ l of GSSG and 0.12 mM 50 μ l of NADPH were added. Samples were placed on the spectrophotometer (Shimadzu, UV-1280) and absorbance changes were measured at 340 nm for 3 minutes. The activity of the glutathione reductase enzyme was calculated according to Carlberg and Mannervik (1975). The amount of enzyme that needed to oxidize 1 μ M of NADPH / min/g FW defined as 1 unit of the enzyme. The results were calculated by taking the mean of the 3 biological and technical replicates and the error bars indicated the standard deviation.

3.9.5. Determination of Peroxidase (POX) Enzyme Activity

Peroxidase enzyme activity was determined according to Quesada et al. (1990). 1.5 ml quartz cuvette used for experiment. For the reaction in the experiment; 50 mM 850 μ l of potassium phosphate buffer, 50 μ l of protein extract, 10 mM 50 μ l of H₂O₂ and 0.02% 50 μ l of dianizidine were used. For the blank; 50 mM 900 μ l potassium phosphate buffer, 10 mM 50 μ l of H₂O₂ and 0.02% 50 μ l dianizidine were used. The absorbance changes of the samples were measured in a spectrophotometer (Shimadzu, UV-1280 at 460) nm for 1.5 min. The activity of the peroxidase enzyme calculated according to Quesada et al. (1990). The amount of enzyme that needed to oxidize 1 μ M of H₂O₂ defined as 1 unit of the enzyme. The

results were calculated by taking the mean of the 3 biological and technical replicates and the error bars indicated as the standard error.

3.9.6. Determination of Ascorbate Peroxidase (APX) Enzyme Activity

Ascorbate peroxidase enzyme activity was performed according to the method proposed by Nakano and Asada (1981). 1.5 ml quartz cuvette used for experiment. For the reaction in the experiment; 800 µl of potassium phosphate buffer, 50 µl of 0.1 mM EDTA, 50 µl of 0.5 mM ascorbic acid, 50 µl of H₂O₂ and 50 µl of protein were used. For the blank; 850 µl of 50 mM potassium phosphate buffer, 50 µl of 0.1 mM EDTA, 50 µl 0.5 mM ascorbic acid and 50 µl of 0.1 mM H₂O₂ were used. The absorbance changes of the samples were measured at 290 nm for 3 min.

The activity of enzyme was calculated according to Nakano and Asada (1981). Ascorbate peroxidase catalyzes the reduction of H₂O₂ by using substrate. High concentrations of leaf cells are found in chloroplast, cytosol, and vacuole. The amount of enzyme that needed to oxidize 1 µM of Ascorbate defined as 1 unit of the enzyme. The results were calculated by taking the mean of the 3 biological and technical replicates and the error bars indicated the standard error.

3.10. Transcriptional Analysis

3.10.1. Determination of Growth Related miRNAs, and their Predictable Target Genes and Cell Cycle Genes

For transcriptional analysis of the growth related miRNAs, and their predicted target genes involved in the growth of maize seedlings were identified by looking at similar studies in the literature. Finally, mir408, mir396, mir319, mir160 and mir169 were determined as possible growth regulatory ones. The coding sequence of the genes (CDS) were obtained from the maize genetics and genomics database (MaizeGDB; www.maizegdb.org) and the sequences of the maize plant miRNA genes were obtained from the miRBASE (The microRNA database; <http://www.mirbase.org/>) database. The possible target genes of the selected miRNAs; *Laccase*(Lac), *Basic blue protein*(BBP), *Teosinte branched1*, *cycloidea*

transcription factors 38(TCP38), *Auxin response factor 17*(ARF17), *Growth regulation factor15*(GRF15) and *Nuclear transcription factor Y subunit A-1* (NF_YA1) were predicted by psRNATarget (A Plant Small RNA Target Analysis Server, 2014) web service (Table 3.2) [Dai and Zhao, 2011].

For the cell cycle genes; CDKA, CDKB2, CycB2_1, CycD5_1, CycD1 and CycA2 were selected for this study according to literature studies (Table 3.3) [Rymen et al., 2007].

3.10.2. Primer Desining

The sequence information of specific primer for miRNA target genes was obtained from Maize GDB (<https://www.maizegdb.org/>) database and design was done with SCiTools Oligo-Analyzer 3.1 (<https://eu.idtdna.com>). The designed primers checked for; melting temperature (T_m) at 58-61 °C, hair hairpin formation at 50 °C with $\Delta G > -3$ kcal/mol and self and counter dimer formation at 50 °C with $\Delta G > -6$ kcal/mol by using SCiTools OligoAnalyzer 3.1. For primer specificity BLASTn algorithm was used. The universal reverse primer were used to convert miRNAs to cDNA which is proposed by Varkonyi-Gasic et al. (2007).

Table 3.2: Primers used in qRT-PCR for analyzing the expression of miRNAs and their targets.

miR408a	miRNA sequence	5'-CUGCACUGCCUCUUCCUGGC-3'
	Stem-loop RT	5'- GTCGTATCCAGTGCAGGGTCCGAGGTATTGCACTGGATACGAC GCCAGG -3'
	Forward	5'- GCCTCT CTGCACTGCCTC -3'
<i>Lac</i> (Laccase)- Zm00001d012477	Forward	5'-CACCACCATCAGTTCGTT-3'
	Reverse	5'-CCCTCTCTGACCTCCAG-3'
<i>BBP</i> (Basic blue protein) Zm00001d051333	Forward	5'-ATTTCACTCGTTCCACACC-3''
	Reverse	5'-CCTTGCACTGCCTCTTC-3'
miR319a-3p	Reverse	5'- AGA CCA CGA CGT CGA TAA -3'
	Stem-loop RT	5'- GTCGTATCCAGTGCAGGGTCCGAGGTATTGCACTGGATACGAC GGGAGC -3'
	Forward	5'- GCG ACT TGG ACT GAA GGG T -3'

Table 3.2 continued

TCP38 (<i>TEOSINTE BRANCHED1, CYCLOIDEA</i> transcription factors 38)- Zm00001d022256	Forward	5'- CAGCCTCACCACGCAATC -3'
	Reverse	5'- AGGCACGCATGCTGAGA -3'
miR160d-5p	miRNA sequence	5'-UGCCUGGCUCCUGUAUGCCA-3'
	Stem-loop RT	5'- GTCGTATCCAGTGCAGGGTCCGAGGTATTGCACTGGATACGAC TGGCAT -3'
	Forward	5'- CCT CTT GCC TGG CTC CC -3'
Arftf17 (Auxin Response Factor 17) - Zm00001d014013	Forward	5'-AGCCCACTTCGCTGTTTCT-3'
	Reverse	5'-GCTGGTTAAGCTGGTAGTCTGT-3'
miR396c	miRNA sequence	5'-UUCCACAGGCUUUCUUGAACUG-3'
	Stem-loop RT	5'- GTCGTATCCAGTGCAGGGTCCGAGGTATTGCACTGGATACGAC CAGTTC -3'
	Forward primer	5'- TGC GTT CCA CAG GCT TTC TT -3'
GRF15 (Growth Regulation Factor15) - Zm00001d007465	Forward	5'-CGGTGCTCTAGAGATGCTGTC-3'
	Reverse	5'-TTGCCTTTCGGCCTTCCA-3'
miR169f-5p	miRNA sequence	5'-UAGCCAAGGAUGACUUGCCUA-3'
	Stem-loop RT	5'- TCGTATCCAGTGCAGGGTCCGAGGTATTGCACTGGATACGAC TAGGCA -3'
	Forward	5'- CGG GTT AGC CAA GGA TGA CTT -3'
NF_YA1 (Nuclear transcription factor Y subunit A-1)- Zm00001d006835	Forward	5'-ATGCAGCAGCGTGTTC-3'
	Reverse	5'-CAACAGCCAAGGATGAGTTG-3'
Universal	Reverse 2	5' CA GGG TCC GAG GTA TTC G 3'
β -TUB (Beta Tubulin) - NP_001105457	Forward	5'-CTACCTCACGGCATCTGCTATGT-3
	Reverse	5'-GTCACACACACTCGACTTCACG-3'

Table 3.3: Primers used in qRT-PCR for analyzing the expression of cell cycle genes.

CDKA	sequence	
Zm00001d027373	Forward	5'-GTCTGTGGGCTGTATCTTTG-3'
	Reverse	5'-CGGTGTACCTAGTATCCTGAA-3'
CDKB2		
Zm00001d027374	Forward	5'-CTCCACCGTGATCTGAAAC-3'
	Reverse	5'-TCGTGCGTGTACTTCTTTAC-3'
CycB2_1		
Zm00001d036360	Forward	5'-AAACTGAGGGTGCAAGC-3'
	Reverse	5'-GCCAGTCAATTAGAATGCCA-3'
CycD5_1		
Zm00001d048143	Forward	5'-GGTGTCGCAGAGTGATTT-3'
	Reverse	5'-CGTTGTTCTGTCGTCTT-3'
CycD1		
GRMZM2G140633	Forward	5'-GTTACACGTGCTACGGTTTC-3'
	Reverse	5'-GTCATCCAAGCCTTGCCCTC-3'
CycA2		
GRMZM2G017081	Forward	5'-GTGCTTCCTACGTTGTGGAGA-3'
	Reverse	5'-GTGATGTCCCGTTGCAAACC-3'

3.10.3. RNA Isolation

RNA extraction was performed according to TRIzol method. The growth zones representing meristem (1th cm), elongation (4th cm) and maturity (10th cm) of the fourth leaf of Sasa139 were harvested after 48 hours its emergence (Figure 3.4). The leaf segments were quickly fixed in liquid nitrogen and stored in ultra-freezer at -80 °C.

