

FUNCTIONAL ANALYSIS OF A miRNA PUTATIVELY INVOLVED IN  
POWDERY MILDEW DISEASE SUSCEPTIBILITY IN BARLEY

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POWDERY MILDEW DISEASE SUSCEPTIBILITY IN BARLEY**

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## ABSTRACT

### FUNCTIONAL ANALYSIS OF A miRNA PUTATIVELY INVOLVED IN POWDERY MILDEW DISEASE SUSCEPTIBILITY IN BARLEY

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Barley is one of the most important crop species in Turkey and powdery mildew is one of the most common pathogen decreasing yield in barley. For this problem, agricultural biologists apply breeding technologies in order to select and propagate resistant barley cultivars. However, this is not a permanent solution since pathogens evolve rapidly to overcome plant resistance mechanisms. On the other hand, molecular plant pathologists are trying to understand basic mechanisms underlying plant-pathogen interactions by using molecular tools in order to develop long term solutions for preventing yield loss.

In this thesis, miR159 mediated regulation of barley GAMyb transcription factor is studied. According to microRNA microarray results regarding to infection with powdery mildew pathogen *Blumeria graminis f.spp hordei* (Bgh) at different time points, miR159 expression level showed significant differences. Bioinformatics analysis revealed that miRNA159 targets GAMyb gene in barley. In order to investigate this relationship, both miRNA and miRNA target were cloned into GFP containing expression vectors through Gateway cloning and resulting vectors were transformed into *Nicotiana benthamiana* through Agrobacterium mediated gene transfer. Observations based on GFP expression showed that miRNA159 targets and decreases the expression of GAMyb *in vivo*.

To conclude, this study can be evaluated as a distinctive study for two aspects; (i) it is the first study assessing a “putative” barley miRNA function biologically and (ii) developed a practical and effective functional assay for miRNA studies in plants.

**Key Words:** microRNA, powdery mildew, *Blumeria graminis f.spp. hordei*, miR159, Hv-GAMyb, Gateway cloning, Agrobacterium mediated transient gene transfer, *Nicotiana benthamiana*.

## ÖZ

### ARPADA KÜLLEME HASTALIĞI OLUŞUMUNDA GÖREV ALDIĞI VARSAYILAN BİR miRNA'NIN FONKSİYONEL ANALİZİ

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Arpa Türkiye'deki en önemli tahıllardan biridir ve arpa verimini düşüren külleme etkeni en yaygın patojendir. Bu problem karşısında ziraat mühendisleri çözüm olarak ıslah teknolojilerine başvurarak dirençli arpa türlerini seçer ve çoğaltırlar. Fakat bu kalıcı bir çözüm sunmaz çünkü patojen bitki direçliliğini yenmek için hızla evrilimektedirler. Moleküler bitki patalogları, ürün kaybına yönelik uzun vadeli çözümler üretmek için moleküler yöntemler kullanarak bitki-patojen ilişkisinin altındaki temel mekanizmaları anlamak için çalışırlar.

Bu çalışmada, miR159 aracılığıyla arpa GAMyb transkripsiyon faktör regulasyonu sunulmuştur. Külleme patojeni inokulasyonundan değişik zamanlarda alınan örneklerle yapılan mikrodizin sonuçlarına göre, miR159'un ekspresyon seviyesi önemli bir farklılık göstermiştir. Biyoinformatik analizler sayesinde miRNA159'un arpada GAMyb genini hedeflediği ortaya çıkmıştır. Bu ilişkiyi araştırmak için hem hedef hem de miRNA Gateway klonlama yöntemi ile GFP içeren ekspresyon vektörlerine klonlanıp, Agrobakteriyum aracılığıyla *Nicotiana benthamiana*'ya (tütün bitkisi) transfer edilmişlerdir. GFP ile yapılan gözlemlere göre miRNA159'un *in vivo* olarak GAMyb'yi hedefleyerek bu genin anlatımını

düşürdüğü saptanmıştır.

Bu çalışma iki yönden önemli bir çalışma olarak değerlendirilebilir; (i) bu, varsayılan bir arpa miRNAsının fonksiyonunu biyolojik yönden öne koyan ve (ii) aynı zamanda oldukça pratik ve kullanışlı moleküler tekniklerin uygulandığı bir çalışmadır.

**Anahtar kelimeler:** mikroRNA, külleme, *Blumeria graminis f.spp. hordei*, miR159, Hv-GAMyb, Gateway klonlama, Agrobakteriyum aracılığıyla gen aktarımı, *Nicotiana benthamiana*.

To my family;  
supportive, ingenuous and special...



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## LIST OF ABBREVIATIONS

AGO	: Argounote protein
AGT	: Appressorial Germ Tube
APP	: Appresorium
As	: Anti sense
Ath	: <i>Arabidopsis thaliana</i>
Att	: Attachment
Avo	: Avocet
Avr	: Avirulence
Bgh	: <i>Blumeria graminis hordei</i>
DCL1	: Dicer Like1
DNA	: Deoxyribonucleic acid
dNTP	: Deoxy-nucleotidetriphosphate
Dpi	: Day post inoculation
ds	: Double stranded
DTT	: Dithiothretiol solution
EDTA	: Ethylenediaminetetraacetic acid
ESH	: Elongating secondary hyphae
ETI	: Effector Triggered Immunity
ETS	: Effector Triggered Susceptibility
GFP	: Green Flourescent Protein
h.	: Hour
Hai	: Hour after inoculation
HR	: Hypersensitive response
kb	: Kilobase
LRR	: Leucine rich repeat
M	: Molar
mg	: Miligram
min.	: Minute



miRNA : microRNA  
mL : Mililiter  
Mla : Mildew Locus A  
mM : Milimolar  
ng : Nanogram  
nm : Nanometer  
Os : *Oryza sativa*  
p : plasmid  
PAMP : Pathogen Associated Molecular Pattern  
PCD : Programmed Cell Death  
PCR : Polymerase Chain Reaction  
pmol : Picomole  
PTGS : Post Transcriptional Gene Silencing  
PTI : PAMP Triggered Immunity  
PTI : PAMP Triggered Immunity  
R : Resistance  
RIN4 : RPM1- interacting protein  
RISC : RNA Induced Silencing Complex  
RNA : Ribonucleic acid  
RNAi : RNA interference  
RT-PCR : Reverse transcriptase polymerase chain reaction  
Ss : single stranded  
*Taq* : *Thermus aquaticus*  
T-DNA : Transfer DNA  
TIR : Toll Like Interleukine  
U : Unit  
 $\mu\text{g}$  : Microgram  
 $\mu\text{L}$  : Microliter  
 $\mu\text{M}$  : Micromolar

## CHAPTER I

### INTRODUCTION

#### 1.1 Powdery mildew of barley

##### 1.1.1 The barley (*Hordeum vulgare*)

Barley (*Hordeum vulgare ssp. vulgare*) is an important cereal of which production rate comes fourth after wheat, rice and maize around the world (Zeybek et al., 2008). The crop is used in various means such as animal feed, beer production and sometimes in human feed. Turkey is the sixth country (Table1, FAO, 2005) in ranking between barley producing countries.

**Table 1.1.** Top ten barley producing country in the world.

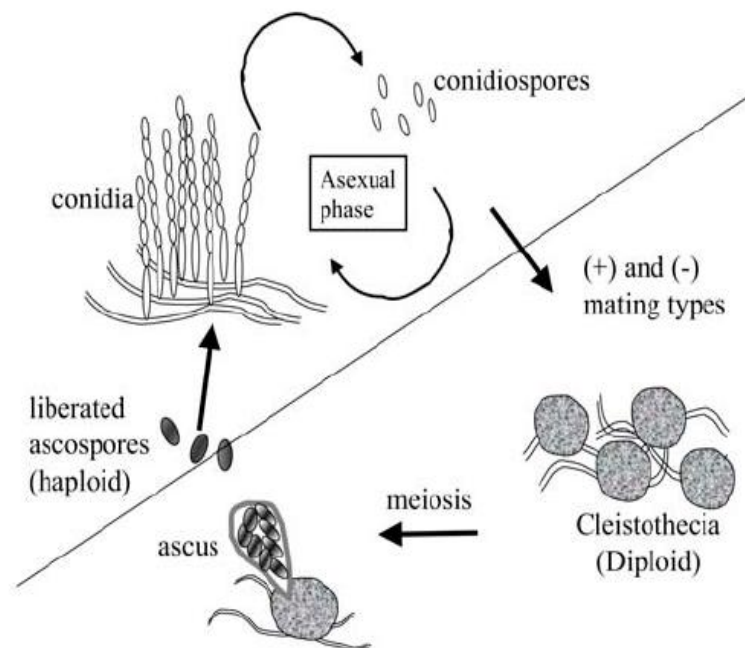
<b>Top Ten Barley Producers — 2005</b> (million metric tonne)	
<a href="#">Russia</a>	16.7
<a href="#">Canada</a>	12.1
<a href="#">Germany</a>	11.7
<a href="#">France</a>	10.4
<a href="#">Ukraine</a>	9.3
<b><a href="#">Turkey</a></b>	9.0
<a href="#">Australia</a>	6.6
<a href="#">United Kingdom</a>	5.5
<a href="#">United States</a>	4.6
<a href="#">Spain</a>	4.4
<b>World Total</b>	<b>138</b>

Source: [UN Food & Agriculture Organization](#) (FAO)

### 1.1.2 *Blumeria graminis f.sp. hordei*

*Blumeria graminis f.sp. hordei* (*Bgh*), causal agent of powdery mildew disease of barley, is an ascomycete which is one of the true obligate biotroph meaning that it grows and reproduces on host epidermal cells and obtains its food by forming an intracellular organ namely 'haustorium' (Ridout et al, 2008) inside the host cell. Because of the economic importance of barley, *Bgh* is said to be most intensively studied powdery mildew fungi (Zhang et al, 2005).

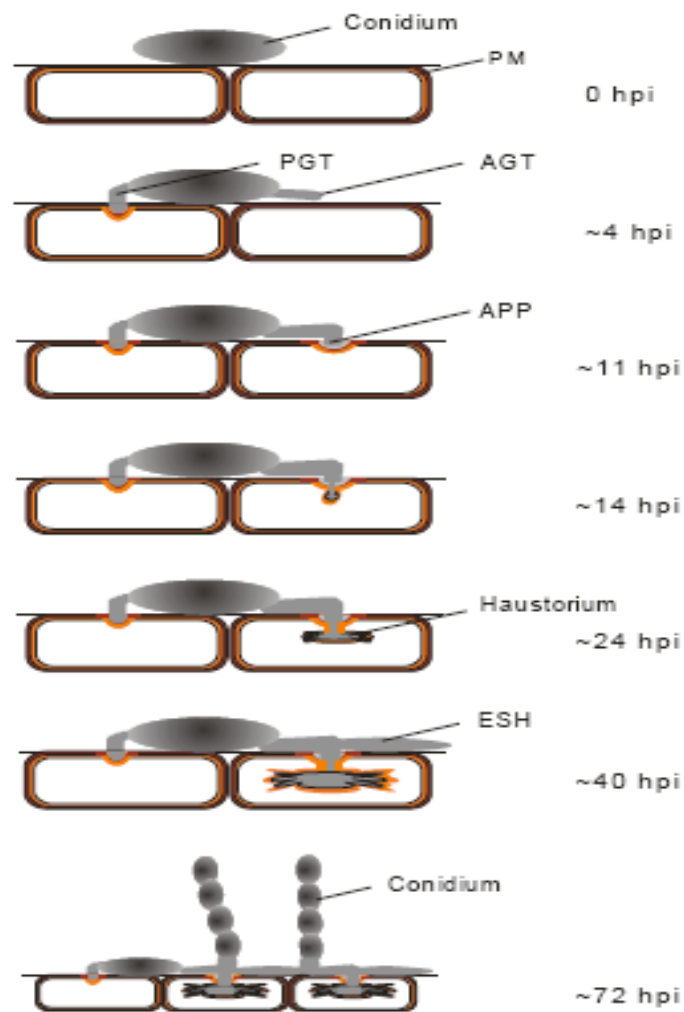
The life cycle of *Bgh* is summarized in Figure 1 (Ridout et al., 2006). The fungus is dependant on a living host in the haploid stage in which it is dispersed as an ascospore, grown and propagated on the barley epidermal cells. (+) and (-) types of conidiospores mate with each other to form diploid cleistothecia.



**Figure 1.1** The life cycle of *Bgh*. Haploid and diploid stages of the fungus development are separated with a diagonal line. The fungus is truly obligate to plant in the asexual stage in which conidia are dispersed through epidermal surface and form colonies by feeding through epidermal cells (Ridout et al., 2006).

### **1.1.3 The powdery mildew disease**

Whitish velvety appearance covering plant leaves is one of the most characteristics of the disease of Powdery mildew (Eichmann et al, 2008). Figure 2 explains how fungal development takes place on epidermal tissues of the barley schematically. The conidium completes its haploid cycle in 5-6 days. As shown in the figure, disease development starts with germination in which the conidia forms of a primary and appressorial germ tube (PGT and AGT). Next, appressorium (APP) is formed by swelling of the AGT. In order to penetrate the plant cell wall and make contact with the plasma membrane of a leaf epidermal cell, appressorial infection peg is formed. If the fungus penetrates into host cell successfully, it develops the feeding organ haustorium in order to acquire nutrients of the host cell. Afterwards, elongating secondary hyphae appears at the leaf surface leading to new generation of conidia (Thordal- Christensen et al., 2000).



**Figure 1.2 Development of *Bgh* on barley epidermal cells.** The figure was taken from Thordal –Christensien et al. 2000. Abbreviations: AGT, appressorial germ tube; APP, appressorium; ESH, elongating secondary hyphae; hpi, hours post infection; PGT, primary germ tube; PM, plasma membrane.

## 1.2 Plant-pathogen interactions

### 1.2.1 General overview

Two models of pathogen recognition in plant disease resistance, namely ‘gene for gene’ (Flor, 1971) and ‘guard’ hypothesis (Dangl and Jones, 2001), are the accepted current basic models of the plant pathogen studies.

According to the gene-for-gene model, there must be direct physical interaction between R-protein of the host and Avr-protein of the pathogen for plant disease resistance. Extensive studies on R-protein structure revealed that LRR domain of the protein participates in protein interaction. On the other hand, although many ‘avr-R gene pairs’ studies were carried out, only a couple of direct interaction could have been shown experimentally between these proteins ( Jia et al., 2000; Deslandes et al., 2003, Dodds et al., 2006). This implies that there must be other types of interaction in plant recognition of pathogen.

Another common model is called as guard hypothesis in which there occur an indirect interaction between R-protein of the plant and Avr-protein of the pathogen. Avr targets and modifies another molecule or complex of the host and then corresponding R protein functions upon this interaction to achieve plant resistance. In a study related with *Arabidopsis- Pseudomonas* interaction “guardee” model is presented very well with interacting host and pathogen molecules (Mackey et al, 2002). The guardee is RIN4 (RPM1- interacting protein) of which phosphorylation is induced by AvrRPM1 and AvrB. Due to phosphorylation, RPM1 becomes functional and pathogen HR response occurs and pathogen growth is restricted.

### **1.2.2 *Hordeum vulgare* - *Blumeria graminis fsp. hordei* relationship**

Related with barley- *Bgh* interaction, many plant R genes and pathogen Avr genes found and their relationship are said to be progressing in gene-for-gene manner. When the plant R protein recognize the pathogen through corresponding Avr molecule, a programmed cell death also called hypersensitive response (HR), usually takes place as plant defense in order to restrict pathogen growth further (Jones and Dangl, 2006). From those of the R-proteins found in barley genome, only seven Mla (Mildew locus allele) genes were isolated yet (Shenet al., 2003; Halterman and Wise, 2004). Likewise, only two Avr genes, Avr-a10 and Avr-k1 ,were isolated from *Bgh* (Skamnioti et al., 2008).

