

THE EFFECT OF VIRUS INDUCED GENE SILENCING OF FAS-
ASSOCIATED FACTOR 1 IN *BLUMERIA GRAMINIS* INFECTED BARLEY

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THE EFFECT OF VIRUS INDUCED GENE SILENCING OF FAS-ASSOCIATED FACTOR 1 IN *BLUMERIA GRAMINIS* INFECTED BARLEY

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ABSTRACT

THE EFFECT OF VIRUS INDUCED GENE SILENCING OF FAS-ASSOCIATED FACTOR 1 IN *BLUMERIA GRAMINIS* INFECTED BARLEY

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Cereal loss due to fungal pathogens is an ongoing setback in agriculture. Elucidating plant's resistance and susceptibility mechanisms against these cereal killers, promises progress in agriculture. In the way of understanding barley resistance against fungus *Blumeria Graminis* we silenced FAS-Associated Factor 1 (FAF1) gene in its mRNA level with Virus Induced Gene Silencing (VIGS) technique. Previous research in our lab had shown an augmentation in mRNA levels of FAF1 gene in fungus infected wheat, suggesting a role of this gene in the resistance mechanism. We hypothesized that the apoptotic role of FAF1 protein in metazoan is conserved in plants by including FAF1 as a factor in hypersensitive response. Barley lines Pallas01 and Pallas03 which are respectively resistant and susceptible against fungus *Blumeria graminis hordei* 103 (*Bgh103*) were used for fungal inoculations after FAF1 silencing, to test if the hypersensitive response against fungus *Bgh103* was prevented. In this aspect the formation of death lesions on the Pallas01 leaf due to fungal resistance was not prevented demonstrating that FAF1 silencing with VIGS in the resistant Pallas01 line of barley is not sufficient to stop apoptosis. On the other hand the FAF1-silenced barley susceptible line

Pallas03 became more sensitive to fungal stress based on conidia (body part of the fungus) counting after trypan blue staining of the infected leaves. In the C-terminus of FAF1 an ubiquitin like domain-X (UBX) is found, which is the cause of stress sensitivity based on the reported data obtained about this domain's loss of function in other proteins. These results suggest that FAF1 is a catalyst in the hypersensitive response and its loss of function makes barley more susceptible to fungal stress. On the other hand a short mRNA homology was found among FAF1 and many pathogen disease related proteins making this homology a possible target site for VIGS of *FAF1* generated siRNAs, which might cause some other protein to be responsible for the barley susceptibility against the fungus.

Key Words: BSMV, VIGS, *FAF1*, barley fungal disease, *Blumeria graminis f.spp. hordei*.

ÖZ

VİRÜS İLE SUSTURULMUŞ FAF1 GENİNİN BLUMERIA GRAMİNİS İLE ENFEKTE OLMUŞ ARPADAKİ ETKİSİ

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Patojenler sebebiyle olan tahıl kaybı tarımda devam eden bir problemdir. Bu patojenlere karşı bitkinin rezistans ve hassasiyet mekanizmaları açıklamak tarımda ilerleme vaat ediyor. Arpanın *Blumeria Graminis hordei* 103 (*Bgh* 103) mantarına karşı rezistansı anlama yolunda fas ile ortak faktör 1 (*FAF1*) genini Virüsle indükte edilmiş gen susturması (VİGS) tekniği ile susturduk. Labımızda olan önceki çalışmalarda *FAF1* geninin mantar ile enfekte olmuş buğdaydaki mRNA seviyeleri yükselmiş, böylece bu genin mantara karşı rezistans mekanizmasında rol oynadığını öne sürüyor. Hipotezimiz *FAF1*'i bitkinin Aşırı Tepki mekanizmasında dahil ederek *FAF1*' in hayvanlardaki apoptik rolü bitkilerde de korunmuş olması idi. Arpadaki *FAF1* genini Vigs ile susturduk ki arpanın *Blumeria graminis hordei* 103 mantarına karşı Aşırı Tepkiyi engel olmuş olalım ve bunun için arpanın Pallas01 (mantara rezistant) ve Pallas03 (mantara hassas) dizileri kullanıldı. Sonuçlarımıza göre *FAF1* in susturulması Pallas01 dizisinde lezyonların rezistanstan dolayı oluşması engellenmedi . Bu da *FAF1* in VİGS ile susturulması apoptosisi durdurmak için yeterli olmadığını gösterir. *FAF1* in susturulduğu arpanın hassas olan dizisinde ise konidia sayısının yükseldiğini gördük. Bu sonuçlar gösteriyor ki arpadaki *FAF1* geninin susturulması bitkiyi mantar stresine

karşı daha hassas yapıyor fakat apoptosisi durduracak seviyeye kadar değil. *FAF1* in rezistans mekanizmasındaki rolü bir katalizatör olması bu da apoptotik yolunda bu genin protein degradasyonunda yardım etmesidir. *FAF1* in sonunda *UBX* domainin bulunması bu rolü daha fazla konfirme ediyor. Başka proteinlerde çalışılmış bu domain hakkında veriler *FAF1* in protein degradasyonunda rol oynadığını ıspatlıyor. *FAF1* ile rezistanla ilgili bir çok protein'in mRNAsında kısa bir homoloji bulduk. Bu da *FAF1*'in VIGS ile susturulmasından ortaya çıkan kısa engeleyici RNAların bir hedefi olabilir. Böylece arpanın mantara karşı olan hassasiyet hakkında başka bir protein de sorumlu olabilir.

Anahtar kelimeler: BSMV, VIGS, *FAF1*, Arpa mantar hastalığı, *Blumeria graminis f.spp. hordei*,

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LIST OF ABBREVIATIONS

μg	: Microgram
μL	: Microliter
As	: Anti sense
Bgh	: <i>Blumeria graminis hordei</i>
BSA	: Bovine Serum Albumin
BSMV	: Barley Stripe Mosaic Virus
CC	: Coiled Coil
DNA	: Deoxyribonucleic acid
dNTP	: Deoxy-nucleotidetriphosphate
Dpi	: Day post inoculation
DTT	: Dithiothretiol
ETI	: Effector Triggered Immunity
GFP	: Green Flourescent Protein
hr.	: Hour
HR	: Hypersensitive response
HSP90	: Heat Shock Protein 90
LiCl	: Lithium Chloride
LRR	: Leucine rich repeat
min.	: Minute
ml	: Mililiter
Mla	: Mildew Locus A
NB	: Nucleotide binding
NEB	: New England Biolabs
PAMP	: Pathogen Associated Molecular Pattern
PCD	: Programmed Cell Death
PCR	: Polymerase Chain Reaction
<i>PDS</i>	: Phytoene Desaturase

PR	: Pathogenesis Related
PTGS	: Post Transcriptional Gene Silencing
PTI	: PAMP Triggered Immunity
R	: Resistance gene
RAR1	: Required for Mildew LocusA Resistance 1
RISC	: RNA Induced Silencing Complex
RNAi	: RNA inhibition
ROS	: Reactive oxygen species
RT-PCR	: Reverse transcriptase polymerase chain reaction
SCF	: Skp1-Cullin-Fbox
SGT1	: Suppressor of G2 allele of Skp1
SIRNA	: Short inhibitory RNA
Skp1	: Suppressor of kinetochore protein 1
<i>Taq</i>	: <i>Thermus aquaticus</i>
TIR	: Toll Like Interleukine
Ub	: Ubiquitin
UBC	: Ubiquitin conjugating enzyme
VIGS	: Virus Induced Gene Silencing

CHAPTER 1

INTRODUCTION

1.1 Barley and its pathogens

Hordeum vulgare is a member of the grass family and its abundance among the cereal grains is ranked as fourth in the world (FAO 2005). This plant is diploid with 14 chromosomes and it is a self-pollinating species. Its two subspecies are spontaneum (wild) and vulgare (domesticated) with the difference of brittle spike in the seeds of wild barley.

Resistance or susceptibility towards fungal pathogens classifies barley lines further as black hulles or pallas varieties which contain different forms of Mildew locus associated (Mla) resistance genes.

Many pathogens are able to infect barley. Among those important to the purpose of this thesis are fungal and viral pathogens but bacteria and nematode are also present as pathogens in nature.

One of the key viral pathogens is barley stripe mosaic virus (BSMV), whose genome was first sequenced in 1989 (Gorbalenia, Kunin et al. 1989) and is explained in detail in the following sections.

Among fungal pathogens, rusts are of interest to our laboratory. They are named according to the organ they infect or the phenotype they cause. Crown, leaf, stem and stripe or yellow rusts are the ones known.

In our experiments we used *Blumeria graminis* f. sp. *hordei* (synonym: *Erysiphe graminis* f. sp. *Hordei*) which causes powdery mildew disease in barley. *Bgh103* is the fungus variety to which *Hordeum vulgare* Pallas01 and Pallas03 lines are resistant and susceptible respectively.

1.2 Mechanism of fungus infection.

Infection occurs with 6 morphological phases (Hein, Barciszewska-Pacak et al. 2005), 2 of which are visible with a light microscope in a range of few millimeters (Fig 1.1). The arrow shows the conidium which is the body part of the spore and the arrowhead shows the formation of haustorium which is the “sign” of infection and the organ of asexual reproduction for the spore from where hostility for nutrition starts.

Haustorium is formed after the spore has penetrated the epidermal cell.

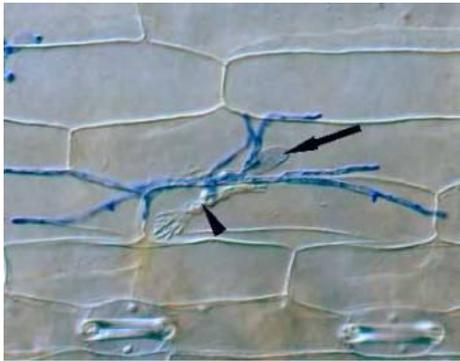


Figure 1.1 Spore penetration into barley leaf. The arrow shows the conidium which is the body part of the spore and the arrowhead shows the formation of haustorium which is the nutrition organ of the spore. (http://www.uni-giessen.de/ipaz-alt/abt_phytopath/ag-phytopath/DFG-Nachwuchs/Nachwuchs-2005-en.htm).

In Fig 1.2 are shown the 6 stages of infection. The formation of small sacs (red ones) comes before the haustoria formation. And it is only after 36 hours that these haustoria can be formed. It is of relevance to mention here that if any gene that plays a role in main resistance is silenced, then the haustoria must not appear.

The spore is spread across the leaf till the plant dies.

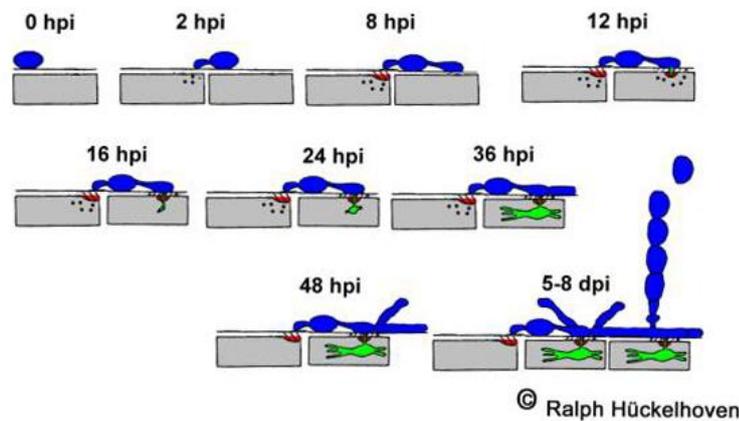


Figure 1.2 The spreading of the spore upon the leaf. Haustorium is starting to form at 16-24 hours post inoculation (hpi) and it multiplies 5-8 days post inoculation (dpi). At 0, 2, 8 hpi only the spore and rear sacs are present. After the full formation of haustorium at 36 hpi the plant either causes a localized death of its own cells or not. If the plant cell dies at 36 hpi then fungus growth stops meaning that the spore can not infect the plant. The spore in the picture instead can infect the plant because of secondary haustorium formation and so on.

(http://www.uni-giessen.de/ipaz-alt/abt_phytopath/ag-phytopath/DFG-Nachwuchs/Nachwuchs-2005-en.htm)

1.3 Resisting the impact

The plant immunity has evolved with many barriers against pathogens

Plants do not possess an adaptive immune system as animals; instead they have an innate immune system for protection against pathogens. This system is usually classified into basal defense called Pathogen associated molecular patterns-triggered immunity (PTI) and gene specific resistance defense called Effector Triggered Immunity (ETI).

1.3.1 Basal defense (PTI)

Pathogen associated molecular patterns-triggered immunity (PTI) in Fig 1.4A is the one that involves those proteins located in the extracellular region and recognize the pathogen associated molecular patterns (PAMPs). These patterns are conserved pathogenic characteristics such as bacterial flagellin or fungal chitin, ergosterols, cerebrosides.

Beside PAMPs, there are also patterns that do not correspond to the pathogen but are consequences of plant cell wall degradation due to pathogenic enzymes. Such forms are cutin monomers, pectin and cellulose fragments (Hardham and Jones, et al. 2007).

A very recent work of Berkley and Max Planck (Liu, Elmore et al. 2009) institutes describe that both defense systems PTI and ETI are connected to each other. The first protein that was found to have such a function is RIN4 (RPM1 INTERACTING PROTEIN 4) where RPM1 is a disease resistance gene in arabidopsis.

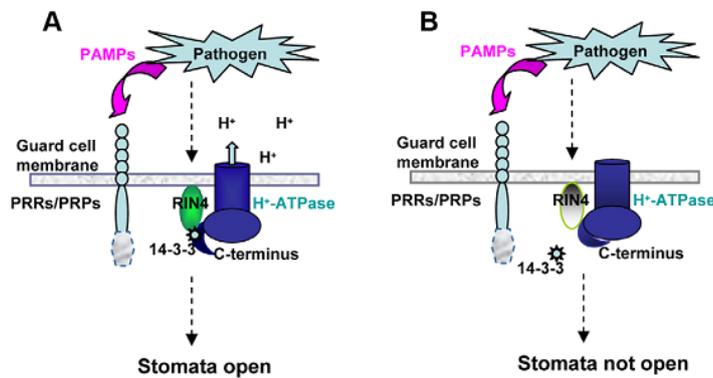


Figure 1.3 Rin4 role in stomata closure A) Susceptible *A.thaliana* line where pathogen associated molecular patterns (PAMPs) are recognized by pathogen recognizing receptors or proteins (PRRs/PRPS) and this event does not trigger the loss of function modification of Rin4 H⁺ATPase by 14-3-3 protein causing the stomata to remain open and allowing pathogen entrance. B) Resistant line where the pathogen contact closes the stomata by Rin4 loss of function and inhibits pathogen penetration.

(<http://ukpmc.ac.uk/articlerender.cgi?tool=pubmed&pubmedid=19564897>)

RIN4 negatively regulates both defenses in Arabidopsis. As the name implies RIN4 interacts with R genes RPM1 and RPS2 (not shown in Fig 1.3) to inhibit their action. When a pathogenic attack occurs RIN4 is hyper-phosphorylated and allows the resistance to be R-gene mediated.

On the other defense PTI, RIN4 interacts with proton pumps (ATPases AHA1 and AHA2) of guard cells that control stomata. In Fig 1.3A stomata are open with an active form of RIN4 interacting with the pump allowing the entrance of the pathogen. In figure 1.3.B upon pathogen pattern recognition post-translational modification of RIN4 occurs that stops the interaction with the pump and therefore the entrance of the pathogen through stomata.

Examples of basal defense genes in barley that mediate the PTI-resistance against powdery mildew are syntaxins, mlo proteins and a new class of small peptides recently discovered called blufensins.

Blufensins (Meng, Moscou et al. 2009) are peptides whose function is not known beside some hypotheses put forward such as playing a role similar to mlo transmembranal proteins. VIGS silencing of blufensin1 gene in barley increased the resistance towards fungus *Blumeria Graminis hordei* and the over-expression decreases this resistance.

Among negative regulators such as blufensins are Lsd1, MAPK4 and EDR1.