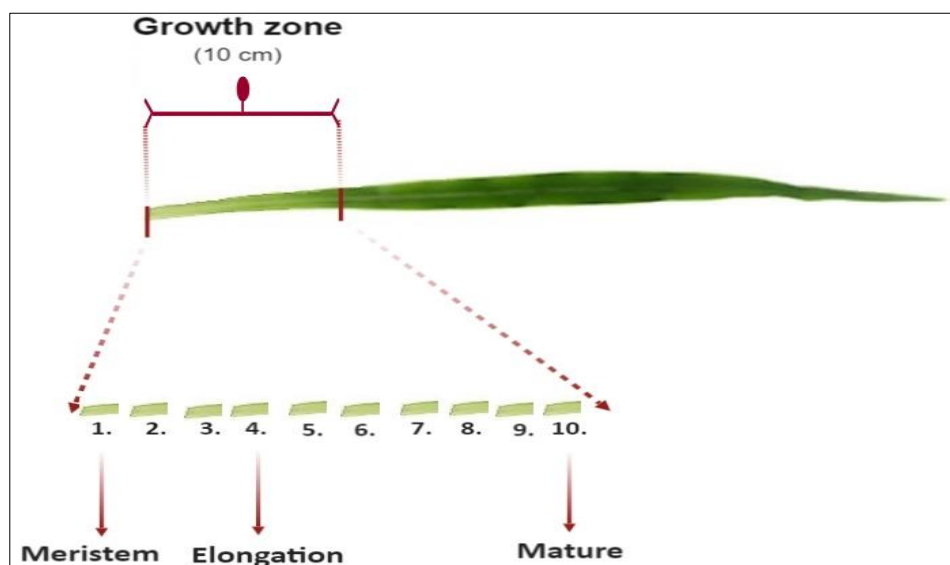


Figure 3.4. Preparation of samples from the growth zone of maize leaf.

The total RNA isolation from these tissue samples was performed according to the TRIzol method. Leaf segments of control and bacteria inoculated groups were taken from -80 °C into liquid nitrogen and three metal beads were placed into ependorf for homogenization. The ependorf tubes were placed in the homogenizer blocks and the homogenizer was set up to 30 seconds at 30 frequencies. When the process was over, ependorf tubes were quickly replaced into liquid nitrogen. 1 ml of TRIzol (Invitrogen) solution added into each tubes. Subsequently, tubes were vortexed well and allowed to incubate at room temperature for 5 minutes. When incubation is over, 200 µl of chloroform added into the ependorf corresponding to 1ml TRIzol tubes were mixed slightly by hand and then mixture was allowed to incubate for 3 min. The samples were centrifuged at 11,000 rpm at + 4 °C for 15 minutes and supernatant (phase of liquid) transferred to the new ependorf tubes. 450 µl of isopropanol was added onto supernatant which was taken to the new ependorf tubes (for 450 µl of supernatant). Samples were allowed to incubation at room temperature for 10 minutes. After incubation, the samples were centrifuged at 11,000 rpm at 4 °C for 10 minutes. Centrifuged supernatants in the ependorf tubes were discarded. The pellets were washed with 1ml of 75% EtOH (for 1ml TRIzol). The washed pellets were re-centrifuged at 11,000 rpm + 4 rpm for 5 min. When the centrifugation were done, EtOH was removed from tubes. The pellet in the tubes was allowed to dry. Finally, 35 µl of ddH₂O was added into ependorf tubes and the samples were stored at -20 °C. Concentrations of isolated RNAs were determined by Nano-Drop (Thermo Scientific, NanoDrop Lite Spectrophotometer).

3.10.4. cDNA Synthesis and Stem-loop Reverse Transcription

The cDNAs of the miRNA target genes were synthesized by using the protocol described in the kit SuperScript III First-Strand Synthesis Kit (Invitrogen). Here, mRNAs transformed into cDNA by using reverse transcriptase enzyme (RNA-dependent DNA polymerase) and oligo (dT) primers as a template. Isolated total RNAs were used for cDNA synthesis. For each reaction; 12.2 µl ddH₂O, 2 µl 10x RT buffer, 0.8 µl 25x dNTP mix (100mM), 2 µl 10x random primer, 1 µl Rtase (50u/ml) and 2 µl total RNA were used. The PCR conditions were as follows: 10 minutes at 25

°C, 120 minutes at 37 °C, 5 minutes at 85 °C and forever at 4 °C. After the reaction was finished, the samples were stored at -20 °C to be used.

The cDNA of miRNA were synthesized according to the stem-loop RT protocol that described by Varkonyi-Gasic et al. (2007) (Figure 3.5). For each reaction; 2 µl RT buffer(10x) , 0.2 µl dNTP (100mM), 2 µl stem-loop primer (5mM) , 4 µl of RNA(1000 µg/µl),0.8 µl of RTase (50m/ul) and 11 µl of ddH₂O (nuclease free) were used. Prior to the PCR reaction, stem-loop primers were pretreated at 65 °C for 5 minutes and on ice for 2 minutes. PCR device (PTC-200, MJ Research) was set up to 60 cycle in total; each cycle has 3 minutes at 16 °C, 30 seconds at 30 °C, 30 seconds at 42 °C and 1 second at 50 °C with an end stage that has 5 minutes at 85 °C. After the procedure is done, the samples were taken from the device and stored at -20 °C for later use.

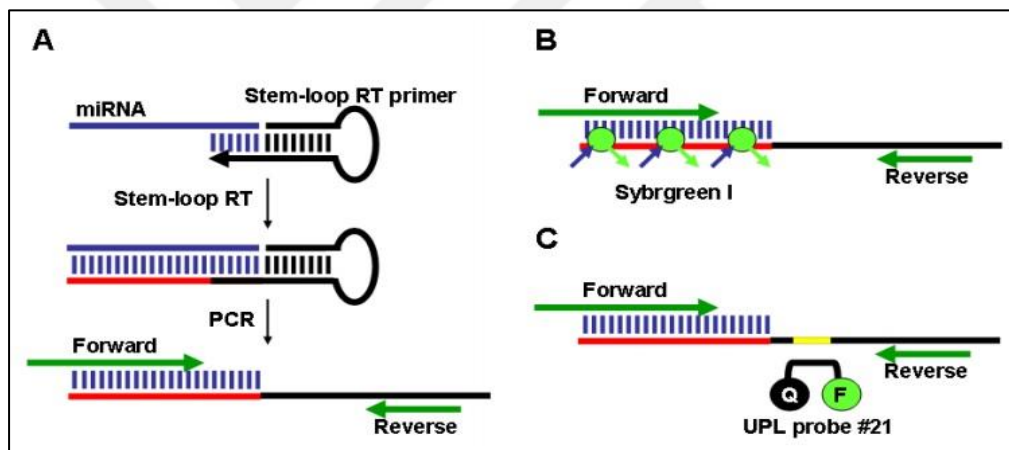


Figure 3.5: Schematic description of SYBR green I miRNA assays, SYBR green I-based real-time quantification of miRNAs includes two steps: A; stem-loop RT and B; SYBR green I real-time PCR assay.

3.10.5. Determination of Transcript Level by Quantitative RT-PCR (Quantitative-Real-Time-PCR) Method

The expression level of growth related miRNAs and their predicted target genes were determined by quantitative RT-PCR (qRT-PCR) (LightCycler® 480, Roche) method. Platinum® SYBR® Green qPCR SuperMix UDG (invitrogen) was used for qRT-PCR. For miRNAs; 8 µl of Syber Green, 1 µl of forward primer (10 mM), 1 µl of universal reverse (10 mM), 2 µl of c-DNA and 8 µl of ddH₂O were added. miRNA that target growth; 8 µl of Syber green, 1 µl of forward primer (10 mM), 1 µl of

reverse primer (10 mM), 1 μ l of cDNA and 9 μ l of ddH₂O were added. qRT-PCR conditions were set up to as: pre-denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 5 s, 60 °C for 10 s and 72 °C for 0.01 s. The melting curve was adjusted as 95 °C for 15s, 60 °C for 1 min and 95 °C for 15 s.

Relative amount of relative the expression was determined according to delta-delta Ct ($2^{-\Delta\Delta Ct}$) method. Normalization was performed with the β -tubulin gene (Housekeeping gene). Analysis were performed as 3 biological and 2 technical replicate. The relative transcript amount was compared to the meristem zone.

3.11. Statistical Analysis

Each experiment was repeated twice and each data included results from three biological replicates. The results were represented as means and error bars were shown as standard error in the graphs. The significance of the difference between the control and inoculated groups was calculated using the Microsoft excel Student T-test. $P < 0.05$ was considered the differences between groups was significant.

4. RESULTS

4.1. Visualization of KT2440 from Maize Root surface

Surfaces of the inoculated maize root (15 days old) were rinsed with cleaning solution and obtained elution were planted on LB Agar medium on a petri dish. After overnight incubation at 37°C, the petri dish were visualized via Imaging system (Azure Biosystem C600) to observe colonization of green fluorescence protein (GFP) labeled KT2440 (Figure 4.1.).

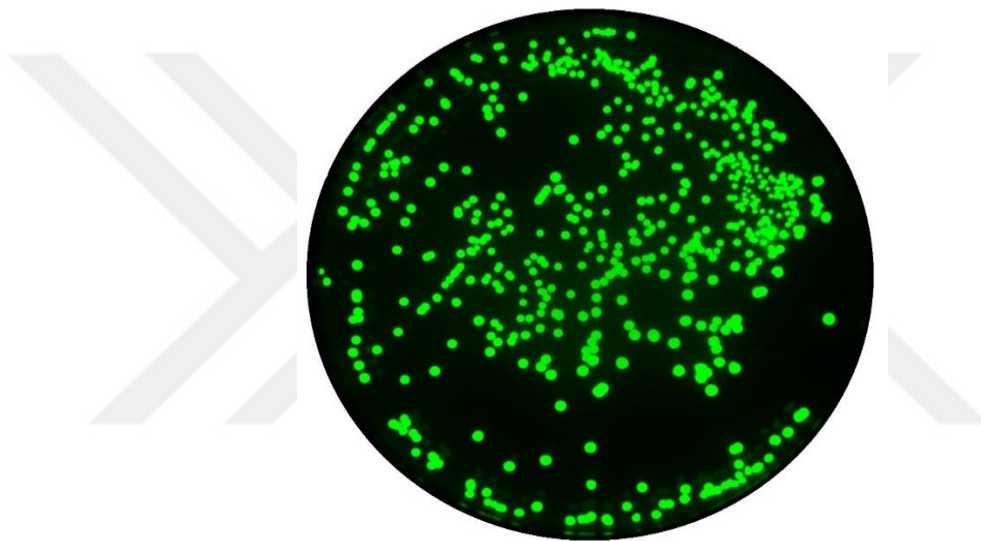


Figure 4.1: Colonization of the GFP-labeled *P. putida* KT2440 from inoculated roots surfaces.

P. putida KT2440 from the root surface of 15 days old seedlings were visualized by using DIC microscope (Differential Interference Microscope, Leica DM2500) (Figure 4.2).

