A NOVEL AP2 DOMAIN TRANSCRIPTION FACTOR FROM *Lycopersicon esculentum*, FUNCTIONS IN TOBACCO MOSAIC VIRUS PATHOGENESIS

by

BURCU DARTAN

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

Sabancı University

July 2004

A NOVEL AP2 DOMAIN TRANSCRIPTION FACTOR FROM *Lycopersicon esculentum*, FUNCTIONS IN TOBACCO MOSAIC VIRUS PATHOGENESIS

APPROVED BY:

Assoc. Prof. Zehra Sayers …….

(Dissertation Supervisor)

Dr. Damla Bilgin …….

(Co-Supervisor)

Prof. Meral Yücel …….

Dr. Alpay Taralp …….

Assistant Prof. Cengiz Yakıcıer …….

DATE OF APPROVAL: …….

ABSTRACT

In this work, one of the established plant defense proteins namely that corresponding to the class EREBP-like transcription factors containing the AP2 domain was examined to identify a possible role in Tobacco Mosaic Virus Pathogenesis. In particular, a putative AP2-domain EREBP-like transcription factor –JERF1- from tomato cDNA was subcloned into various expression vectors in order to test for its potential role in virus response in *Solanacaea.* The gene silencing experiments, in which death was observed subsequent to silencing of the *JERF1* gene and TMV infection, have shown that this particular gene might be involved in the TMV infection cycle in *Solanacaea*. The results of a yeast mating assay conducted in this study has also implied that JERF1 is an interactor of plant $P58^{IPK}$.This possibility would in turn suggests that JERF1 may be involved in plant-virus interaction. Overexpression, yeast-protein expression, and GFP-fusion constructs have also been prepared to support functional analyses of this putative transcription factor. It would follow to reason that this study of the recently sequenced JERF1 describes the first attempt to understand the function of this protein in plant-virus interaction.

ÖZET

Bu çalışmada, EREB protein benzeri transkripsiyon faktörlerine karşılık gelen, AP2 alanına sahip olan ve bitki savunma proteinlerinden olan bir protein, Tütün Mozaik Virüsü (TMV) patojenesisindeki muhtemel rolünün tanımlanması için incelenmiştir. Özellikle, AP2 alanına sahip EREB protein benzeri bu transkripsiyon faktörü-JERF1-domates cDNA'sından klonlanmış ve virus duyarlılığında ya da dirençliliğinde rolünü bulabilmek için değişik vektörlere takılmıştır. *JERF1* geninin susturulduğu ve tütün mozaik virüsünün enfeksiyonunun yapıldığı gen susturulması deneyleri, bu genin *Solanacea* ailesinde TMV enfeksiyon döngüsünde görev aldığını gösterir. Maya çiftleştirmesi deneyleri de JERF1 proteininin bitki $P58^{IPK}$ proteini ile etkileştiğini göstermiştir ve bu JERF1'in bitki virus etkileşiminde yer alabileceğini belirtir. Ayrıca, bu transkripsiyon faktörünün fonksiyonal analizi için bu gen, overekspresyon, maya-protein ekspresyonu ve GFP-bileşik vektörlerine takılmıştır. Bu çalışma, yakın zamanda dizisi belirlenen JERF1 proteininin, bitki-virus etkileşimindeki işlevini tanımlamak için atılan ilk adımı oluşturmaktadır.

To my family with all my heart,

Rest & be thankful in a world where nothing is random…

ACKNOWLEDGEMENTS

I am grateful to Prof. Dr. Zehra Sayers for her supervision and to Dr. Damla Bilgin for her endless support, advise and help throughout this study.

I also would like to express my appreciation to Dr. Alpay Taralp who has taught me how to think, analyze and criticize in the way paving through being a scientist. My special thanks go to Dr. Metin Bilgin who has given me new inspirations when I was stuck during my study. I also would like to thank to Dr. Fahriye Ertuğrul and Dr. Sedef Tunca who have taught me how to conduct a scientific experiment and helped me with my research.

My deepest appreciation goes to my parents Belgin and Besim Ömer Dartan, my aunts, my uncles, my cousins and my grandparents who have gifted me my life and personality.

Moreover I would like to thank to my sisters Didem Demirbaş, Funda Güngör, Gözde Vrana, Işın Nur Cicerali, Senem Tuğ and Tuğçe Güleryüz for their valuable friendship during my undergraduate and graduate education.

Thanks also go to my colleagues Çetin Baloğlu, H.Ümit Öztürk, Kıvanç Bilecen, Özgür Gül and Özgür Kütük for their help in this study.

I also would like to offer my sincere appreciation to my dearest SU GIRLS- Burcu Kaplan, Elanur Şireli, E. Süphan Bakkal, Filiz Dede and Yasemin Türkeli- for their encouragement and friendship throughout my study. Finally, I would like to express my intense gratitude to my friend A. Barış Karagözler without whom I would not bring about this thesis.

TABLE OF CONTENTS

ABBREVIATIONS

Aba : Absisic Acid

ADR : Activated disease resistance

AP2 : Apetala2

ARC : Apoptosis gene products,Resistance, CED-4

Avr : Avirulence

CC : Coiled coil

DRE : Drought-responsive element

dsRNA : Double-stranded RNA

EREBP : Ethylene-responsive element binding protein

ERF : Ethylene-responsive factor

GDP : Guanosine diphosphate

GTP : Guanosine triphosphate

HR : Hypersensitive response

IFN : Interferon

IL : Interleukin

JA : Jasmonic Acid

LRR : Leucine-rich-repeat

MAPK : Mitogen-activated protein kinase

MAPKKK : Mitogen-activated protein kinase kinase kinase

NBS : Nucleotide-binding-site

PCD : Programmed cell death

PKR : Interferon-induced protein kinase

PR : Pathogenesis-related

PVX : Potato virus X

R : Resistance

RBM : Regulatory domain-binding motif

ROS : Reactive oxygen species

Rp : Receptor protein

SA : Salicylic Acid

SAR : Systemic acquired resistance

SIPK : Salicylic Acid-inducible protein kinase

SOD : Superoxide dismutase

ssRNA : Single-stranded RNA

TEV : Tobacco etch virus

TF : Transcription factor

TIR : Toll- and Interleukin like receptor

TM : Transmembrane

TMV : Tobacco mosaic virus

TPR : Tetratricopeptide repeat

WIPK : Wound-inducible protein kinase

LIST OF FIGURES

A NOVEL AP2 DOMAIN TRANSCRIPTION FACTOR FROM *Lycopersicon esculentum*, FUNCTIONS IN TOBACCO MOSAIC VIRUS PATHOGENESIS

by

BURCU DARTAN

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

Sabancı University

July 2004

A NOVEL AP2 DOMAIN TRANSCRIPTION FACTOR FROM *Lycopersicon esculentum*, FUNCTIONS IN TOBACCO MOSAIC VIRUS PATHOGENESIS