Another important resistance which lies in all plants is non-host resistance. An interesting example of this resistance is the *hml* gene which resists the infection of *Cochliobolus carbonum* race 1 (CCR1) in maize (Sindhu, Chintamanani et al. 2008). *Hml* gene is the first plant resistance gene to be discovered and it encodes a reductase enzyme towards *Helminthosporium carbonum* (HC) toxin, a tetrapeptide that causes virulence in susceptible maize. The conserved form of *hml* is found in rice, barley and wheat and surprisingly enough when this gene is silenced with VIGS technique in barley causes this plant to become susceptible to CCR1 which normally is a maize pathogen.

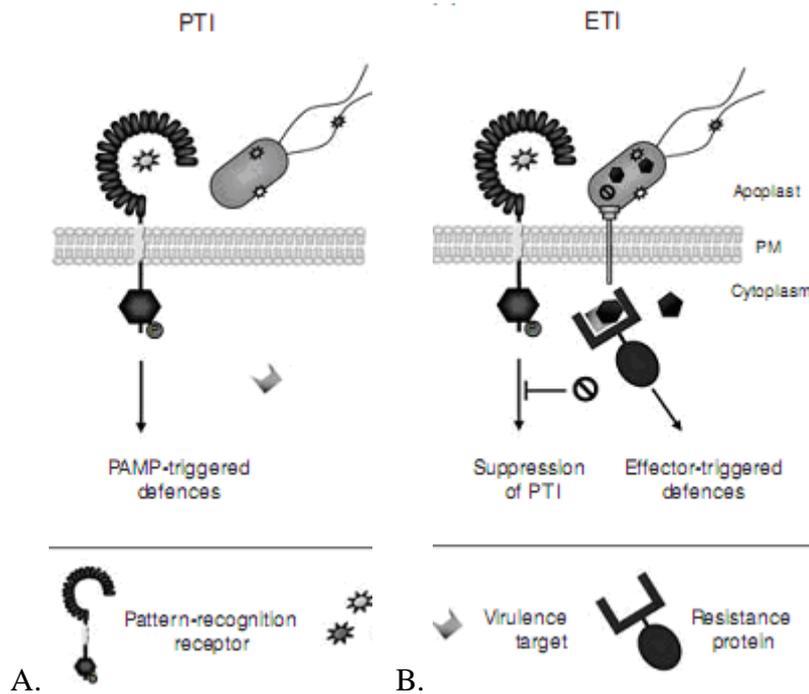


Figure 1.4 PTI and ETI immunities (Annual Plant Reviews (2009) 34, 18).

A. The PAMP triggered immunity (PTI) is related with the barrier defense of the plant against the pathogen and it includes pattern recognition proteins. B. Effector triggered immunity fights the pathogenic effectors by releasing resistance (R) proteins specific for each effector.

As it is shown in Fig 1.4A PTI is a barrier system that activates the signaling pathway of mitogen activated protein kinases (MAPK) and causes the levels of reactive oxygen species (ROS) to increase (Feys and Parker 2000). Callose deposition and transcriptional reprogramming are other consequences of this defense type. ETI is the defense system that utilizes R (resistant) genes against the invading effectors.

1.3.2 Effector triggered immunity (ETI) and R genes

Effector triggered immunity (Fig 1.4B) is conferred by disease resistant genes (R). These R genes distinguish the effector molecules either directly or indirectly. The pathogenic effectors are usually encoded by *Avr* genes. An immediate response that these R genes perform as a resistance sign is the hypersensitive response (HR) that is accompanied with production of reactive oxygen species (ROS) rapid ion fluxes and cell death. The R proteins are present mostly in the cytosol and they possess nucleotide binding domains and leucine rich repeats (NB-LRR). These domains are probably needed for recognition of effector molecules (Jones 2001).

Beside these common domains other R proteins have been found to have coiled coils or Toll and interleukin like N-terminal domains (CC- NB-LRR) or (TIR- NB-LRR) respectively (Bieri, Mauch et al. 2004).

Barley contains a *mildew resistance a (Mla)* locus in chromosome 5 with 28 alleles against different lines of powdery mildew and therefore specific to different pathogenic *Avr* proteins (Caldo, Nettleton et al. 2004). Even though direct interactions between *Mla* and *Avr* have been found in the receptor-ligand pattern, Research has shown that R genes (*Mla*) form complexes with many proteins in the absence of pathogenic *Avr*. Therefore indirect interactions between *Mla* and *Avr* may also be possible.

Pallas01 (*Mla*1) gives a fast and absolute resistance in barley associated with local cell death against fungus Bgh103, whereas Pallas03 (*Mla*6) gives other responses by allowing the formation of haustoria and growing of the pathogen.

The *CS motif* is the additional amino acid sequence that is found in metazoan Rar1 (Required for Mla12 resistance) but not plant Rar1 (Fig1.5). This motif is also found in Sgt1 protein (suppressor of G-two allele of *skp1* (suppressor of kinetochore protein 1)) (Shirasu and Schulze-Lefert 2003).

What Rar1 in plants possesses are domains of CHORD-I and II (cysteine and histidine rich domains) with which the interaction of this protein with Hsp90 and *sgt1* respectively occurs (Fig 1.6).

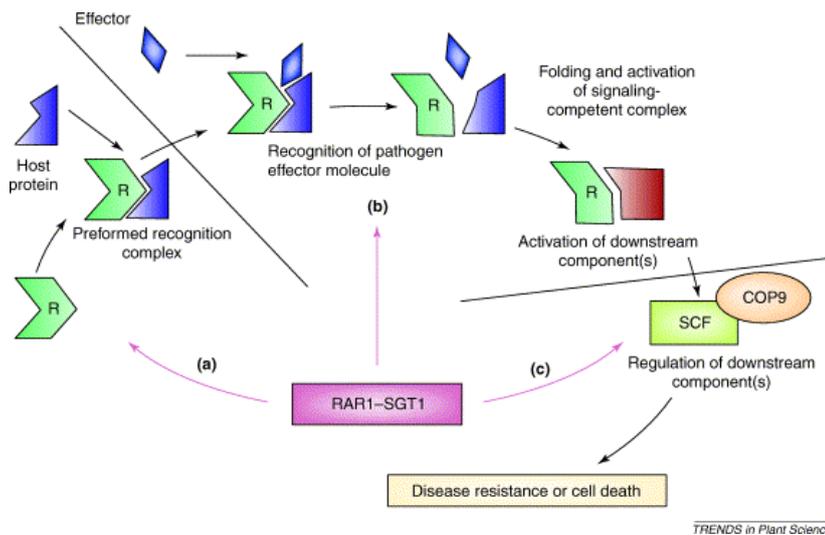


Figure 1.5 Rar1 and Sgt1 role in defense. The possible roles of these 2 proteins during resistance are: a) to help in the formation of R protein complexes. b) To activate R proteins after the complex of recognition is formed. c) To regulate downstream components such as SCF and COP9 complexes.

[http://www.cell.com/trends/plant-science/abstract/S1360-1385%2803%2900104-](http://www.cell.com/trends/plant-science/abstract/S1360-1385%2803%2900104-3)

[3](#))

Sometimes Rar1 sometimes Sgt1 or both or none of them are needed to confer resistance against fungus in different lines of barley therefore the real mechanism behind the participation of these proteins in resistance remains subtle. The R proteins of barley (Mla) themselves may or may not need the interaction with these proteins in order to provide resistance (Zhou and Kurth, et al. 2001). As their name implies for example Rar1 (required for Mla12 resistance) is necessary for Mla12 and Mla6 to confer resistance but is not necessary for Mla1. The Mla genes contain an extra C terminal non-LRR region (CT) and therefore they are CC-NB-LRR-CT type resistance proteins. It is the CC-NB region of these proteins that decides about the necessity of including Rar1-Sgt1 in resistance while LRR-CT recognizes the pathogenic effector Avr.

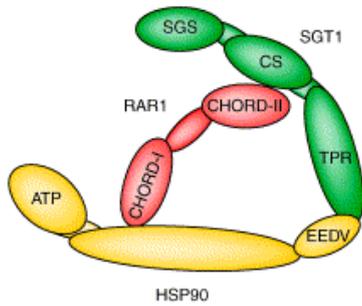


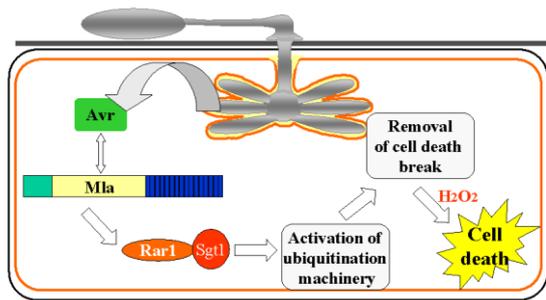
Figure 1.6 The interaction of Rar1 with Sgt1 and Hsp90. CHORD-I domain of RAR1 interacts with HSP90 and CHORD-II interacts with the CS motif of SGT1. This interaction causes the engagement of SCF (SKP1/CULLIN/F-box) complex in disease resistance by protein degradation.

(<http://www.cell.com/trends/plant-science/abstract/S1360-1385%2803%2900104-3>)

In this context Sgt1 may serve as a chaperone to assemble or regulate the structure of multiprotein complexes. Sgt1 is known to interact with SKP1 (suppressor of kinetochore protein 1) where the latter is needed for centromere-binding factor 3 (CBF3) and kinetochore assemblage and is part of the SCF (SKP1/CULLIN/F-box) ubiquitin ligases. This interaction between Sgt1 and SCF has been seen also in yeast and animals. Silencing of SKP1 with VIGS technique causes loss of resistance against TMV. Therefore it can be concluded that SCF complex plays a role in resistance by regulating the degradation of proteins by the proteasome (Lu and Malcuit, et al. (2003).

COP9 signalosome (CSN) is similar to the 19S regulatory lid of the proteasome and both contain a metalloprotease activity. COP9 plays role in regulating the activity of SCF and COP9 subunits silenced in tobacco affect the disease resistance to TMV (Liu, Schiff et al. 2002).

Therefore, there are many experiments showing that ubiquitination pathway is involved in plant disease resistance either in regulating the system by protein degradation or sending the cell to death as it is the case of hypersensitive response (HR). In Fig 1.7 hypersensitive response shows the relationship with ubiquitin and Mla-related antifungal resistance.



Ubiquitin-dependent removal of cell death repressors?

Figure 1.7 Hypersensitive response (HR). When avirulent (Avr) effectors of the fungus enter the plant cell the Mla resistant gene of barley triggers Rar1 and Sgt1 interaction which eventually engage the SCF complex of the ubiquitination pathway in the degradation of those proteins that prevent cell death. Finally the cell dies and stops the nutrition of the fungus and therefore its growth. That is why the effector is called avirulent.

(http://www2.mpiz-koeln.mpg.de/schlef/PSL_webpage.html)

Penetration resistant proteins:

Most of the non-host and a part of host pathogens fail to penetrate the cell wall of plant's leaf. The molecular aspects of this basal resistance are not understood well. In barley ROR2 (required for penetration resistance against powdery mildews) is a gene that confers the basal resistance against fungus *Blumeria Graminis*. When *A.thaliana* is infected with the same fungus, a protein similar to ROR2 was identified and called as Pen1 for penetration protein. These proteins are shown to be syntaxin proteins (Collins et al. 2003). Furthermore, in the same work synaptosome-associated protein (SNAP) has been shown to form SNAP receptor (SNARE) complex with ROR2. Altogether, these proteins facilitate the formation of H₂O₂-containing vesicles that will be the cause of hypersensitive response.

PEN1 (syntaxin), PEN2 (glycosyl hydrolase) (Lipka, Dittgen et al. 2005) or PEN3 (ABC transporter) (Stein, Dittgen et al. 2006) are arabidopsis proteins responsible for penetration resistance. An elegant research was performed in this respect with PEN mutants. As mentioned previously Mlo is a negative regulator of basal defense. Mlo mutants therefore provide resistance against fungus. When loss of function mutants of PEN1, PEN2, PEN3 are prepared in Mlo mutants the resistance decreases (Miklis, Consonni et al. 2007).

1.3.3 Auxin hormone dependent resistance

Auxin hormone is the major hormone in plant and it has been shown to be related with plant immunity. It is known that plant hormone auxin is induced from the pathogen to manipulate the developmental process of the plant. Plants repress the

auxin levels as a defense mechanism. It has been shown that it is the salicylic acid that controls the auxin repression (Wang, Pajerowska-Mukhtar et al. 2007). Therefore salicylic acid is considered part of the defense mechanism against bacteria in arabidopsis.

Another way of repressing auxin is performed by plant miR-399 in arabidopsis which is activated from bacterial flagellin penetration and thereafter this microRNA inhibits the expression of F-box auxin receptors TIR1, AFB2, and AFB3 (Navarro, Dunoyer et al. 2006).

1.4 Virus induced gene silencing

Virus induced gene silencing is an effective gene function analysis method in plants.

There are many viruses used for this purpose and for different reasons they are not all efficient. DNA viruses are incorporated into the nucleus while RNA viruses will replicate in the cytosol with their own helicases. The inoculation is made by mechanical means by hand, agroinfiltration or microprojectile bombardment (Robertson 2004).

Barley Stripe Mosaic Virus (BSMV) was first sequenced in 1989 and its 7 proteins and their role were identified up to 1994. In 2002 the BSMV was used as an efficient silencing construct for the barley's endogenous gene Phytoene Desaturase (*PDS*) which results in the photo bleaching of barley leaves and is used as a positive control. Another positive control is the expression of green fluorescent protein by being inserted in the construct of the virus also (Holzberg et al. 2002).

1.4.1 Barley stripe mosaic virus genes

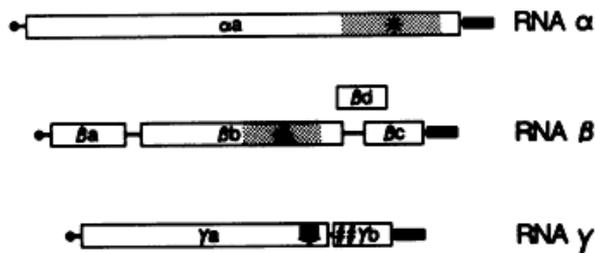


Figure 1.8 The representation of the BSMV genome with its three RNAs. Positive sense single stranded RNAs α , β and γ with 5' methylguanosine cap structures (solid circles) and a common tRNA like sequence at their 3' ends (solid rectangles). The genome has 7 open reading frames (white rectangles) with helicases (*), a polymerase (black downward arrow at the end of γa) and (#) cysteine rich regions.

<http://www.pubmedcentral.nih.gov/picrender.fcgi?artid=160546&blobtype=pdf>

α and γ can replicate on their own independently of RNA β . The ORF of RNA α itself is only one protein encoding the viral replicase. The ORF of γa also encodes a replicase (Gorbalenia et al. 1989). γb instead has two cystein rich regions which are common to many viruses and it is the presence of this region that causes the efficiency of the spreading to increase as discovered by mutant forms of γb . The ability of the γb bicistronic protein to increase spreading is indirect by increasing

the expression levels of β proteins (Donald and Jackson 1994). It is β_b , β_c and β_d that are responsible for cell to cell and vascular movement and each of them is required. The coat protein β_a is not important for infective spreading.

1.4.2 BSMV genome modifications

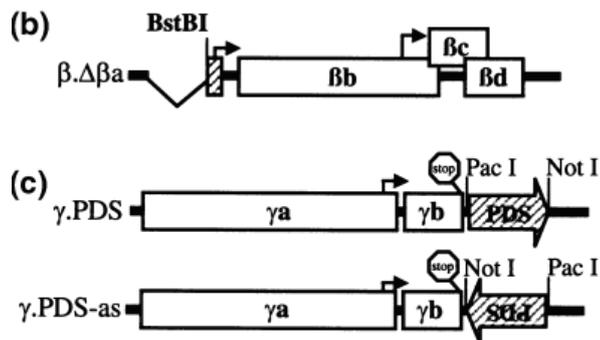


Figure 1.9 Modified BSMV genome for gene silencing. b) The mutant form of β genome without the coat encoding region β_a . BstBI is a restriction site where a subgenomic promoter for β_b is inserted. c) The insertion of Phytoene Desaturase gene in two orientations, sense and antisense. In the end of γ_b there are stop codons that do not allow the translation of *PDS* fragments. The arrows show the region of subgenomic promoters.