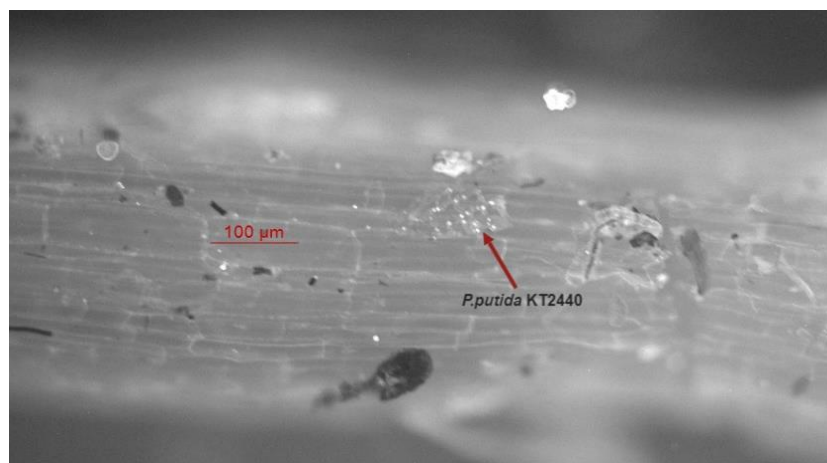


Figure 4.2: Visualization of the GFP-labeled *P.putida* KT2440 from the inoculated-root surfaces under DIC microscope.

4.2. Evaluation of Maize Hybrids against *Pseudomonas putida* KT2440 at Morphological, Cellular and Physiological Level

This part of the study aimed to show the effectiveness of possible PGPR indicators for selection of the best genotype across hybrid populations and to found out best hybrid line for transcriptional and enzymatic analysis. For this aim, the leaf elongation rate (LER), the final leaf length (FLL), the leaf area (LA), the shoot length (SL), the shoot fresh weight (SFW), the shoot dry weight (SDW), the mature cell length (MCL), the cell production (CP) and the chlorophyll content (CC) of the 3th leaf was measured comparatively with the bacteria-inoculated versus mock seedlings for 9 hybrids. As morphological parameters; final leaf length, shoot length, leaf area and leaf elongation rate were analyzed. For cellular level; mature cell length were measured and cell production were calculated by means of kinematic analysis. Lastly, chlorophyll content and shoot dry/fresh weight were analyzed for physiological parameters. Data were collected from phenotypic and microscopic observation and colorimetric measurement analyzed by statistical tools.

4.2.1. Evaluation of Maize Hybrids at Morphological Level

In the study, significant differences were observed between the final leaf length (FLL) and the shoot length (SL) of maize hybrids grown under mock and inoculated conditions. The final leaf length of hybrids grown under inoculated

conditions were not show significant differences in Sasa5, Sasa152 Ada1650 and Adasa16. However, significant increases were observed for FLL in Sasa99 (4%), in Sasa137 (8%), in Sasa139 (3%) and in Sasa166 (5%) ($P<0,05$). The final leaf length of Sasa186 were significantly reduced 10% due to inoculation ($P<0,01$) (Table 4.1). The shoot length (SL) of inoculated Sasa99, Sasa139, Adasa16, Sasa5, Sasa166 and Sasa137 showed similar significant increased profile ranging from 10% to 40% ($P<0,01$) (Table 4.1). Sasa186 and Ada1650 were showed reducing profile ranging from 15% to 35%. Sasa152 were not show significant differences between mock and inoculated conditions ($P<0,01$) (Table 4.1).

Leaf area (LA) of Sasa166, Ada1650 and Adasa16 were not show significant differences in both mock and inoculated conditions ($P>0,05$). However, LA of Sasa137 was increased 7% in inoculated conditions ($P<0,05$). Other hybrids displayed similar reduction profile ranging from 7% to 24% ($P<0,05$) (Table 4.1). The leaf elongation rate (LER) were significantly increased in inoculated Sasa139 (15%), Sasa166 (11%), sasa5 (5%), Sasa99 (7%) and Adasa16 (11%). LER of Sasa137 and Ada1650 were not show significant differences between mock and inoculated conditions ($P>0,05$). Mock treated Sasa152 and Sasa186 showed 8% reduction in LER ($P<0,05$) (Table 4.1).

Table 4.1: The final leaf length (FLL), the shoot length (SL), the leaf area (LA) and the leaf elongation rate (LER) of third leaf of maize hybrids grown under inoculated and mock condition.

		FLL(mm)		SL(mm)		LA(mm ²)		LER(mm/h)	
Sasa99	M	455,3	± 15	116	±6	45	±6	2,6	±0,2
	I	477,3	± 12	129	±8	36	±6	2,8	± 0,1
	%	(+4)**		(+11)**		(-19)**		(+7)*	
Sasa5	M	408,4	± 22	102	±4	38	±5	2,2	±0,1
	I	414,1	± 33	123	±10	35	±4	2,4	±0,08
	%	NS		(+21)		(-7)		(+5)**	
Sasa166	M	514,1	± 46	114	±14	52	±8	2,5	±0,3
	I	543,3	± 14	138	±8	51	±4	2,8	±0,1
	%	(+5)*		(+20)**		NS		(+11)**	
Sasa139	M	407,6	± 14	97	±8	42	±2	2,2	±0,06
	I	420,3	± 16	112	±4	33	±4	2,6	±0,1
	%	(+3)*		(+15)**		(-23)		(+15)**	
Sasa152	M	424,3	± 22	103	±6	50	±5	2,2	±0,2
	I	425,1	± 41	110	±10	40	±8	2,1	±0,6
	%	NS		NS		(-19)*		(-7)	

Table 4.1 continued

Sasa137	M	503,9	± 23	115	±11	54	±5	2,5	±0,2
	I	547,2	± 10	149	±7	58	±3	2,5	±0,1
	%	(+8)**		(+30)		(+7)*		NS	
Sasa186	M	472,2	± 26	122	±10	48	±7	2,5	±0,1
	I	425,2	± 31	95	±4	41	±7	2,2	±0,2
	%	(-10)**		(-33)		(-16)*		(-8)*	
Ada1650	M	443,9	± 29	122	±10	47	±5	2,6	±0,1
	I	455,1	± 22	104	±9	44	±5	2,6	±0,08
	%	NS		(-15)**		NS		NS	
Adasa16	M	373,9	± 32	86	±11	46	±3	2,1	±0,2
	I	389,6	± 15	123	±8	45	±3	2,3	±0,2
	%	NS		(+41)		NS		(+11)*	±0,2

M: Mock; I: Inoculated; ave ± sd; n= 9; NS: Not Significant; %: Percent changes between mock and inoculated conditions (-% represent percent reduction, +% represent percent increased); * Significant at p < 0.05, ** Significant at p < 0.01.

4.2.2. Evaluation of Maize Hybrids at Cellular Level

The mature cell length (MCL) of Sasa5, Sasa186 and Adasa16 were not show significant differences between inoculated and mock conditions. However, Sasa99 and Ada1650 showed significant reduction for 10% at cellular level under the inoculated condition (P<0,01) (Table 4.2). On the contrary, Sasa139 and Sasa166 showed significant increase on MCL about 9% compared to mock conditions (P<0,01) (Table 4.2). The cell production (CP) was decreased 9% and 15% in Sasa137 and Sasa186 respectively (P<0,05) (Table 4.2). CP in inoculated Sasa99, Sasa5, Sasa139 and Ada1650 seedlings were accelerated 17%, 6 %, 7%, 6%, respectively (P<0,01) (Table 4.2). Besides these, CP kept constantly in inoculated Sasa166, Sasa152, Sasa137 and Adasa16 seedlings compared to mock ones (P>0,05) (Table 4.2).

Table 4.2: The mature cell length (MCL) and the cell production (CP) of third leaf of maize hybrids grown under inoculated and mock condition.

		MCL(mm)		CP (cells/h)	
Sasa99	M	149	± 14	0,017	±0,003
	I	135	± 19	0,020	±0,001
	%	(-10)**		(+17)**	
Sasa5	M	145	± 6	0,015	±0,000
	I	144	± 11	0,016	±0,001
	%	NS		(+6)*	
Sasa166	M	140	± 6	0,018	±0,002
	I	153	± 10	0,018	±0,001
	%	(+9)**		(+2)	
Sasa139	M	137	± 6	0,016	±0,000
	I	147	± 8	0,017	±0,001
	%	(+7)**		(+7)*	
Sasa152	M	134	± 11	0,014	±0,006
	I	137	± 11	0,017	±0,003
	%	NS		(+15)	
Sasa137	M	139	± 12	0,017	±0,002
	I	148	± 11	0,015	±0,006
	%	(+6)		(-15)	
Sasa186	M	153	± 14	0,016	±0,001
	I	153	± 11	0,014	±0,001
	%	NS		(-9)*	
Ada1650	M	160	± 7	0,016	±0,001
	I	150	± 9	0,017	±0,001
	%	(-7)*		(+6)*	
Adasa16	M	138	± 15	0,015	±0,001
	I	145	± 9	0,016	±0,001
	%	NS		(+5)	

M: Mock; I: Inoculated; ave ± sd; n= 9; NS: Not Significant; %: Percent changes between mock and inoculated conditions (-% represent percent reduction, +% represent percent increased); * Significant at p < 0.05, ** Significant at p < 0.01

4.2.3. Evaluation of Maize Hybrids at Physiological Level

Shoot Fresh Weight (SFW) and Shoot Dry Weight (SDW) of inoculated hybrids showed significant increase of shoot fresh/dry weight profile ranging from 12% to 74% (P<0,01 and P<0,05, respectively). Ada1650 was only hybrid that reduced weight about 17% for SFW and 25% for SDW (P<0,01) (Table 4.3). CC were increased 10% (P<0,01) in Sasa5, 11% in Sasa166, 9% in Sasa139, 27% in Sasa137 and 6% in Adasa16 (P<0,05) (Table 4.3). However, CC were reduced 14% in Ada1650 (P<0,01) but remained unchanged in Sasa186 (P>0,05).

Table 4.3: The chlorophyll content (CC) and the shoot dry/fresh weight (SDW, SFW) of the third leaf of maize hybrids grown under inoculated and mock condition.