### **1.3 Plant microRNAs and their role in biotic stress**

MicroRNAs are small, 21-24 nt long, noncoding molecules that regulate gene expression in eukaryotes. Despite close similarity in structures, mode of action of the miRNAs differs in animals and plants. In animals, translational repression occurs mostly and miRNAs bind to 3'UTR region of transcripts. In addition, there could be several mismatches between miRNA and its target. On the other hand, in plants miRNAs have role in post transcriptional stage of the mRNA and miRNA target sites are generally located in the coding region of the gene. Also, there is only a few or no mismatch between miRNAs and its targets in plants. (Schwab R. et al., 2005 ).

Investigations on plant miRNAs started with cloning of small RNAs (Llave et al., 2002b). Then more specific steps were added to the miRNA cloning protocols. Many novel miRNAs have been described by cloning small RNAs processed by an endogenous RNAIII endonuclease Dicer-like1 (Dcl1) (Llave et al., 2002b; Mette et al., 2002; Park et al., 2002; Reinhart et al., 2002; Xie et al., 2003; Sunkar and Zhu, 2004 ). Due to extensive work and limited data gathered from experimental approaches, studies about plant miRNA search turned to use computational

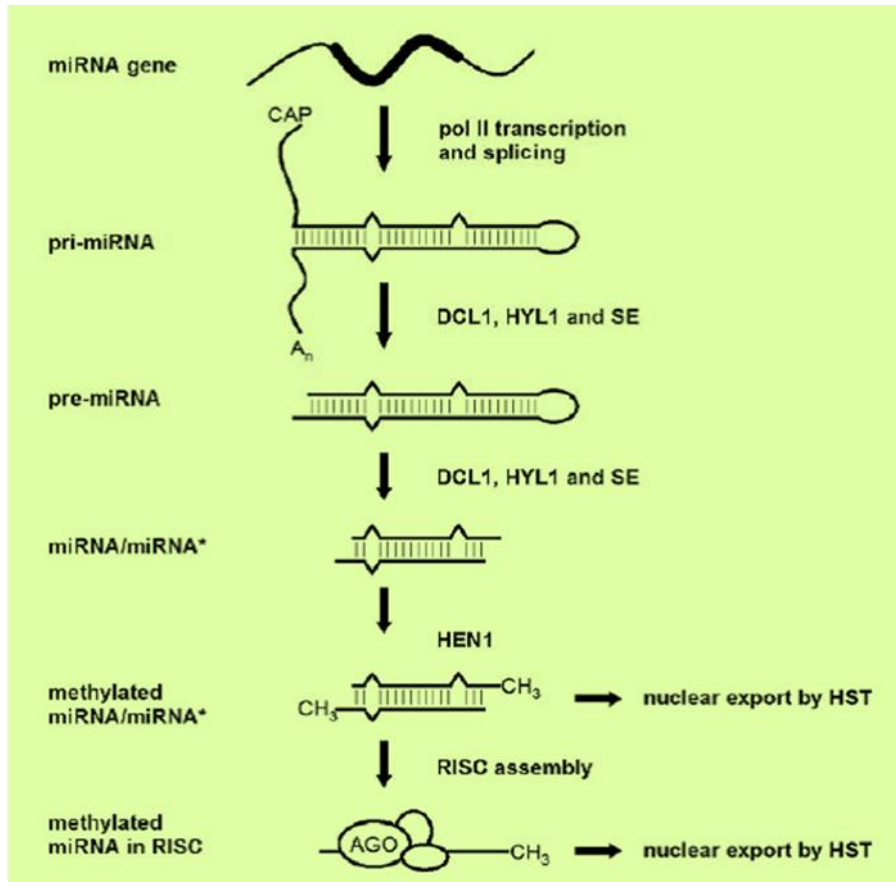
approaches (Jones-Rhoades and Bartel, 2004). The first study was performed by comparing genomes of the Arabidopsis and Oryza in order to identify conserved miRNA genes. Seven miRNA gene families were assigned in this study. Then, computational approach was improved with criteria for prediction of miRNAs (Wang et al., 2004 ; Bonnet et al., 2004; Adai et al., 2005).

Up to date, 320 plant miRNA families most of which identified in the fully sequenced genomes of Arabidopsis, Populus, and Rice have been found in plants (miRBase database available at <http://microrna.sanger.ac.uk/cgi-bin/sequences/browse.pl>).

### **1.3.1 miRNA biogenesis**

MIR genes encode for microRNAs. A maturation process takes place in order to have a functional miRNA, so called mature miRNA. Maturation is a stepwise process starting with the synthesis of the primary miRNA (pri-miRNA) and ending with the synthesis of the mature miRNA (Yan Lu et al., 2008). MIR gene is transcribed into pri-miRNA by RNA Polymerase II (Pol II). Stem loop structured pri-miRNAs may also be modified by splicing, capping and polyadenylation (Xie et al., 2005). Pri-miRNA is then cleaved by Dicer-like1 (DCL1) with two additional protein Serrate (SE) and Hyponastic leaves1 (HYL). After two cleavages by DCL1, miRNA/miRNA\* duplex having two nucleotide overhangs at 3' site (Papp et al, 2003) is formed. The 5' end of the miRNA\* is less stable than other strand. This feature is determinant for the strand selection for incorporation into the RISC complex (Khvorova et al, 2003). Methylation step is a very important step in miRNA biogenesis since methyl group protects the duplex against an endonucleolytic attack (Li et al., 2005a). HUA ENHANCER1 (HEN1) is responsible in methyl group addition to miRNA duplex. Methylated miRNA duplex either stays in nucleus or is transported to the cytoplasm in order to participate in RNA-Induced Silencing Complex (RISC). ARGONAUT1 (AGO1) is the most important molecule of this complex (Figure 1.3, Bartel, 2004).





**Figure 1.3 miRNA biogenesis in plants.** The pri-miRNA is transcribed by RNA pol II in an hairpin form with a CAP and polyA tail. Next, pre-miRNA is produced from cleavage of pri-miRNA by DCL1 and help of the SE and HYL1 proteins. Then, pre-miRNA is cleaved in the same manner in order to produce miRNA/miRNA\* duplex. HEN1 methylates the duplex by adding methyl groups to the ribose of the last nucleotide in either strand. Methylated miRNA duplex is transported by HST to the cytoplasm in order to participate into RISC. Some of miRNAs participate into RISC in the nucleus rather than cytoplasm. Abbreviations: DICER-LIKE1 (DCL1), HYPOPLASTIC LEAVES1 (HYL), SERRATES (SE) and HUA ENHANCER1 (HEN1), HASTY (HST), RNA-induced Silencing Complex (RISC) ( Bartel, 2004).

### 1.3.2 Functions of miRNA in plant defense

MicroRNAs have roles in various events such as development, signal transduction, cell cycle control, cellular differentiation, protein degradation and response to biotic and abiotic stresses (Yan-du Lu et al., 2008; Wang et al., 2007).

There is a few miRNA identified having role in biotic stress (Table 2, from Lu et al., 2008). From the miRNAs of *Physcomitrella*, miR1-39 targets a mucin-like protein which is an crucial protein acting during pathogen invasion (Isam et al., 2007). In the same study, miR160-3 was found to targeting pathogenesis related protein. Also an electron transporter was targeted by miR408. In Arabidopsis, it was found that during bacterial infection miR393 is induced by flg22 and auxin receptor TIR1 targeted by miR393 decreases upon this induction. As a result of this, auxin signalling is repressed (Navarro et al., 2006).

**Table 1.2** Plant miRNAs having role in plant defense system.

Host plants	miRNA	Target protein or virus	Reference
<i>Physcomitrella</i>	miR1-39	A mucin-like protein	Isam et al. (2007)
<i>Physcomitrella</i>	miR160-3	Pathogenesis-related	Isam et al. (2007)
<i>Physcomitrella</i>	miR408	Electron transporter	Isam et al. (2007)
<i>Populus</i>	miRNAs	Defense response	Lu et al. (2005)
Arabidopsis	miR393	Auxin signaling	Navarro et al.
<i>V. vinifera</i>	miR171e	RGA1	Not published
<i>V. vinifera</i>	mir166a	OLP precursor	Not published
Rice	Artificial	Rice dwarf virus	Ma et al. (2004)
Arabidopsis	Artificial	TYMV and TuMV.	Niu et al. (2007)
<i>Nicotiana</i>	Artificial miRNAs	Plum pox virus	Simo ´n-Mateo and García (2006)

### **1.3.3 Mechanism of miRNA action**

miRNAs regulate gene expression through two mechanisms: cleavage of target mRNA (Llave et al., 2002b ) and translational repression of mRNA (Aukerman and Sakai, 2003; Chen, 2004; Arteaga-Vazquez et al., 2006; Gandikota et al., 2007)

Among these mechanisms, mRNA cleavage is the principal way of the miRNA action in plants. miRNA binds to its target and guides the slicing activity of the Argonaute (AGO) protein in the RISC. miRNA function studies applying mutations in miRNA pathway revealed overaccumulation of mRNAs which are target messages of miRNA (Vaucheret et al., 2004, Vazquez et al., 2004). These studies show that miRNA negatively regulates expression of its target (Jhoanes- Rhodes et al., 2006). In addition, 3' cleaved products of miRNA targets were detected by means of either Northern blot assays (Kasschau et al, 2003, Llave et al, 2002, Mallory et al, 2005) or 5' RACE (Voinnet, 2009). These studies support that there is a negative relation between expression levels of miRNA and its target.

Another miRNA action mechanism occurs at translational level. This mode of action is less known with respect to cleavage of the transcript. However there are several supportive studies on this issue. Mir172 which targets APETALA2 (AP2) protein is found to affect the protein accumulation of the target (Aukerman et al., 2003; Chen, 2004). In another study, the function of AGO1 were assessed in translational repression of miRNA target as well as transcriptional cleavage (Baumberger and Baulcombe, 2005). In addition to these, RISC and target miRNA are found to localized in "P bodies", where translation is repressed (Liu et al., 2005b; Sen and Blau, 2005; Chu and Rana, 2006).

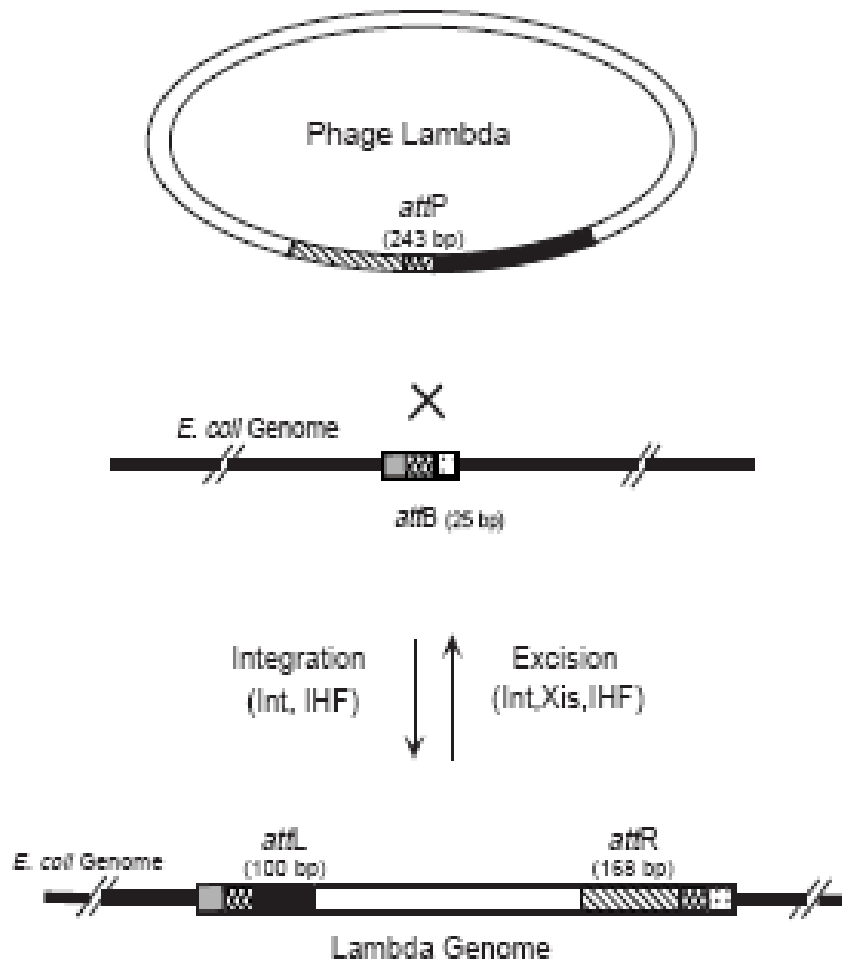
### **1.3.4 Prediction of miRNA targets**

Mature microRNA sequences show nearly perfect match with its target message as stated in section **1.3**. This feature comprises basis for miRNA target search in

plants. Due to this feature many miRNA targets in plants were predicted and validated (Bartel, 2004). Arabidopsis miRNA targets were predicted through computational approaches were applied by using high sequence complementarity (Rhoades, 2002). 49 miRNA targets were predicted by this approach. Further studies were held in miRNA target prediction in Arabidopsis and other plant species by using high complementarity between miRNA and its target (Park et al., 2002; Reinhart et al., 2002; Sunkar and Zhu, 2004; Adai et al., 2005). Target search algorithms were improved further by modifying the criteria of number of mismatch and presence of bulged nucleotides (Palatnik et al., 2003; Jones-Rhoades and Bartel, 2004; Wang et al., 2004).