<http://www3.interscience.wiley.com/cgi-bin/fulltext/118956961/HTMLSTART>

When γ vectors are double digested with *PacI* and *NotI* restriction enzymes the γ vectors are then available for the ligation of your gene of interest. It is in this region that fragments of barley *FAFI* gene are inserted for endogenous silencing.

When the gene of interest is inserted into γ in the sense orientation and after that given to the plant as a single stranded RNA, the plant immune system forms a second strand of RNA upon the first. The double stranded RNA then becomes a template for post transcriptional gene silencing mechanism which cleaves the dsRNA into siRNAs and the latter become tools for further silencing of the same gene (Holzberg et al. 2001).

This mechanism of silencing occurs differently when the gene is oriented antisense inside γ . In this case when the single stranded RNA antisense form is given to the plant, the silencing will occur only if the complementary mRNA therefore the gene itself is present in the cytosol. Only after the formation of this double stranded RNA the silencing mechanism starts.

Phytoene desaturase is an enzyme that inactivates phytoene hormone which is responsible for the bleaching effect in many plants. *PDS* is replaced by green fluorescent protein (GFP) in the GFP control with GFP in antisense orientation.

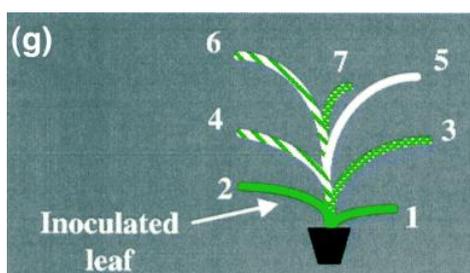


Figure 1.10 Viral spreading in barley leaves. The modified virus with *PDS* fragment is inoculated on leaf 2 and the maximum bleaching of phytoene due to *PDS* silencing is observed on leaf 5. Leaves 4 and 6 show partial bleaching while leaves 3 and 7 show minimum bleaching.

<http://www3.interscience.wiley.com/cgi-bin/fulltext/118956961/HTMLSTART>

In Fig 1.10 is shown the spreading of the virus together with phytoene (substrate of *PDS*). The *PDS* in leaf 4, 6 is partially silenced while in leaf 5 is totally silenced. In Table 1.1 is shown the orientation of sequences inside the modified γ vector. Beta lactamase gene for ampicillin resistance flanks the sequence upstream and downstream. The region in the second column is the one that will be in vitro translated. γ bsp stands for γ b subgenomic promoter. BssHIII is the restriction site from where the vectors are linearized. The T7 in the left is the one that is used by DNA dependent RNA polymerase for in vitro transcription. Fragments from FAS associated factor 1 are inserted in the same region of *PDS* after *PDS* is removed with double digestion by restriction enzymes.

Table 1.1 Sequence representation of the γ plasmid

5' M13F(-20,-40)	T7	γ a5'utr	γ a	γ bsp	γ b	PDS	d(A) ₁₅	BssHIII	T7 M13R 3'
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1.5 Post transcriptional gene silencing after virus entrance to the plant cell

After the BSMV modified virus is incorporated into the 2nd leaf, the plant starts a mechanism known as Post transcriptional gene silencing (PTGS) in order to defend itself. In the section below is shown the relationship between VIGS and short inhibitory RNAs (siRNAs). Because siRNAs are very close to microRNAs (miRNAs) in function and biogenesis (Fig 1.11 and Fig 1.12) they are both explained by being compared with each other.

1.5.1 Double stranded RNA in the cytosol triggers the PTGS

It is the presence of the dsRNA in the cytosol that triggers the mechanism of RNA silencing. Those mRNAs that overpass their threshold which might occur from

different reasons become template for RNA dependent RNA polymerases (RdRP) that form the second strand of the dsRNA. Thereafter the dsRNA is cleaved into siRNAs (Fig 1.11). It is important to understand that these siRNAs are not transcribed from their genomic regions in the plant nucleus as are miRNAs (Fig 1.12). Therefore the whole mechanism is explained below to better understand the difference.

Post transcriptional gene silencing is the name of the mechanism that explains the silencing of genes in their mRNA levels. Shortly PTGS in plants, RNAi for RNA inhibition in metazoan and quelling in fungi, is a mechanism involving the silencing of any organism's mRNA itself or that of any infector's (viruses). Short inhibitory RNAs (siRNAs) and microRNAs are the key players in this mechanism and are mainly triggered by stress and development.

siRNAs and miRNAs are transcribed in the nucleus, processed with Dicer proteins and transported to the cytosol as 21-24 ribonucleotides long (in plants). In animals siRNAs and miRNAs are processed to their mature form (21-24 nt. long) in the cytosol. Then they enter the RNA Induced Silencing Complex (RISC) which recruits them to their complementary regions of the genes targeted for silencing. It is the Argonaut proteins that perform the cleavage of the target mRNA exactly in the 10th nucleotide of the miRNA or siRNA (starting from 5' end). Mutants of argonaute protein in *A.thaliana* had argonaute form, therefore its name. Briefly microRNAs and siRNAs differ in their biogenesis and they are single stranded and double stranded oligos respectively. In plants cleavage is more common than repression which in mammals is vice versa. In plants, the coding and in animals the 3'UTR are usually the targeted regions (Bartel 2004).

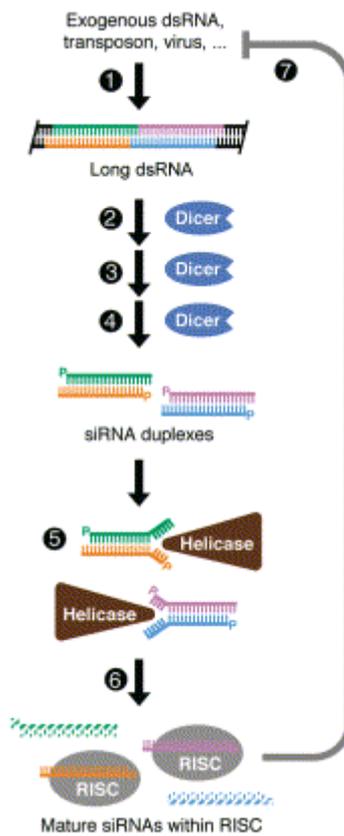


Figure 1.11 siRNA biogenesis. The dsRNA template present in the cytosol is cleaved by Dicer protein into siRNAs. An helicase separates the two strands and each strand is incorporated into a different RISC.

<http://www.cell.com/retrieve/pii/S0092867404000455>

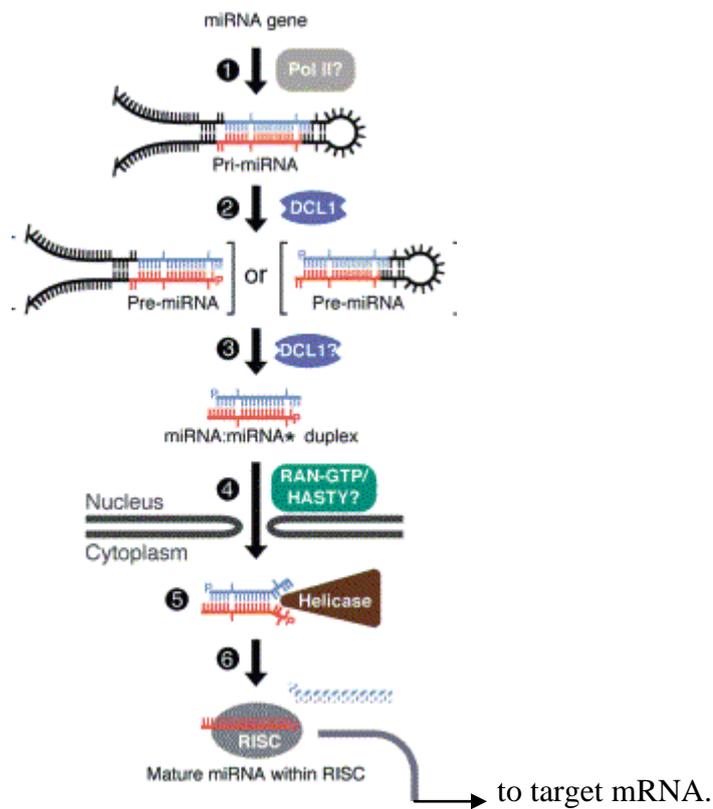


Figure 1.12 MicroRNA biogenesis. RNA polymerase II transcribes the miRNA gene. Dicer like proteins form the mature form of miRNA and RAN-GTP/HASTY proteins transport it to the cytosol. After the helicase separates the 2 strands, one of them usually the one which has a mismatch nearest to its 5' end is incorporated into RISC.

<http://www.cell.com/retrieve/pii/S0092867404000455>

1.6 FAS associated factor 1 (*FAFI*)

1.6.1 FAS associated factor 1 in plants

FAFI's mRNA has been sequenced completely in rice and partially in tobacco, wheat and in our work in barley. No function related to this protein has yet been identified in plants.

1.6.2 FAS associated factor 1 in metazoan as a death inducer

The signal of apoptosis starts with FAS receptor and passes from plasma membrane to caspases. FAS is a tumor necrosis factor receptor and contains a death domain (DD) in its cytosolic region. The FAS-associated death domain protein (FADD) binds to FAS and has a DED at its N-terminus that interacts with caspase 8 and brings the latter one to death induced silencing complex (DISC) (Chu, Niu et al. 1995). FAF1 enhances the apoptosis started by FAS but is not able to initiate it (Ryu et al, 1999). The DED-similar region of FAF1 interacts with FAS and functions as part of FAS-DISC interaction. (Ryu et al, 2003)

Another interesting interaction of FAF1 is Aurora-A, a kinase whose highest levels are in between G2 and M phase (Ouchi et al. 2004). It is found that Aurora-A phosphorylates FAF1 at Ser289,291 and this causes FAF1 to degrade Aurora-A via a proteasome dependent but not ubiquitin dependent degradation. Aurora A in itself has a ubiquitin ligase binding domain (Jang, Sul et al. 2008).

FAF1 is also shown to be upregulated by anticancer drugs such as the proteasome inhibitor bortezomib. This drug beside FAF1, upregulates some heat shock proteins, a heme-oxygenase which is a target of hypoxia inducible gene 1 alpha and also a target of heavy metal protoporphyrins. Other works have demonstrated that this apoptotic drug activates endoplasmic reticulum stress and production of reactive oxygen species (ROS) (Hamamura, Ohyashiki et al. 2007). The proteins mentioned in this paragraph are all related positively with disease resistance in plants demonstrating a conserved mechanism in plants of FAF1 with these proteins. Another interaction which is worth to be mentioned is the interaction of FAF1 with Hsp70. The Hsp70 chaperone refolds the denatured proteins after a heat shock or any other stress. When FAF1 interacts with HSP70 where N terminuses of both proteins are responsible for the tight interaction, the role of Hsp70 is inhibited. Therefore it has been put forward that FAF1 prevents the cells from recovery after a certain stress and procures their death (Kim, Song et al. 2005). The interaction of FAF1 with Casein kinase 2 (CK2) seems to be of relevance in disease resistance (Litchfield 2003) because many pathogens are known to induce CK2 expression in plants (Matsushita et al. 2003).

1.6.3 FAF1 role in the degradation of proteins

In previous sections we mentioned that the protein degradation pathway proteins are included in disease resistance in plants. The mammalian FAF1 protein contains a UAS domain with unknown function and a UBX domain. The UBX domain is similar to ubiquitin with some exception in its C-terminus, in the region where ubiquitin activating enzymes (E1) operate. Therefore FAF1 is not affected by E1 (Schuberth et al. 2008). Nevertheless the UBX domain has been shown to have a role in ubiquitination pathway. FAF1 binds through UBX to valosin, a protein which is part of the AAA ATPase family (ATPase associated with different cellular activities). The valosin in itself regulates the degradation of proteins by binding to multi-ubiquitinated ones. In the same work authors conclude that hFAF1 is a scaffolding protein in the ubiquitin-proteasome mechanism (Kim, Song et al. 2005).

1.7 A homology between *FAF1* and other immunity mRNAs in plants

After sequencing a region of FAF1 mRNA in barley we searched for computational resemblance of the region with other mRNAs and we found more than 20 genes related with disease resistance sharing the same homology making it a possible short inhibitory target zone. In order to verify this, 5' rapid amplification of cDNA ends 5'RACE must be performed for cleaved mRNAs that share this homology. The conventional 5'RACE used for this purpose is shown below.

1.8 The 5'RACE for amplifying miRNA/siRNA targets

Post transcriptional gene silencing (PTGS) in plants or RNA inhibition (RNAi) in metazoans is a mechanism unraveled in the past decade where RNA oligos differing in size 18-24 nucleotides inhibit the expression of proteins. These oligos such as microRNA or short inhibitory RNAs (siRNAs) provide this inhibition in the cytosol by entering the RISC (RNA induced silencing complex) and altogether they bind to the mRNAs of the genes in those regions where miRNAs or siRNAs are complementary or almost-complementary to the mRNAs (Bartel 2004). The presence of the complex itself upon the mRNA is an obstacle for the ribosome

which causes the translational inhibition, but in plants usually this complex cleaves the mRNA in a specific point which is between 10-11 nucleotides starting from the 5' end of the miRNA or siRNA (Allen, Xie et al. 2005). What is left behind from the mRNA is the region from polyA up to the 5' cleavage point nucleotide. You can find these cleaved mRNAs in the total RNA and they are definitive proof for the silencing of the corresponding gene by microRNAs (Llave, Xie et al. 2002). Sequencing of these cleaved mRNAs and identification of the 10 nucleotides downstream the cleavage point enables scientists to identify the silencing miRNA due to complementarity. The amplification and therefore sequencing of these cleaved targets is enabled by RNA Ligase Mediated-RACE (RLM-RACE) which ligates a known RNA oligo to the cleaved mRNA; this ligation made possible by the presence of a monophosphate in the 5' end nucleotide of the cleaved mRNAs (Elbashir, Martinez et al. 2001). The primer(s) inside the known RNA oligo and the gene specific primer(s) will perform the amplification of the cleaved mRNA alone and will not amplify the capped full length mRNA of the same gene. The conventional protocol to this approach and the novelty to this approach are described in the Results and Discussion session.

CHAPTER 2

MATERIALS AND METHODS

2.1 RNA Isolation from Barley Leaf Tissue for mRNA sequencing of FAF1 gene

Total RNA from 10 day old Pallas01 (resistant to fungus Bgh103) line of *Hordeum Vulgare* leaves grown in long day conditions (16+8hrs.) was extracted with Trizol reagent (Invitrogen). The mortar, pestle and spoons were placed overnight in distilled water with 0.1% DEPC (diethylpyrocarbonate) in a dark container and autoclaved for 20 min (50Hz). Then they were dried in 200 °C overnight. After procuring an RNase-free environment, 100 mg of leaf tissue was frozen in liquid nitrogen and the grinding was performed with mortar and pestle. Filtered tips were used in all steps of the procedure. The frozen powder was transferred in 2 ml eppendorf tubes with 1 ml of ice cold Trizol reagent and incubated for 10 minutes at 25 °C. Then 0.2 ml of ice cold chloroform was added and the tubes were vigorously shaken for 15 seconds and waited for 5 minutes at 25 °C. The samples were centrifuged at 15300 rpm at 4 °C for 15 min. The total RNA is found in the upper phase which is 60% of the Trizol amount added and therefore approximately 600 µl was taken from the upper solution and transferred in a new 2 ml eppendorf tube. 0.5 ml of ice cold 2-propanol was mixed with the transferred total RNA and the tube inverted slowly for 8-10 times. For precipitation to take place the tube was left at 25°C for 10 min. and centrifuged at 15300 rpm. for 10 min. The supernatant was separated from the white pellet in the bottom of the tube which is the total RNA. The washing step was performed with 1 ml of ice cold 75% ethanol. The pellet was floated in the ethanol and centrifuged again at 15300 rpm for 5 min. Ethanol was taken with a pipette and the tube let open for all the remained ethanol to dry. The pellet was diluted with 50 µl. dd water and mixed well in order to be dissolved. Concentration was measured in nanodrop as 560 ng/ µl.