		CC (Spad)	SFW (g)	SDW(g)
Sasa99	M	41 ± 3	5,8 ±0,98	1,9 ±0,09
	I	42 ± 4	8,3 ±1,75	2,5 ±0,01
	%	NS	(+43)**	(+30)**
Sasa5	M	40 ± 2	5,1 ±0,70	1,4 ±0,06
	I	44 ± 3	9,3 ±1,31	2,1 ±0,27
	%	(+10)**	(+80)*	(+50)**
Sasa166	M	33 ± 3	5,3 ±01,47	1,3 ±0,15
	I	37 ± 5	8,5 ±0,711	2,1 ±0,04
	%	(+11)*	(+59)	(+53)**
Sasa139	M	33 ± 3	4,9 ±0,57	1,4 ±0,08
	I	36 ± 2	7,6 ±1,03	2,3 ±0,06
	%	(+9)*	(+56)*	(+55)*
Sasa152	M	37 ± 4	6,4 ±1,04	1,5 ±0,41
	I	40 ± 2	8,46 ±2,70	1,7 ±1,00
	%	(+9)*	(+30)*	(+12)
Sasa137	M	30 ± 2	5,5 ±0,99	1,5 ±0,18
	I	38 ± 3	9,1 ±0,95	1,6 ±0,29
	%	(+27)	(+65)*	(+10)
Sasa186	M	37 ± 3	6,4 ±1,29	1,6 ±0,22
	I	34 ± 4	11,1 ±1,53	2,6 ±0,37
	%	(-8)	(+73)*	(+58)*
Ada1650	M	35 ± 3	7,0 ±0,48	1,9 ±0,15
	I	30 ± 3	5,9 ±0,84	1,4 ±0,12
	%	(-14)**	(-17)**	(-25)**
Adasa16	M	36 ± 3	7,0 ±1,75	1,9 ±0,18
	I	38 ± 3	9,1 ±1,01	2,0 ±0,24
	%	(+6)*	(+29)* *	NS

M: Mock; I: Inoculated; ave ± sd; n= 9; NS: Not Significant; %: Percent changes between mock and inoculated conditions (-% represent percent reduction, +% represent percent increased); * Significant at p < 0.05, ** Significant at p < 0.0

4.2.4. Growth Parameters of Selected Hybrid Sasa139

According to evaluated data from screening studies, Sasa139 were selected among nine hybrids because as being represent growth promoting. To better understanding of the accelerated growth of Sasa139 leaf, detailed analysis were conducted on phenotype, cellular, molecular and enzymatic.

The length of the third leaf of mock and inoculated maize seedlings were measured daily from soil level as a reference point. The leaf elongation rate (LER) (mm/h) of the third leaf was calculated as derivative of the leaf length over time. It was observed that the third leaves of the mock and inoculated Sasa139 were grown at a constant rate for four days and then the growth rate decreased and stopped on

the tenth day . Third leaves of the inoculated seedlings showed better elongation rate at first 4 days when compared to the mock groups ($P < 0.05$) (Figure 4.3).

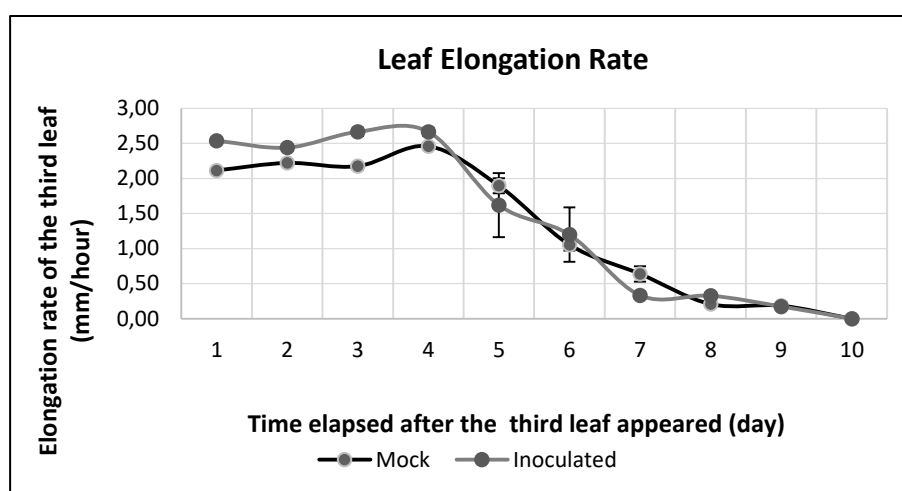


Figure 4.3: Leaf elongation rate (LER) of third leaf of maize seedlings grown under mock and inoculated conditions. LER (mmh^{-1}) was plotted against time period. Error bars indicate the standard error ($n = 9$, $P < 0.05$).

The cell length profile in leaf growth zone (10th from leaf base to tip) of Sasa139 grown under mock and inoculated conditions were determined by microscopic observation. Both mock and inoculated seedlings showed similar cell length profile at growth zone. The cell length at meristem zone was significantly short compared to elongation and maturity zones ($P < 0.05$) (Figure 4.4). The length of meristematic cells were calculated 19,2 μm in mock and 18,7 μm in inoculated leaves. The length of elongation cells were calculated 81 μm in mock and 87 μm in inoculated leaves. The length of mature cells were calculated 137 μm in mock and 132 μm in inoculated leaves ($P < 0.05$) (Figure 4.4). Mature cell length of inoculated groups were significantly higher (7%) than mock groups ($P < 0.01$) (Table 4.4).

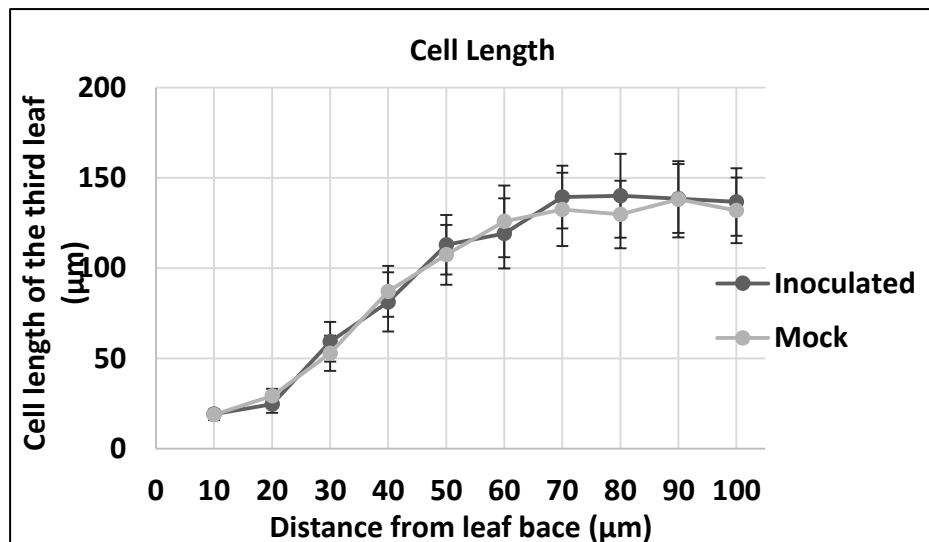


Figure 4.4: The cell length profile of growth zones of sasa139 grown under mock and inoculated conditions. Average $\pm$ standard error ($n=3$, $P<0,05$).

4.3. The expression Analysis of Cell Cycle Genes Response to *Pseudomonas putida* KT2440

To observed the promoted growth in maize leaf whether affect cell cycle or not, the expression of cell cycle genes controlling switch between cell cycle checkpoints were analyzed in meristem, elongation and mature zones. For this aim, *CDKA*, *CycB2_1*, *CDKB2*, *CycD5_1*, *CycD1* and *CycA2_1* were selected for analysis.

CDKA, displayed significant high level of expression profiles in meristem zone and low level expression in elongation and maturity zones under both inoculated and mock conditions. It has been observed that inoculated leaves showed a significant expression level of *CDKA* in mature zone compared to the mock conditions. Furthermore, the expression level of *CDKA* was high in elongation in mock leaves compared to the inoculated ones ($P<0,05$) (Figure 4.5).

CDKB2 expression level was found significantly up-regulated at meristem and mature zones in inoculated leaves. For mock conditions, the expression level of *CDKB2*, displayed high level of expression at meristem, elongation and mature zones. Comparative expression analysis showed that *CDKB2* highly expressed in elongation zones in mock leaves compared to the inoculated ones ($P<0,05$) (Figure 4.5). *CycB2_1* was significantly up-regulated at meristem zone and lowly expressed in elongation and mature zones in both mock and inoculated leaves. The expression

of *CycB2_1* showed significant differences level between growth zones ($P<0,05$) (Figure 4.5). *CycD5_1* showed high expression level at meristem zone in mock and inoculated conditions. On the contrary, the expression level was low at elongation and mature zones. At meristem zone, the expression level of *CycD5_1* was higher in inoculated conditions compared to the mock ($P<0,05$) (Figure 4.5). *CycD1* and *CycA2_1* were significantly high in meristem zone of the fourth leaves of mock and inoculated leaves. For both *CycD1* and *CycA2_1*, expression level was found to be significantly down-regulated at elongation and maturity zones compared to the meristem.

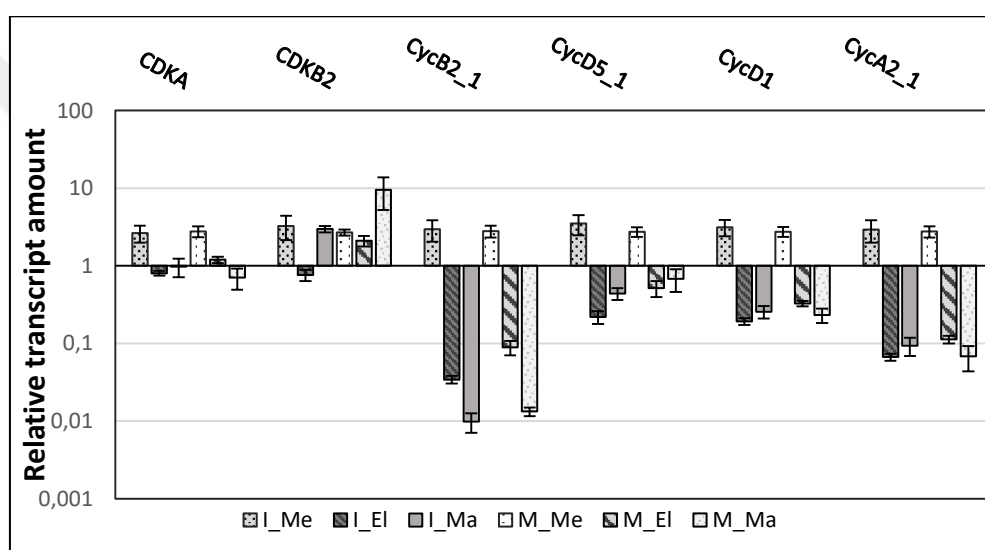


Figure 4.5: The expression analysis of selected cell cycle genes in the growth zones of the fourth leaf of maize seedlings grown under the mock (M) and inoculated (I) conditions. Me, El and Ma represents meristem, elongation and mature zones, respectively. Three biological and two technical replicates were used in the analyses. The error bars indicate the standard error and the Y-axis is logarithmic scale. ($n=3$, $P<0,05$).