APPROVED BY:

Assoc. Prof. Zehra Sayers …….

(Dissertation Supervisor)

Dr. Damla Bilgin …….

(Co-Supervisor)

Prof. Meral Yücel …….

Dr. Alpay Taralp …….

Assistant Prof. Cengiz Yakıcıer …….

DATE OF APPROVAL: …….

ABSTRACT

In this work, one of the established plant defense proteins namely that corresponding to the class EREBP-like transcription factors containing the AP2 domain was examined to identify a possible role in Tobacco Mosaic Virus Pathogenesis. In particular, a putative AP2-domain EREBP-like transcription factor –JERF1- from tomato cDNA was subcloned into various expression vectors in order to test for its potential role in virus response in *Solanacaea.* The gene silencing experiments, in which death was observed subsequent to silencing of the *JERF1* gene and TMV infection, have shown that this particular gene might be involved in the TMV infection cycle in *Solanacaea*. The results of a yeast mating assay conducted in this study has also implied that JERF1 is an interactor of plant $P58^{IPK}$.This possibility would in turn suggests that JERF1 may be involved in plant-virus interaction. Overexpression, yeast-protein expression, and GFP-fusion constructs have also been prepared to support functional analyses of this putative transcription factor. It would follow to reason that this study of the recently sequenced JERF1 describes the first attempt to understand the function of this protein in plant-virus interaction.

ÖZET

Bu çalışmada, EREB protein benzeri transkripsiyon faktörlerine karşılık gelen, AP2 alanına sahip olan ve bitki savunma proteinlerinden olan bir protein, Tütün Mozaik Virüsü (TMV) patojenesisindeki muhtemel rolünün tanımlanması için incelenmiştir. Özellikle, AP2 alanına sahip EREB protein benzeri bu transkripsiyon faktörü-JERF1-domates cDNA'sından klonlanmış ve virus duyarlılığında ya da dirençliliğinde rolünü bulabilmek için değişik vektörlere takılmıştır. *JERF1* geninin susturulduğu ve tütün mozaik virüsünün enfeksiyonunun yapıldığı gen susturulması deneyleri, bu genin *Solanacea* ailesinde TMV enfeksiyon döngüsünde görev aldığını gösterir. Maya çiftleştirmesi deneyleri de JERF1 proteininin bitki $P58^{IPK}$ proteini ile etkileştiğini göstermiştir ve bu JERF1'in bitki virus etkileşiminde yer alabileceğini belirtir. Ayrıca, bu transkripsiyon faktörünün fonksiyonal analizi için bu gen, overekspresyon, maya-protein ekspresyonu ve GFP-bileşik vektörlerine takılmıştır. Bu çalışma, yakın zamanda dizisi belirlenen JERF1 proteininin, bitki-virus etkileşimindeki işlevini tanımlamak için atılan ilk adımı oluşturmaktadır.

To my family with all my heart,

Rest & be thankful in a world where nothing is random…

ACKNOWLEDGEMENTS

I am grateful to Prof. Dr. Zehra Sayers for her supervision and to Dr. Damla Bilgin for her endless support, advise and help throughout this study.

I also would like to express my appreciation to Dr. Alpay Taralp who has taught me how to think, analyze and criticize in the way paving through being a scientist. My special thanks go to Dr. Metin Bilgin who has given me new inspirations when I was stuck during my study. I also would like to thank to Dr. Fahriye Ertuğrul and Dr. Sedef Tunca who have taught me how to conduct a scientific experiment and helped me with my research.

My deepest appreciation goes to my parents Belgin and Besim Ömer Dartan, my aunts, my uncles, my cousins and my grandparents who have gifted me my life and personality.

Moreover I would like to thank to my sisters Didem Demirbaş, Funda Güngör, Gözde Vrana, Işın Nur Cicerali, Senem Tuğ and Tuğçe Güleryüz for their valuable friendship during my undergraduate and graduate education.

Thanks also go to my colleagues Çetin Baloğlu, H.Ümit Öztürk, Kıvanç Bilecen, Özgür Gül and Özgür Kütük for their help in this study.

I also would like to offer my sincere appreciation to my dearest SU GIRLS- Burcu Kaplan, Elanur Şireli, E. Süphan Bakkal, Filiz Dede and Yasemin Türkeli- for their encouragement and friendship throughout my study. Finally, I would like to express my intense gratitude to my friend A. Barış Karagözler without whom I would not bring about this thesis.