2.2 1st strand cDNA synthesis

1,5 µg total RNA was primed with a coding sequence adapter (CDS 3M) from Trimmer Direct kit. (Evrogen)

5'AAGCAGTGGTATCAACGCAGAGTGGCCGAGGCGGCCd(T)₂₀VN3'

and the 1st strand cDNA synthesis was performed according to Clontech protocol with a *native* MMLV-RT (Promega). In a 0.5 ml eppendorf tube the followings were mixed: 1,5 µg total RNA, 10pmol 3'CDS and ddH₂O up to 5 µl. The solution was mixed and the tube placed in a lid at 70 °C for 2 min. followed directly by 2 min. in ice. The tubes were spinned briefly and the followings added: 2µl 5x first strand buffer; 1µl DTT(20 mM); 1µl dNTP mix (10mM each); 1µl MMLV reverse transcriptase (200u/ µl). The content of the tube mixed and spinned and placed at 42 °C for 1,5 hours in a hot-lid. Inactivation of the enzyme was performed at 72 °C for 7 min and the mixture diluted to 150 µl.

2.3 3' Rapid Amplification of cDNA Ends (3'RACE)

The following amplification was performed with the first cDNA strand as a template and cDNA polymerase Mix (Clontech) as an amplifying enzyme. The primers were M1 which anneals upon the CDS strand and serves as a reverse primer and forward primer 1 which anneals with a known expressed sequence tag from wheat.

NCBI EST Accession # FE900151

Ubx domain containing protein in *Triticum Aestivum*

GAGCGGCCGCGAGTCTAAGAGGAAGCGTATAGAAACGAGGAAGCACAAAGCCAGGGCAGTC Fwd. 1
 CAAGAAGCAGCTGAAAAGCAAGCGCTCTTGCAAGGAGACGGCAAGAGAGGCAATGGCT Fwd. 2
 CTGGGCGCTGAGCCAGAGAAAGGGCCTGACATTACTCGAGTTCTTATAAGATTTCCAACCT
 GGAGAGCGCAAAGAAGGAGATTCCACGGTTCGCCACCATTACCTCGATATATGACTAT Rev. 1
 GTTGATTCTTTGGATTGCTTCAAAGCGGAAAAGTACAGCCTAGTTTCTAATTTTCCAAGG Rev. 2
 GTAACATACGGTCTGAAAAGAACTCCCAAAGCTGGTGAAGCAGGTTTGCACCCACAA
 GCAAGCCTATTTATCGAGATAGAACAATGAATGTCCCAATTTTCTAAGTTATATGACT
 GAAACTGCAAGTATAGGTGCTCCITTTTTACTCTATTTGACAAACAGTGGGGACTTCCA
 GCTGTATGGCAATATACTTGACTTCCGTGCAAAAAAANNAGATATCACTCAGCATAATGA

Figure 2.1 *FAF1* (FAF1 domain-containing) protein in *Triticum aestivum*

Forward primer 1: 5'GACGAGGAAGCACAAAGCCAGGGC 3'

M1: 5' AAGCAGTGGTATCAACGCAGAGT 3'

Table 2.1 1st amplification conditions for *FAF1* in barley

Component	Conc.	Volume (μ l)	Amplification Condition			
Template		3	Initial denaturation	94°C	2 min	1 cycle
R. Buffer	10X	5	Denaturation	94°C	30 sec	5 cycles
dNTP	10 mM	1	Annealing	64°C	30 sec	
F. Primer 1	10 pM/ μ l	1	Elongation	68°C	30 sec	
M1 Primer	10 pM/ μ l	1	Denaturation	94°C	30 sec	30 cycles
Enzyme	1 U/ μ l	1	Annealing	58°C	30 sec	
ddH ₂ O	-	38	Elongation	68°C	30 sec	
Total		50				

1 μ l was taken from the first amplification and used as a template in a second PCR in order to walk upon the unknown sequence from both sides.

The following primers were used:

Forward primer 2: 5'GCAAGGAGACGGCAAGAGAA 3'

M2: 5' AAGCAGTGGTATCAACGCAG 3'

The PCR reaction was as follows:

Table 2.2 2nd amplification conditions for *FAF1* in barley

Component	Conc.	Volume (μ l)	Amplification Condition			
Template		1	Initial denaturation	94°C	2 min	1 cycle
R. Buffer	10X	5	Denaturation	94°C	30 sec	5 cycles
dNTP (mix)	10 mM	1	Annealing	64°C	30 sec	
F. Primer 2	10 pM/ μ l	1	Elongation	68°C	30 sec	
M2 Primer	10 pM/ μ l	1	Denaturation	94°C	30 sec	30 cycles
Enzyme	1 U/ μ l	1	Annealing	58°C	30 sec	
ddH ₂ O	-	40	Elongation	68°C	30 sec	
Total		50				

2.4 Sequencing of the amplified product

The band was run in a 1,5% agarose gel with TBE buffer (Tris-Boric acid-EDTA) 1x and cut.

The DNA in the cut fragment was purified with Qiagen gel extraction kit.

Briefly, the cut band was placed in a 1.5 ml eppendorf and mixed with 3 volumes of buffer QG. It was placed in a hot bath for 10 min. The liquefied solution was poured into filter containing tubes and centrifuged at 14 000 rpm for 1 min. The remnants discarded. 0.75 ml of buffer PE was poured this time upon the filter and

centrifuged for 1 min at 14 000 rpm. The remnants were discarded and the tube was centrifuged again to totally get rid of buffer PE. Then 30 μ l was poured directly in the center of the filter, waited for 1 min and centrifuged for 1 min. The concentration was measured in nanodrop and was suitable for ligation.

Caution: After centrifugation 3-4 μ l from buffers are collected upon the circular plastic ring found upon the filter. Get rid of that buffer especially if it is PE. 2 μ l of EB are absorbed by the buffer, therefore 28 μ l must be the amount in which the DNA is collected.

Because cDNA polymerase mix (Taq DNA polymerase) adds adenine residues to the DNA tails, the purified DNA was directly ligated into pGEM-T Easy with Promega kit as 3 Weiss units of T4 DNA ligase, 2 μ l 5x buffer, 10 ng of pGEM-T Easy vector and 3 μ l of DNA. The mixture was let o/n at +4 °C then it was transformed into E.Coli Dh5 α .

The cells were made competent as follows: Dh5 α cells were grown in 10 ml Luria Bertani (LB) medium o/n at 37 °C. 1ml was taken and poured into 100 ml LB medium and grown up to 0,5 OD₆₀₀ which usually takes 2,5-3 hrs. Cells centrifuged at 4000 rpm for 10 min. and the pellet suspended in 3 ml of 0,1 M CaCl₂ and waited in ice for 30 min. The tubes were centrifuged again at 4000 rpm for 10 min. The pellets were suspended and stored in 0,6ml of 0,1 M CaCl₂ at +4 °C.

Transformation was performed as follows: 50 μ l of competent cells were mixed with the ligation solution in a 2 ml eppendorf tube and placed in ice for 20 min. Then for 50 sec. in a hot bath at 42 °C and directly in ice for 2 min. 400 μ l of LB was poured and the tube placed for 1,5 hrs at 37 °C with 150 rpm in an air incubator. The contents were poured into ampicillin (100mg/ml), 20 μ l X-Gal (50 mg/ml) and IPTG (0,1M) 100 μ l per plate. White colonies were selected and grown in ampicillin containing LB medium o/n. 4 ml of LB+cells were used for plasmid isolation with Qiagen plasmid isolation kit.

Briefly 4ml was centrifuged at 14 000 rpm for 1 min. with the followings added to the pellet: 250 µl buffer P1 and vortexed; 250 µl buffer P2 and 350 µl buffer N3 inverted several times after each buffer. The tube was centrifuged at 17 800 g for 10 min. The supernatant was poured into a filter containing tube and centrifuged at 14 000 rpm for 1 min. The filtered cleaned with 0,75 ml buffer PE and centrifuged twice at 14 000 rpm for 1 min. and 30 µl of buffer EB was placed in the filter to dilute the plasmid. The inserted fragment was sequenced with M13 forward primer.

2.5 Preparation of vectors for virus induced gene silencing

2.5.1 Preparation of the 3' UTR fragment to be used for silencing

The region to be silenced is part of the gene's 3'UTR and it was amplified with primers containing overhangs of Pac1 and Not1 restriction sites. The region of ~500 bp. which was already sequenced and cloned for barley, persisting to the end of the gene including 3'UTR was used as a template.

In the UTR region (171 bp)

NotI FAF1 UTR Fwd. 5' ATAGCGGCCGCTTTCTAAGTTATAT 3'

PacI FAF1 UTR Rev. 5' ATATTAATTAAAATTTATGCTGAGTGATATC 3'

Hot Start Kod XL kit (Toyobo; Novagen) was used for amplification

Table 2.3 Amplification conditions of 3'UTR *FAF1* with *PacI*, *NotI* primers

Component	Conc.	Volume (μl)	Amplification Condition			
Template		3	Initial denaturation	95°C	2 min	1 cycle
R. Buffer	10X	5	Denaturation	95°C	20 sec	35 cycles
dNTP	2 mM	5	Annealing	40°C	10 sec	
UTR Fwd	10 pM/μl	1.5	Elongation	70°C	10 sec	
UTR Rev	10 pM/ μl	1.5				
MgSO ₄	25mM	3				
Enzyme	1 U/ μl	1				
ddH ₂ O	-	30				
Total		50				

The PCR product was gel extracted (Qiagen) as previously mentioned. Adenine residues were added with GenTaq polymerase at 72 °C for 30 min. The product was ligated into pTZ57R/T vector with TA cloning (Fermentas) for it is a vector that does not contain *PacI* and *NotI* sites.

Table 2.4 Ligation of *PacI*, *NotI*-containing 3'UTR in pTZ57R/T

Component	Concentration	Volume (μl)
PCR product	-	25
pTZ57R/T	0.054μg/μl	1
Buffer	10X	3
T4 DNA Ligase	5 U/μl	1
Total		30

After 1 hr. at 22 °C with subsequent transformation and spreading onto plates ampicillin resistant colonies were taken for plasmid isolation (Qiagen).

Plasmids (~15µg) were double digested with Pac1 10U and Not1 15U restriction enzymes (New England Biolabs) with NEB2 buffer 10x and BSA (100x) for 16 hrs at 37 °C. The contents were run in an agarose gel and the Pac1, Not1 sticky end-containing UTR fragment was cut from the gel and purified (Qiagen). The concentration was measured in nanodrop as 85ng/µl.

2.5.2 Preparation of viral RNAs

The Barley Stripe Mosaic Virus DNA vectors $p\alpha$, $p\beta\Delta\beta\alpha$, $p\gamma$ sense *PDS4*, $p\gamma$ antisense *PDS4*, with 10ng/µl each were all transformed into E.Coli DH5 α . Plasmids (~25µg each) were isolated with Qiagen plasmid isolation kit as previously mentioned.

$p\gamma$ sense *PDS4* and $p\gamma$ antisense *PDS4* (8 µg each) were double digested with Pac1 10u and Not1 15u restriction enzymes (New England Biolabs) with NEB2 buffer 10x and BSA (100x) for 16 hrs at 37 °C. The “empty” non-*PDS* gamma vectors were used for insertion of 3’UTR region of *FAF1* already double digested, with the following sticky end ligation:

Table 2.5 Ligation of double digested 3’UTR of *FAF1* in γ plasmid

Component		Concentration	Volume (µl)	
3’UTR fragment		85 ng/µl	13	15
$p\gamma$ vectors	sense	54 ng/µl	4	-
	antisense	118 ng/µl	-	2
Buffer		10X	2	2
T4 DNA Ligase		5 U/µl	1	1
Total			20	20

The tubes were placed in a PCR lid for 16 hr. at 22 °C

The ligated vectors were transformed into DH5 α and selected for ampicillin resistance.

The clones were called p γ sense FAF1 and p γ antisense FAF1 and plasmid isolations were subsequently performed (Qiagen).

All isolated plasmids were made linear with their respective enzymes as follows:

p α with MluI (Fermentas) at 37 °C for 16 hrs and gel extracted

p $\beta\Delta\beta\alpha$ with SpeI (Fermentas) at 37 °C and gel extracted.

p γ sense *PDS4*, p γ antisense *PDS4*, p γ sense FAF1 and p γ antisense FAF1 with BssHI (NEB) at 50 °C and gel extracted.

0,8 μ g of each linear vector was transformed to DH5 α and colonies were counted to measure the amount of circular plasmid and therefore to evaluate the efficiency of linearization and it was seen that ~95% of plasmids were linear.

80 ng of each linear plasmid were used as templates for in vitro transcription (IVT) with mMessage mMachine T7 kit (Ambion) according to manufacturer instruction.

In vitro transcription reactions:

Table 2.6 In vitro transcription of p α and p β viral RNAs

Component		Concentration	Volume(μ l)	
BSMV plasmids	p α	110 ng/ μ l	0,75	-
	p β	110 ng/ μ l	-	0,75
NTP mix		2X	1,25	1,25
Buffer		10X	0,25	0,25
Enzyme mix		10X	0,25	0,25
Total		-	2,50	2,50

Table 2.7 In vitro transcription of p γ viral RNAs

Component			Conc.	Volume(μ l)				
Bsmv plasmid	p γ	<i>PDS4-s</i>	110 ng/ μ l	0,75	-	-	-	-
		<i>PDS4-As</i>	110 ng/ μ l	-	0,75	-	-	-
		<i>FAF1-s</i>	110 ng/ μ l	-	-	0,75	-	-
		<i>FAF1-As</i>	110 ng/ μ l	-	-	-	0,75	-
		<i>BSMV-0</i>	110 ng/ μ l	-	-	-	-	0,75
NTP mix			2X	1,25	1,25	1,25	1,25	1,25
Buffer			10X	0,25	0,25	0,25	0,25	0,25
Enzyme mix			10X	0,25	0,25	0,25	0,25	0,25
Total			-	2,50	2,50	2,50	2,50	2,50

2,50 μ l is the standard amount for one reaction and 30 reaction amounts (3 tubes x 10 rxn) were prepared for alfa and beta vectors each, while 5 reactions were prepared for each gamma type.

2.5.3 DNase treatment and LiCl precipitation

All in vitro reactions were treated with Turbo DNase (AMBION) from mMessage mMachine T7 kit as 0.5 μ l (2u/ μ l) of enzyme at 37 °C for 20 min with 5x 1st strand

buffer which should normally degrade 1 µg of DNA. DNase was inactivated at 65 °C for 15 min. Lithium Chloride precipitation of in vitro RNA was performed for RNA purification. LiCl (7.5M) from the same kit was mixed with ddH₂O and in vitro reaction in a ratio of 1:1:1 to give a final concentration of LiCl 2.5 M. The mixture is left o/n in -20 °C and after that centrifuged at 15 300 rpm for 20 min. The supernatant taken and the pellet was washed in 75% ethanol, centrifuged at 15 300 rpm for 5 min and diluted in ddH₂O depending on the pellet amount. The tube was placed in 55 °C water bath till the pellet was dissolved completely which lasted more than 10 min. The concentrations before being spread onto each leaf were 1.5 µg each as p α , p β and each of *PDS4*-s; *PDS4*-As; FAF1-s; FAF1-As; BSMV-0.