4.4. Evaluation of Antioxidant Enzyme Activity of Sasa139 Response to *Pseudomonas putida* KT2440

4.4.1. Evaluation of Glutathione reductase (GR) Enzyme Activity

Studies have done with fourth leaves of maize seedlings that grown under inoculated and mock conditions showed that glutathione reductase enzyme activity

significantly increased following inoculation in compared to the mock condition at both meristem, elongation and mature zones. On the contrary, the glutathione reductase activity of inoculated samples decreased approximately three fold from meristem to the mature zone, while the mock group decreased approximately 2,5 fold ($P<0,05$) (Figure 4.6). A significant increase of glutathione reductase activity was observed in growth regions of the fourth leaves of inoculated maize seedlings compared to the mock conditions. It was observed that the catalase activity of inoculated samples was four times higher in the meristem zone compared to the mock groups. In the elongation zone this rate was observed as 1,5 fold higher in the countenance of inoculated treatment. Catalase activity of maturity zone of inoculated group was 3,5 fold higher compared to the mock group. ($P<0,05$) (Figure 4.6).

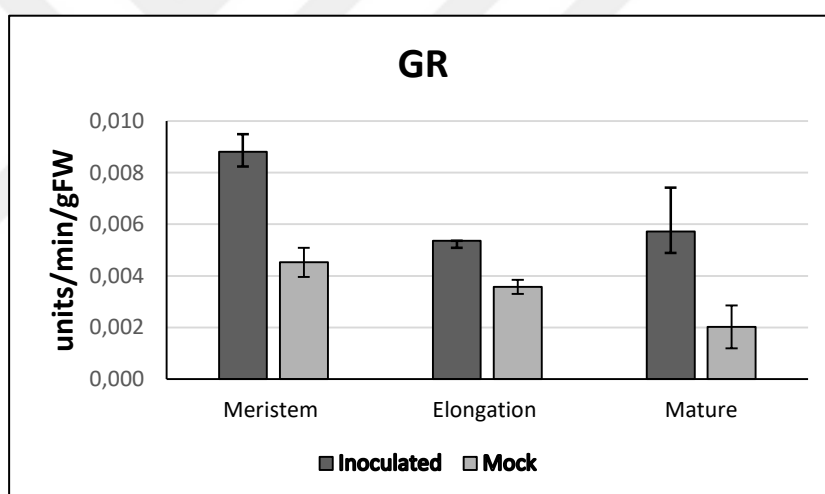


Figure 4.6: Glutathione reductase enzyme activity in growth zones of Sasa139 fourth leaf under inoculated and mock conditions. The data represented as mean, and error bars indicated as $\pm$ standard error ($n=3$, $P<0,05$).

4.4.2. Evaluation of Ascorbate Peroxidase (APX) Enzyme Activity

Maize seedlings that were grown under inoculated and mock conditions showed significantly high activity of ascorbate peroxidase enzyme at meristem and elongation zone following inoculated treatment in comparison to the mock. Also, the figure indicated that ascorbate peroxidase (APX) enzyme activity in the fourth leaves of maize seedlings increased in the meristem zone and showed a decreasing

profile as it through the elongation and mature zones for both mock and inoculated leaves. This showed that, the ascorbate peroxidase activity of inoculated samples decreased approximately 3 fold from meristem to the maturity zone ($P<0,05$)(Figure 4.7).

For the meristem zone, a significant increase of APX activity was observed in fourth leaves of inoculated maize seedlings compared to the mock treatment. It was observed that the APX activity of inoculated samples was approximately 4,5 fold higher in the meristem zone compared to the mock groups. Inoculated treatments also showed significant increase like 5 fold higher activity of ascorbate peroxidase in the elongation zone compared to the mock. ($P<0,05$)(Figure 4.7).

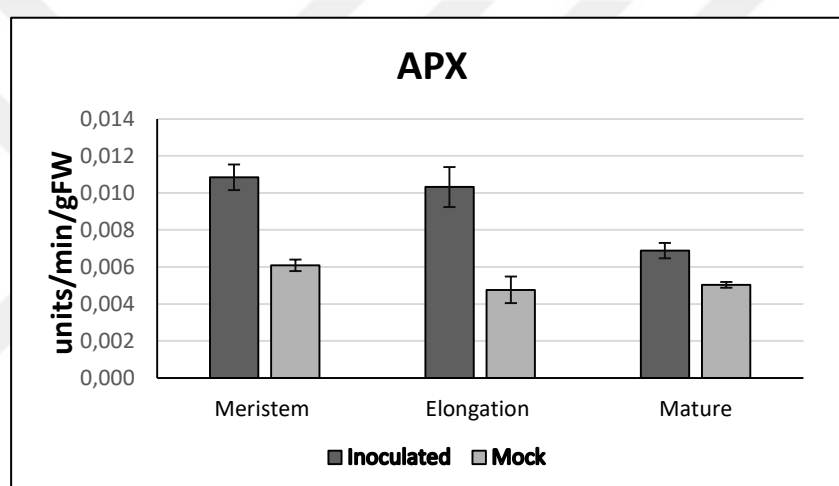


Figure 4.7: Ascorbate Peroxidase enzyme activity in growth zones of Sasa139 fourth leaf treated with *Pseudomonas putida* KT2440 and mock. The data represented as mean, and error bars indicated as $\pm$ standard error ($n=3$, $P<0,05$).

4.4.3. Evaluation of Catalase (CAT) Enzyme Activity Results

Activity of catalase enzyme from meristem to the maturity zone showed a profile that decreases gradually in mock group while inoculated group has not changed. The catalase enzyme activity of the mock group compared to the inoculated maize seedlings showed a significant increase; following 30% higher in the meristem zone and 15% higher in the elongation. ($P<0,05$) (Figure 4.8).

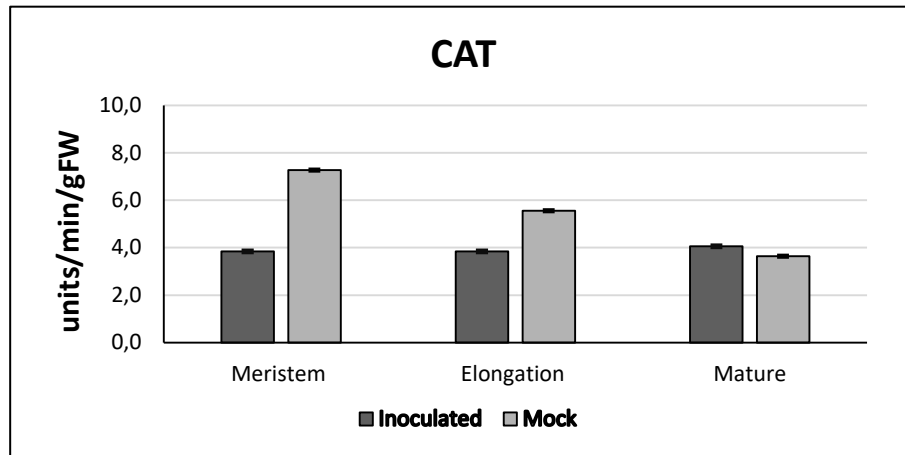


Figure 4.8: Catalase enzyme activity in growth zones of Sasa139 fourth leaf treated with *Pseudomonas putida* KT2440 and mock. The data represented as mean, and error bars indicated as $\pm$ standard error (n=3, P<0,05).

4.4.4. Evaluation of Peroxidase (POX) Enzyme Activity Results

It has been observed that peroxidase (POX) enzyme activity is gradually decreasing from the meristem to the elongation zone, and showed an increasing profile from the elongation to the maturity zone for both inoculated and mock groups. In peroxidase enzyme assay no significant difference and increase observed at the meristem and elongation zones of the fourth leaves of the maize seedlings for both conditions. However, the peroxidase activity in the region of maturity zone significantly increased approximately 1,5 fold compared to the control (P<0,05) (Figure 4.9).

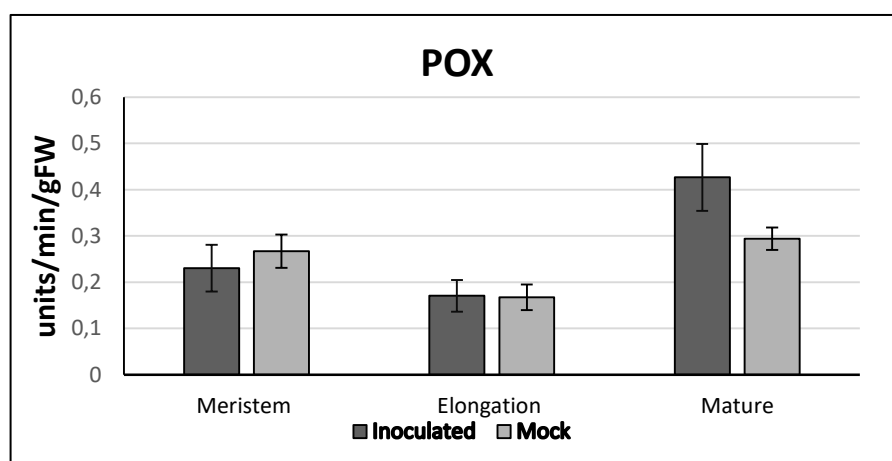


Figure 4.9: Peroxidase enzyme activity in growth zones of Sasa139 fourth leaf treated with *Pseudomonas putida* KT2440 and mock. The data represented as mean, and error bars indicated as $\pm$ standard error (n=3, P<0,05).