TABLE OF CONTENTS

ABBREVIATIONS

Aba : Absisic Acid

ADR : Activated disease resistance

AP2 : Apetala2

ARC : Apoptosis gene products,Resistance, CED-4

Avr : Avirulence

CC : Coiled coil

DRE : Drought-responsive element

dsRNA : Double-stranded RNA

EREBP : Ethylene-responsive element binding protein

ERF : Ethylene-responsive factor

GDP : Guanosine diphosphate

GTP : Guanosine triphosphate

HR : Hypersensitive response

IFN : Interferon

IL : Interleukin

JA : Jasmonic Acid

LRR : Leucine-rich-repeat

MAPK : Mitogen-activated protein kinase

MAPKKK : Mitogen-activated protein kinase kinase kinase

NBS : Nucleotide-binding-site

PCD : Programmed cell death

PKR : Interferon-induced protein kinase

PR : Pathogenesis-related

PVX : Potato virus X

R : Resistance

RBM : Regulatory domain-binding motif

ROS : Reactive oxygen species

Rp : Receptor protein

SA : Salicylic Acid

SAR : Systemic acquired resistance

SIPK : Salicylic Acid-inducible protein kinase

SOD : Superoxide dismutase

ssRNA : Single-stranded RNA

TEV : Tobacco etch virus

TF : Transcription factor

TIR : Toll- and Interleukin like receptor

TM : Transmembrane

TMV : Tobacco mosaic virus

TPR : Tetratricopeptide repeat

WIPK : Wound-inducible protein kinase

LIST OF FIGURES

1 INTRODUCTION

Several proteins have been shown to be involved in plant defense systems opposing biotic and abiotic stresses. One subgroup of these proteins is the AP2 domain containing EREBP-like transcription factors and moderate responses by regulation of various signaling pathways in plants. The AP-2 domain is unique to plants and found to be evolutionarily conserved in *Solanacaea* and *Brassicaceae.* Besides the role of AP2-domain transcription factors in biotic and abiotic stress resistance, they are found to be playing a role in developmental and metabolic processes of plants (Reviewed by Zhang, 2003).

A recently sequenced putative transcription factor has been found to contain an AP2 domain and named as JERF1. This putative transcription factor might be functioning as Jasmonic acid and Ethylene Response Factor (Huang *et al.*).

A partial clone of JERF1 has been found to be interacting with plant $P58^{IPK}$ (Bilgin D.D, unpublished data). This implies that JERF1 might be involved in plant-virus interaction. However, there has been no other published data about the role of this putative transcription factor.

In order to obtain functional information about this putative transcription factor, it has been subcloned into various expression vectors. The silencing, overexpression, protein-protein interaction and GFP-fusion protein expression constructs prepared in this work have been used for functional analyses of this putative transcription factor. Silencing experiments in which death was observed in TMV infected nn *N.benthamiana* plants succeeding *JERF1* silencing implies that JERF1 might be involved in TMV susceptibility of plants.

2 OVERVIEW

Plant diseases are the most important obstacles in agriculture. In 1994, Brears and Ryals have estimated that the worldwide crop loss due to plant diseases have exceeded $ 100 billion. Increased human populations and the demand for food bring about the extensive use of chemical pathogen control. Applications of fungicides and pesticides help control plant diseases but chemical control is not only economically costly but also environmentally undesirable. Therefore, more effective utilization of natural genetic disease resistance mechanisms with novel and environmentally friendly active ingredients for chemical control has been of much concern for research. This results in the study of plant diseases and resistance mechanisms in a much more detailed manner and development of new strategies based on plant's own defense mechanism for disease control.

Plants are sessile organisms and they have to survive in various environmental conditions during growth and development. Plants use various environmental signals such as light, temperature and water availability in order to regulate their normal growth, germination and flowering. However, severe conditions such as drought, low temperature, heat, high salinity of the soil or flooding have adverse effects on plant growth and development and constitute the abiotic stresses. Plant gene expression levels change in response to different environmental signals. Plants perceive the environmental signal and start a cascade of events finally altering gene expression and physiological responses (Shinozaki and Dennis, 2003) (See Fig: 2.1).

Fungi, bacteria, nematodes, insects and viruses create biotic stress in the plants by using their photosynthetic products, replication machinery etc. Plants have evolved highly sophisticated species and pathogen specific response mechanisms to perceive the attacks from pathogens and in turn give an adaptive response. For the past century, it is

known that plants own genetically inherited resistance mechanisms to combat phytopathogenic fungi, bacteria and viruses, etc. However, with the development of molecular biology, the understanding of the basis of these relationships has improved significantly.