#### **1.4 Gateway cloning**

The Gateway cloning system makes easy and accurate cloning of a gene of interest (GOI) in a desired order, orientation and correct frame. Site specific recombinations of phage  $\lambda$  in *E.coli* comprises the basis of Gateway cloning. The bacteriophage can enter lytic or lysogenic states depending on environmental conditions. During lysogenation the phage integrates its genome into *E.coli* genome site specifically. When the conditions becomes favourable, it is excised from the bacterial genome and enter the lytic state (Figure 1.4, from Ptashne, 1992). Integration and excision reactions take place in a site specific manner between attBxattP and attLxattR (Landy, 1989)

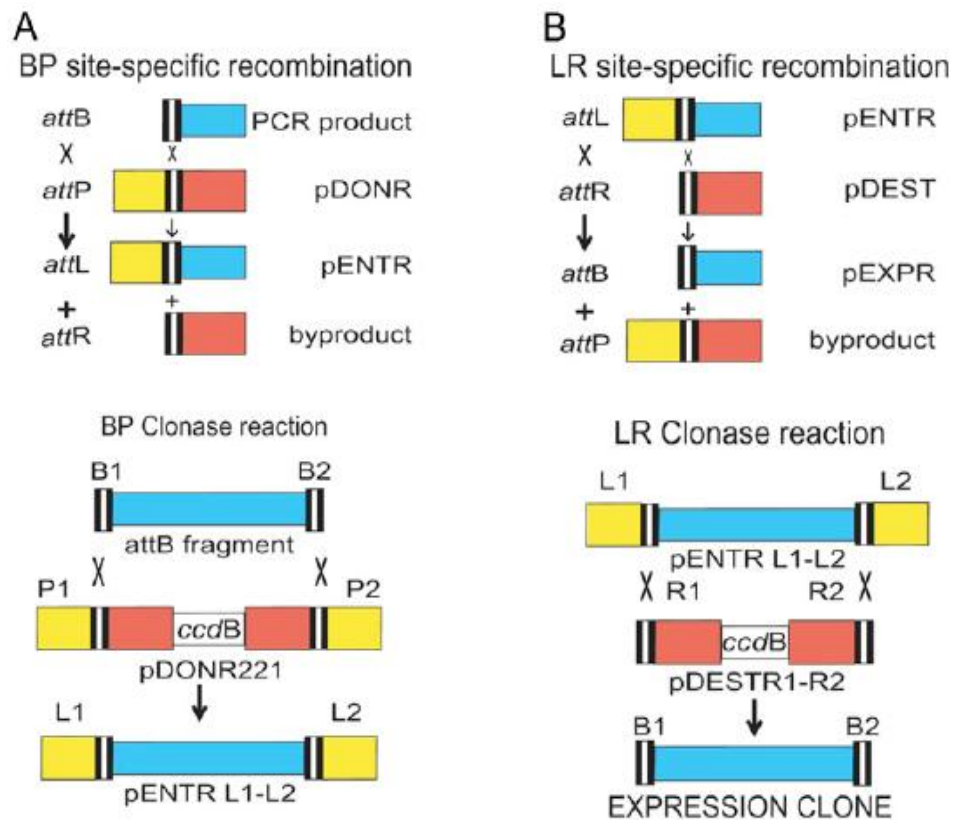


**Figure 1.4 Site specific recombination of bacteriophage  $\lambda$  in *E. coli*.**  $\lambda$  has *attP* site and *E. coli* has *attB* site. Integrase (Int) and excionase (Xis) are phage coded factors having role in integration and excision reactions. Integration Host Factor (IHF) is also take a role in these reactions and encoded by *E. coli*. (Ptashne, 1992)

Site specific recombination requires special recognition sites as *attP*, *attB*, *attL* and *attR*. In Gateway cloning BP and LR reaction are applied in order to clone the GOI into an entry vector and into expression vector subsequently (Figure 1.5).

There are four types of vectors namely; entry vector (having *attL*), donor vector (having *attP*), destination vector (having *attR*) and expression vector (having *attB*). The core of the Gateway cloning is to obtain an entry clone. After having an entry clone containing the GOI, an appropriate destination vector is used and expression clone is produced very practically.

Gateway cloning is not just practical but also highly accurate cloning method which makes it more advantageous over the other cloning methods. In Gateway cloning, in addition to an antibiotic resistance marker, vectors also include a *ccdB* gene, known as counter selectable marker, between the recombination sites as a second selection. This gene product induce gyrase-mediated double-stranded DNA breakage (Bernard et al., 1992) and kills the cells unless they have *ccdB* resistance. Due to two selection system, transformants will be only desired clones having GOIs.



**Figure 1.5 Schematic summary of the Gateway recombination reactions.** (A), BP clonase reaction; a PCR product or plasmid having *attB* sites recombines with a donor vector (pDONR) having *attP* sites and an entry vector (pENTR) having *attL* sites and a byproduct having *attR* are produced. (B), LR reaction, entry vector (pENTR) having *attL* sites recombines with a destination vector (pDEST) and an expression vector having *attB* sites and a byproduct with *attP* sites are produced. Blue boxes, DNA fragments of interest assembled in BP and LR clonase reactions; black box with vertical white stripe, *attB* sites, also at the core of the *attP*, *attL*, and *attR* sites; yellow box, portion of the *attP* and *attL* sites; red box, portion of the *attP* and *attR* sites. B1 to B4, *attB*1 to *attB*4 sites; L1 to L4, *attL*1 to *attL*4; R1 to R4, *attR*1 to *attR*4 (Karimi et al., 2002).

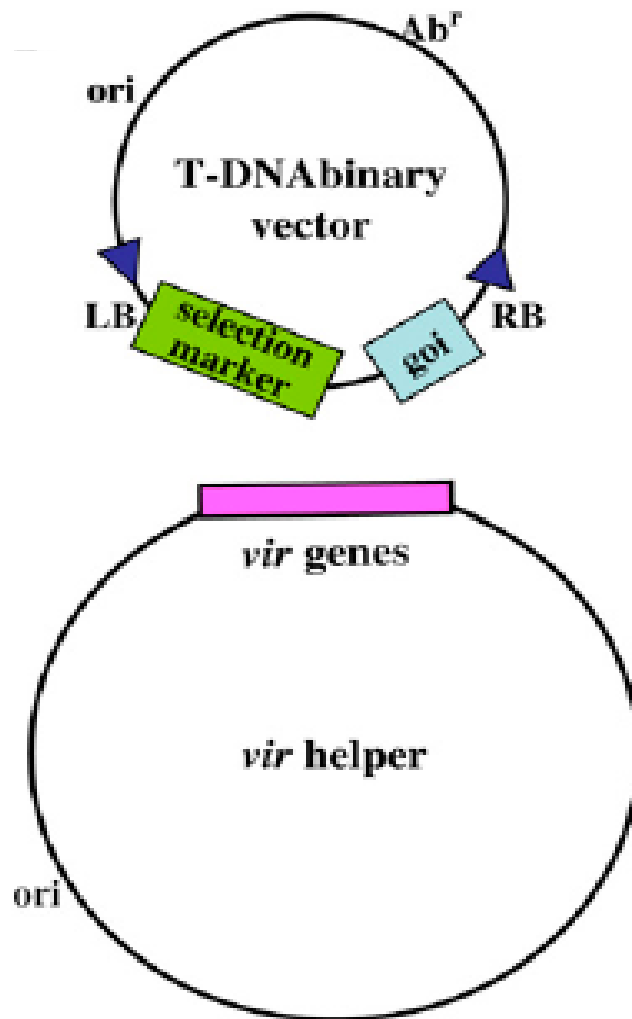
## 1.5 Agrobacterium mediated gene transformation

Agrobacterium mediated gene expression is a rapid, low cost, easy to handle process and very common in plants due to wide host range of the bacterium of *Agrobacterium tumefaciens* (Wydro *et al*, 2006).

*Agrobacterium tumefaciens* is a pathogenic soil bacterium causing crown galls or tumors in the host (Zupan, 2000). The bacterium itself transfers genes to the host in order to establish tumor and synthesize opines (unusual amino acids). Opines are important source of carbon and nitrogen source for Agrobacterium growth (Hooykaas and Beijersbergen, 1994). The bacterium infects its host through transferring a particular portion of its DNA, so called Transfer DNA (T-DNA). The bacterium has a Tumor inducing (Ti) plasmid in which T-DNA and ~35 virulence genes (*vir* region) are found. *Vir* genes have very critical roles during bacterial infection and transfer of T-DNA. Agrobacterium can sense host phenolic compounds by vir proteins (VirA and virG), T-DNA processing (VirD1 and VirD2) and secreting into host (VirB and VirD4) are done by vir proteins. Participation of T-DNA is also achieved by vir proteins(virD2,virD5, virE2,virE3,virF) (Lee *et al.*, 2008).

In order to improve Agrobacterium mediated gene transfer binary Ti vector plasmids were developed (Figure 1.6). In this system, genes for tumor induction and opine synthesis were removed. Then, T-DNA and *vir* genes are separated into two vectors binary Ti vector including GOI and a *vir* helper plasmid (Hoekema, 1983). *Vir* genes act combinatorily and achieve T-DNA transfer into the host nucleus (Jouann *et al*, 1985). For Agrobacterium mediated transformation, Agrobacterium strains had been engineered into new commercial strains (LBA 4404, GV3101 MP90, AGL0, EHA101, and its derivative strain EHA 105) containing non-oncogenic *vir* helper plasmids (Gelvin, 2003).

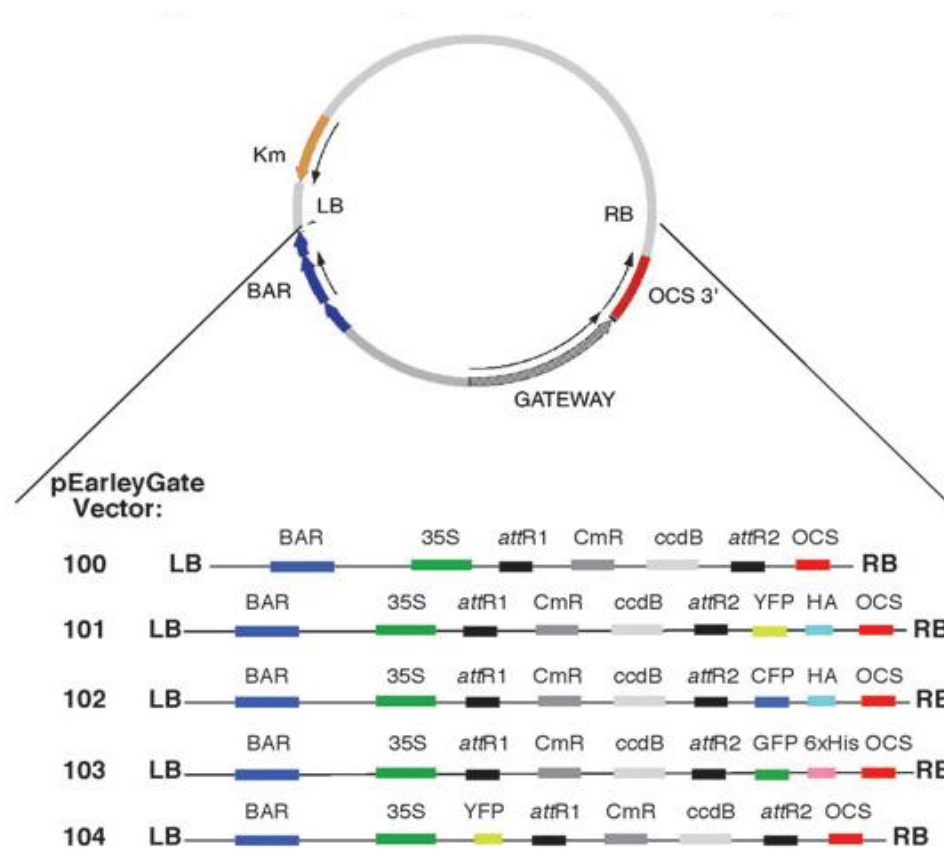




**Figure 1.6 Schematic representation of T-DNA binary vector system.** Gene of interest (goi) is cloned into T-DNA region of the binary vector. T-DNA region is limited with Right and Left Borders (RB and LB). *Vir* genes are found in the other vector, so called *vir* helper. Other components such as selection marker (important for control of plant transformation), origin of replication (ori), Antibiotic resistance (Ab<sup>r</sup>) are also present in the binary vector system (Lee et al., 2008).

## **1.6 pEarleyGate vectors for Agrobacterium mediated transformation**

In Gateway cloning system, among various destination vectors, a set of 'pEarleyGate' plasmid vectors provide researchers to utilize benefits of Gateway cloning and Agrobacterium mediated gene transfer for plant functional genomics and proteomics studies. The vector contains a T-DNA region with RB and LB. There are many vector components in between T-DNA region according to applications. For example pEarleyGate101 contains YFP gene to be fused with GOI in order to screen gene expression fluorescently, as well as HA tag in order to purify expressed protein if desired (Figure 1.7).



**Figure 1.7 pEarleyGate plant transformation vectors.** pEarleyGate vectors are binary vectors having RB and LB sites for *Agrobacterium*-mediated T-DNA transfer. T-DNA vector components are presented for each vector. Standard components such as attR1 & 2, a chloramphenicol resistance gene (CmR), the ccdB killer gene, the cauliflower mosaic virus 35S promoter and its upstream enhancer, OCS, the 3' sequences of the octopine synthase gene, including polyadenylation and presumptive transcription termination sequences are the same for all vectors. BAR, the Basta herbicide resistance gene for selection of transgenic plants. Km, the bacterial kanamycin resistance gene within the plasmid backbone. Different pEarleyGate vectors allow engineering and expression of proteins fused in frame with HA, FLAG, cMyc, AcV5 or tandem affinity purification (TAP) tags and/or yellow, green or cyan fluorescent proteins (YFP, GFP or CFP, respectively) at either the amino-terminal or carboxy-terminal end of the target proteins (Earley *et al.*, 2006).

## **1.5 Aim of the Study**

Crop species occupy important sources for the human needs. Therefore, It is important to grow high quality cereals. To do this, any biotic and abiotic stress should be minimized. Barley is an important cereal and powdery mildew disease of the barley is an common biotic stress.

In order to eliminate powdery mildew disease of barley, in the long run the basics of the plant pathogen interaction should be understood. For example, the question of which gene products act during infection should be answered. Another question to be answered is how they interact with each other? In addition to these, regulation of gene expression is one of the most important aspect of the resistance mechanism.

The present work aimed to assign putative barley microRNAs that have a role in powdery mildew disease susceptibility and\or resistance in barley by regulating disease related molecules. Our objective was to apply novel molecular biology techniques for investigating the relationship between miRNA and its target message. These novel techniques including Gateway cloning and Agrobacterium mediated gene transfer would make the experiments to be more practical and accurate.

## CHAPTER II

### MATERIALS AND METHODS

#### 2.1 Plant and fungal materials

*Nicotiana benthamiana* and *Arabidopsis thaliana columbia* plants were planted to soil at 16 hour light/ 8 hour dark intervals at 22 °C constant temperature (Boyes, et. al., 2001). Barley plants of Pallas 03 (P03) and fungal isolates on detached leaves were maintained in separate growth chambers at 18 °C (16 h of light/8 h of dark periods )(Wise R.P. et al, 2003).

#### 2.2 Barley infection and sample collection

Barley leaf segments of 2-3 cm in length from the plants grown for 10-14 days, were placed on water-agar containing 0.1% benzimidazol and infected with two different isolates of *Blumeria graminis f.sp. hordei* (Bgh) isolates; Bgh 95 and 103 heavily as explained in Schultheiss et al. (2008). Plates with inoculated plants were incubated at 18 °C 16/8 light / dark periods with 60% humidity growth chamber. Adequate number (approximately 5-6) of leaf segments were collected at 6, 12, 24, and 48 (hours after inoculation ) hai and stored at -80 °C for RNA isolations to be conducted later.

#### 2.3 Visualization of infections

Infected barley leaves at different time points as described in 2.2 were stained with trypan blue solution (10 g phenol, 10 mL glycerol, 10 mL lactic acid, 10 mL water and 0.02 g of trypan blue 1:2 v/v diluted with 96 % ethanol). Trypan blue solution stains death plant cells and fungal structures. Staining protocols were performed

according to Vogel et al. (2000). Infected leaves were incubated methanol-acetic acid solution (1:1) for 1 day and subsequently in chloral hydrate solution (25 mg in 10 mL ddH<sub>2</sub>O) for 1 day. Next, the leaf samples were incubated in trypan blue solution overnight and observed by light microscope (Leica DM4000B).