4.4.5. Evaluation of Superoxide Dismutase (SOD) Enzyme Activity Results

Activity of superoxide dismutase (SOD) enzyme from meristem to the mature zone showed a profile that decreases gradually in control group while inoculated group has not changed. It has been observed that superoxide dismutase (SOD) activity for the meristem and elongation zone showed a significant increase in fourth leaves of mock maize seedlings compared to the inoculated treatment. However, at maturity zone significant difference were not observed for both inoculated and mock groups. It was observed that the superoxide dismutase activity of mock leaves were approximately 4 fold higher in the meristem zone and 2,5 fold in elongation zone compared to the inoculated leaves ($P < 0,05$) (Figure 4.10).

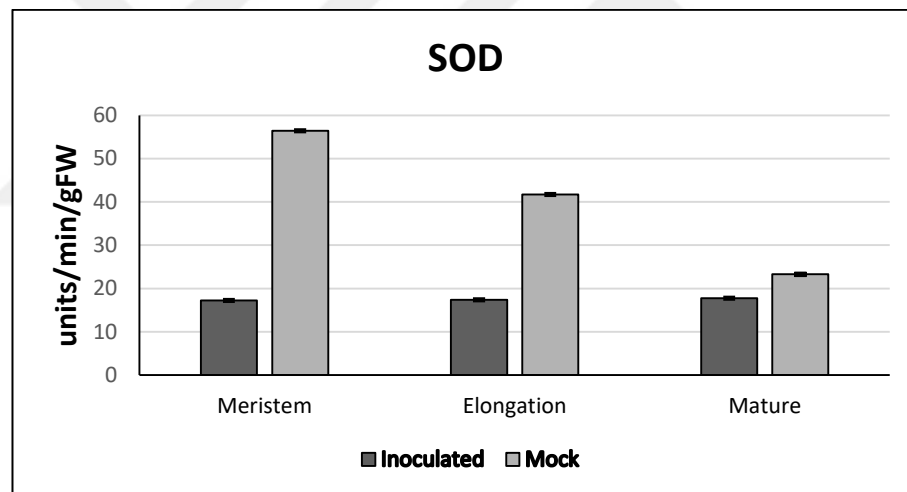


Figure 4.10: Superoxide Dismutase enzyme activity in growth zones of Sasa139 fourth leaf treated with *Pseudomonas putida* KT2440 and mock. The data represented as mean, and error bars indicated as $\pm$ standard error ($n=3$, $P < 0,05$).

4.5. The expression Analysis of Putative Growth-related miRNAs and Their Predicted Targets Response to *Pseudomonas putida* KT2440

4.5.1. The Expression of miR160 and *ARF17*

The expression level of miR160 was high in meristem, elongation and maturity zones for both mock and inoculated plant leaves. In mature zone this expression level was high compared to the meristem and elongation zones for both conditions. It was observed that miR160 significantly highly expressed in mature zone of inoculated plant leaves compared to the meristem and elongation zones. When mock and inoculated leaves were compared; miR160 down-regulated at meristem and elongation zones response to *P. putida* ($P < 0,05$) (Figure 4.11).

The expression level of predicted target *ARF17* showed the same highly expressed profile as mir160 at meristem and elongation zones in both mock and inoculated leaves. When the inoculated conditions were compared by the expression level at meristem, elongation and mature zones it was observed significant differences between themes. Concordantly, the expression level of *ARF17* was significantly high at meristem zone compared to the elongation zone both conditions. There was seen reciprocal expression pattern between miR160 and its predicted target at meristem and mature zones ($P < 0,05$) (Figure 4.11).

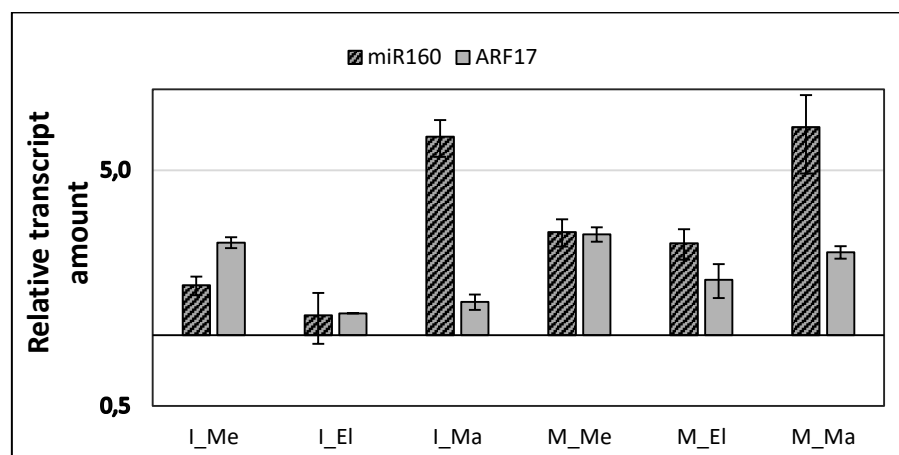


Figure 4.11: The expression analysis of miR160 and its predicted target gene *ARF17* in leaf growth zone of maize seedlings grown under the mock (M) and inoculated (I) conditions. Me, El and Ma represents meristem, elongation and mature zones, respectively. Three biological and two technical replicates were used. The error bars indicate the standard error and the Y-axis is logarithmic ($n=3$, $P < 0,05$).

4.5.2. The Expression of miR169 and *NF_YA*

It has been observed that the expression level of miR169 in the fourth leaves of the inoculated maize seedlings were high at mature zone and was low at meristem and elongation zones. In inoculated leaves, miR169 were significantly up-regulated only at mature zone. For mock leaves, the expression level of miR169, displayed high level of expression at meristem, elongation and mature zones. The expression of miR169 was significantly high at mature zone compared to the elongation zone ($P < 0,05$) (Figure 4.12).

The expression of *NF_YA* which are predicted as target for miR169 was up-regulated at meristem and mature zones for both mock and inoculated leaves. Contrastingly, *NF_YA* was down-regulated at elongation zone in inoculated leaves. The expression of miR169 was low at meristem and elongation zone while the expression of *NF_YA* was higher in inoculated leaves. This antagonist expression profile between miR169 and *NF_YA* indicated that there might be an regulation in meristem and elongation zones ($P < 0,05$) (Figure 4.12).

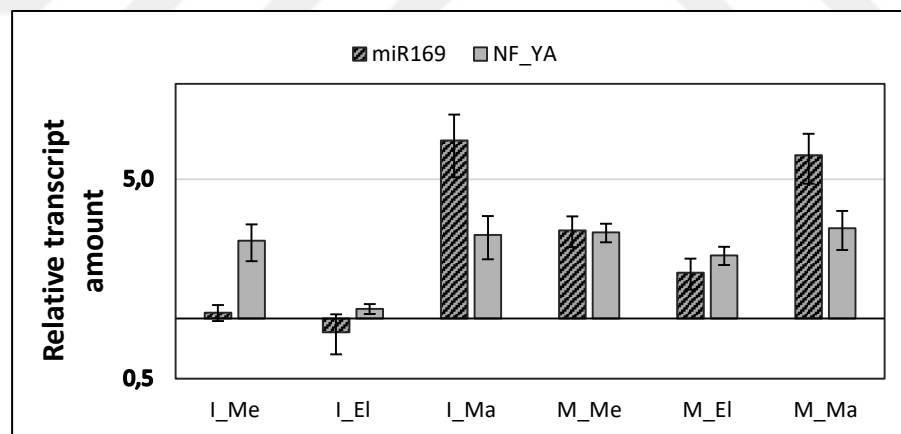


Figure 4.12: The expression analysis of miR169 and its predicted target gene *NF_YA* in leaf growth zone of maize seedlings grown under the mock (M) and inoculated (I) conditions. Me, El and Ma represents meristem, elongation and mature zones, respectively. For each expression, three biological and two technical replicates were used. The error bars indicate the standard error and the Y-axis is logarithmic.

($n=3$, $P < 0,05$).

4.5.3. The Expression of miR319 and *TCP38*

The expression of miR319 was significantly higher at meristem zone when compared to the elongation and mature zones in mock and inoculated leaves. Concordantly, a notable down-regulation was observed at elongation and maturity zones. ($P < 0,05$) (Figure 4.13).

The expression of *TCP38* was high expression meristem, elongation and mature zones. In inoculated and mock conditions, *TCP38* was significantly up-regulated at elongation compared to the meristem and maturity zones. However comparative expression analysis of *TCP38* between inoculated and mock conditions did not show significant differences at growth zones ($P < 0,05$) (Figure 4.13).

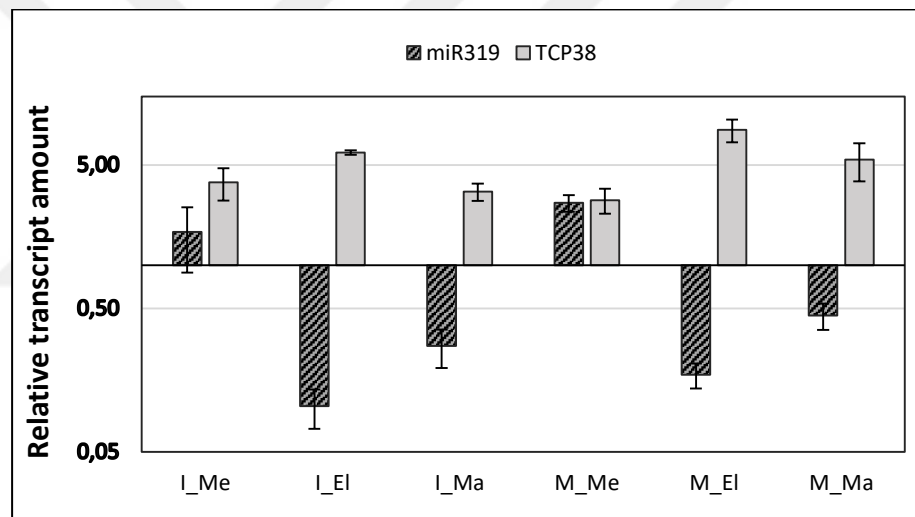


Figure 4.13: The expression analysis of miR319 and its predicted target gene *TCP38* in leaf growth zone of maize seedlings grown under the mock (M) and inoculated (I) conditions. Me, El and Ma represents meristem, elongation and mature zones, respectively. For each expression, three biological and two technical replicates were used. The error bars indicate the standard error and the Y-axis is logarithmic.