2.5.4 FES buffer preparation

5 ml 10X GP (18.77 g Glycine, 26.13 g K₂HPO₄, ddH₂O upto 500 ml), 0.25 g Sodium pyrophosphate, 0.25g Bentonite, 0.25 g Celite and ddH₂O up to 25 ml

2.5.5 Application of viral vectors onto plants

15 plants of P01 (resistant to Bgh103 fungus) and 15 plants of P03 (susceptible to Bgh103) lines of *Hordum Vulgare* were grown in long day (16+8 hrs.) conditions and watered daily. Inoculations of the BSMV viral vectors with modified gamma vectors for each silencing (*PDS* and FAF1 with sense and antisense orientations and BSMV0) were performed onto the second leaves when they reached 6-7 cm length. A negative control was applied as only FES buffer.

45 µl of FES buffer was applied manually mixed with the combinations of p α , p β , and respective p γ and they were applied in a ratio of 1:1:1 as 1.5 µg each.

2.5.6 Green fluorescent protein expression in barley

Green fluorescent protein sequence combined with BSMV viral vectors already prepared by Holzberg et al. was also used as a positive control for two plants beside Phytoene desaturase (*PDS*) silencing control. GFP gamma vectors were linearized for 16 hrs with BssHIII at 50 °C and gel extracted with Qiagen. GFP linearized DNA's in vitro transcription was performed for a total of 10 µl and therefore with

5µl for each plant. Alfa and beta was kept as 1.5µg each similar to other silencing combinations.

The expression of GFP was observed under fluorescence microscope (Leica) in the 2nd day after application for one plant and 3rd day for the other.

2.6 Evaluation of silencing

After RNA application onto 2nd leaves, the 5th leaf of *PDS* sense, the 3rd leaf of FAF1-antisense were cut and total RNA isolation was performed as previously mentioned.

1st strand cDNAs were constructed with oligod(T)₂₅NV according to Clontech protocol and the amount of total RNA started was 1 µg each.

The cDNA construction was controlled with actin primers that amplify a 133 bp region near the start codon of actin mRNA.

All qRT-PCR amplifications were performed with Brilliant SYBR Green qPCR Master Mix (Stratagene) as 2 µl cDNA, 1 µl each primer (10 µM), 10 µl SYBR (10x) and completed to 20 µl with ddH₂O.

Real time primers:

Actin: Fwd: AATGGTCAAGGCTGGTTTCGC
Rev: CTGCGCCTCATCACCAACATA

PDS: Fwd: CTGGATGAAAAAGCAGGGTGTTC
Rev: CTACTTTCAGGAGGATTACCATCC

FAF1 Fwd: GCAAGGAGACGGCAAGAGAA
Rev: TTGGATTGCTTGAAAGCGG

Viral RNA inoculated barley 3rd leaves of resistant line Pallas01 and susceptible line Pallas03 to *Bgh103* (*puccinia graminis hordei* 103) fungus, were placed in agar plates as Pallas01 FAF1-silenced and Pallas01 FAF1-BSMV00; Pallas03 FAF1-silenced and Pallas03 FAF1-BSMV00. *Bgh103* was blown onto the plates and the effect of infection was observed after 10 days.

2.7 Trypan blue staining

In order to observe the conidia growth of the pathogen infected leaves were placed in 2 ml eppendorf tubes with trypan blue (20 ml of ethanol, 10 ml of phenol, 10 ml of water, 10 ml of lactic acid (83%), and 10 mg of trypan blue) and left in boiling water for 2 min according to Koch and Slusarenko protocol (1990). Then the leaves were cleaned o/n in other eppendorf tubes containing chloral hydrate (2.5 g dissolved in 1 ml of water).

The penetration of the spore was observed under camera connected to Leica microscope.

2.8 Quantitative Real Time PCR

All of 6 total RNAs were isolated with Trizol as previously mentioned and cDNA was constructed with oligod(T)₂₅NV according to Clontech protocol and the amount of total RNA started was 1 µg each and the cDNAs were diluted from 10µl to 20µl with ddH₂O.

Normalization of cDNA was performed with actin primers as follows:

Actin: Fwd: AATGGTCAAGGCTGGTTTCGC

Rev: CTGCGCCTCATCACCAACATA

2µl from the cDNA reaction was used as a template with 10 pmol of each primer and 10µl of 2x Brilliant SYBR Green qPCR Master Mix (Stratagene) in a total of 20 µl.

Real time measurements with real time primers as shown above were performed for *PDS* and *FAFI* silenced leaves (treated) vs. their non-silenced leaves as a calibrator (untreated). The fold change was measured as:

$$\text{fold change} = 2^{-\Delta\Delta C_T}$$

with $\Delta\Delta C_T = (C_{T\text{sample}} - C_{T\text{sample-ref}})_{\text{treated}} - (C_{T\text{sample}} - C_{T\text{sample-ref}})_{\text{untreated}}$

where;

$C_{T\text{sample}}$ = the average of C_T values for the transcript of interest (*PDS*, *FAF1*)

$C_{T\text{sample-ref}}$ = the average of C_T values for β -actin transcript

More information for the derivation of the formula and the assumptions made to derive it is found in Applied Biosystems User Bulletin No.2 (P/N 4303859) (Livak and Schmittgen 2001).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Amplification of *FAF1* 3'UTR in barley

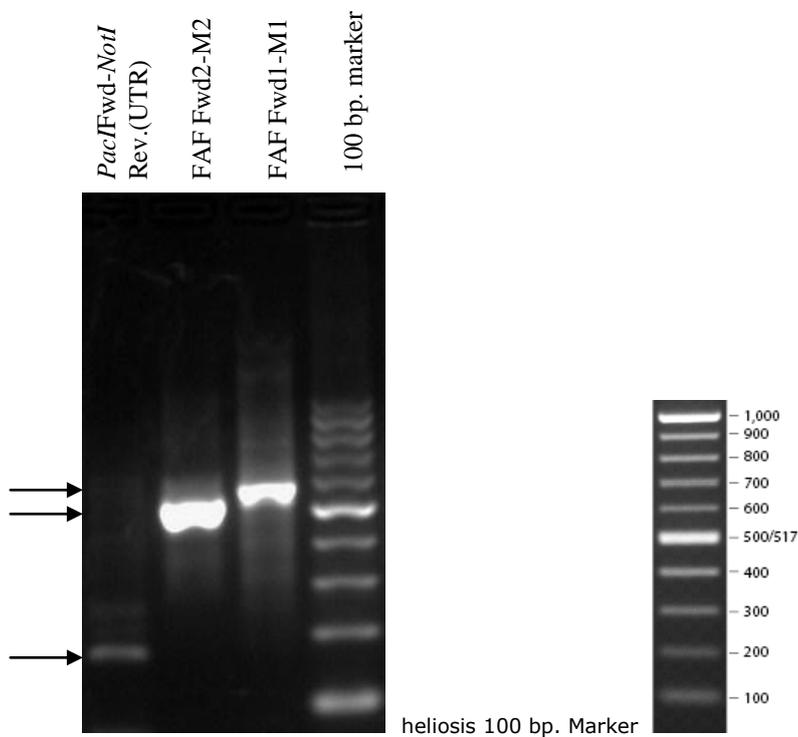


Figure 3.1 Amplification of 3'UTR of *FAF1* in barley (lower arrow). 1st amplification was performed with a primer annealing upon the CDS adapter and the other annealing in the coding region (upper arrow). 1 μ l from the first amplification was used as a template in a second amplification by walking with primers upon the adapter and the coding sequence (middle arrow). 1 μ l was taken from this band too and used to amplify the 3'UTR of *FAF1* with gene specific primers attached to *PacI* and *NotI* (lower arrow).

In Fig 3.1 are shown the amplified bands of the unknown regions of *FAF1* mRNA that were used for silencing. From 10-day old barley leaf total RNA the cDNA was constructed with oligod(T) and attached to a known 3'adapter sequence (CDS).

3.2 Sequencing of the unknown regions of *FAF1* in barley

The region in barley of *FAF1* mRNA from forward primer 2 down to polyA (middle arrow in Fig3.1) is shown in Fig 3.2. The underlined Fwd. primer2 and stop codon are also shown.

GCAAGGAGACGGCAAGAGAAGGCAATGGCTCTTTGGCGCTGAGCCTGAGAAAGGGCCTGACGTTACTC
GGGTTCTTATAAGATTTCCAACCTGGAGAGCGCAAAGAAAGGAGATTCCGCAGTTCCGCCACCATTAC
CTCCATATATGACTATGTTGATTCTTTGGATTGCTTGAAAGCGGAGAAGTACAGCCTAGTTTCTAAT
TTTCCGCGGGTAACATACGGTCCCGAAAAGAAGTCCCAAACGCTCGTGGAAGCAGGTTTGCACCCAC
AAGCAAGCCTATTTATCGAGATAGAACATGAATGTCCCCAATTTTCCAAGTTATATGTACTGAAAC
TTGCAAGTATAGGTGCTCCTTTTTTACTCTATTTGACAAACAGTGGGGAAGTCCAGCTGTATGGCA
ATATACTTAGACTTGACTTTTTGTGCGAAATGCTGCTCTTGTGGTTATCATTCGTTAAATAAATTCACC
TTCTGCCGGACCAAAAAAAAAAAAAAAAAAAAAA

Figure 3.2 The mRNA sequence of *FAF1* in barley from fwd. primer 2 down to polyA. Fwd. primer 2 and the stop codon are shown underlined.

3.3 Confirmed sequence of 3'UTR of *FAF1*

In Fig 3.1 the lower arrow's sequence which was used for *FAF1* silencing with VIGS came as follows:

TTTCTAAGTTATATGTACTGAAACTTGCAAGTATAGGTGCTCCTTTTTACTCTATTTGACAAACAGT
GGGGAAGTCCAGCTGTATGGCAATATACTTAGACTTGACTTTTTGTGCGAAATGCTGCTCTTGTGGAT
ATCACTCAGCATAA

In bold green are shown the primers attached to *PacI* and *NotI* restriction sites. Because the primers were designed according to the EST sequence of *FAF1* in *T.aestivum* they were not 100% complementary in barley. In blue is shown a sequence homology between many proteins related with disease resistance.

3.4 A putative miRNA/siRNA inside 3'UTR of *FAF1* and its computational targets in plants and metazoan

The sequenced 3'UTR region of barley *FAF1* was aligned with *Zea Mays FAF1* and a conserved region of 28 nt. came out from the comparison (shown in blue inside the sequence).

When a 21 nt. complementary sequence pertaining to this region, the putative miRNA/siRNA aaaaaggagcaccuauacuug was searched for other possible targets in “mirU plant target finder” program (<http://bioinfo3.noble.org/miRNA/miRU.htm>), inside barley and wheat gene indexes, many proteins related in function to *FAF1* came out.

Some of them with their respective functions are as follows:

- Mucin protein which binds to pathogens and plays role in immunity
- Ubiquitin conjugating enzyme E1 involved in protein degradation
- ABA induced plasma protein which helps in the formation of vesicles with a role similar to cation channels that play role in basal resistance (Trouverie, Vidal et al. 2008).
- Myb like transcription factor that activates the programmed cell death
- Ca⁺⁺ proton antiporter which plays role in vesicle formation and therefore in the basal defense (Han, Chen et al. 2009)
- SET1 histone methyl transferase which opposes the role of aurora kinase in chromosome segregation. *FAF1* also degrades Aurora-A kinase as mentioned in the introduction section (Zhang, Lin et al. 2005).
- Oxygen evolving enhancer protein 1 which is a thioredoxin. In humans it has been shown that a thioredoxin induces caspase 8-dependent cell death and the RNA inhibition of this thioredoxin inhibited cell death (Heide,

Kalisz et al. 2004). In the introduction we mentioned heme-oxygenase playing role in the same pathway with FAF1.

When this putative microRNA sequence was compared in miRBase program with SSearch (<http://microrna.sanger.ac.uk/sequences/search.shtml>) for whether being an already available miRNA, the only miRNA that resembled was mmu-mir-294. This microRNA is part of mmu-mir-290 cluster composed of 5 microRNAs and it includes ~70% of the miRNAs during embryonic development in mouse. It is also called as the stem cell miRNA cluster playing the biggest role in cell differentiation (Houbaviy et al. 2003). Even though the predicted targets of mmu-mir-294 in metazoan are too many because of the similarity in only the seed region some of the targets related to cell death and therefore to FAF1 in MIRANDA search are:

- Paired box gene 5 whose aberrations are reason for many lymphomas Vaccinia related kinase 3 (VRK3). This is also called casein kinase 1 and it phosphorylates p53 to disrupt the p53/mdm2 interaction inhibiting therefore p53 degradation and promoting the programmed cell death (Barcia, Lopez-Borges et al. 2002). VRKs are members of the casein kinase family. We already mentioned in the introduction that casein kinase 2 (CK2) in humans interacts with FAF1. The similarity in amino acids of maize and human CK2 is 65% (Guerra and Issinger 1999). Even though CK2 is not a computational target of mmu-miR-294 it would be logical to be targeted by mir-290 cluster which would be a switch for the cell between dying and proliferating (Judson et al. 2009). It is known that p53 positively regulates the apoptotic mir-34 and therefore it can be assumed that the cell differentiating mir-294 opposes the action of the apoptotic mir-34 through this pathway.
- F-box and leucine-rich repeat protein 4 which play role in plant defense against fungal pathogens and LRR is a domain of many resistance genes in

plants that recognizes the fungal AVR effectors (Shirasu and Schulze-Lefert 2003).

- Transient receptor potential cation channel, subfamily V which in plants helps the SNARE in basal defense.
- Cell division cycle associated 4 (Wang, Y., S. Baskerville, et al. 2008)
- Apoptosis-inducing factor
- Heat shock protein 110
- Cell death-inducing DNA fragmentation factor
- Proteasome (prosome, macropain) 26S subunit, ATPase 3, interacting protein
- COP9 which is part of the proteasome and whose silencing in tobacco inhibited resistance against Tobacco mosaic virus (Liu, Schiff et al. 2002).
- Ubiquitin carboxy-terminal hydrolase L1 similar to PEN2 mentioned in the introduction
- Coiled-coil and C2 domain containing 1A similar to CC-domain of R proteins
- ATPase, Class V (in intro we mentioned valosin ATPase that interacts with FAF1).
- DNA methyltransferase
- Ubiquitin-like 7
- Mitogen activated protein kinase kinase kinase 1
- Cyclin-dependent kinase inhibitor 2D
- Bcl-2-related ovarian killer protein
- Programmed cell death 1
- ATP-binding cassette, sub-family A (ABC1) (similar to PEN3)
- Heat shock protein 90Ae. HSP90 silencing in barley decreased resistance against *B. graminis* (Hein, Barciszewska-Pacak et al. 2005).
- Ariadne ubiquitin-conjugating enzyme E2 binding protein
- Histone acetylase
- Bromodomain known to regulate aurora-B and it is also a domain found in AAA ATPases (You et al. 2009).

- SNAP-associated protein (part of SNARE) related to PEN1
- SHQ1 which is a homologue of HSP90

and many proteins involved in kinetochore function. We mentioned in the introduction the suppressor of kinetochore protein 1 as part of the SCF that mediates resistance against *B.graminis* in barley.