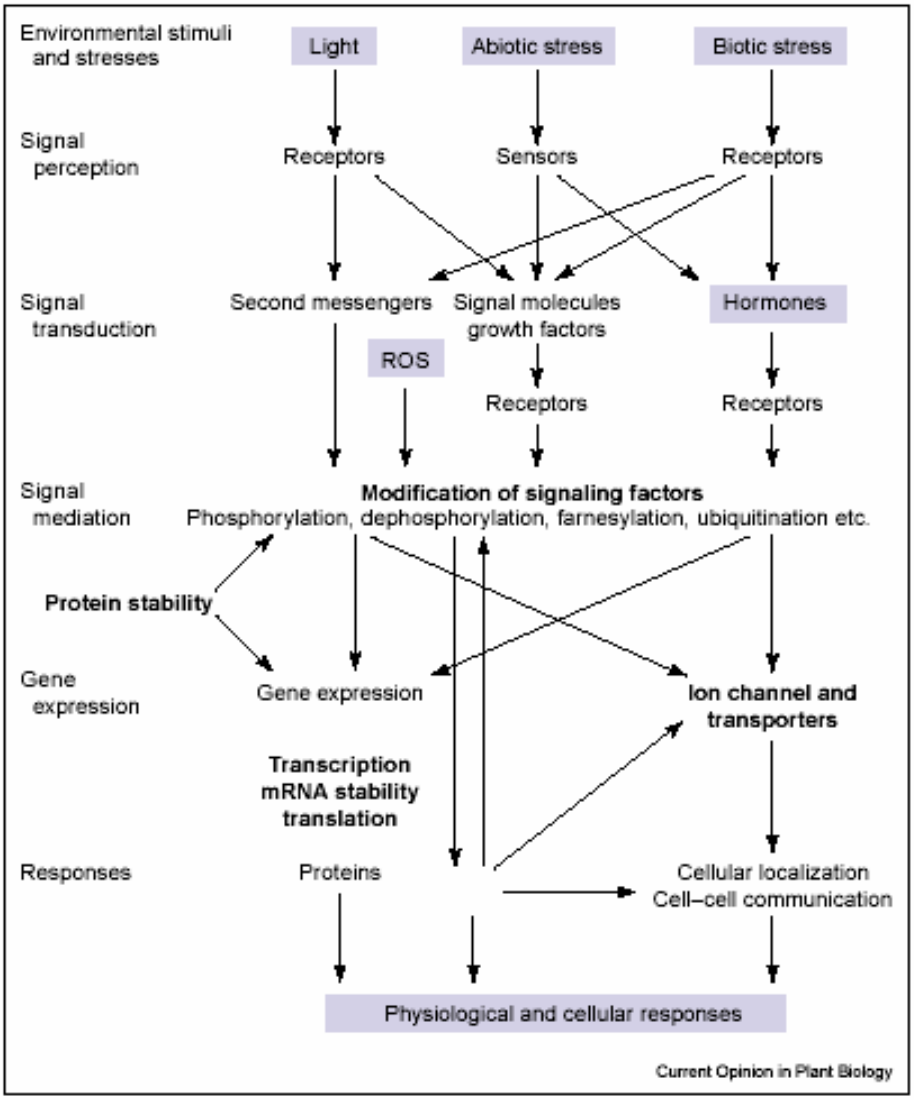


Figure 2.1: Schematic description of signal transduction in plants (Shinozaki and Dennis, 2003)

Combined genotypes of host and pathogen govern the plant resistance and susceptibility that are dependent on a complex exchange of signals and responses occurring under specific environmental conditions. The long process of host-pathogen co-evolution has made plants develop various elaborate mechanisms to avoid pathogen

attack. Defense mechanisms may be preformed, such as the physical and chemical barriers to hinder pathogen infection, or induced only after pathogen attack. Induced plant defense responses include a network of signal transduction and rapid activation of gene expression following pathogen infection just like animal immune responses (Yang *et al*., 1997).

2.1 Signal perception and transduction in plant defense responses

Plants can be divided into two categories according to the response that they administer upon a pathogen attack. Plant's nomination as "Resistant" or "Susceptible" depends on the timely recognition of the invading pathogen and rapid and effective activation of host defense mechanisms. A plant is referred as "Resistant" if it is capable of rapidly invoking a wide variety of defense responses in order to prevent pathogen colonization. On the other hand, a "Susceptible" plant is generally severely damaged or even killed by the pathogen infection. Pathogen-encoded molecules that are able to activate defense responses in plants when recognized by the host are called "Elicitors". The interaction of pathogen elicitors with host receptors activate a signal transduction cascade that might include protein phosphorylation, ion fluxes, reactive oxygen species (ROS) generation and other signaling events (Yang *et al*., 1997).

The resistance (R) genes encode the receptors for recognition of specific elicitors or ligands. The potential damage that can be caused when a plant is infected by a pathogen can be limited by the recognition of the pathogen. The interaction between a dominant avirulence (*avr*) gene in the pathogen and the corresponding dominant resistance (*R*) gene in the host is explained by the so-called "gene-for-gene interaction". This model proposes that the activation of defense responses requires the expression of a matching pair plant R gene and pathogen avr gene (Flor, 1971).

2.1.1 Resistance Genes and Resistance Protein Function

Plant-pathogen interaction is provided by specific interactions between pathogen avr gene loci and alleles of corresponding plant disease resistance (R) locus. Disease resistance occurs when the avr and corresponding R genes are present in both host and pathogen. On the other hand, disease results when either is absent or inactive. Recognition of avr-dependent signals by R gene products and initiation of the signal-transduction events that activates defense mechanisms and arrest of the pathogen growth is the simplest model accounting for the genetic interaction between avr and R genes. It has been found that functional R genes isolated so far demonstrate resistance to a wide range of pathogen taxa including bacteria, viruses, fungi, nematodes, oomycetes and even insects. However, R gene products share striking structural similarities. This suggests that certain signaling events are held in common in plant defense (Reviewed by Dangl and Jones, 2001and Martin *et al*., 2003).

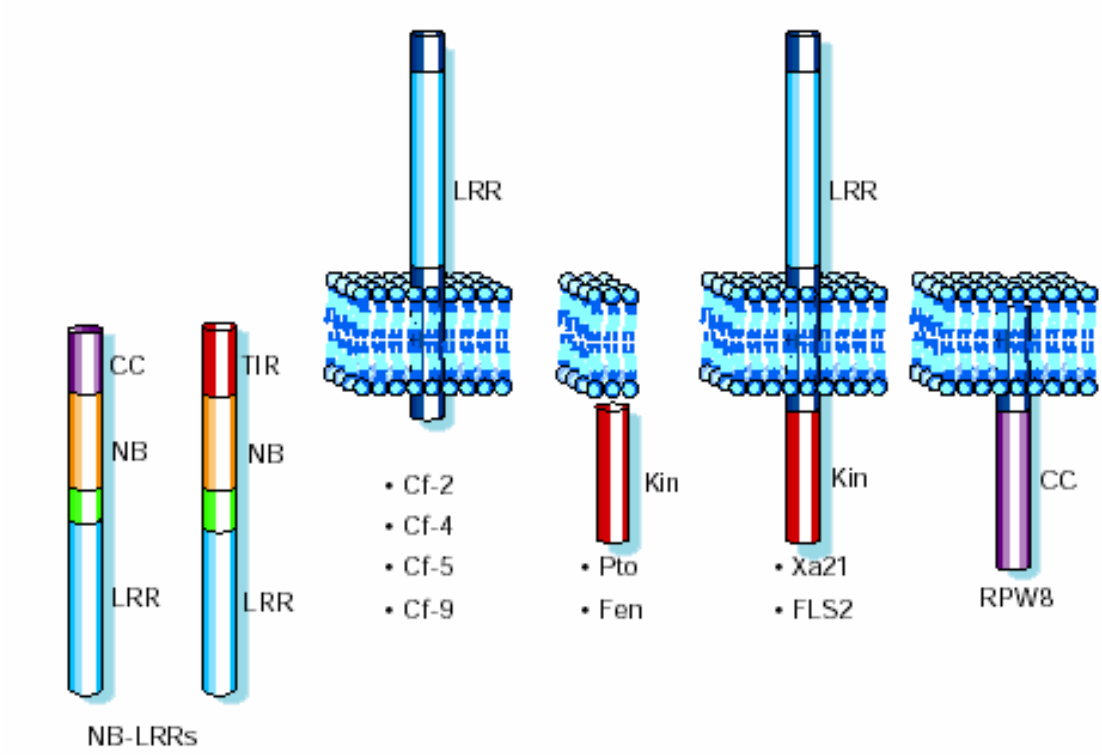


Figure 2.2: Representation of the location and structure of the five main classes of plant disease resistance proteins. (Dangl and Jones, 2001)

:

Numerous R genes have been cloned from several plant species and they can be grouped into 5 classes according to the protein they code (See Fig: 2.2). The first class encodes a cytoplasmic receptor-like protein that contains a Leucine-rich-repeat (LRR) domain and a nucleotide-binding site (NBS).