#### **2.4 Total RNA isolation from infected barley leaves**

High level of precaution must be met before and during the RNA isolations since contaminating RNase may result in degradation of RNA samples. Experiment equipment is treated with active 0.1 % DEPC solution for overnight and sterilized after treatment by autoclaving. RNA isolation was performed in 5 stages. In homogenization step, plant tissues (50-100 mg) were disrupted in liquid nitrogen using DEPC treated mortar and pestles. To the frozen plant powder, 1 mL of trizol reagent was added to further homogenize tissue samples. Homogenized samples were incubated for 5 min at 15-30 °C to permit the complete dissociation. In phase separation step, 0.2 mL of chloroform per 1 mL of trizol reagent was added. The samples were shaken vigorously and incubated at 15-30 °C for 2 to 3 min. Then, the samples were centrifuged at 12,000xg for 15 min at 4 °C. Next step is called as precipitation step. Following the centrifugation, the aqueous phase which is about 60% of the volume of trizol used, was transferred to a new tube and 0.5 mL of isopropylalcohol per 1 mL of trizol used was added to the sample for precipitation. The samples were incubated at 15-30 °C for 10 min and centrifuged at 12,000xg for 10 min at 4 °C. The RNA precipitate was obtained as a gel like formation. After centrifugation, in the RNA wash step, the supernatant was decanted and the RNA pellet was washed in 1 mL of 75% ethanol per 1 mL of trizol used. The pellet was disturbed by tapping and then re-centrifuged at 7500 g for 5 min at 4 °C. The RNA pellets were air-dried. The pellets were then dissolved 50 µL of PCR graded sterile water.

#### **2.4.1 Quality control of Total RNA**

For assessing total RNA quality, 1% agarose gel in 1X Phosphate Buffer (PBS, pH 5.5) was prepared. 1,5  $\mu$ L of the RNA sample, 1,5  $\mu$ L 6X RNA loading dye and 7  $\mu$ L ddH<sub>2</sub>O were combined for each sample electrophoresis in each well. The electrophoresis was performed in 1X PBS Buffer by refreshing the buffer for every 20 minutes during electrophoresis.

#### **2.4.2 Concentration measurements on NanoDrop**

Concentrations of RNA samples were measured by using Nanodrop ND-1000 Spectrophotometer which utilize the ND-1000 v3.1.2 program. The program was run and spectrophotometer was initialized according to program instructions. In order to minimize background effects measurements were done comparing with blank solution. After that, 1  $\mu$ L of the sample was loaded and “measure” comment was clicked.

#### **2.5 First strand cDNA synthesis from total RNA of infected barley samples**

After the isolation and the quality control assessments of total RNAs of various time points of infected barley leaves, first strand cDNAs were synthesized from total RNAs according to Invitrogen product manual. In the beginning, 2  $\mu$ g of total RNA, 50 pmol oligo-d(T)<sub>20</sub>, 0.1 mM of dNTP mix and sterile water up to 12  $\mu$ L were combined in a sterile PCR tube and incubated at 65 °C for 5 min. Next, the mixture was transferred onto ice immediately and kept on ice for 2 min. Then, 4  $\mu$ L of 5X first strand cDNA buffer, 1  $\mu$ L of 0.1 M DTT and 1  $\mu$ L of 40 U RNase inhibitor (Invitrogen CA, USA) were added to the tube. Tube contents were spinned and centrifuged briefly. The mixture was incubated at 50 °C for 2 min. Finally, 200 units of reverse transcriptase enzyme, SuperScript III<sup>TM</sup> (Invitrogen CA, USA), was added. Reactions were carried out at 50 °C for 90 min and then incubated at 70 °C for 15 min for enzyme inactivation.

## **2.6 Purification of genomic DNA from Arabidopsis leaves**

For genomic DNA isolation Qiagen DNeasy plant mini kit was used. Buffer AP1 and Buffer AP3/E of this kit were warmed up to 65 °C. The required amount of absolute ethanol (96-100 %) was added to the Buffer AW and Buffer AP3/E before using. A water bath was kept at 65 °C for the later stages of the isolation procedure. Plant material used was approximately 100 mg in weight as required for the kit procedure. Sterile mortar and pestle were cooled in liquid nitrogen and plant sample was added to the mortar containing liquid nitrogen and disrupted into powdery particles. After collecting the frozen powdered tissue into a fresh 2 mL centrifuge tube, 400 µL of Buffer AP1 and 4 µL RNase A stock solution (100 mg/mL) were added to each 100 mg homogenized plant tissue and the mix was vortexed vigorously. The mixture was incubated for 10 min at 65 °C by inverting tubes 2 or 3 times during incubation. To the mix, 130 µL of Buffer AP2 was added to the mix and the mix was incubated on ice for 5 min. As a recommended step, the lysate was centrifuged at 20,000xg for 5 min in order to get rid of undissolved particles. Next, the supernatant was applied to QIAshredder mini spin column in a 2 mL collection tube, and centrifuged at 20,000xg for 2 min. Flow-through fraction which was 450 µL was transferred into a new tube. To which 1,5 volumes of Buffer AP3/E was added to the lysate and mixed by pipetting. Then, 650 µL of the mixture was pipetted into a mini spin column and centrifuged at 6000xg for 1 min, the flow through was discarded. This step was repeated with remaining lysate. After that, the mini spin column was placed onto a new 2 mL collection tube, 500 µL Buffer AW was added and centrifuged at 6000xg for 1 min. Flow-through was discarded. 500 µL of Buffer AW was added to the spin column again and centrifuged at 20,000xg for 2 min in order to dry the membrane completely. In the final step, elution step, spin column was taken in a new eppendorf tube and 100 µL of Buffer AE was pipetted directly onto the DNeasy spin column and incubated for 5 min at room temperature. Finally, the spin columns were centrifuged at 6000xg for 1 min to elute the genomic DNA.



Genomic DNA was analyzed on 1 % agarose gels. 0.5 g agarose was weighed and dissolved in 50 µL 1X TBE buffer (Tris-base 10,8 g, Boric-acid 5.5 g, EDTA 0.58 g in 1L ddH<sub>2</sub>O) by boiling in microwave oven. After cooling it on the benchtop 30 µL EtBr was added to the gel and gel was poured on the tray. 1.5 µL of gDNA, 1,5 µL of 6X DNA loading dye (25 mg bromophenol blue (0.25%), 25 mg xylene cyanol (0.25%), 4 g sucrose (40%), adjusted volume to 10 ml with H<sub>2</sub>O) and 7 µL of ddH<sub>2</sub>O were brought together and applied to each well and electrophoresed with 100 bp DNA ladder.

## 2.7 Primer design

Total RNAs isolated from plant material as described in 2.4, were sent to microarray service in order to survey for changing miRNA expression levels at defined time points of *Bgh* infection. According to microarray data, miRNAs that show significant difference at changing time points were assessed and the difference was confirmed by quantitative PCR (Dagdaz Y., supervised by Mahinur Akkaya). From those miRNA and their targets, miRNA159 and GaMyb gene were chosen for doing further biological assays.

Full sequence of the target genes were obtained from NCBI nucleotide database. Target gene sequences are gathered from barley, *Hordeum vulgare*, genome. However for mir159 sequence, *Arabidopsis thaliana* genome sequence was used since its pri-mirna sequences were not available for barley. Primers were designed by using NCBI primer service (available at <http://www.ncbi.nlm.nih.gov/tools/primer-blast/> which uses Primer3 software) (Table 3.1). Primers were synthesized by Bioneer Oligo synthesis service in 3.2 nmole scale.

**Table 2.1** attB linked primer sequences of genes of interests (GOI) required for Gateway cloning. Primer names were assigned according to organisms; Hv is *Hordeum vulgare*, Ath is *Arabidopsis thaliana*. attB sites are in small case and bold.

<b>Primer</b>	<b>Primer sequence 5'→3'</b>	<b>Length (bp)</b>
Hv-GaMyb F	<b>ggggcaactttgtacaaaaaagttgtc</b> ATGGATCAAG TAAACCAAGC	47
Hv-GaMyb R	<b>ggggcaactttgtacaacaaagttgtc</b> GAGCTGCTGA TTCTTCCCAG	47
Ath-mir159a F	<b>ggggacaagttgtacaaaaaagcaggcttc</b> ATTCTCA AATTATAGCGAATAATCCT	57
Ath-mir159a R	<b>ggggaccactttgtacaagaaagctgggtc</b> TAATGTAA GAGATTTACCTCTTTTGG	56

## 2.8 PCR application

### 2.8.1 PCR protocol by using Gentaq DNA Taq polymerase

The reaction components and their required amounts for a single reaction are given in the table 2.2 and 2.3. PCR by using GenTaq Polymerase was applied for routine control PCRs such as colony PCRs.

**Table 2.2** PCR reaction components and their optimum volumes.

<b>Component</b>	<b>Volume (<math>\mu\text{L}</math>)</b>
10X GenTaq DNA Poly. Buffer	5
25mM $\text{MgCl}_2$	4
10mM dNTP each	1
Template DNA	2
Forward primer (10 $\mu\text{M}$ )	1
Reverse primer (10 $\mu\text{M}$ )	1
GenTaqDNA Polymerase (5 U/ $\mu\text{L}$ )	0.2
Sterile water	35.8
Total	50

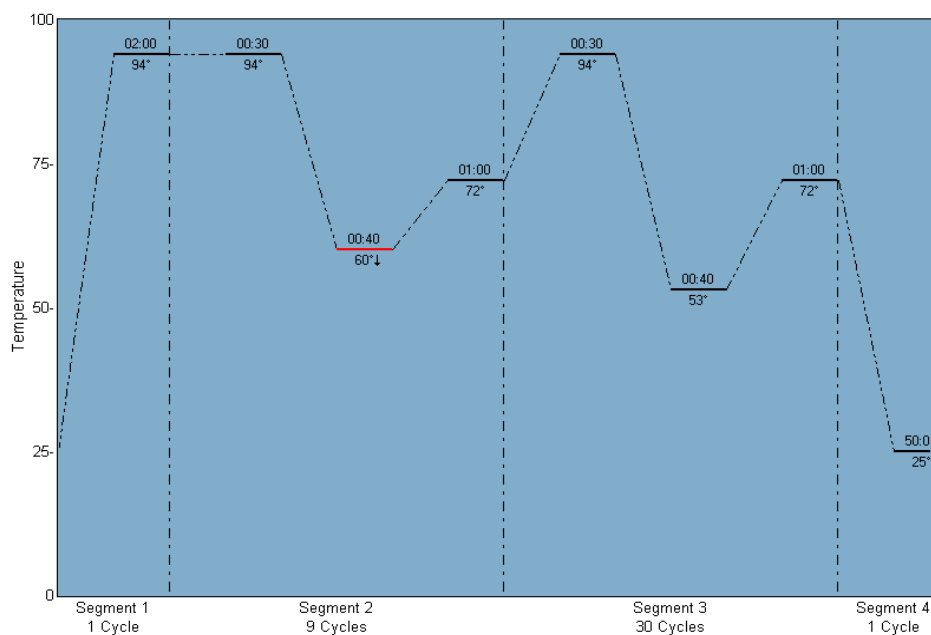
A master mix was prepared for multiple reactions in order to minimize ingredient loss and provide accurate pipetting.

**Table 2.3** Reaction conditions for PCR

<b>Temperature (<math>^{\circ}\text{C}</math>)</b>	<b>Time</b>	<b>Cycle</b>
94	2 min	1
94	15-30 sec	} 30
55	15-30 sec	
72	1 min	
72	10 min	1

## 2.8.2 Touchdown PCR

PCR reaction setup can be changed according to type of *Taq* Polymerase and its buffer. In this reaction, conditions were optimized by testing different annealing temperatures. This PCR was used when the gene of interest was in small amounts in the template. Touchdown PCR was used to amplify miRNA and miRNA target genes.



**Figure 2.1** Reaction conditions for Touchdown PCR. In this PCR, conditions were set with varying annealing temperatures. Red line in segment 2 indicates 1 °C decrease in each cycle of this segment.

## 2.9 Gel extraction of the positive PCR products for cloning into pDONRzeo

After screening PCR products, putative fragments were determined comparing with the sizes of DNA marker bands. 30  $\mu$ L of each PCR product was loaded on agarose gel (1%) and excised from the agarose gel with a sterile sharp scalpel. Gel extraction was performed according to QIAquick Gel Extraction Kit (Qiagen). The

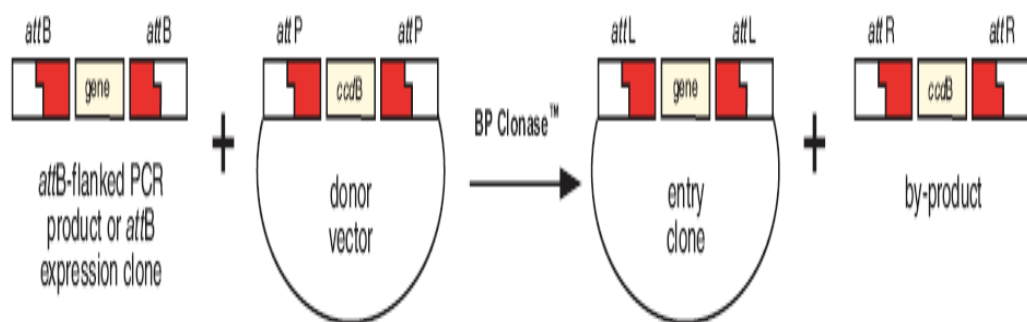
gel slice was weighed and 3 volumes of Buffer QG was added to a 1 volume of gel (100 mg~100  $\mu$ L). The mixture was incubated at 50 °C for 10 min. Mix was vortexed every 2-3 min to dissolve gel, during incubation. After dissolving the color of the mixture was yellow as described in the protocol. A QIAquick spin column was placed in a 2 mL collection tube and the sample was applied to the spin column and centrifuged for 1 min in order to bind DNA to the membrane. Flow-through was discarded and as an optional step, 0.5 mL of Buffer QG was added to the column and centrifuged. Next, 0.75 mL of buffer PE was added to the column and centrifuged for 1 min to wash the membrane. The flow-through was discarded and the column was centrifuged at 10,000xg for an additional 1 min. The QIAquick spin column was placed into a new 1.5 mL eppendorf tube and 50  $\mu$ L of buffer EB or water was added to the center of the membrane. Before centrifugation, the column was standed for a 1 min for better DNA concentration. Then the tube was centrifuged and the resulting concentration was measured by NanoDrop.

## **2.10 Gateway cloning**

For Gateway cloning, it is important to create an entry clone with gene of interests (GOI). To do this, GOIs were amplified with attB flanked primers firstly through PCR. Next, BP reaction was performed between the donor vector pDONR-Zeo and attB flanked PCR product. Sequencing of clones were performed by Refgen Biotechnology (Ankara, Turkey).

### **2.10.1 BP clonase reaction**

BP clonase enzyme mix catalyzes this reaction in which recombination of the attB-PCR product with an PDONR-Zeo to obtain an attL containing entry clone (Figure 2.2).

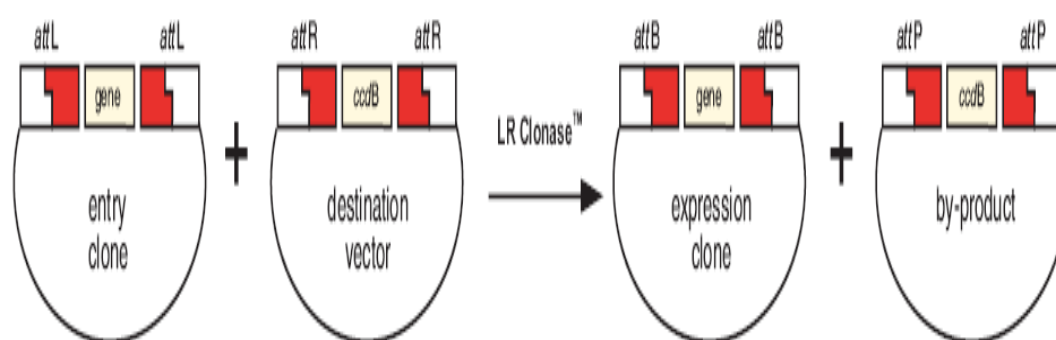


**Figure 2.2** BP clonase reaction.

BP clonase reaction was performed according to Invitrogen BP clonase mix kit protocol. In a 1.5 mL tube, 1-7  $\mu\text{L}$  of attB-PCR product (=10 ng/ $\mu\text{L}$ ; final amount ~15-150 ng) and 1  $\mu\text{L}$  of donor vector (150 ng/ $\mu\text{L}$ ) were combined at room temperature. Next, TE buffer (pH 8.0) was added up to 8  $\mu\text{L}$ . The BP Clonase™ enzyme mix was thawed on ice for about 2 min and vortexed briefly twice, 2 sec for each time. 2  $\mu\text{L}$  of BP Clonase™ enzyme mix was added to each reaction tube and mixed well by vortexing briefly twice. Reaction tube was microcentrifuged briefly. The reaction was incubated at 25 °C for 1 hour. 1  $\mu\text{L}$  of Proteinase K solution was added to each sample vortexing briefly to terminate the reaction. Samples were incubated at 37 °C for 10 minutes. Resulting vectors were used to transform *E.coli* and plated on LB-zeocin agar.