3.5 Insertion of 3' UTR of *FAF1* into γ As

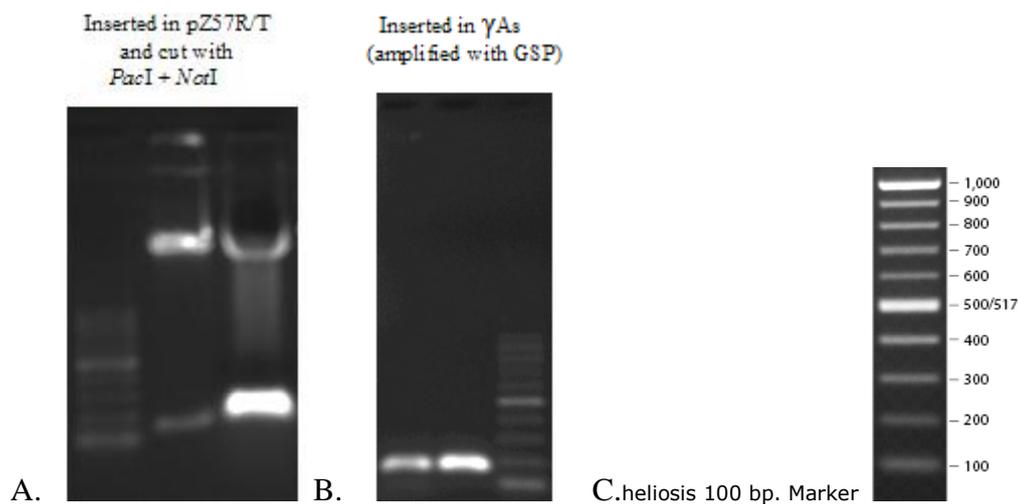


Figure 3.3 Double digestion of 3'UTR and ligation into γ plasmid. A. The 3'UTR fragment already ligated inside pTZ57R/T and double digested with *PacI* and *NotI*. (left 2 μ g and right 18 μ g loaded). B. The double digested 3'UTR was ligated to viral γ As vector and the insertion was confirmed for 2 clones by amplifying the 3'UTR region with gene specific primers. C. 100bp marker

3.6 Linearization of viral plasmids with BssHII

After the fragment of *FAF1* is inserted into γ As, the γ As plasmid was linearized with *BssHII* restriction enzyme for in vitro RNA transcription and so were α and β viral DNA plasmids.

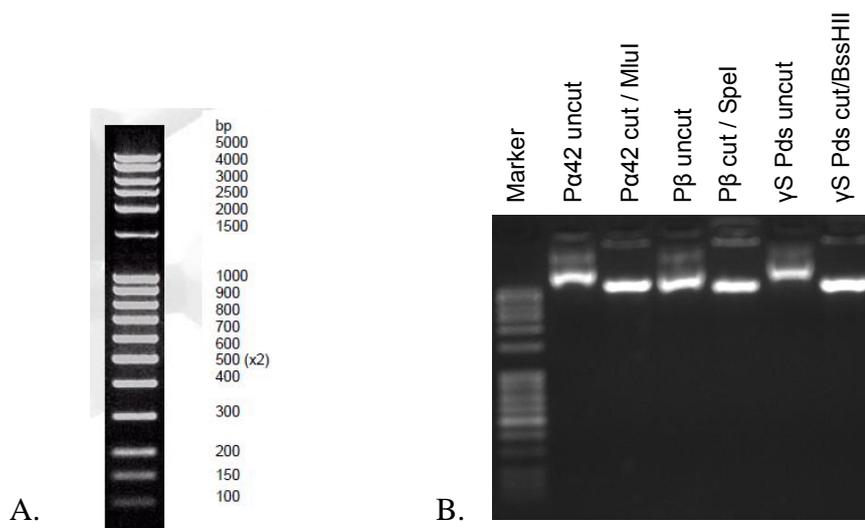


Figure 3.4 Circular and linear forms of α , β , γ SPDS plasmids. A. DNA Ladder Mix 100-5000 (lyophilized) B. Starting from left as shown above the figure α , β , γ SPDS were respectively cut with MluI, SpeI and BssHII restriction enzymes and run as 150 ng each in agarose gel (1.5%) with TBE buffer.

Even though not shown all viral vectors after being linearized with *BssHII* were transformed to *E.coli* DH5 α and observed for % of linearization based on the fact that circular vectors would transform the bacteria and survive in ampicillin-containing LB plates. By colony counting we observed that after 16 hrs of linearization ~95 % of plasmids were linearized.

3.7 Effectiveness of mMessage mMachine in vitro RNA kit

After linearization of viral sequence containing DNA plasmids, in vitro RNA transcripts were produced as in Fig 3.6 and before that the effectiveness of mMessage mMachine in vitro RNA kit was measured first upon X-ref control DNA that contains 3 promoters as T7, SP6 and SP3 as shown in Fig 3.5.

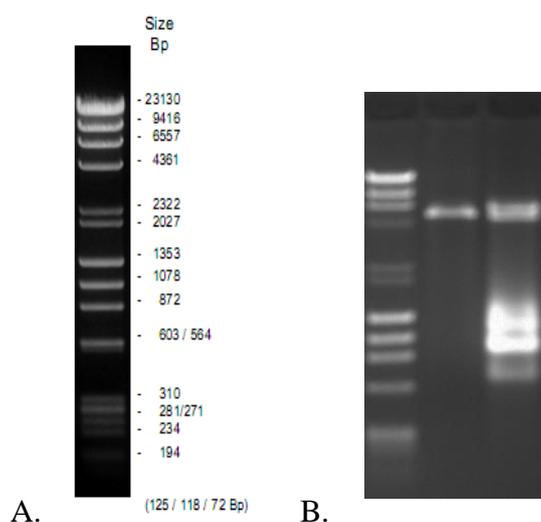


Figure 3.5 X-ref DNA and X-ref in vitro RNA control. A. Lambda HindIII/phix174 HaeIII DNA marker. B. From left 1st well: marker; 2nd well: X-ref DNA plasmid from mMessage mMachine in vitro RNA kit (100ng loaded) with a length of ~6kb which contains T7, T3, SP6 promoters; 3rd well: X-ref DNA (6kb) and 3 in vitro RNA bands between 1.8 and 1.9 kb that are produced from the RNA polymerase enzyme mix of in vitro RNA kit confirming enzyme's in vitro activity for the 3 different promoters found inside X-ref DNA.

3.8 Observation in agarose gel of in vitro RNA for viral vectors

In Fig 3.6 is shown a gel picture of in vitro RNAs transcribed from the original DNA vectors that contain the viral mRNAs. We ran it in a gel in order to control

the efficiency of T7 promoter inside these vectors. After verifying this, we went on to produce viral RNAs as shown in Fig 3.7.

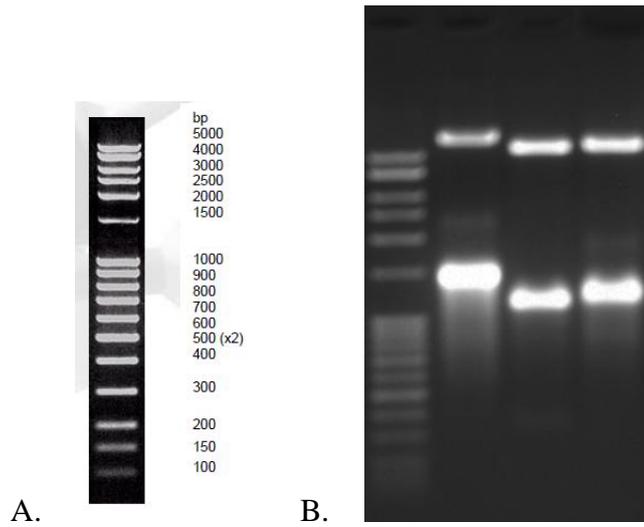


Figure 3.6 In vitro RNA of viral α , β , γ -*PDS*. A. DNA Ladder Mix 100-5000 (lyophilized). B. Starting from left beside marker, 1st well: α DNA plasmid (80 ng and ~6kb) with viral α RNA (~3kb); 2nd well: β DNA plasmid (80 ng and ~6kb) with viral β RNA (~3kb); 3rd well: γ *SPDS* DNA plasmid (80 ng and ~6kb) with viral γ *SPDS* RNA (~3kb); The RNA amount loaded for each well is the outcome of a single in vitro RNA reaction started with 80 ng plasmid. 1% agarose gel with phosphate buffer (pH6.8) was used for the run.

3.9 DNase treatment and LiCl precipitation of viral RNAs

Before being spread onto the leaves after DNase treatment and LiCl precipitation the RNAs were once again observed in an agarose gel as shown in Fig 3.7

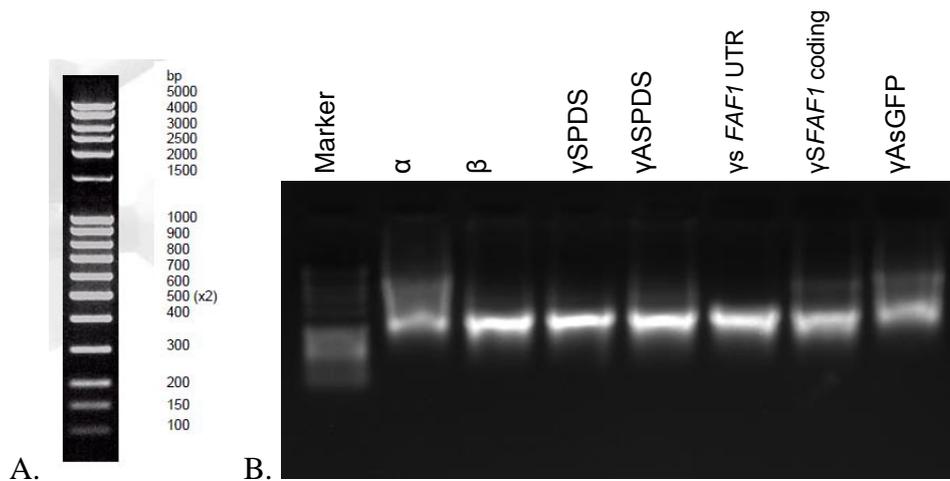


Figure 3.7 In vitro RNAs of all viral vectors before inoculation. B. Above the figure are written the names of the respective viral RNAs that have been already DNase treated and LiCl precipitated. $\sim 1 \mu\text{g}$ RNA is loaded in each well and run in an agarose gel (1%) with phosphate buffer (pH6.8). γ AS-PDS and γ S-*FAF1* coding with a region of *FAF1* from its coding region did not give a silencing effect probably due to RNA degradation before application on the leaf.

3.10 GFP observation under Leica light microscope in the 3rd day

The GFP expression as shown in Fig 3.8 and Fig 3.9 was used as a positive control to demonstrate that the cell wall disrupting FES buffer had worked properly and viral RNAs expressing GFP could penetrate the plant cell. Viral RNAs with GFP were spread with FES buffer in the 2nd leaves of barley.

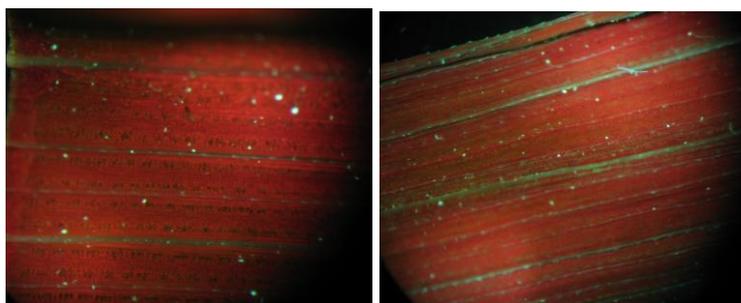


Figure 3.8 GFP expressions in the 3rd day taken from the second leaf. A light microscope was used to detect the GFP signaling.

3.11 GFP observation with camera-connected Leica microscope in the 4th day

In order to make sure that GFP was expressed, we monitored it also under camera connected Leica microscope at 40X magnification on the 4th day after spreading

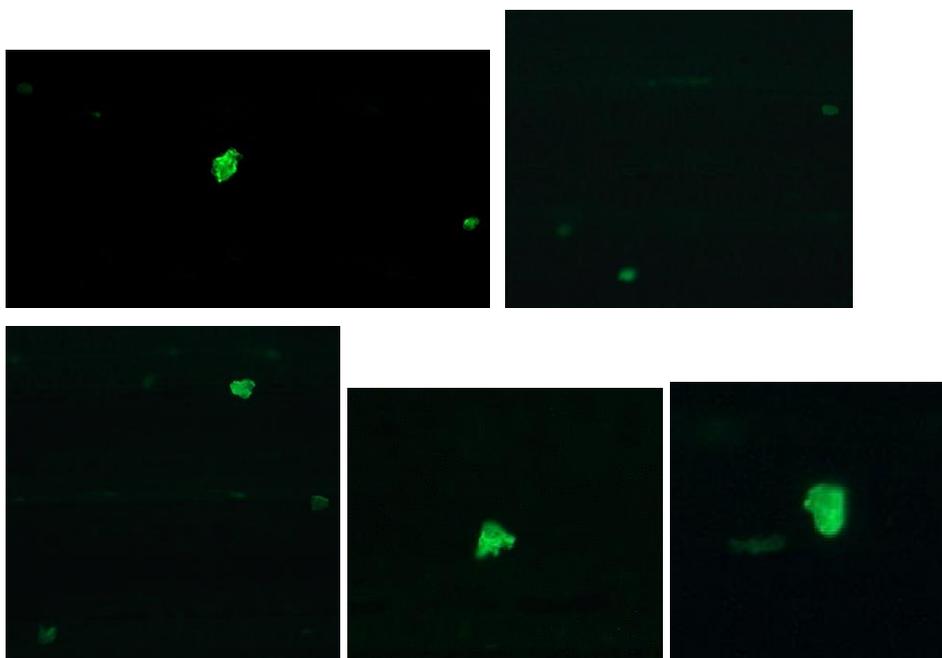


Figure 3.9 GFP expressions in the 4th day at 40X under Leica microscope.

3.12 The stripe symptoms of the BSMV:00 virus

When the virus is spread on the second leaf of barley we expect that the virus will replicate itself and perform a systemic movement in the plant and therefore be observed in the leaves that later emerge. The BSMV:00 which is the natural unmodified form of the virus was used as a positive control for this purpose.



Figure 3.10 Stripe symptoms of BSMV: 00 in the third leaf of barley.

The natural form of the virus (BSMV: 00) was spread on the 2nd leaf of Pallas03 barley line and its stripe symptoms were observed in the third leaf and captured in a photograph.

3.13 The bleaching of Phytoene Desaturase silencing

After the systemic movement of the virus was verified with BSMV:00 we silenced the phytoene desaturase gene whose mutant form has an obvious phenotypic property by bleaching the leaves of barley.

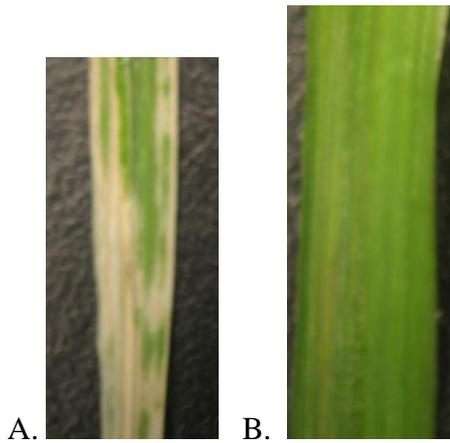


Figure 3.11 Bleaching of barley leaf due to phytoene desaturase (*PDS*) silencing. A. α , β , γ -*PDS* in vitro RNAs were mixed with FES buffer and spread by hand on the 2nd leaf of barley. The phenotypic bleaching of *PDS* silencing with VIGS was observed in the 5th leaf. B. The green color of a *PDS* non-silenced leaf infected with a natural virus is shown.

3.15 Total RNA isolation of viral inoculated leaves

In order to measure the fold difference due to silencing of the *PDS* and *FAFI* mRNAs via VIGS in barley leaves, we isolated total RNAs of the *PDS*-silenced leaf that seemed to have bleached most and *FAFI*-silenced barley pallas03 leaf that seemed to have been infected most by fungus as shown in Fig 3.13. The barley resistant pallas01 line total RNA was also isolated even though resistance against fungus was not disrupted by *FAFI* silencing. The total RNA of respective BSMV:00 controls of these 3 silenced leaves was also isolated and run in a gel as shown in Fig 3.12.