C-terminus of NBS domain is found in APAF-1 and CED-4 proteins, which have a role in programmed cell death among animal cells, and is called as ARC (apoptosis, R gene products and CED-4) (Van der Biezen and Jones, 1998). There is an N-terminal sub-domain containing a consensus kinase 1a (P-loop), kinase 2 and kinase 3 motifs which are found in a large variety of nucleotide-binding proteins. This suggests that R proteins might control plant cell death by their NBS- ARC domains when activated via LRR-dependent recognition of the plant pathogen.

The NBS-LRR group can be subdivided into two. The first subgroup contains a TIR (Toll-IL-1R homology region) domain that shows homology to cytoplasmic domains of the Drosophila developmental gene Toll and the mammalian immune response gene encoding the interleukin-1 receptor (IL-1R). Drosophila Toll and mammalian and avian IL-1R not only show structural similarities but also share homologous downstream signaling components including adaptor proteins, kinases and transcription factors. Toll and IL-1R are membrane-bound proteins that have role in innate cellular resistance responses of animals. (Lemaitre *et al*., 1996; Medzhitov *et al*., 1997) Upon binding of ligand to the extracellular domains of Toll and IL-1R, the intracellular domains of these receptors activate the signaling pathway in order to avoid the pathogen attack. (Volpe *et al*., 1997; Yang and Steward, 1997). R proteins containing N-terminal TIR domains are; *N* gene from tobacco (Whitham *et al*., 1994), L6 of flax (Lawrence *et al*., 1995), RPP, RPP10, RPP14 (Botella *et al*., 1998), RPP5 (Parker *et al*., 1997), RPS4 (Gassmann *et al*., 1999) and M (Anderson *et al*., 1998) from *Arabidopsis thaliana*. LRR domain is present in many proteins of diverse function and is found to be involved in protein-protein interactions (Kobe and Deisenhofer, 1994). Domain swaps and TIR and NBS comparisons of flax rust resistance L gene have shown that L regions are involved in recognition specificity of the R protein with its effectors (Ellis *et al*., 1999; Luck *et al*., 2000).

The second subgroup contains putative coiled-coil domains (CC-NB-LRR). The CC structure is a repeated heptad sequence has interspersed hydrophobic amino acid residues. There are two or more alpha helices whose interaction form a super coil and is thought to function in protein-protein interactions, oligomerization and oligomerization-dependent nucleic acid binding (Reviewed by Martin *et al.*, 2003). Reconstruction of a functional Rx protein by co-expression of CC-NBS domain with LRR region and CC domain with NBS-LRR show that intramolecular interactions are important for response. Upon pathogen recognition, conformational changes in Rx occur that cause the disruption of intramolecular interactions and initiate the signaling cascade (Moffett *et al*., 2002).

The third class proteins encode a serine-threonine kinase with homology to mammalian Raf, IRAK and Drosophila Pelle kinases in IL insensitive response pathways. *Pto* gene from tomato is an example for this group (Sessa *et al*., 2000). Pto kinase confers resistance to strains of *Pseudomonas* bacteria that express *avrPto* that directly interacts with Pto kinase. It has been found that *Prf* gene located in *Pto* gene cluster is also required for Pto-specified responses. The function of Prf is not exactly known but is supposed to be guarding the Pto upon avrPto interaction and activating the host defense. Pto has been found to interact with other protein kinases such as Pti1 and Pti 4/5 and 6 transcription factors which have sequence similarity to ethylene-responsive factors (ERFs) and regulate expression of PR proteins (Reviewed by Pedley and Martin, 2003)

The fourth class encodes a putative transmembrane receptor with an extracellular LRR domain and an intracellular serine-threonine kinase domain. *Xa21* gene from rice is an example for this type of *R* genes (Song *et al*., 1995)

The newest found *R* gene forms the fifth group that encodes a small, probable membrane protein with a possible coiled-coil domain and essentially no other homology to known proteins. First and third group of *R* genes lack transmembrane (TM) domains and are thought to be localized intracellularly.

Few R proteins do not fit into these five classes. *Hs1pro-1* nematode resistance gene from sugar beet has been found to represent a novel resistance gene class (Ellis and Jones, 1998).

It is suggested that R proteins in general colocalize with their pathogen effectors. All of the R proteins belonging to group 1 and their effectors are found to be associated with the plasma membrane. Effectors of the second group R proteins carrying transmembrane and extracellular LRR domains are found to be extracellular. Since localization of R proteins depends on the effectors localization, R proteins that recognize more than one effectors may localize to more than once subcellular location and translocation of some R proteins may take place during signaling (Reviewed by Martin *et al*., 2003).

Due to the low abundance of R proteins in plant cells the patterns of protein-protein interactions of R protein-mediated recognition of effectors are largely unknown. An important model for these interactions is named as "guard" hypothesis whose essential concept is the recognition of an effector-taking place indirectly as recognition of an interaction of that effector with a target of its virulence function (See Fig: 2, 3). When guard is absent, plant defense is somehow down-regulated by interaction of the effector and its target. This releases nutrients to the apoplast or contributes to pathogenesis. For plant- pathogen systems in which guard model can apply, new members of R protein recognition complexes might be uncovered by identification of host targets or virulence factors.