### 2.10.2 LR Clonase reaction

LR Clonase™ enzyme mix catalyzes this reaction and recombination of an attL containing entry clone with an attR containing destination vector was performed to create an attB-containing expression clone (Figure 2.3).



**Figure 2.3** LR clonase reaction

After obtaining an entry vector by BP clonase reaction, LR clonase reaction was performed in order to obtain expression vector. LR clonase was performed according to invitrogen manual. In a 1.5 mL tube, 1-7  $\mu\text{L}$  of entry clone (15-150 ng) and 1  $\mu\text{L}$  of destination vector (150 ng/  $\mu\text{L}$ ) were added at room temperature. Next, TE buffer (pH 8.0) was added up to 8  $\mu\text{L}$ . The LR Clonase™ enzyme mix was thawed on ice for about 2 min and vortexed briefly twice, 2 sec each time. 2  $\mu\text{L}$  of LR Clonase™ enzyme mix was added to each reaction tube and mixed well by vortexing briefly twice. Reaction tube was microcentrifuged briefly. The reaction was incubated at 25 °C for 1 hour. 1  $\mu\text{L}$  of Proteinase K solution was added to each sample vortexing briefly to terminate the reaction. Samples were incubated at 38 °C for 10 min. Resulting vectors were used to transform *E.coli* and plated on LB-Kan agar.

## **2.11 CaCl<sub>2</sub> mediated transformation of *E.coli***

It is important to propagate the vectors in *E.coli* by transforming the bacteria with the related vectors through CaCl<sub>2</sub> mediated transformation. Preparation of chemically competent cells and transformation of competent cells were performed (Ausebel I., et al, 1994).

### **2.11.1 Competent cell preparation**

Competent cell preparation one colony from LB plate is taken into 2 mL LB liquid medium and grown at 37 °C overnight while shaking. 1 mL overnight cell culture was inoculated into 100 mL LB medium and grown at 37 °C shaking vigorously until A<sub>600nm</sub> ~ 0.25-0.3. The culture was incubated on ice for 15 min. The cells were centrifuged at 3300xg for 10 min. The supernatant was discarded and the cell pellet was resuspended in 30-40 mL ice cold 0.1 M CaCl<sub>2</sub>. The cells were kept on ice for 30 min. The cells were centrifuged again. The supernatant was removed and the pellet was resuspended in 6 mL 0.1 M CaCl<sub>2</sub> solution plus 15% glycerol. The cell suspension was aliquoted into eppendorf tubes and stored at -80 °C.

### **2.11.2 Transformation of bacterial cells**

Transformation of competent cells was performed according to *Current Protocols in Molecular Biology* (Ausebel I., et al, 1994). Competent cells, 100 µL, and 10 ng ligation product were combined in a sterile 2 mL microcentrifuge tube. The mixture was swirled gently and incubated on ice for 10 min. The cells were heat shocked by placing in 42 °C waterbath for 2 min. The sample was taken and 1 mL of LB was added. Then, the cells were grown at 37 °C for one hour while shaking at 250rpm. 50 µL of transformation culture was aliquoted and plated on LB/antibiotic media. The plates were incubated at 37 °C for 12-16 hours.



## **2.12 Plasmid isolation**

After transformation of *E.coli* cells and plating them on selective media various number of single colonies were selected randomly from the BP and LR recombined products transformed *E.coli* cells. Cell cultures were centrifuged at 4000 rpm for 10 min. Plasmids were purified according to QIAprep Spin Miniprep Plasmid DNA purification Kit (Qiagen) protocol. Bacterial cell pellet was resuspended in 250  $\mu$ L buffer of P1 and transferred to a microcentrifuge tube. 250  $\mu$ L of Buffer P2 was added and the tube mixed thoroughly by inverting the tube 4-6 times. Then 350  $\mu$ L of Buffer N3 was added and the mixture was inverted 4-6 times. The mixture was centrifuged at 13,000 rpm for 10 min. The supernatant was applied to the QIAprep spin column and the tube was centrifuged for 30-60 sec. The flow-through was discarded. 0.75 mL buffer PE was added to the QIAprep spin column and it was centrifuged for 30-60 sec . The flow through was discarded and the tube was centrifuged for an additional min in order to remove residual wash buffer. In order to elute DNA, 50  $\mu$ L Buffer EB was applied to the center of the spin column and incubated for 1 min, then centrifuged for 1 min. Concentration of the elute was measured by NanoDrop.

## **2.13 Agrobacterium transformation and agroinfiltration of *N. benthamiana***

Resulting expression vectors including GOIs after LR clonase ( 2.10.2), were used to transform Agrobacterium *EHA105* strains. Then, agrobacterium colonies were used to transform *N.benthamiana* leaves through agroinfiltration. Procedures were taken from Lindbo Lab Ohio State University protocols.

### **2.13.1 Preparation of electro-competent cells**

The original culture of the agro cells were plated on LB-(antibiotic)plate and grown 2-3 days at 28 °C. One colony was taken and grown in 5 mL LB-antibiotic medium overnight at 28 °C while shaking. Next, 1 mL of start culture was taken and grown

in 200 mL LB media overnight at 28 °C while shaking. OD<sub>600</sub> value was measured and it must be in the range of 0.5-0.7. The culture was divided into two centrifuge bottles and keep on ice at least 30 min. The cultures were centrifuged at 3500 rpm at 4 °C for 15 min. The supernatant was poured off and the pellets are resuspended in 50 mL ice cold 10 % glycerol. The suspension is centrifuged at 3500 rpm at 4 °C for 15 min. The supernatant was discarded and pellets are washed with 50 mL ice cold 10% glycerol again. The same centrifuge was done again and the pellets were resuspended in 200 µL GYT medium (%10 glycerol, 0,125% yeast extract, 0,25% tryptone). The resuspended competent cells were aliquoted into 1.5 mL eppendorf tubes kept on ice and stored at -80 °C for up to a year.

### **2.13.2 Electroporation**

50 µL competent cell and 2-3 µL ligation reaction were mixed and cooled on ice for 10 min. The mixture was transferred to a precooled pulser cuvette. Then the cuvette was zapped at 2.5 kV (Cellject duo, Thermo corporation) 1 mL of SOC medium was added and the mixture was transferred to 1.5 mL microcentrifuge tube. The cells were grown at 28 °C for 1 hour. Then, 100 µL of cells was plated on to LB with appropriate antibiotic plate and incubated at 28 °C for 2 days. Putative colonies were screened for insert by colony PCR. Colonies positive for inserts could be grown in LB freeze or by mixing 930 µL of an overnight culture with 70 µL of DMSO and storing in the -80 °C freezer.

### **2.13.3 Agroinfiltration**

For agroinfiltration process LB media (10 g bacto-tryptone, 5 g yeast extract, 5 g NaCl per L), 0.5 M MES pH 5.7, 0.1 M Acetosyringone in DMSO, 1 M MgCl<sub>2</sub>, L-MESA media (10 mM MES pH 7.5, 10 mM MgCl<sub>2</sub> 150 µM Acetosyringone) were prepared. Acetosyringone/per L media is added just before use, Agroinduction media.

Agrobacterium colonies from the plates were picked and inoculated into 3 mLs L-Kan-Rif-MES broth. The culture is grown in shaker at 28 °C 24 to 30 hours, 250 rpm, until culture is very dense in late log or stationary phase. 500 µL of agro culture is inoculated 5 mLs L-MESA (acetosyringone is added freshly) and grown in shaker at 28 °C, 250 rpm, about 6-8 hours. OD<sub>600</sub> is recorded after 6-8 hours of culturing. (Target A<sub>600</sub> is between 0.5 and 1.0, or more preferably between 0.8 and 1.0). after recording, bacterial cells are harvested by centrifuging at 3500 g for 10 min. Liquid part is poured off without disturbing the cell pellet. Cell pellet is resuspended in appropriate volume of agroinduction media to a final OD<sub>600</sub> of about 1.0- 1.1. The table below (2.6) shows the appropriate volume to resuspend the cell pellet.

**Table 2.6** Calculation of Agroinduction media volume. Agrobacterium cultures with corresponding A<sub>600nm</sub> are resuspended in Agroinduction media to a final A<sub>600nm</sub> of 1.

Starting Culture A <sub>600nm</sub>	1ml cell pellet resuspended in Agroinduction media (µL)	Final A <sub>600nm</sub>
0.2	160	1
0.25	220	1
0.3	275	1
0.35	320	1
0.4	380	1
0.45	410	1
0.5	500	1
0.55	540	1
0.6	590	1
0.65	625	1
0.7	680	1
0.75	740	1
0.8	790	1
0.85	840	1
0.9	880	1
0.95	940	1
1	1000	1

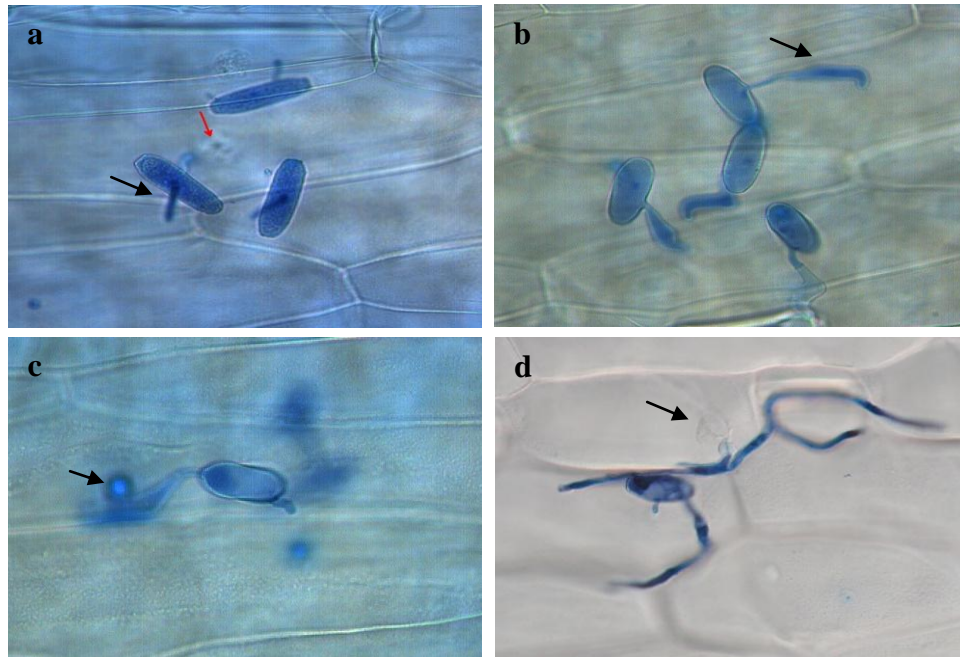
Resuspended cells were incubated in induction media for at least 2-3 hours to overnight at room temperature on bench top. Overnight incubation is preferred for more efficient results. Leaves were labelled with sharpie marker. Small hole was poked in each leaf half with toothpick. Leaves were infiltrated from the underside using 1 mL syringae with no needle at site of small hole, blocking hole from other side with a glooved finger. There were two infiltration zones on a leaf; one for a mix of miRNA and miRNA target and the other was for just the target. miRNA functional analysis was performed by comparing these two zones in each leaf. GFP reporter activity was observed in infiltrated zones by 30 hours postinfiltration under dissecting microscope with appropriate filter.

## CHAPTER III

### RESULTS AND DISCUSSION

#### 3.1 Imaging of Powdery Mildew infections

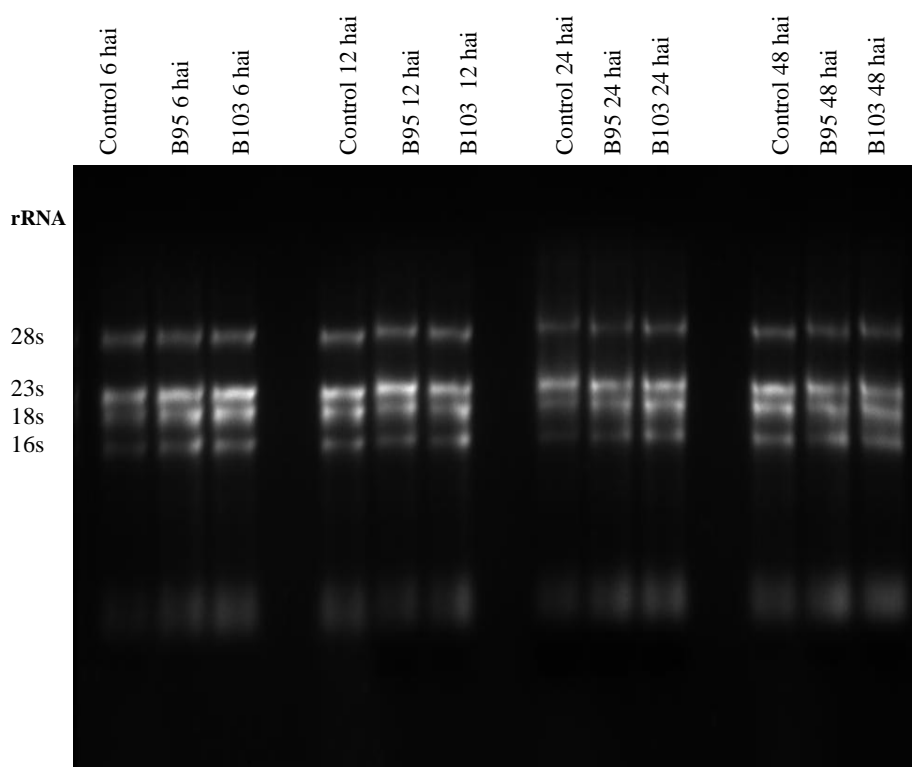
In order to observe infection stages on barley epidermal cells and also determine the appropriate time points for collecting samples, the infected P03 (having Mla6 & Mla14) leaves with virulent *Bgh103* at different time points were stained with trypan blue. In the images presented (Figure 3.1), infection stages and time points were consistent with the time points as in other studies (Both M. et al., 2005).



**Figure 3.1** Infection stages of *Bgh103* on P03 epidermal tissue. **(a)**, 6 hai, germinated conidia; primary and secondary germ tubes (arrow) were visible. **(b)**, 12 hai, germinated conidia; appresorium (arrow) delimited from secondary hyphae by a septum was observed. **(c)**, 24 hai, penetration by hyphae; penetration (arrow) region seems shiny due to host cell depositions. **(d)**, 48 hai, feeding organ haustorium (arrow); branched haustorium was formed and epiphytic mycelium formation was observed along epidermal tissue. All images presented here were taken under light microscope, at 40X.