($n=3$, $P < 0,05$).

4.5.4. The Expression of miR396 and *GRF15*

Expression analysis of miR396 predicted as target for cell proliferation in leaves showed significant high level of expression profiles from meristem to the maturity zones in both inoculated and mock conditions. For both conditions,

significant high expression level was observed at elongation and maturity zones compared to the meristem zone. MiR396 showed significant high expression at maturity zone in inoculated condition compared to the mock. Expression level of miR396 was high at elongation zone in mock compared to the inoculated condition ($P<0,05$) (Figure 4.14).

Predicted target *GRF15* was found to be highly expressed at meristem and down-regulated at elongation and maturity in both conditions. In the case of miR396 targeting a predicted target GRF, an antagonist association is expected. Concordantly, expression of *GRF15* was down-regulated while miR396 up-regulated at elongation and maturity zones. Results indicate that miR396 and *GRF15* found to be differentially expressed under inoculated and mock conditions at elongations and maturity zones ($P<0,05$) (Figure 4.14).

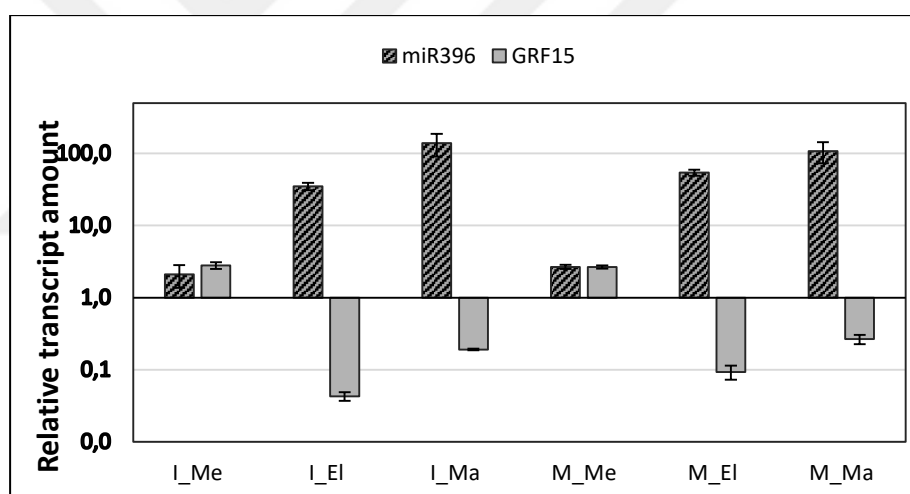


Figure 4.14: The expression analysis of miR396 and its predicted target gene *GRF15* in leaf growth zone of maize seedlings grown under the mock (M) and inoculated (I) conditions. Me, El and Ma represents meristem, elongation and mature zones, respectively. For each expression, three biological and two technical replicates were used. The error bars indicate the standard error and the Y-axis is logarithmic.

($n=3$, $P<0,05$).

4.5.5. The Expression of miR408 and Laccase/Basic Blue Protein

MiR408 showed persistently increased expression at meristem, elongation and mature zones in both inoculated and mock conditions. For both group,

expression of miR408 was found to be significant at elongation and maturity when compared to the meristem. Mir408 showed higher expression at elongation and mature zones in mock conditions compared to the inoculated ($P<0,05$) (Figure 4.15). The predicted targets *BBP* (basic blue protein) and *Lac* (laccase) showed differentially expression profile at growth zones. *BBP* were significantly up-regulated at meristem compared to elongation and maturity zones in both conditions. However, *BBP* showed remarkable down-regulation at elongation and maturity zones. Expression profile of *Lac* was high at meristem, elongation and maturity zones. Laccase showed increased expression profile following meristem through the maturity zones. In mock and inoculated conditions, expression of *lac* was significantly higher at maturity zone compared to the meristem. Expression of *lac* was high at maturity zone in mock compared to inoculated conditions. Result promote that, mir408 and lac highly expressed at growth zones while *BBP* down-regulated at elongation and maturity zones. This reciprocal expression of *BBP* indicate that being of a putative target for miR396 ($P<0,05$) (Figure 4.15).

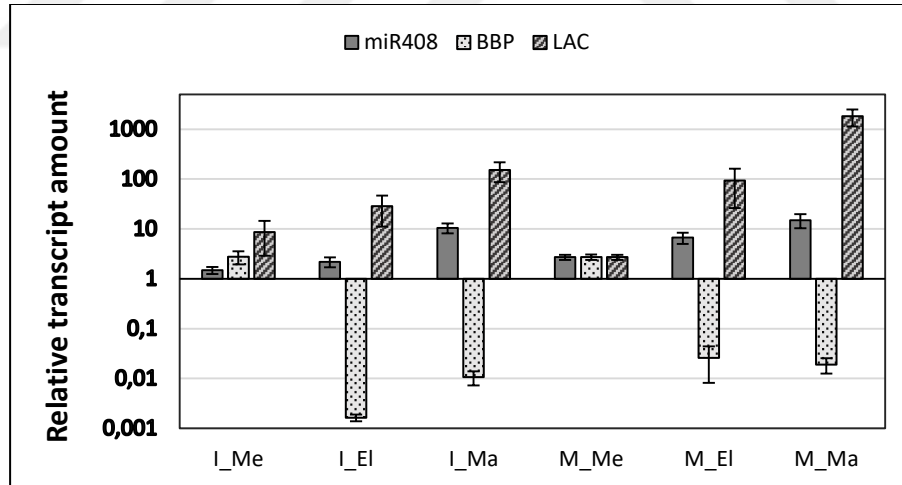


Figure 4.15: The expression analysis of miR408 and its predicted target gene *BBP* and *LAC* in leaf growth zone of maize seedlings grown under the mock (M) and inoculated (I) conditions. Me, El and Ma represents meristem, elongation and mature zones, respectively. For each expression, three biological and two technical replicates were used. The error bars indicate the standard error and the Y-axis is logarithmic. ($n=3$, $P<0,05$).

5. DISCUSSION AND CONCLUSION

Growth of an organ is a result of cell division and cell expansion processes and the size of plant organs depends on the number and size of their own cells. The leaf elongation rate (LER), the final leaf length (FLL), the leaf area (LA), the shoot length (SL), the shoot fresh weight (SFW), the shoot dry weight (SDW), the mature cell length (MCL), the cell production (CP) and the chlorophyll content (CC) of the 3th leaves were measured comparatively in KT2440 inoculated versus mock seedlings for screening of nine hybrids. When LER was considered between hybrids, Sasa139 and Sasa166 showed significant increase with 15% under the inoculated conditions ($P<0,01$). Sasa186 showed 8% reduction ($P<0,05$) under mock conditions. Sasa137 and Sasa139 FLL were best expanded under inoculated conditions whereas Sasa186 leaves were mostly affected with 10% reduction. LA of Sasa137 was higher in inoculated conditions with 7% ($P>0,05$). LA of other hybrids displayed similar reduction profile ranging from 8% to 23%.

MCL of Sasa139 and Sasa166 were longer in inoculated conditions with 8% percent compared to the mock ($P<0, 01$). Sasa99 showed shortened MCL with 10% reduction ($P<0,01$). CP value was increased for Sasa99, Sasa5, Sasa139 while it was reduced for Sasa137 and Sasa186 ($P<0,05$). Considering all results from growth-related indicators, Sasa139 drew attention because LER, SL, MCL, CP ,CC, SFW and SDW were higher under the response of *P. putida* KT2440.

After morphological and cellular growth, plants try to keep homeostasis via physiological rearrangements for growth development processes. In this context, SFW, SDW and CC were assigned as possible physiological parameters for growth. The results showed that, SFW and SDW of Sasa139, Sasa5, Sasa99, and Adasa16 were higher in inoculated conditions. When CC data were evaluated, photosynthesis activity of the Sasa166, Sasa139 and Sasa137 hybrids were significantly high in inoculated conditions. These results indicate that KT2440 inoculated seedlings had showed better growth parameters compared to the mock. These finding together indicated that bacteria inoculated seedlings experienced beneficial effects on growth processes of maize via direct or indirect metabolism.

Quantifying parameters used in screening is always obstacle. Because each hybrid displays different performance according to different parameters. In this case, according to a screening study, Sasa139 was selected as the best hybrid for transcriptional studies under response of KT2440.

It is well known that plant growth promoting rhizobacteria increase the growth and development of plants by colonizing the roots. These beneficial rhizobacteria have been comprehensively studied for their capacity to adhere to maize seeds [Espinosa and Ramos, 2004] and to regulate colonization with cell to cell communication [Fernández et al., 2012].

Previous studies have reported changes in the expression of several genes in response to PGPR inoculation during root colonization, metabolic changes and various abiotic stresses [Wei et al., 2014; Ram et al., 2018]. However, direct involvement of PGPR on miRNA expression has not been reported in literature, but it has been shown that the expression of miRNAs contributes to growth and development in plants [Mallory et al., 2005; Jones et al., 2006]. Detailed analysis of the genome of KT2440 conducted by Wu et al., (2010) revealed that the presence of genes in pathways dedicated to PGPRs for promoting growth such as the production of phytohormones by their own mechanisms. In this context, it might be strongly possible that KT2440 could alter the regulation of miRNAs and may induce the adaptive responses related to growth and development of maize. Therefore, this study mainly investigated the maize-miRNA and KT2440 interactions during leaf growth by analyzing the changes in the expression levels of 5 growth related miRNAs and their predicted target genes in maize seedlings.

The possible miRNAs involved in the growth and development mechanisms of maize-bacteria interaction have been identified by searching literature studies. The target genes of these miRNAs in maize plant were identified through *in-silico* analyses by bioinformatic tools. From bioinformatics analysis, miR160 predicted target gene was determined as auxin response factor 17 (*ARF17*) gene; miR169's was nuclear transcription factor Y subunit alpha (*NF_YA*); miR319's was transcription factor (*TCP38*), miR396's was growth-regulating factor 15 (*GRF15*) and miR408's were laccase (*Lac*) and basic blue protein (*BBP*).

Transcriptional analyses were performed, in order to demonstrate the relationship between miRNAs and their predicted target genes in plant growth and development. For this purpose, their expression level in Sasa139 leaf growth zones which includes meristem (1th cm), elongation (4th cm) and mature (10th cm) were analyzed by qRT-PCR and determined by DeltaDeltaCt ($2^{-\Delta\Delta Ct}$) methods.