The second model is the simplest model interpreting the gene-for-gene hypothesis. In this model R protein and Avr protein directly interact and activate the defense mechanism. However, there has been no evidence showing the direct interaction of effectors with R proteins.

In the third model named as Bridge model, binding of the effector independently to the R protein and to a third protein recruits one to another. Downstream signaling for defense is activated by the effector-dependent interaction of the two proteins. In order to

confirm this model, interaction of the effector with both plant proteins by means of distinct domains has to be shown.

The fourth model is called the Matchmaker that proposes that effector induces direct interaction between the R protein and a third protein by a conformational change in one or both of the proteins.

In Affinity Enhancement model the interaction of the effector with the R protein, a third protein or both stabilize a pre-existing, weak interaction between the two plant proteins. The downstream signaling is then activated by the increased abundance of the complex and defense response is induced.

The Derepression model proposes that the effector disrupts an interaction of the R protein and the effector derepresses a third protein that negatively regulates the activity of the R protein and by this way defense response. In order to apply this model to a given system, there must be the interaction of the R protein and a third protein and down regulation or mutagenesis of the third protein should activate defense in the absence of the effector.

In the last model, which is called the Dual Recognition, model independent interactions between the effector and the R gene and the third protein are required for the resistance (Reviewed by Martin *et al*., 2003)

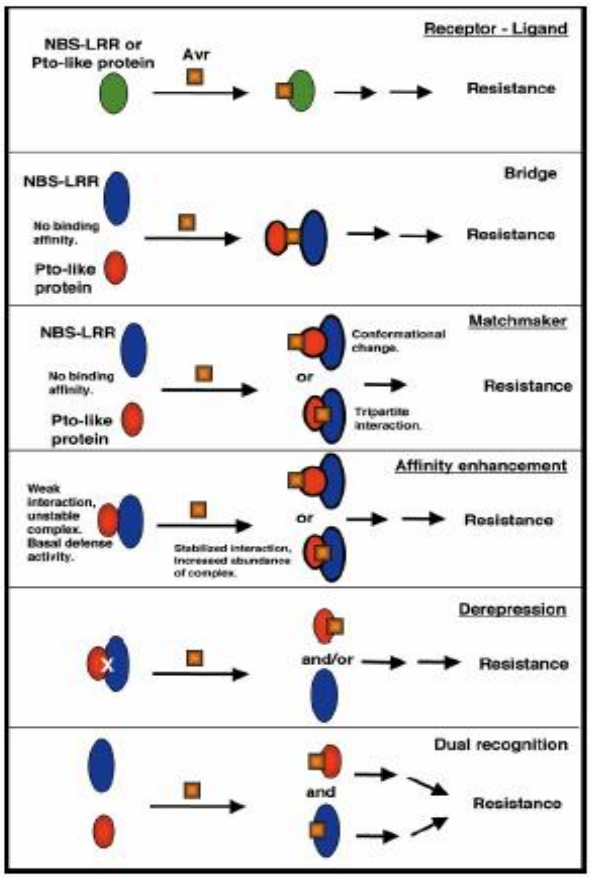


Figure 2.3: Models for protein-protein interactions that might underlie plant-pathogen"gene-for-gene" recognition. Models that encompass interactions that could be consistent with the "guard" hypothesis are underlined (Martin *et al*., 2003).

2.2 Resistant and Susceptible Host Responses

Upon recognition of an avirulence protein, a signal transduction cascade that leads to the induction of a number of plant defenses, which either directly or indirectly inhibit pathogen growth and multiplication, is activated. These defenses involve the hypersensitive response (HR), ROS generation, cell wall fortification, benzoic acid and salicylic acid accumulation, pathogenesis-related (PR) and other defense related protein induction, lipoxygenase enzyme activity increase and phytoalexin accumulation (Hammond-Kosack and Jones, 1996) (See Fig:2.4).

Within minutes of pathogen attack, local plant defense responses are activated. Sometimes defense responses also arise in tissues far from the invasion site and even in

neighboring plants within hours of infection. Pathogens activate systemically a specific subset of PR (Pathogenesis-related)-type genes by a mechanism known as "Systemic Acquired Resistance" (SAR). In order to confer SAR, necrotic lesions must form due to the initial infection as a part of Hypersensitive Response (HR) or as a symptom of disease (Buchanan, Gruissem, Jones, 2000). Although the exact mechanisms are not known in detail, lignifications, induction of PR proteins and conditioning are the basic mechanisms involved in SAR. Lignification causes the strengthening of cell walls so that plant cells become more resistant to enzymes of pathogens. During SAR, *PR-1, PR-2* and *PR-5* transcripts were found to be accumulated in Arabidopsis thaliana and tobacco. PR proteins which are activated during SAR are different than the ones in HR and are therefore named as systemically induced (SAR) proteins. Although the exact function is not known, plants whose PRs are activated have shown increased resistance to pathogens. Several studies have shown that pathogen pretreated plants react more rapidly and more efficiently to a challenge by second infection. In these manners, SAR similar to immunization in mammals, is specific for broad range of pathogens, and is important as much as the innate immune response of plants (Reviewed by Sticher *et al*., 1997).

Pathogen infection is generally unlikely to results in a diseased plant. There are four main reasons for failure of pathogens to infect plants successfully. These are: pathogens are generally recognized in the plant as nonhost which prevents the support of the life-strategy requirements of the pathogen; nonhost resistance of the plant which possess a preformed structural barrier or toxic compound oppose the pathogen infection; defense mechanisms activated upon recognition of the attacking pathogen and environmental changes that kill the pathogens whereas plants have already adapted to these changes and can survive. As mentioned before, pathogen infection can be sensed by the plant, which subsequently activates its defense responses. One of the most important responses given by the plant is the rapid activation of defense reactions in association with host cell death and called the "Hypersensitive Response (HR)". Since dead cells contain high levels of antimicrobial, antifungal and antiviral molecules they are not subsequently attacked by pathogens anymore. Moreover, protective secondary metabolites can also be synthesized and cell walls can be reinforced around the HR site so that pathogen is controlled at the area of infection. HR is programmed genetically in

the plant and is a consequence of new host transcription and translation (Buchanan, Gruissem, Jones, 2000).