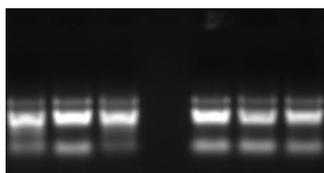
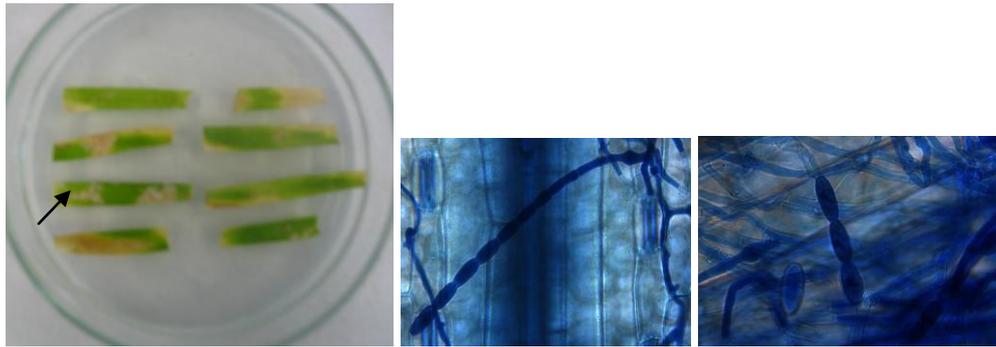


Figure 3.12 Total RNAs of silenced leaves and their controls.

In the left 3 wells are shown total RNAs of leaves that are infected with the natural unmodified form of the virus BSMV:00 and in the right of the gel the 3 total RNAs of the silenced leaves as barley pallas03 leaf 5 silenced *PDS*, barley Pallas01 leaf 3 silenced *FAF1* and barley Pallas03 leaf 3 *FAF1*. cDNA was constructed from these total RNAs in order to perform qRT-PCR measurement for *PDS* and *FAF1*.

3.14 *FAF1*-silenced gene and fungal inoculation

The barley pallas01 line resistant against fungus *Bgh103* was supposed to loss resistance when *FAF1* gene was silenced by VIGS but we could not observe any susceptibility of the *FAF1*-silenced leaves towards the fungus beside the normal hypersensitive response zones as the form of resistance. The susceptible line pallas03 instead showed an increased susceptibility towards the fungus especially in one of the silenced leaves as shown in Fig 3.13A and B. The *FAF1*-silenced leaf that seemed to be infected most was stained with trypan blue for conidia (body part of the pathogen) counting and was counted as 8 (Fig 3.13B). At the same time its respective *FAF1*-non silenced BSMV:00 control was counted as 3 (Fig 3.13C).



A)

B)

C)

Figure 3.13 A) *FAF1*-silenced leaves and fungal inoculation. In the left part of the figure are the Pallas 03 lines of barley whose *FAF1* gene was silenced. In the right it is the BSMV:00 of the same line. In the *FAF1*-silenced barley leaves it can be seen the increased susceptibility towards the fungus *Blumeria graminis* showing a possible role in the resistance mechanism of this gene. B) 10 day old *FAF1* silenced Pallas03 (8 conidia); C) 10 day old BSMV:00 control (3 conidia).

3.16 Real time measurement of *PDS* and *FAF1* silencing

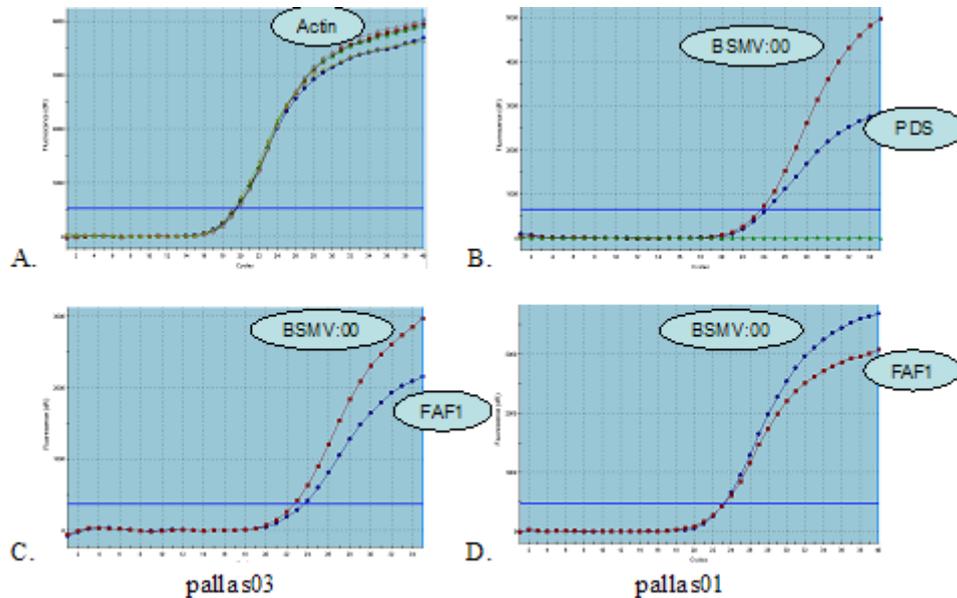


Figure 3.14 Real time measurements. A. cDNAs were constructed from 1 μ g of each total RNA shown in Fig 3.12 and actin was chosen for calibrating real-time measurements. B. Real time measurement of *PDS*-silenced leaf and its reference control (BSMV:00). C. Real time measurement of *FAF1*-silenced barley pallas03 vs. reference control (BSMV:00). D. Real time measurement of *FAF1*-silenced barley pallas03 vs. reference control (BSMV:00).

We measured the fold change according to Livak and Schmittgen (2001). In their work, the threshold cycle (C_T) is defined as “the fractional cycle number at which the amount of amplified target reaches a fixed threshold”.

$$\text{fold change} = 2^{-\Delta\Delta C_T}$$

with $\Delta\Delta C_T = (C_{T\text{sample}} - C_{T\text{sample-ref}})_{\text{treated}} - (C_{T\text{sample}} - C_{T\text{sample-ref}})_{\text{untreated}}$

where;

$C_{T\text{sample}}$ = the average of C_T values for the transcript of interest (*PDS*, *FAF1*)

$C_{T\text{sample-ref}}$ = the average of C_T values for β -actin transcript.

Treated stands for *PDS* and *FAF1* silenced leaves while untreated stands for non-silenced control leaves (BSMV:00). The non-silenced control is the calibrator and β -actin is the endogenous reference gene. Actin was chosen as an internal reference based on the fact that it amplifies in the same way as *FAF1* does, with E_x value ~ 1 . (Bozkurt et al. 2007).

Table 3.1 C_T values of Real Time measurements

Name of the sample	$C_{T_{\text{sample}}}$ value	$C_{T_{\text{actin}}}$ value
PDS silenced P03	24.47	19.14
control PDS silenced P03	23.69	20.26
<i>FAF1</i> -silenced P03	24.04	19.09
Control <i>FAF1</i> -silenced P03	22.68	19.33
<i>FAF1</i> -silenced P01	23.13	19.42
Control <i>FAF1</i> -silenced P01	22.18	19.48

Therefore:

$$\text{PDS: } \Delta\Delta C_T = (24.47 - 19.14) - (23.69 - 20.26) = 1.9$$

$$\text{FAF1 Pallas03: } \Delta\Delta C_T = (24.04 - 19.09) - (22.68 - 19.33) = 1.6$$

$$\text{FAF1 Pallas01: } \Delta\Delta C_T = (23.13 - 19.42) - (22.18 - 19.48) = 1.01$$

This would imply a $2^{-1.9}$ therefore 3.7 fold decrease in *PDS* mRNA in the *PDS* silenced leaves. For *FAF1*-silenced Pallas03 that would be a 3 fold decrease and for *FAF1* Pallas01 the fold decrease ratio is ~ 2 .

3.17 When the primer is not fully complementary

In green in section 3.3 are shown the primers attached to *PacI* and *NotI* restriction sites. Because the primers were designed according to the EST sequence of *FAF1* in *T.aestivum* they did not anneal entirely in barley. This was overcome with 3'RACE where the 3'UTR sequence of the gene was forced to be amplified after

walking from one side upon the gene twice and twice from the 3'RACE adapter. The template that came out from 3'RACE was used for a third amplification with 3'UTR primers at an annealing temperature of 40°C with Hot Start Kod-XI polymerase. Another polymerase (Gentaq) used in the same manner failed to amplify the product. Also from other amplifications we can say that when the primer is partially complementary with the annealing region Kod-XI is a good choice as an amplifying enzyme. The 500 bp. region was amplified with cDNA polymerase mix (*Taq*. Clontech.), which is a common enzyme used in rapid amplification of cDNA ends.

For 3' RACE we used 3 different adapters from different companies Ambion, Clontech, Trimmer-Direct. Trimmer Direct seemed to serve the function of 3'RACE best. This might be because of the longer d(T) oligo attached to it as d(T)25. Clontech adapter also contains d(T)25 and seems to attach fine to polyA but a combination of primers short and long is used for wrong adapter binding suppression and according to our experiments it is hard to obtain any amplification at all. The Ambion adapter instead with its d(T)15 region seems to bind more non-specific than the Trimmer-Direct adapter.

3.18 The upstream polyA region of *FAFI* not similar among monocots

The sequenced 500 bp. region of *FAFI* in barley was compared with the one in wheat and rice. The similarity between genes was more than 85% and it was interesting that this similarity declined to zero ~100 bp upstream the polyA. It can be said that this 100 bp. region might be considered a fingerprint for orthologous genes. If the conservation exists in the genomic level but not in the mRNA it might even be that one of them has escaped RNA inhibition machinery by a different splicing form.

3.19 The double digestion

pTZ57R/T is a T/A cloning vector and the reason we used it is that there are no PacI and NotI restriction sites. Care must be shown in this aspect with very commonly used other T/A vectors such as pGEM-T and pGEM-T Easy vectors that

contain Pac1 and Not1 restriction sites directly upstream and downstream the insertion site and therefore double digest in 6 different forms.

3.20 SCAR (Simultaneous control and reaction).

In worldwide VIGS works the phytoene desaturase control, where a fragment of this gene as 200 bp. is inserted into γ , is performed separately from the silencing of the gene of interest. If the fragment of gene of interest is ligated together with the fragment of phytoene desaturase, that would make a SCAR. Possible silencing SCAR constructs of up to 500 bp. are possible and that would guarantee the silencing of gene of interest in photo-bleached regions of leaves.

3.21 Plasmid linearization

8 μ g of a 6kb long plasmid will be completely digested in a double digestion with 10 units of enzyme each. Transformation of the linear product will show no colony in a selective media. In our experiment it is important to totally get rid of the *PDS* insert from the gamma vector before ligating the insert of gene interest otherwise colonies of *PDS* vector will appear in the ampicillin selective LB plates and it will be confusing to isolate the colonies of your gene of interest.

3.22 In vitro RNA production

It would be interesting to evaluate the efficiency of in vitro RNA production by increasing the sequence of T7 promoter from 18 to 20 nt. and allow overnight production in the presence of an RNase inhibitor.

3.23 Efficiency of silencing

According to real time results you can see a 4-fold decrease in the *PDS* and 2-3 fold decrease in *FAF-1* silenced leaves. According to previous data the infection causes the plant to increase the expression of *FAFI* gene 3 folds.

Because levels of FAF-1 are high in a normal plant the decrease is considered sufficient to observe the effect of silencing.

3.24 Possible roles of FAF1 in the resistance mechanism

We already mentioned in the introduction that casein kinase 2 (CK2) in humans interacts with FAF1. In fact this is a very tight interaction and inhibits CK2 to perform its functions even though this interaction has not been observed in only embryonic cells and not differentiated ones. Other CK2 inhibitors have also been studied where the inhibition stimulates a caspase 8 dependent apoptotic action (Litchfield 2003). It is amazing the similarity in amino acids of maize and human CK2 as 65%. Many data exist that pathogens in plants stimulate the expression of CK2 to perform their functions (Matsushita, Ohshima et al. 2003). Therefore while the plant cell tries to shut down, the pathogen tries to reverse it. CK2 is also known to phosphorylate Hsp105 (Hsp110 family) which is a negative regulator of Hsp70 and with this action it relieves Hsp70 from its inhibition and let it continue with the protein folding process (Ishihara, Yamagishi et al. 2003). CK2 is also known as the kinase of 1001 targets therefore the loop of FAF1 actions can be zoomed-out in this interaction.

In the introduction we mentioned that FAF1 also inhibits the process of Hsp70 by tight interaction of UBA domains of both proteins. We can not say for certain that this interaction also occurs in plants. Hsp70 is known to play a positive role in resistance against tobacco mosaic virus in tobacco like FAF1 against fungus and they should not interfere with each other at the time point where they are both useful.

It is interesting the relationship of FAF1 with Hsp90 and Hsp110 where they seem to be targeted by the same putative miRNA/siRNA as shown in the Results section and other works have shown that VIGS silencing of Hsp90 increases resistance against *Blumeria Graminis* in barley. Other putative targets beside FAF1 such as coiled coil domain, leucine rich repeat-containing protein, E1, E2, F-box, COP9, SNAP, PEN2, PEN3 further confirms a common role of FAF1 with these proteins. The presence of UBX c-terminal domain in FAF1 seems to be responsible for

barley's increased susceptibility against fungus. UBX (ubiquitin like X) domains are similar to ubiquitin but with a small difference in their ends conserved among UBX as Arg20, Arg26 and Val85 that inhibits them to conjugate with other proteins (Buchberger, Howard et al. 2001). 7 UBX domain-containing proteins are found in *Saccharomyces cerevisiae* and they all interact with cdc48 (Rancour and Park 2004). Mutants of 2 of these UBX proteins p47 and ubx2 show defects in the protein degradation pathway and are highly sensitive to stress (Schuberth, Richly et al. 2004). p47 is the most studied UBX protein. p47 is a cofactor of p97 (AAA ATP-ase) and it is the only cofactor known in the p97-regulated membrane fusion pathway. It helps the interaction between p97 and syntaxin 5 (PEN1 a member of the SNARE receptors). The p47-p97 is supposed to disassemble the t-t SNARE to prepare it for other rounds of membrane fusion and it does this through binding of ATP (Yuan, Shaw et al. 2001). This process positively regulates the barrier defense in plants as mentioned in the introduction where factors that help in vesicle formation are included in barrier defense against pathogens. The FAF1 in plants also contains an ATP-binding domain and the presence of UBX domain seems to relate its function with p47 making it a possible barrier defense factor.

It must have occurred to the reader that all the proteins mentioned in the last paragraph are also computational targets of the same putative siRNA. For such an important sequence not to have been found in plants derives us to the conclusion that this siRNA might be an effector sent by the pathogen itself to immediately disrupt all forms of resistance against it.

According to our results shown in Fig 3.13 *FAF1* VIGS silencing in the susceptible line Pallas03 of barley made the plants more susceptible to the pathogen *Blumeria Graminis*. The fungus infection was measured as 8 conidia in one of the *FAF1* silenced leaves vs. 3 conidia in the control leaf which is a considerable increase in the growth of the pathogen. Again this result seems to relate the function of FAF1 with p47, where the mutant forms of these domains make the plant more sensitive to stress. The difference in their internal regions makes the difference, where the one in FAF1 plays a role in the death-inducing action.

Another theory for our results is that other proteins might be responsible for the increase in susceptibility of barley against fungus. During VIGS silencing of *FAF1* siRNAs are generated from the fragment of *FAF1* that we inserted inside the virus for silencing. One of these siRNAs might be generated from the region that contains the homology as shown in section 3.4. The figure below shows the cleavage after VIGS of *FAF1* and a combined primer which is explained in detail later and which can possibly amplify the degraded forms of *FAF1* and other mRNAs that contain the same homology and in case any of them has been degraded too. If this were the case then with *FAF1* a protein inside the ubiquitination pathway or any from the barrier defense would also be silenced and this would explain the susceptibility against fungus.

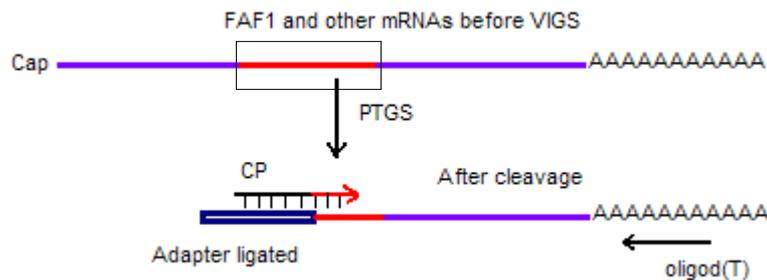


Figure 3.15 Possible siRNA generated inside the homology

Those mRNAs that contain the homology might have been silenced during *FAF1* silencing with VIGS. A siRNA might have been generated from the box region which contains the homology. A combined primer (CP) that will amplify in a single reaction only valid siRNA-cleaved mRNAs that contain the homology based on the fact that siRNA cleaves between 10 and 11 nt. is shown and is explained in detail in the following sections.