A study done by Mallory et al. (2005), indicated that miR160 and its predictable target gene *Auxin response factor (ARF)* has crucial role in growth and development for plants. From this analysis, miR160 expression was found to be highly expressed at meristem and maturity zones in both the control and inoculated groups. Statistically significant high expression level was observed at maturity zone of inoculated and mock conditions. It has been observed that miR160 can suppress the level of expression by targeting the *ARF17* at maturity zone. Up-regulation of *ARF17* at meristem zone indicated that it has critical role in cell proliferation which is rich in daughter cells.

The study conducted by Xu et al., (2014) indicated that the miR169 and its predicted target *NF-YA* has a significant role in plant growth and plant responses to abiotic stress. The over-expression of *rNF-YA2* gene resulted in late flowering, indicating that miR169 regulates stress-induced flowering by suppressing the expression of NFYAs in *Arabidopsis thaliana*. From our analysis, it was observed that the expression level of miR169 were not change in the zone of meristem and elongation in the inoculated group, while up-regulated in the mature zone. However, in the inoculated condition negative regulation were observed at elongation zone. Expression level of *NF_YA* was low at meristem and elongation zone in inoculated conditions. This low-expression level of *NF_YA* might indicate that inoculated seedlings would have early flowering.

The study conducted by Koyama et al. (2017) indicated that miR319 and its predicted target gene (*TCP*) played a crucial role in leaf development in *Arabidopsis thaliana*. Suppression of *TCPs* caused proliferation and induced the differentiation of cells in *Zea mays* [Schommer et al., 2014]. In this study, expression analysis exhibited opposite profiles at the elongation and maturity regions in hybrid Sasa139, where maize *TCP38* was estimated as the target for miR319. *TCP38* were down-regulated in the meristem and up-regulated in elongation and maturity zones

in both groups. As expected, expression level of miR319 was significantly high at meristem than in elongation and mature zones in inoculated and non-inoculated conditions.

Rodriguez et al., (2010) indicate that miR396 and its predicted targets *GRF15* plays role in cell proliferation in leaves of *Arabidopsis*. In the case of miR396 targeting a predicted target *GRF15*, an antagonist association is expected. Collaterally, expression of *GRF15* was down-regulated while miR396 up-regulated at elongation and maturity zones ($P < 0.05$). The results indicate that at maturity zones inoculated groups showed significant high expression at elongation (cell expansion) and maturity (cell differentiation) compared to the mock condition. In this context, interaction between maize and *P. pseudomonas* KT440 were not show any negative affect on plant growth. The significant high expression level of mir396 under the inoculated conditions revealed that this beneficial interaction helped the growth processes in maize seedlings.

Zhaoqing et al. (2017) showed that targeted over-expression of miR408 in *Arabidopsis* changed plant morphology by significantly increase leaf area, petiole length, and plant height. Therefore, the results of this thesis revealed that miR408 probably has a pleiotropic effect on growth and development in plants. In this study, for both group, expression level of miR408 was found to be significantly up-regulated at elongation (cell expansion) and maturity (cell differentiation) zones compared to the meristem (cell proliferation). Up-regulation of miR408 at elongation and maturity zones indicate that the increase in plant size was mainly due to cell expansion rather than cell proliferation. Predicted targets of miR408; *BBP* (*Basic blue protein*) and *Lac* (*Laccase*) showed differential expression profile between the growth zones. *BBP* showed significant high expression at meristem and low expression level at elongation and maturity zones in both inoculated and mock conditions. The result indicated that the predicted target *BBP* is meristem specific while *Lac* was elongation and maturity specific.

Glutathione reductase (GR) enzyme activity in maize seedlings grown under inoculated conditions showed significantly high and close activity as following four times in the meristem zone, 1,5-fold in the elongation zone and 3,5-fold in the mature zone, compared to mock groups ($P < 0.05$). Sudhir et al., (2011) indicate that,

antioxidant enzymes such as APX, CAT and GR in leaves of wheat inoculated with PGPR helped to reduce activities as compared to control plants growing under salinity stress. In this study, activity of glutathione reductase was not significantly high at growth zones when compared to mock groups.

In this study, it was observed that ascorbate peroxidase (APX) activity in the fourth leaves of maize seedlings was increased in meristem zone and decreasing profile as it progressed to elongation and maturity zones. In addition, it was determined that enzyme activity was statistically higher in all growth segments of inoculated maize seedlings than in mock groups. However, in general this activity levels was at lowest level for both group when compared to the other studies in literature [Sandhya et al., 2010]

In this study, it was found that the activity of catalase (CAT) enzyme exhibited an unchanged profile in the growth regions of maize seedlings grown under mock and inoculated conditions. CAT activity was low in maize seedlings grown in inoculated conditions compared to mock group. Based on the observed data, it can be speculate that maize plant roots were not perceive these beneficial bacteria as a stress. A study conducted by Sandhya et al., (2010) showed similar results under PGPR inoculation in maize.

In peroxidase (POX) enzyme assay, no significant difference was observed at the meristem and elongation zones of the fourth leaves of the maize seedlings for both conditions. Avramova et al. (2015) indicated that activity of POX was highest at meristem zone in drought stress conditions. In this study, it was observed that peroxidase (POX) enzyme activity was decreased from the meristem to the elongation zone, and showed opposite increasing profile from the elongation to the mature zone in both conditions. POX enzyme activity is found in the meristem zone and is known to trigger cell metamorphisms and growth [Crevecœur et al., 1997]. In this experiment, the activity of POX was found to be twice as higher at mature compared to the meristem and elongation zones ($P < 0.005$).

Activity of superoxide dismutase (SOD) enzyme from meristem to the mature zone showed a gdecreasing profile in mock group while inoculated group was not changed. Neto et al., (2016) found that regulation of SOD enzyme increased and decreased in some plant lines under abiotic stress conditions. In this study, SOD

activity in mock group showed decreasing profile from meristem to the mature zone while it was low at all zones in inoculated conditions ($P < 0,005$).

Meristem zone of plant cells has high division ability for plant growth. This zone has complex growth processes mechanism that regulated by cell cycle genes. The selected cell cycle genes were used in this study indicate that all cell cycle genes were significantly up-regulated at meristem zone ($P < 0,05$) (Figure 4.5).

Regulation of the cell cycle is crucial for plant growth and development. The cell cycle is mainly regulated by cyclin, cyclin-dependent kinases (CDK) which belongs to serine / threonine protein class and cyclin-dependent kinase inhibitors (CDI). Normally, CDKs are inactive when they are present on their own but they become activated when bound to cyclin proteins. In this context, the homologs of the genes involved in the cell cycle of the model organism *Arabidopsis* [De Jager, 2005].

CDKA protein which plays role in transition between G1 and S phase, showed high expression level at meristem zone and low-expression level at elongation and mature zones for both inoculated and non-inoculated conditions. It has been observed that inoculated group showed a significant expression level of *CDKA* in mature zone compared to the mock conditions ($P < 0,05$).

CDKB2 protein involved in G2 phase of mitosis in cell cycle. *CDKB2* were upregulated at all growth zones of inoculated and mock conditions. However, *CDKB2* were down-regulated at elongation zone of inoculated conditions.

Cyclins used in this study (*CycB2_1*, *CycD5_1*, *CycD1*, and *CycA2_1*) showed high expression at meristem and slightly expressed at elongation and mature zones in both mock and inoculated conditions ($P < 0,05$). Meristem zones are place for cell division and cell cycle processes. Results indicate that cell cycle regulator at meristem zone actively regulate cell cycle processes. Inoculated groups showed positive response of cell cycle regulators.

As a conclusion, maize hybrids displayed different growth response to *P. putida*. Sasa139, Sasa166 and Sasa137 hybrids growth were increased while Ada1650 and Sasa186 were inhibited. Growth of Adasa16, Sasa5 and Sasa152 hybrids were not affected by inoculation of *P. putida*. A mutualistic interaction was observed between Sasa139 and *P. putida*. Leaf elongation rate (LER) of Sasa139

was 15% increased under *P. putida* inoculation. Chlorophyll content of Sasa139 were 10% higher in inoculated conditions compared to the mock. Shoot length of inoculated Sasa139 was 15% taller than mock group. Fresh and dry weight of inoculated Sasa139 were 56% more than mock. Mature cell length (MCL) and cell production (CP) of inoculated Sasa139 were 8% higher when compared to mock.

The cell cycle genes that controlling switch between cell cycle checkpoints were highly up-regulated at meristem zones under both mock and inoculated conditions. The expression level of *CycD5_1* at meristem zone was higher in inoculated Sasa139 seedlings.

P. putida caused fluctuation on redox regulation. Antioxidant enzymes activity differentiated between growth zones. Ascorbate peroxidase (APX) and glutathione reductase (GR) enzyme activity were significantly increased in meristem, elongation and mature zones of *P. putida* inoculated Sasa139. Catalase (CAT) and superoxide dismutase (SOD) enzyme activity were decreased in inoculated Sasa139.

MiRNA genes and their predicted targets differentially expressed between growth zones response to *P. putida* inoculation. The expression level of miR160 and miR169 were low at meristem and elongation and high at mature zone in inoculated Sasa139. Reciprocal regulation was observed between miR169 and *NF_YA* in inoculated conditions. MiR319 were up-regulated at meristem and down-regulated at elongation and mature zone in both conditions. The expression analysis of mir319 showed similar results between mock and inoculated conditions. MiR396 showed significant high expression (30% higher) at maturity zone in response to *P. putida*. MiR396 and *GRF15* found to be differentially expressed at elongation and mature zone in both conditions. MiR408 were up-regulated at all growth zones. Predicted targets; lac highly expressed at meristem zones of inoculated Sasa139. BBP was up-regulated at meristem and down-regulated at elongation and maturity zones in both conditions. Growth related miRNAs and their predicted targets involved in the growth and development mechanisms of maize and *P. putida* KT2440 interaction were differentially expressed in this study.

The results of this study will revealed the use of *Pseudomonas putida* KT2440 as an additional nutrient (bio-fertilizer) supplement to the soil. In

accordance with the information, it will provide an alternative solution to prevent wrong fertilization in agriculture. Plant-microbe interaction examined at the gene level will provide insight into biotechnological applications in future breeding studies and will serve as a model study in monocots.

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