### 3.2 Total RNA isolation from *Bgh95*, *Bgh103* and mock inoculated P03 leaves

Infected leaves collected at 6 hai, 12 hai, 24 hai and 48 hai were used to isolate total RNA in order to perform miRNA target cloning studies. Quality control of the total RNAs was assessed with 1% RNA gel (Figure 2).



**Figure 3.2** Total RNA profile of the infected P03 samples. P03 barley leaves were inoculated with avirulent *Bgh95*, virulent *Bgh103* strains at defined time points; 6, 12, 24, 48 hai. In addition to pathogen inoculations, mock inoculations (controls) were also performed for the same time points. 1% RNA gel is prepared with 1X Phosphate Buffer (pH 5.5).

Half of these total RNA samples (20  $\mu$ L of each) were aliquoted for microarray analysis in order to detect miRNA expression levels during powdery mildew infection. The rest was used to synthesize cDNA for qRT-PCR and miRNA target

cloning procedures. According to microarray data analysis by LC Sciences service, osa-miRNA159 expression profile is one of the miRNAs showing significant change in response to powdery mildew disease (Table 3.1).

The osa-miRNA159 expression level was found to be the most changed one upon pathogen attack. Its mature sequence was available at mirBASE (<http://microrna.sanger.ac.uk/cgi-bin/sequences/browse.pl>). Mature miRNA sequence of osa-miR159a was used to predict its potential target in barley (*hordeum vulgare*, *Hv*) by Plant miRNA target finder software (available at <http://bioinfo3.noble.org/miRNA/miRU.htm>). Based on the target search, the potential target of miRNA159 was found to be *Hv*-GaMyb (Lacombe et al, 2008).



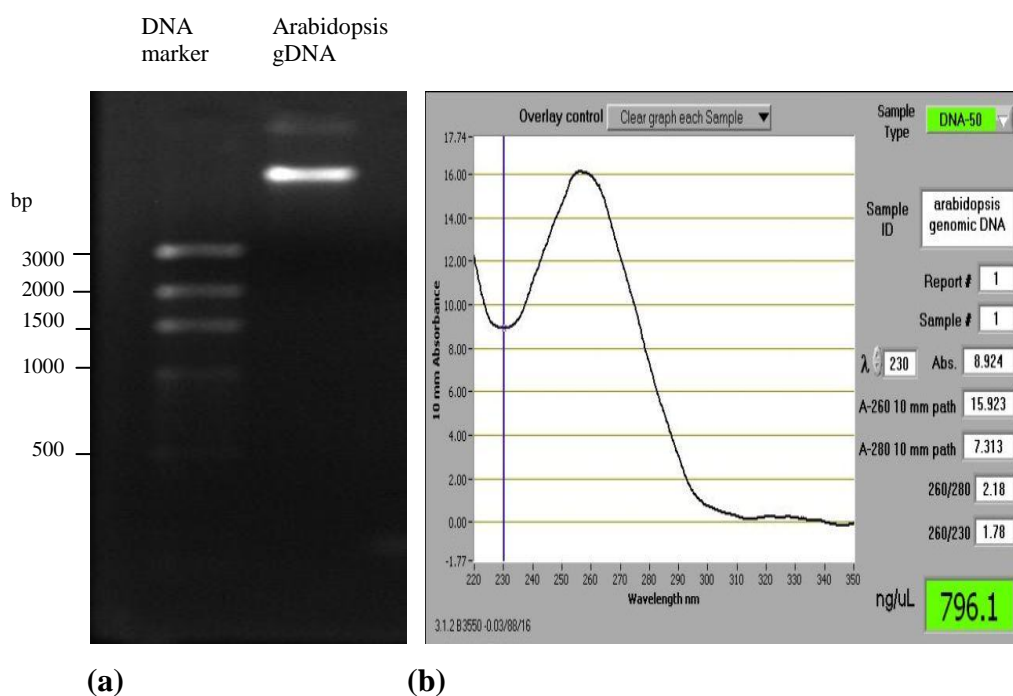
**Table 3.1** Pair t-test data for miRNA expression levels. These data are from 24 hai of compatible and incompatible interactions between barley and Bgh.

<b>miRNA ID</b>	<b>Bgh103 infected</b>	<b>Bgh95 infected</b>
<b>Sbi-miR156e</b>	795	542
<b>Osa-miR159d</b>	4,322	4,025
<b>Ath-miR156g</b>	945	699
<b>Osa-miR168a</b>	797	1,217
<b>Osa-miR396d</b>	478	769
<b>Osa-miR156l</b>	612	356
<b>Ath-miR156a</b>	938	704
<b>Bna-miR156a</b>	837	622
<b>Ath-miR159b</b>	7,590	6,445
<b>Osa-miR159f</b>	7,375	6,365
<b>Ath-miR159a</b>	7,608	6,377
<b>Osa-miR159e</b>	3,825	3,638
<b>Osa-miR166m</b>	80	149
<b>Ptc-miR166n</b>	41	87
<b>Ppt-miR166j</b>	31	49
<b>Zma-miR171c</b>	24	32
<b>Osa-miR166k</b>	63	109
<b>Vvi-miR166d</b>	56	110
<b>Vvi-miR166b</b>	62	125
<b>Sbi-miR166a</b>	79	130
<b>Osa-miR408</b>	23	40
<b>Ptc-miR167h</b>	231	209
<b>Ppt-miR896</b>	25	80

Abbreviations: Ath: *Arabidopsis thaliana*, Osa: *Oryza sativa*, Ptc: *Populus trichocarpa*, Bna: *Brassica napus*, Gma: *Glycine max*, Mtr: *Medicago truncatula*, Sof: *Saccharum officinarum*, Sbi: *Sorghum bicolor*, Ppt: *Physcomitrella patens*, Zma: *Zea mays*, Pta: *Pinus taeda*, Tae: *Triticum aestivum*, Vvi: *Vitis vinifera*.

### 3.3 Genomic DNA (gDNA) isolation from *Arabidopsis thaliana* leaves

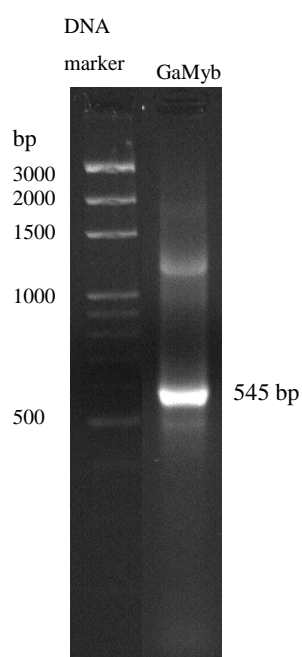
For miRNA cloning, pri-miRNA sequences is needed since mature miRNA is very short molecule (21-24 nt) and could not be amplified by PCR. In this situation, we had to amplify *Arabidopsis* pri-miRNA159 because pri-miRNA sequences are highly variable and there is no barley pri-miRNA identified yet. On the other hand, by using pri-miRNA, miRNA biogenesis would taken place and this will be an advantage for the biological assays. In order to clone pri-miRNA159, genomic DNA (gDNA) was isolated and its quality was assessed in both DNA gel (1%) and NanoDrop spectrophotometer (Figure 3.3).



**Figure 3.3** Agarose gel (1%) electrophoresis image and concentration profile of *Arabidopsis* gDNA. 1  $\mu\text{g}$  of gDNA was loaded and compared with 100 bp DNA marker (a). Absorbance spectrum of gDNA. The concentration of gDNA was 796,1 ng/ $\mu\text{L}$  (b).

### 3.4 Hv-GaMyb gene amplification

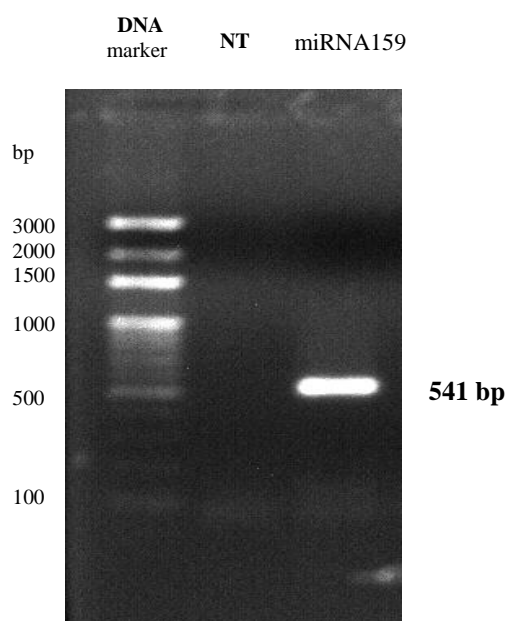
Hv-GaMyb gene (AY008692), as a potential miRNA159 target in barley, was amplified through PCR from barley cDNA. Figure 3.4 shows the GaMyb fragment on DNA gel. The expected size was 545 bp and a dense band corresponding to the expected size was considered to be the putative GaMyb gene fragment of RT-PCR product.



**Figure 3.4** PCR amplification of Hv-GaMyb gene from barley cDNA and profile of the band on agarose gel (1%). 100 bp DNA marker (Fermentas, USA).

### 3.5 Pri-miRNA159 amplification from Arabidopsis gDNA

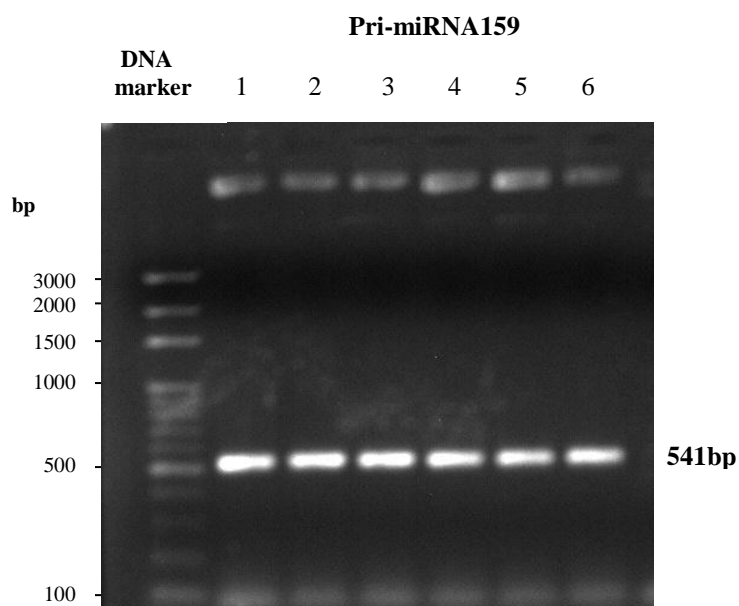
Pri-miRNA159 was amplified by touchdown PCR –varying annealing temperatures between 63-55 °C- with attB flanked primers from Ath-genomic DNA. Expected pri-miRNA159 fragment size was 541 bp and that was the PCR product size observed on the gel ( Figure 3.5).



**Figure 3.5** PCR amplification of pri-miRNA159 using Arabidopsis gDNA as a template. The first well is 1  $\mu$ L of 100 bp DNA marker (Fermentas,USA) and second well is ‘no template’ sample as a negative control of the PCR reaction. The third well is the PCR product of putative pri-miRNA159.

### 3.6 Construction of the pEntry clones

In order to express amplified fragments of pri-miRNA159 and GAMyb through Gateway cloning technology, an entry clone having gene of interest was constructed. In this part, BP clonase reaction was performed by combining attB-flanked PCR fragments of GAMyb and pri-miRNA159 with donor vector (having attP sites), pDONR-Zeo in separate reactions. Transformants of BP clonase reaction were assessed by colony PCR and DNA sequencing. Colony PCR of the pri-miRNA159 colonies resulted in expected size (541 bp) of the gene (Figure 3.6). Sequence result of entry clone including GAMyb hit the *Hv*-GAMyb through blast analysis (Figure 3.7).



**Figure 3.6** Pri-miRNA fragments amplified after BP clonase reaction transformed *E.coli*. Lanes 1-6 belong to pDONR-Zeo- Ath-pri-miRNA159/Dh5 $\alpha$ .

```

(a)
>GANYB2-M13F
cgccggggccaataatgaatTTTTTgaactgaatagtgaacctgttcgttgcaacaaaattgaatgagcaatgctTTTTTat
aatgccaaactttgtg[CAAAAAAGCAGGCTTCATGGGATCAAGTAAACCAAGCAGGGATGCTAAACAGTCTGACCCTTTACT
CCCTGGATTGAGCGACACCATCAATGGCGCGCTCTCCTCGGTCGATCAATTCTCAAATGACTCTGAGAAGCTCAAGCAGGCTC
TTGGTTTTGACTATCTCCACGAAGCCAACCTAGCAGCAAGATTATTGCACCATTTGGGGTGCCTTACTGGCAGCCATGCC
TTTTTAAATGGCACCTTCTACTTCTAGGACCATCAATGGTCTTTGAAGATGGAGCTCCCTTCACTCCAAGATACCGAATC
TGATCCGAATAGCTGGCTCAAGTATACCGTGGCTCCTGCGATGACGCTACGGAGTTGGTTGATCCGTACATGCAGTCCCGA
CAGCAACTCCGTGAGTAAATCGGAGTGTGCTTCGCCGAGGAACAGCGGCCTCTTGGAAGAGCTGCTTCATGAAGCTCAGGGA
CTAAGATCTGGGAAGAATCAGCAGCTC]gaccagctttctgtacaaaagtggcattataagaagcattgcttatcaattt
gttgcaacgaacaggtcactatcagtcaaaaataaaatcattatttgccatccagctgatatcccctatagtgagtcgattac
atggctcatagctgtttcctggcagctctggcccgtgtctcaaatctctgatgttacattgcacaagataaaaataatcatcat
gatcagtcctgctcctcggccacgaagtgcacgcagttgccggccgggtcgcgcagggcgaactcccgcccacggctgctcg
ccgatctcogtcatggccggcccggaggcgtcccgaagtctctggacacgacctccgaacctcggcgctacagttcgt

(b)
H.vulgare mRNA for GAMyb protein Length=2263
Score = 911 bits (493), Expect = 0.0 Identities = 505/510 (99%), Gaps = 3/510
(0%) Strand=Plus/Plus

GAMYB 99 CAAAAA-AGCAGGCTTCATGGGATCAAGTAAACCAAGCAGGGATGCTAAACAGTCTGAC 157
||||| || | ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct 952 CAAAAACTGC-GGCTTCAT-GGATCAAGTAAACCAAGCAGGGATGCTAAACAGTCTGAC 1009

GAMYB 158 CCTTTACTCCCTGGATTGAGCGACACCATCAATGGCGCGCTCTCCTCGGTCGATCAATTC 217
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct 1010 CCTTTACTCCCTGGATTGAGCGACACCATCAATGGCGCGCTCTCCTCGGTCGATCAATTC 1069

GAMYB 218 TCAAATGACTCTGAGAAGCTCAAGCAGGCTCTTGGTTTTGACTATCTCCACGAAGCCAAC 277
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct 1070 TCAAATGACTCTGAGAAGCTCAAGCAGGCTCTTGGTTTTGACTATCTCCACGAAGCCAAC 1129

GAMYB 278 TCTAGCAGCAAGATTATTGCACCATTTGGGGTGCCTTACTGGCAGCCATGCCTTTTTTA 337
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct 1130 TCTAGCAGCAAGATTATTGCACCATTTGGGGTGCCTTACTGGCAGCCATGCCTTTTTTA 1189

GAMYB 338 AATGGCACCTTCTACTTCTAGGACCATCAATGGTCTTTGAAGATGGAGCTCCCTTCA 397
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct 1190 AATGGCACCTTCTACTTCTAGGACCATCAATGGTCTTTGAAGATGGAGCTCCCTTCA 1249

GAMYB 398 CTCCAAGATACCGAATCTGATCCGAATAGCTGGCTCAAGTATACCGTGGCTCCTGCGATG 457
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct 1250 CTCCAAGATACCGAATCTGATCCGAATAGCTGGCTCAAGTATACCGTGGCTCCTGCGATG 1309

GAMYB 458 CAGCCTACGGAGTTGGTTGATCCGTACATGCAGTCCCCGACAGCAACTCCGTCAGTGAAA 517
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct 1310 CAGCCTACGGAGTTGGTTGATCCGTACCTGCAGTCCCCGACAGCAACTCCGTCAGTGAAA 1369

GAMYB 518 TCGGAGTGTGCTTCGCCGAGGAACAGCGGCCTCTTGAAGAGCTGCTTCATGAAGCTCAG 577
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct 1370 TCGGAGTGTGCTTCGCCGAGGAACAGCGGCCTCTTGAAGAGCTGCTTCATGAAGCTCAG 1429

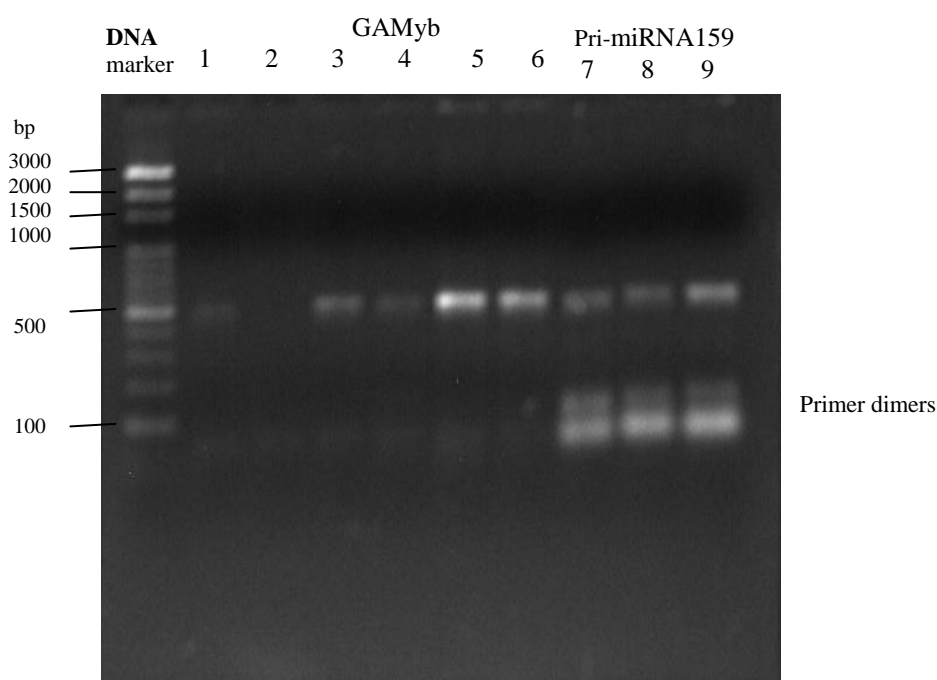
GAMYB 578 GGACTAAGATCTGGGAAGAATCAGCAGCTC 607
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct 1430 GGACTAAGATCTGGGAAGAATCAGCAGCTC 1459