Future work such as immuno-precipitation of *FAF1* would make possible to elucidate the interacting proteins in this pathway.

In our experiment the silencing of *FAF1* in Pallas 01 resistant line to *Blumeria Graminis* where the plant cell of fungus entrance dies to stop infection, did not confer susceptibility (data not shown). This means that silencing of *FAF1* in a 2 fold level only, is not enough to stop apoptosis and allow infection. Total silencing of *FAF1* in the genomic level must be performed to see whether apoptosis can be stopped.

The possible function of FAF1 protein in plants can be summarized based on the domains that it contains. When the 464 amino acid FAF1 protein sequence of *Orizum Sativa* was aligned in NCBI blastp for amino acid sequence similarity the following domains and regions were revealed:

- UBA (ubiquitin associated) domain that interacts with ubiquitin of ubiquitinated proteins by transporting them to the proteasome aiding therefore in protein degradation a pathway known for its influence in the resistance mechanism.
- UAS (ubiquitin associated) domain with unknown function.
- UBX (ubiquitin like X) domain that interacts with AAA ATPases where the latter modulates vesicle trafficking already known to play a role in barrier defense against pathogens. Yeast with mutants of UBX domain containing proteins has been shown to be more susceptible to stresses.
- CC (coiled coil), NB (nucleotide binding) regions that are found in all plant resistance genes and are responsible for RAR1-Sgt1 mediated resistance against fungus.
- LRR (leucine rich repeat) region which is found in all plant resistant proteins and is responsible for recognizing the pathogenic effectors that enter the cytosol.

In the same NCBI blast outcome certain regions of FAF1 proteins are present in other proteins such as: AAA ATPase and intersectin that are both factors in vesicle trafficking and therefore the plant's barrier defense. Intersectin helps endocytosis, exocytosis and actin polymerization in mammals (Ma et al. 2008). Another protein

that showed similarity was a bacterial peptidoglycan hydrolase. In *Arabidopsis thaliana* PEN2 protein which is a glycosyl hydrolase inhibits the penetration of fungus and therefore plays role in the plant's barrier defense.

Therefore FAF1 protein in the plant resistance seems to be related with vesicle trafficking and therefore is part of the plant's barrier defense.

3.25 A 5' RACE approach to amplify cleaved mRNAs of the homology

In Fig 3.16 are shown most of the proteins whose mRNAs contain the same homology which might be a target site for short inhibitory RNAs. In order to verify this, 5' rapid amplification of cDNA ends is performed for any of the mRNAs that contain the homology but the mRNA we chose is not certain to be a valid target until we verify the cleavage point with 5'RACE which might not give an amplification which makes it obvious that we have choosed the non-valid target. The following section deals with this problem and offers a solution to amplify in a single reaction only the valid cleaved mRNAs among the 20 proteins of the homology shown.

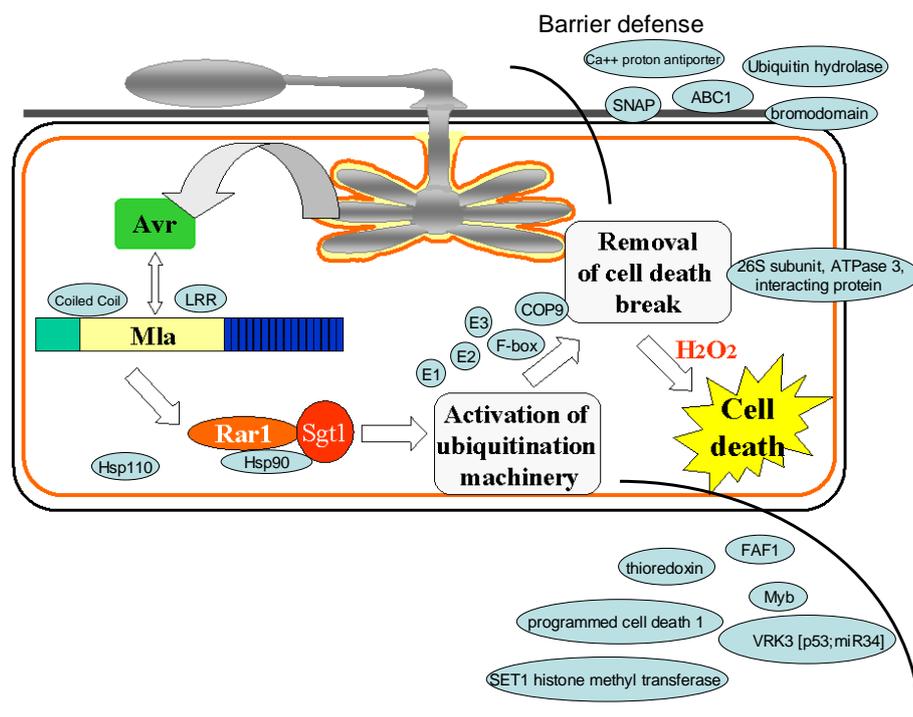


Figure 3.16 Disease resistance related mRNAs with the same homology

Many of the proteins mentioned in the section 3.4 are positioned according to the role they play in disease resistance and they all have in common a homology in their mRNAs which might be a possible siRNA target site for FAF1 generated siRNAs during *FAF1* silencing.

Modified from: (http://www2.mpiz-koeln.mpg.de/schlef/PSL_webpage.html)

We mentioned in the introduction (1.8) the conventional protocol to identify cleaved mRNAs as

Step 1. A gene is chosen whose mRNA is complementary to the selected microRNA or has a maximum score of 3 (for plants) according to algorithms of “mirU plant target finder”. This score may be increased according to Rhoades and Bartel based on the conservation of the mismatch among plants. According to Schwab and Palatnik, again in plants the microRNA and its target is enough to be

complementary between 3-12 nts. of microRNA with one mismatch possible and that mismatch not near the cleavage point.

Step 2. PolyA isolation is made and an RNA adapter is ligated to mRNAs that contain a mono-phosphate, with T4 RNA ligase 1 (CIP-TAB treatment is not performed for this protocol).

Step 3. The amplification is made with primers annealing upon the adapter and with gene specific primers, and the gene chosen from 1.

Step 4. After sequencing, if the amplified mRNA has its upstream 10 nucleotides matching the microRNA's first 10 nucleotide or somewhat obeys the rules mentioned in 1, then the target is identified. (The target is cleaved exactly between 10 and 11 nts. corresponding to miRNA and that is valid for siRNAs, piRNAs etc.)

INSTEAD

All the targets of any microRNA have in common polyA in their 3'ends (tails) and 10 nucleotides matching complementary the microRNA in their 5'end (head). Therefore they can be amplified in a single step and we do not need to have any information for the target if we have the sequence of the microRNA.

As in the normal protocol the RNA adapter will be ligated and the primers will be oligod(T) and a combined primer of 25 nts. where the first 15 nts will anneal to the adapter and the other 10 nts or less than 10 nts of the primer will anneal to the heads of targets.

The Fig 3.17 will make the idea more comprehensible.

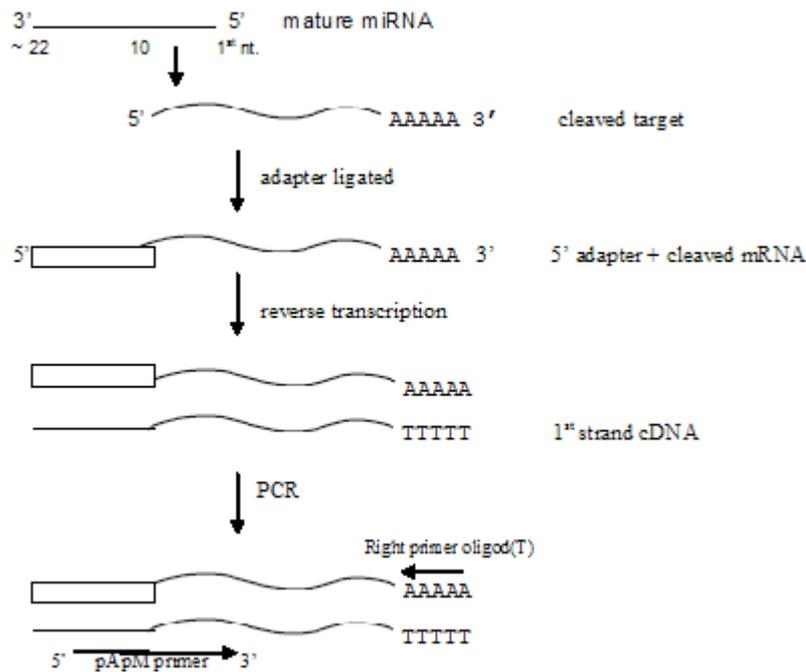


Figure 3.17 The combined pApM primer that amplifies all the targets of a single microRNA. The adapter shown in box is a single stranded RNA and the pApM-primer is the combined partially adapter partially microRNA complementary primer.

If we are to consider the targets of the putative miRNA/siRNA that was discussed in section 3.4 the protocol to amplify the valid cleaved mRNAs of the proteins mentioned in the same section would be:

mature miRNA

5' aaaaaggagcaccuauacuug 3'

All the targets in their cleaved form of this microRNA will have starting from their 5'end

5' gcuccuuuuu(gene sequence) polyA 3'

And after the ligation of an adapter (1) their sequences will be:

5' Adapter(1) gcuccuuuuuu(gene sequence) polyA 3'

oligo(dT) has a low annealing temperature therefore the mRNA can be primed with an oligo(dT) attached to a known adapter (2) sequence. Then before amplification the cDNAs of targets of the putative microRNA will have the following structure:

3' complementaryAdapter1 cgaggaaaaa[gene sequence] d(T) adapter2 5'

where we can walk twice upon adapter2.

We know that miRNA may not be fully complementary to its target especially near the 5' end of the miRNA

If the adapter1 sequence is 5' augcuagugua 3' then for the combined primer to amplify all the targets in one amplification reaction the following sequence will be enough: atgctagtgtagctc and because in rapid amplification of cDNA ends walking twice upon the gene is preferable to get rid of smears and wrong annealing, after the first amplification, a second is performed by extending the primer with two nt. as: atgctagtgtagctcct

3.26 The 5' RACE adapter

In the RLM-RACE (RNA-ligase mediated rapid amplification of cDNA ends) the adapter is attached to monophosphates of 5' ends of cleaved targets. A 5' monophosphate is found also in tRNAs and ribosomal RNAs therefore the polyA isolation is necessary for ligation only to the cleaved mRNAs.

We performed such a ligation starting with 150 µg of total RNA and we obtained 3 µg of mRNA as expected but we did not have any success in amplifying any microRNA target. We might have lost it during washing steps (5 washing steps) of mRNA isolation.

That is why we are considering other approaches to ligate the adapter.

Future work

One of them is to directly ligate concentrated forms of the adapter to total RNA instead of mRNA.

The second approach is, after constructing the cDNA, to ligate a DNA adapter to the 3' ends of cDNAs. This is a poor ligation and is performed with T4 RNA ligase 1 overnight. Yet cDNA purification is necessary for the adapter to ligate only to cDNA 3'ends because the 3' ends of RNAs are good substrates for this ligation. We performed cDNA purification with Zymogen kit, but seems that still there is a great loss of cDNA during purification revealed with actin amplification. (Must show care to use a native MMLV-RT so that no extension to the cDNAs will be performed which will cause the sequence of the cDNA at between the cleavage point and the adapter to change)

3rd approach is the template switch approach, where the property of a mutant form of MMLV-RT only in the presence of manganese can add cytosines after constructing the cDNA. This approach has no intrusions such as polyA isolation or cDNA purification but the manganese concentrations and research related to this approach because of being old are focused upon adding cytosines only to cDNA extremities that match to capped mRNAs. If the mRNA is degraded or cleaved as it is in our case the reverse transcriptase adds only 1 nucleotide and that is not certainly a cytosine, therefore the ligation of the adapter with switch approach will not be performed.

4th and which is the best option and not encountered in literature for microRNA targets is to circularize the cDNA with single stranded DNA Circulase. It works at 60°C and there is no need for polyA isolation or cDNA purification. Priming of cDNA is performed with oligod(T) modified to have a monophosphate in its 5' end. Subsequent circularization of all cDNAs including microRNA targets will occur. After that the combined primer to amplify all targets of a specific microRNA can be designed with the same logic as mentioned and the steps will be: Total RNA isolation - cDNA construction – Circularization – Amplification with no phase intrusions and therefore no target or gene loss.

The property of this enzyme to directly ligate a DNA adapter to 3' ends of cDNAs instead of circularizing the cDNA, even though the company claims that the enzyme does not perform such a reaction, will be searched.

CONCLUSION

The effect of Fas associated factor 1 (*FAF1*) gene in barley was investigated about its possible role in the plant immunity against fungus *Blumeria Graminis hordei*. The transient mutant form with Virus Induced Gene Silencing technique of this gene was performed and the decrease in plant resistance was evaluated based on the increase of fungal infection. The experimental results showed that the silencing of *FAF1* in barley made the plant more susceptible to fungal stress. There are no previous works concerning this gene in plants and the possible interactions of *FAF1* in plant immunity were discussed based on this gene's role in metazoan. In the latter it is found the relationship of this gene with apoptotic proteins. We can say that while the plant tries to perform a local hypersensitive response by sending the cells to apoptosis in the fungal penetration site, the fungus tries to reverse it. According to the results *FAF1* silencing is not enough to disrupt the apoptotic pathway confirmed by mammalian research that this gene induces but does not start apoptosis. The c-terminus of this protein contains a UBX domain which is a domain found in 7 different families of proteins. Some of them play role in regulating the ubiquitination mechanism and some in vesicle formation. Both these mechanisms are known to play a major role in plant immunity. The role of *FAF1* seems to be related with these mechanisms due to the presence of UBX domain and due to the results of our experiments in *FAF1*-silenced barley that showed increased susceptibility against the fungus.

For the first time a homology in the mRNA level was found between proteins that play major roles in plant defenses. This homology was found in the *FAF1* 3'UTR and seemed to be conserved in E1, E2, E3, COP9, Hsp90, SNAP, PEN2, PEN3, CC and LRR-containing proteins and many others where the function of each of these proteins in plant disease resistance has been prescribed in detail in the introduction section. This homology pertaining to the mmu-miR-290 cluster, the major stem cell microRNA in mammals which causes cell proliferation further confirms a conservation of a short inhibitory RNA in all organisms. A short oligo protecting

this homology of mRNAs from being cleaved would maintain active all the defense mechanisms in plants and stop cells from proliferating in mammals such as the cancer cell dedifferentiation. A novel approach has been prescribed for amplifying all degraded mRNAs of a certain short inhibitory RNA in a single polymerization. This will make possible identification of miRNA targets in a large scale and reveal new systems concerning the microRNA-target relationship. With this approach the qRT-PCR of a microRNA's impact on the transcriptome based on the cleaved target(s) concentration and not the microRNA concentration will also be possible. The 5'RACE is a definitive proof of miRNA target identification. Difficulties in the conventional protocol concerning the ligation of the adapter and possible solutions are also evaluated.

Until now the SCF complex proteins and transcription factors seem to be main targets of microRNAs. One of the works of our lab is to identify a new system where a microRNA targets both the transcription factor and the signaling pathway of this transcription factor providing a dual function for the microRNA. That would be miR164-NAC domain TF-Map kinase 2. Another system would be mirtron-splicosome relationship where in literature are already found microRNAs to target splicing proteins. Yet the question to why microRNA does not inhibit all its complementary mRNAs and therefore the real relationship with its targets remains to be elucidated.

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APPENDIX

The genomic sequence of pds4Antisense vector

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