One of the best-characterized plant response mechanisms upon a pathogen infection is Pto-mediated resistance to bacterial speck disease in tomato caused by the bacterial pathogen, *Pseudomonas syringae* pv.tomato. In this model, a transcription factor Pti4 is activated when the pathogen attacks the plant (Gu *et al*., 2000). Pto kinase then phosphorylates the available Pti4 that facilitates its localization into nucleus, DNA binding and interaction with other transcription factors that activate the pathogenesis-related (PR) proteins that control the defense responses (Gu *et al*., 2002).

The final step of activated events is the death of a single cell or group of cells, even sometimes death of the whole organism in order to prevent the pathogen growth and multiplication. In 1965, Lockshin and Williams were the first to mention the phrase "programmed cell death (PCD)" to describe the activation of suicide pathways in response to external or internal stimuli. Then in 1972, Kerr *et al*. gave the name "apoptosis" in which a distinct morphology was observed in programmed cell death (PCD) mechanism. Currently the term apoptosis is the name given to death of an animal cell that results from genetically ordered series of physiological and morphological events. On the other hand, another death type known as "necrosis" occurs in response to injurious environmental stimuli and is not genetically controlled (Birch *et al*., 2000). Morphological features of apoptosis in mammals include the chromatin and cytoplasm condensation, nuclear and cellular convolution, cell shrinkage, nucleus disintegration, DNA fragmentation, apoptotic body formation and phagocytosis of apoptotic bodies. (Reviewed by Birch *et al*., 2000).

A cell suicide pathway is also found to be activated upon recognition of an invading pathogen in plants. This pathway is thought to be involved in defense mechanisms against infection. *AvrRpt2* gene containing *Pseudomonas syringae* has been found to interact with *Rps2* gene of *Arabidopsis thaliana* triggering the coordinated activation of cell death and defense mechanisms including activation of PR proteins (Mittler *et al*., 1997).

Plants have been found to confer similar morphological features of apoptosis in animals. Nuclear fragmentation and formation of membrane-bound structures similar to apoptotic body formations of animals have been observed in response to tomato toxin (Wang, 1996). Moreover, cytoplasm and nucleus condensation was observed in tobacco plants during PCD upon virus infection (Mittler *et al*., 1997).

Similar to mammals, cysteine proteases have been identified in cell death responses in development of plants (Jones *et al*., 1996). The presence of similar NBS domains in the proteins Apaf-1 and R genes (Van Der Biezen and Jones, 1998); and detection of a Bcl-2 homologue localized into mitochondria, chloroplasts and nuclei in tobacco (Dion *et al*., 1997) proposes that similar programmed cell death mechanisms can be involved in defense mechanisms of both plants and animals.

2.2.1 Similarities of PCD between Plants and Animals

Protein phosphorylation in which Mitogen-activated protein kinase (MAPK) cascades are found to be important in apoptosis of animal cells (Jarpe *et al*., 1998). R gene also has been found to contain a serine-threonine kinase domain which can be used for phosphorylation of downstream transcription factors and PR proteins (Zhou *et al*., 1997). Salicylic acid inducible protein kinase (SIPK) a MAPK activated by salicylic acid has been shown to be activated by Tobacco mosaic virus (TMV) infection in plants (Zhang and Klessig, 1998). Another MAPK from tobacco called WIPK- wound inducible protein kinase- is also found to be induced by TMV (Zhang and Klessig, 1998) show that MAPK are also found to be important in PCD of plants.

Increases of extracellular Ca+2 levels in response to pathogen attack (Suzuki *et al*., 1995; Levine *et al*., 1996) suggests that Ca+2 plays an important role in signaling defense responses leading to cytochrome c is released from mitochondria, which is the universal feature of apoptosis in mammals (Krebs,1998). Cytosolic calcium levels have an impact on signaling cascades by activating protein kinases and protein phosphatases to promote modification of proteins involved in PCD of plants (Buchanan, Gruissem, Jones, 2000).

Another striking feature of apoptosis is the accumulation of radicals such as $O^{2.-}$ and H_2O_2 that are known as reactive oxygen species (ROS) that leads to the oxidative burst of the cells in order to prevent pathogen growth. $O^{2.-}$ and H_2O_2 have been detected in tobacco leaves when infected with TMV and necrotic lesions were induced in these plants (Doke and Ohashi, 1988).

2.2.2 Secondary Signaling Molecules in Plant Defense Responses

Secondary signaling molecules including Salicylic acid (SA), ethylene, and jasmonic acid (JA) have been found to be involved in plant defense responses (Reviewed by Yang *et al*., 1997). NahG phenotype of tobacco and Arabidopsis thaliana plants which has lost the ability to accumulate SA have been shown to exhibit poor induction of PR genes and have been more susceptible to normally avirulent pathogens (Gaffney *et al*., 1993; Delaney *et al*., 1994). Jasmonate has been implicated in plant responses to wounding and insect feeding (Reymond *et al*., 2000). The perception and signal transduction of Jasmonate is not fully known yet. COI1 gene encoding an F-box protein is the only cloned defense regulator gene in Jasmonate response (Xie *et al*., 1998).

Ethylene perception and signal transduction is the best-known pathway in plant growth and development. Ethylene receptors have been found to be similar to bacterial two-component histidine kinase receptors. When ethylene is absent, the downstream negative regulator CTR1 that functions as a MAPKKK represses ethylene receptors ETR1, ERS1, ETR2, EIN4 and ERS2. Binding of ethylene inhibits receptor activation of CTR1 by inhibition or promotion of histidine autophosphorylation. Absence of CTR1 activates C-terminus of another protein called EIN2 whose subcellular location is not known yet. Activation of EIN2 then activates several transcription factors, which are either ethylene-responsive element binding proteins (EREBP) or ethylene-response-factors (ERF) that bind to the GCC box promoter element of ethylene-regulated genes (Reviewed by Chang and Shockey 1999).