```

**Figure 3.7** GAMyb sequence analyzed from entry clone. GAMyb sequence was in brackets “[ ]” and remaining sequence belonging to vector was given in small case (a). Blast analysis result confirmed the desired clone (b).

### 3.7 Cloning the pri-miRNA159 and GAMyb into expression vectors

After obtaining entry clones with desired inserts (GAMyb and pri-miRNA159), entry clones (having attL sites) were combined with destination vectors (having attR sites), pEarleyGate100 and pEarleyGate103, in order to obtain expression vectors. Expression clones were obtained through LR clonase reaction. pEarleyGate103 including GFP region to be fused with the insert was used for the target gene, GaMyb, and pEarleyGate100 without GFP fusion was used to clone pri-miRNA159. Colony PCR with sequence specific primers confirmed the positive colonies including desired clones; pEarleyGate100- pri-miRNA159a /Dh5 $\alpha$  and pEarleyGate103-HvGaMyb/Dh5 $\alpha$  (Figure 3.10). Sequence analysis for inserts also hit desired genes in ncbi blast (Figure 3.9,10)



**Figure 3.8** Colony PCR to confirm LR clonase reaction after propagating vectors in *E.coli*. The first well represents 100 bp DNA marker with particular band sizes given at the left side. The wells 1-6 belongs to GAMyb gene fragment, the wells of 7-9 represent pri-miRNA159 bands. Dense bands below the desired gene products are most probably dimeric primers.

```

(a)
>P3-F_A04.ab1

Cgcggcagcgggattatgcttttttcgcttttaactt[AAAATTAAGATGAATCCGAATAAATAATTGTTGAGTAG
AAATAAAGAAAAATAAAAAATAGAGAAGGGGAAAGAAGATGTAGAGCTCCCTTCAATCCAAAGAAGAGTAAAAG
CCATTAAGGGCAAGTTAAAGCTCCTGAGATATGCAATCCATATAACCAAATCCTTTTTTACTTTTACAAGGATT
AGGGCTTATGGGATCCATAGCTTAGCAGCTTTTCTTTACCCTGCTCAACTCATGTTTGAACTTTAAGGAGCTCT
ACTTCCATCGTCAGATCAAGATCTATCGAAAAACAAAGTATCTTGTCAAATATATTATGGATCTGACATAAGCA
AACTGTAAAGACCTTTAACTCTTCATAAAAACAATGAGAAAGTTTTGATGAGAACGTGGATTAACAAAAACACCA
AAAGAGGTAATCTCTTACATTA] acccagcttggttggtgggagggtggtgcctaggtagtctagagagttaa
ttaagaccgggactagtccttagagtccctgcttttaagatgatcgagagcgcctatgatcgcatgatatttg
ctttcaattctggtgacggttgtaaaaaacctgagcatggtgtagctcagatccttaccgcccgttttcggttc
attctaataatataatcaccggttactatcgatatttttatgaataatattctccggttcaatttactgattgtac
cctactacttataatgtaccatattaatgaacatataattgtgctgaatagtttatagcgaaatctatgataga
gcgcaacaataacaacattgctgttttattatacaatccggttaaaaaagcacagaaccggtaacctaaagac
tgattactaatcttatcaatttcaaggtgccccaggggc

(b)
>gb|EU548553.1| Arabidopsis thaliana isolate CS901 miR159a primary transcript
(miR159a) gene, complete sequence Length=480

Score = 785 bits (425), Expect = 0.0 Identities = 430/432 (99%), Gaps = 1/432
(0%) Strand=Plus/Plus

P3-F_ 36   AAAATT-AAGATGAATCCGAATAAATAATTGTTGAGTAGAAAATAAAGaaaaataaaaaaT 94
        |||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||
Sbjct  49   AAAATTAAAGATGAATCCGAATAAATAATTGTTGAGTAGAAAATAAAGAAAAATAAAAAAT 108

P3-F_ 95   AGAGAAGGGGAAAGAAGATGTAGAGCTCCCTTCAATCCAAAGAAGAGTAAAAGCCATTAA 154
        |||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||
Sbjct  109  AGAGAAGGTGAAAGAAGATGTAGAGCTCCCTTCAATCCAAAGAAGAGTAAAAGCCATTAA 168

P3-F_ 155  AGGGCAAGTTAAAGCTCCTGAGATATGCAATCCATATAACCAAATCCTTTTTTACTTTTAC 214
        |||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||
Sbjct  169  AGGGCAAGTTAAAGCTCCTGAGATATGCAATCCATATAACCAAATCCTTTTTTACTTTTAC 228

P3-F_ 215  AAGGATTAGGGCTTATGGGATCCATAGCTTAGCAGCTTTTCTTTACCCTGCTCAACTCAT 274
        |||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||
Sbjct  229  AAGGATTAGGGCTTATGGGATCCATAGCTTAGCAGCTTTTCTTTACCCTGCTCAACTCAT 288

P3-F_ 275  GTTTGAACTTTAAGGAGCTCTACTTCCATCGTCAGATCAAGATCTATCGAAAAACAAAGT 334
        |||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||
Sbjct  289  GTTTGAACTTTAAGGAGCTCTACTTCCATCGTCAGATCAAGATCTATCGAAAAACAAAGT 348

P3-F_ 335  ATCTTGTCAAATATATTATGGATCTGACATAAGCAAACCTGTAAGACCTTTAACTCTTCA 394
        |||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||
Sbjct  349  ATCTTGTCAAATATATTATGGATCTGACATAAGCAAACCTGTAAGACCTTTAACTCTTCA 408

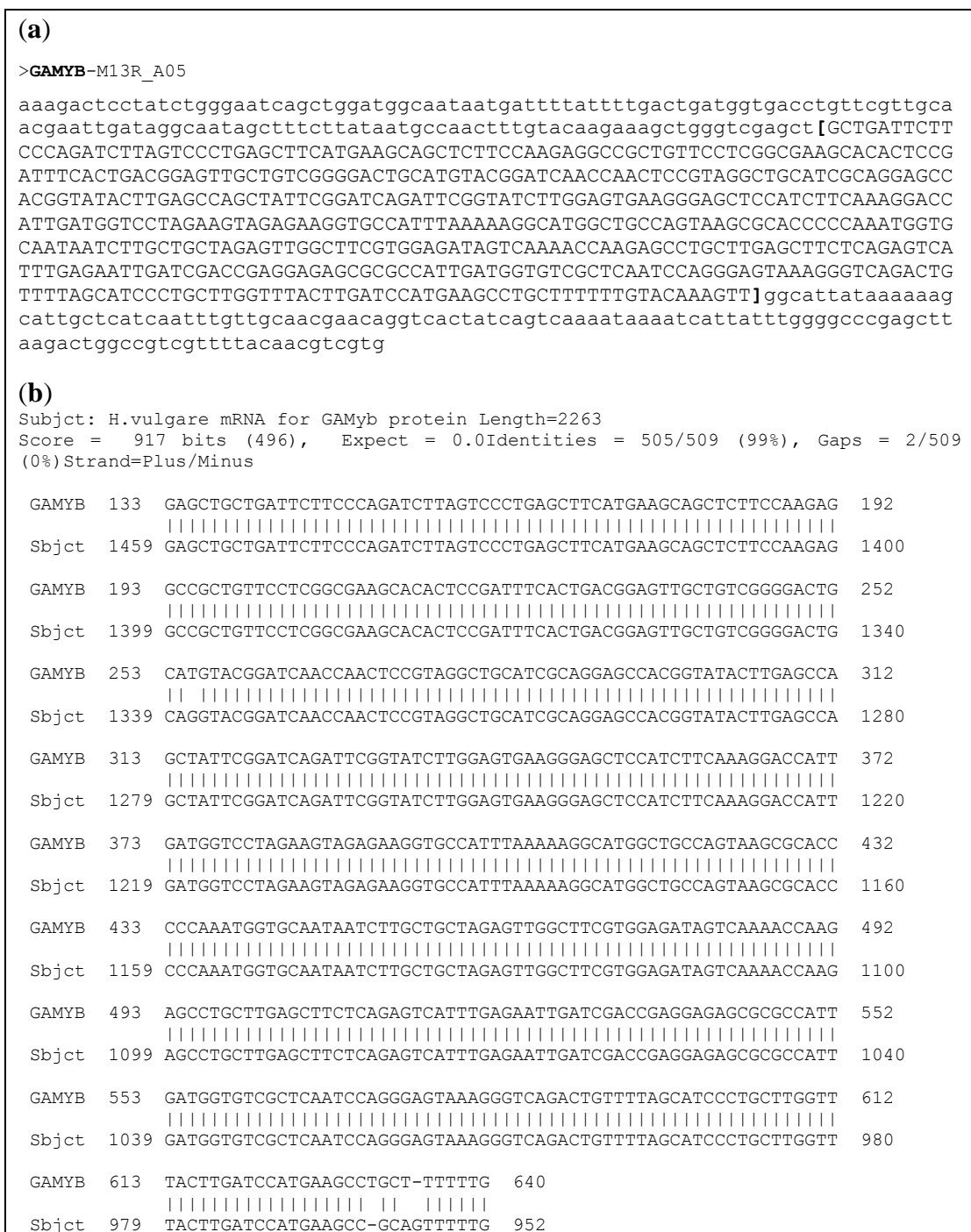
P3-F_ 395  TAAAACAATGAGAAAGTTTTGATGAGAACGTGGATTAACAAAAACACCAAAGAGGTAAA 454
        |||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||  |||||||
Sbjct  409  TAAAACAATGAGAAAGTTTTGATGAGAACGTGGATTAACAAAAACACCAAAGAGGTAAA 468

P3-F_ 455  TCTCTTACATTA 466
        |||||  |||||||
Sbjct  469  TCTCTTACATTA 480

```

**Figure 3.9** pri-miR159 sequence of p3 expression clone. Sequence analysis corresponding blast result were given in the box (a) and (b). Small case letters (a) show vectoral region (pEarleyGate).





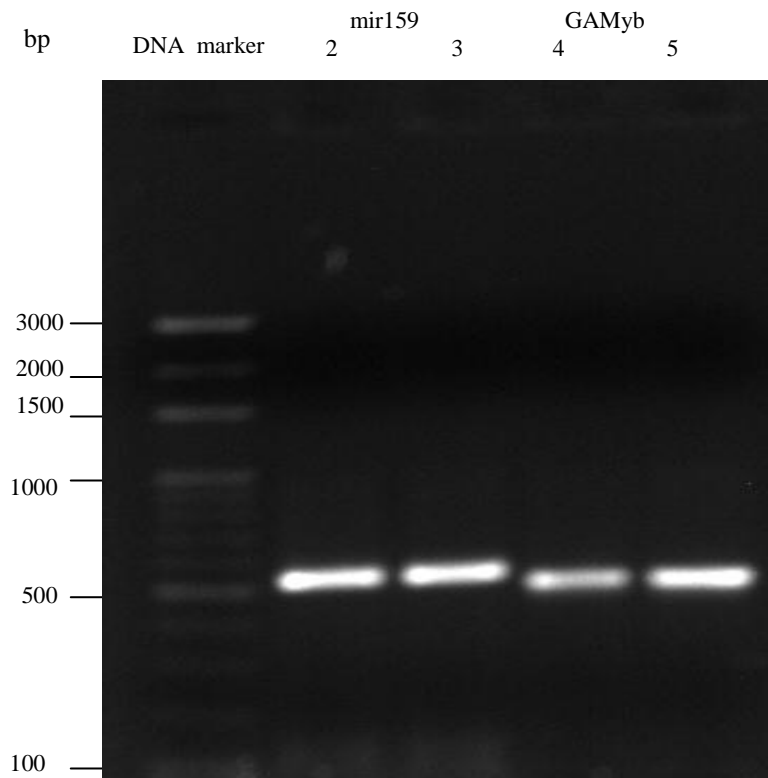
**Figure 3.10** Hv-GAMyB sequence analysis result. The sequence performed with M13 reverse primer and the region delimited by brackets “[ ]” refers to sequences matching to *Hv-GaMyb* sequence (a) of which blast result is also given below the sequence (b). Small cased sequences belong to expression vector backbone.

### **3.8 Transformation of Agrobacterium by electroporation**

Expression clones pEarleyGate100- pri-miRNA159a /Dh5 $\alpha$  and pEarleyGate103- HvGAMyb/Dh5 $\alpha$  were confirmed and transferred into Agrobacterium strain EHA105 through electroporation. Plated cultures gave colonies on LB-Kan-Rif media.

### **3.9 Confirmation of transformation of Agrobacterium**

Positive Agrobacterium colonies on selective media were taken to the LB medium to grow and also tested by colony PCR with sequence specific primers of GAMyb and pri-miRNA159. Reaction result was screened on DNA gel and expected bands were observed (Figure 3.11).



**Figure 3.11** Colony PCR of *Agrobacterium* colonies supposed to have positive inserts: pri-miRNA159 and GAMyb. First well is 100 bp DNA marker, 2&3 belongs to pri-miRNA159 and 4&5 belongs to Hv-GAMyb.

### 3.10 *Agrobacterium* Infiltration of *N. benthamiana*

#### 3.10.1 Growing bacteria and incubating in Agroinduction media

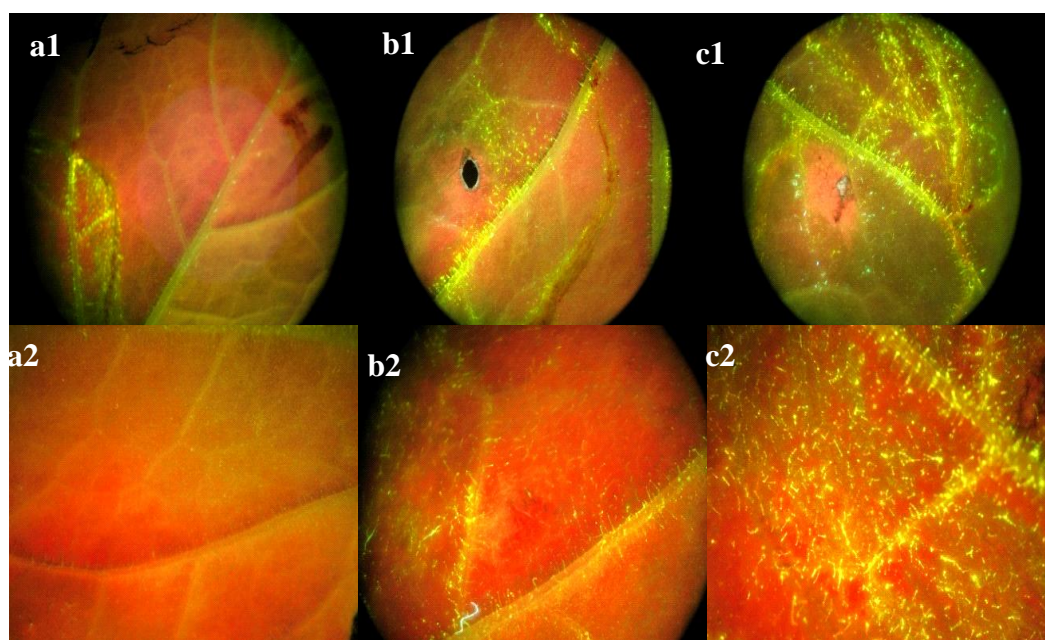
*Agrobacterium* colonies having expression clones were grown in LB media and L-MESA media.  $A_{600nm}$  value was recorded for each sample (Table 3.2). Theoretically  $A_{600nm}$  values between 0.8 and 1.0 are more preferable. From the cultures p1 and p2 were used for pri-miRNA expression and g3 and g4 were used for GAMyb expression.

**Table 3.2**  $A_{600nm}$  values of growing Agrobacterium cell cultures and calculation of agroinduction media volume require for each sample. “p” stands for pri-miRNA159 including Agro cultures, and “g” stands for Hv-GaMyb including Agro cultures in order.

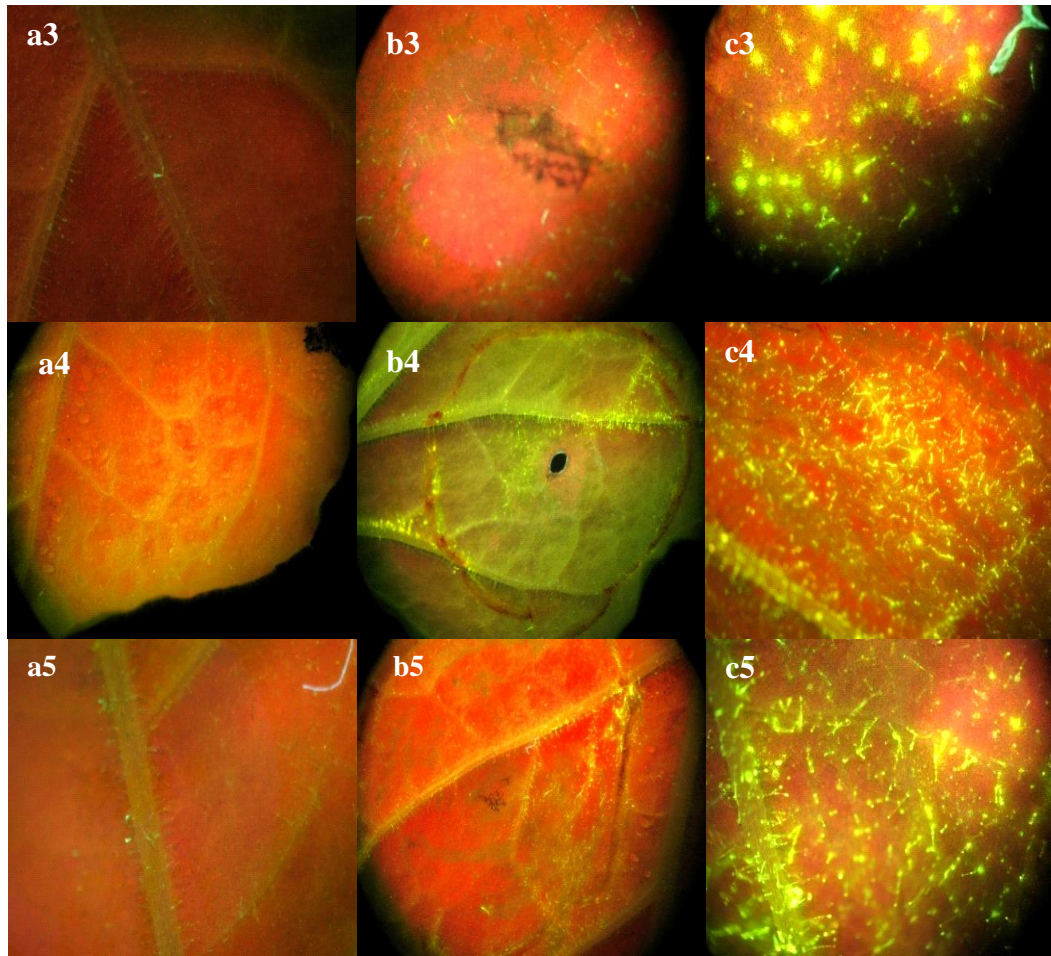
Sample name	Starting $A_{600nm}$	Volume of agroinduction media( $\mu$ L)	Final $A_{600nm}$
p1	0.850	840	1
p2	0.728	700	1
p3	0.596	590	1
p4	0.642	625	1
p5	0.707	680	1
p6	0.677	650	1
p7	0.637	600	1
p8	0.671	650	1
p9	0.714	680	1
p10	0.645	625	1
g1	0.720	680	1
g2	0.749	740	1
g3	1.092	1000	1
g4	1.060	1000	1
g5	0.845	840	1
g6	0.860	840	1
g7	0.965	940	1
g8	0.490	500	1
g9	0.821	800	1
g10	0.916	880	1

### 3.10.2 Imaging the gene expression through Dissecting microscope

Agrobacterium cultures including GOI clones; pEarleyGate100-*Ath*-miRNA159a /EHA105, pEarleyGate103-*Hv*-GAMYb /EHA105 were incubated in agroinduction media overnight. two agroinfiltration zones were marked on each *N.benthamiana* leaf; one zone including miRNA and miRNA target clones and the other zone including only miRNA target clone in order to compare GFP expression in one leaf.



**Figure 3.12** GFP expression in *N. benthamiana* leaves. From left to right; (a1-a5) were taken anywhere on the leaf out of the infiltration zones, called as control regions, (b1-b5) were taken from the infiltration zone where both miRNA and miRNA target clones infiltrated, (c1-c5) were taken from the infiltration zone where only miRNA target clone was infiltrated. Leaves were imaged 30 hours after inoculation (hai) in order to visualize the leaves at the optimum GFP expression time.



**Figure 3.12** cont'd GFP expression in *N. benthamiana* leaves. From left to right; (a1-a5) were taken anywhere on the leaf out of the infiltration zones, called as control regions, (b1-b5) were taken from the infiltration zone where both miRNA and miRNA target clones infiltrated, (c1-c5) were taken from the infiltration zone where only miRNA target clone was infiltrated. Leaves were imaged 30 hours after inoculation (hai) in order to visualize the leaves at the optimum GFP expression time.

### **3.11 Discussion**

In this study miRNA159 was found as a putative barley miRNA targeting barley GAMyb and having role in powdery mildew disease. In this study, according to microarray data, miRNA159 was implied having role in barley powdery mildew since its expression level has changed in resistance and susceptibility cases. By using bioinformatics tools, mature miRNA sequence and its target in barley were predicted. Then, by using overexpression strategy (Krutzfeldt et al., 2006) functional analysis of miR159 was performed *in vivo*.

#### **3.11.1 osa-miRNA159 expression level changed significantly**

Total RNA samples from infected barley were hybridized with plant microRNAs by using microchip screen technology. The spot in the chip showed that osa-miRNA159 expression level was changed significantly (Table 3.1). Previous studies also showing that miR159 is induced by abscisic acid and thereby regulates the expression of two MYB factors (MYB33 and MYB101) during Arabidopsis seedling (Reyes et al., 2005). In this study, Mir159 expression was observed to be increased during incompatible interaction (susceptibility) with respect to control samples. This implies that miRNA159 may be involved in gene regulation during infection. In another study related with tomato leaf disease, miR159 expression was upregulated and its target Myb33 was downregulated during viral infection by tomato leaf curl virus (ToLCV) (Naqvi et al., 2008).

#### **3.11.2 osa-miRNA159 targets Hv-GAMyb**

Osa-miRNA159 sequence was available at miRBase. Mature miRNA sequence which is supposed to bind its cognate sequence in the target message was used to find its potential target in barley. Its potential target was found as barley GAMyb gene. GAMyb which directly binds to specific sequences located in the promoters of GA-responsive genes is a key protein in gibberellin (GA) signalling. Recent

functional studies imply that GAMyb is an important component in floral development (Achard et al., 2004; Kaneko et al., 2004; Millar and Gubler, 2005; Murray et al., 2003). Mutations in GAMyb disrupt internode elongation and floral organ development (Kaneko et al. , 2004). In addition, overexpression of GAMyb causes male sterility (Murray et.al., 2003). To summarize, it is known that GAMyb is regulated by miR159 posttranscriptionally in the plants such as Arabidopsis, rice and tomato (Achard et al., 2004; Millar and Gubler, 2005; Palatnik et al., 2003; Schwab et al., 2005).

### **3.11.3 miRNA159 and Hv-GAMyb cloning**

In a comprehensive study held by Sunkar et al. (2008), mir159 family is identified in barley genome by computational methods. It is known that although pri-miRNA sequences are highly variable even within the same miRNA gene family, mature miRNA sequences were highly conserved between species. (Bartel, 2004). Therefore, it is reasonable to use this homology and synthesize putative miRNA159 from other organisms of which the miRNA sequences available. Although the rest of the sequence is variable, another important alternative is to synthesize pri-miRNA instead of mature miRNA, because it was aimed to utilize miRNA biogenesis within the plant.. *Oryza sativa* (rice) was the first candidate to apply for pri-miRNA sequence since it is in the grass family as *Hordeum vulgare* (barley). However, I could not use rice genomic DNA because we don't have facility to grow rice. Instead, I isolated and used Arabidopsis genomic DNA in order to synthesize pri-miRNA159. In early studies, searching for plant miRNAs were conducted by comparative genomic analysis of Arabidopsis and rice genomes. Among the annotated miRNA families, miR159 was found that it is highly conserved between Arabidopsis and rice genomes (Li et al., 2005). Once pri-miRNA was amplified by PCR from Arabidopsis gDNA, it was cloned into the expression vector with Gateway cloning. For the miRNA target ,GAMyb, cloning barley cDNA was used as template and it was cloned into the expression vector very practically.



#### **3.11.4 Agrobacterium infiltration of *N. benthamiana***

In order to perform functional analysis of miRNA159; miR159 and GAMyb were cloned into pEarleyGate vectors under the control of a constitutive promoter, 35S, providing overexpression of these genes. GFP is a noninvasive and sensitive marker for transient gene expression systems. In our study, GAMyb inserted expression vector had GFP gene and miRNA159 inserted vector did not have. The reason for that when the miR159 targeted GAMyb, expression of GAMyb will be reduced and GFP level will decrease since it is fused with GAMyb.

*N. benthamiana*, an important model organism in plants, was used for *in vivo* procedures. The reason for that agrobacterium mediated transient gene transfer procedure is very efficient for *N.benthamiana* species (Wydro et al., 2006) but not at possible for monocots including barley. Images were obtained namely from three regions of the plant leaves miR159+GAMyb coinfiltrated zone, GAMyb infiltrated zone and no treatment zone. When the images were compared, it was possible to observe that there was no fluorescence in noninfiltrated zones, there was slight but weak fluorescence in miR159+GAMyb coinfiltrated zones and there was bright and strong fluorescence in target, GAMyb infiltrated, zones.

These findings showed that overexpression of miR159 results in decreased level of GAMyb expression. These results support previous studies in which miR159 regulates Myb gene expression (Reyes et al., 2007; Tsuji et al., 2006; Naqvi et. al., 2008). However, those results were shown for Myb factors in Arabidopsis, rice and tomato. According to our study barley GAMyb expression was suppressed during fungal attack and miR159 expression level was increased implying that miR159 regulates barley GAMyb expression during infection. GAMyb is a well studied transcription factor and has role in plant development (Tsuji et al., 2006).

Based on these data and future confirmations to be held, we can assign a novel function for the miRNA159 involving in plant response to biotic stress.

## CHAPTER 4

### CONCLUSION

In this research one of the putative miRNAs of barley having role in powdery mildew disease was addressed through biological assays. Using microarray analysis and bioinformatics tools, miR159 targeting barley GAMyb was found worthy to be analysed functionally. It was revealed that miR159 negatively regulates barley GAMyb expression.

This study is important for two reasons; it is the first study assessing functional analysis of a putative barley miRNA, miRNA159, and secondly it showed that Hv-GAMyb acts during fungal infection and is regulated by miR159.

miR159 was accounted as “putative” barley miRNA targeting Hv-GAMyb because this study needs further confirmation related with miRNA target prediction issue such as northern blotting and/or 3' RACE of cleaved fragments (Voinnet, 2009). Also, in order to prove its role in powdery mildew in barley, gene expression studies should be conducted in barley. In addition to GFP imaging, leaves should be inoculated with Bgh in order to study the effect of pathogen for the function of miRNA *in vivo*.

In addition to presenting crucial data regarding to a putative barley miRNA for future studies, novel biological techniques including Gateway cloning and Agrobacterium mediated gene transfer were applied effectively for miRNA functional analysis.

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