Potentiation experiments that imply the increase of magnitude and kinetics of defense responses associated with different pathways suggest that there might be a cross talk between SA, ET and JA signaling. ET has been found to potentiate the SA-mediated induction of PR-1 gene expression in *Arabidopsis thaliana* (Lawton *et al*., 1994), *PR-1* transcripts of tobacco have been superinduced upon treatment of SA, and Methyl JA compared to single effects of these molecules (Xu *et al*., 1994).

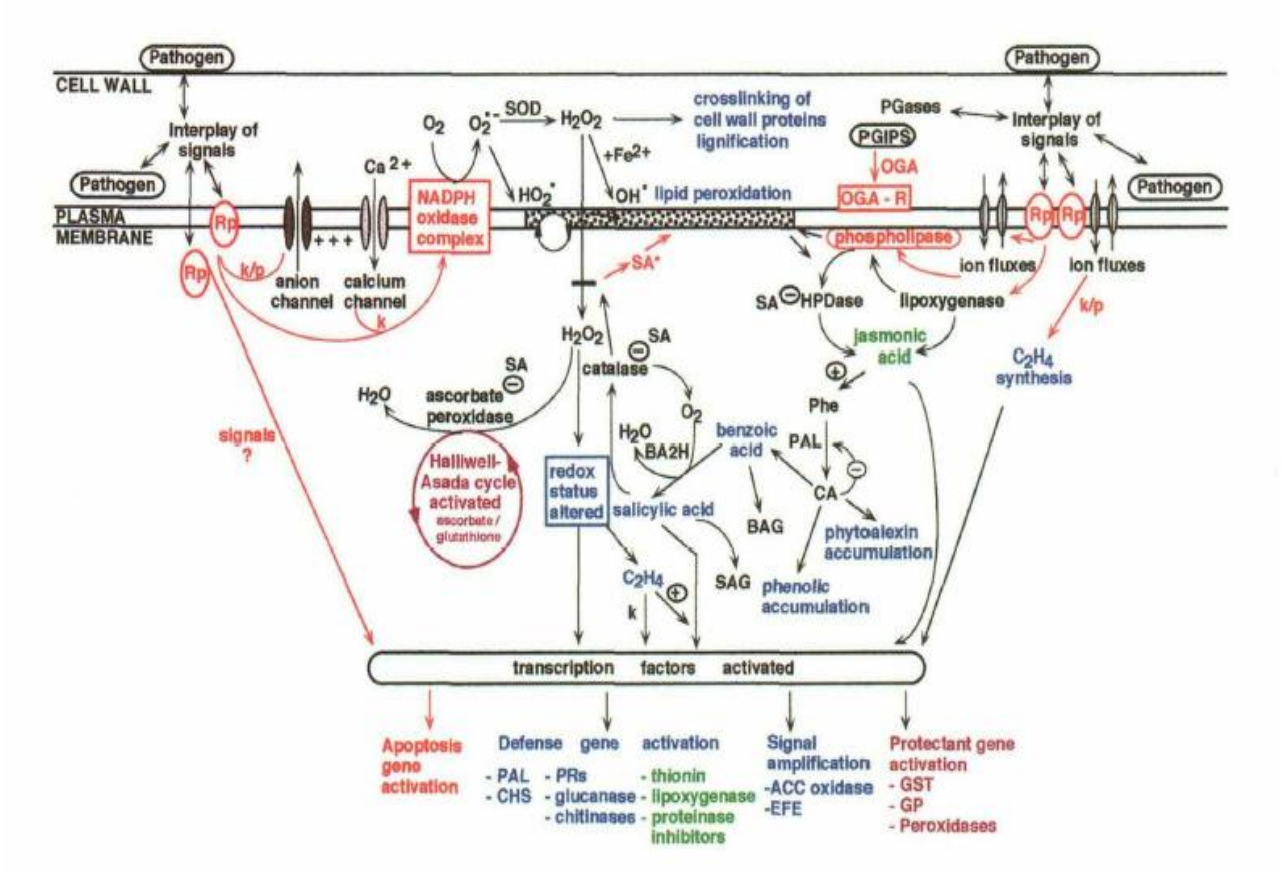


Figure 2.4 Complexity of Signaling Events Controlling Activation of Defense Responses. (Hammond-Kosack and Jones, 1996)

2.3 Viruses and Viral Pathogenicity

As being simple, acellular viruses are a unique group of infectious agents. A complete virus particle consists of one or more molecules of DNA or RNA but not together at the same time (except human cytomegalovirus containing a DNA genome and four mRNAs); carbohydrates; lipids and additional proteins. In extracellular phase

(called virions), viruses cannot reproduce independent of living cells. They exist primarily as replicating nucleic acids that induce host metabolism to synthesize virion components in the intracellular phase and eventually complete virus particles or virions are released from the host.

All virions are constructed around a nucleocapsid core which is composed of a nucleic acid (either DNA or RNA) and held within a protein coat called the capsid.

Viruses can employ all four possible nucleic acid types: single-stranded DNA, double-stranded DNA, single-stranded RNA and double-stranded RNA. Plant viruses generally have single-stranded RNA genomes. Most RNA viruses contain single-stranded RNA (ssRNA) as their genetic material. The RNA strand is called the plus strand or positive strand when RNA base sequence is identical with that of viral mRNA, called minus or negative strand when the viral RNA genome is complementary to viral mRNA. Most of the RNA viruses are known to contain segmented genomes meaning that the genome is divided into fragments each coding for one protein.

An outer membrane layer called an envelope bound many viruses. Sometimes envelope proteins might project from the envelope surface as spikes or peplomers. These spikes are thought to be involved in virus attachment to the host surface.

Although viruses lack true metabolism and can not reproduce independently of living cells they may carry one or more enzymes such as an RNA-dependent RNA polymerase that serves as a replicase and RNA transcriptase, essential to completion of their life cycles (Prescott, Harley and Klein, 2002).

2.3.1 Host Defense Responses against Viruses

Viral infection occurs when the virus enters into the host cell, replicates its genome and moves into the neighboring cells and through the plant by its vascular system (Carrington *et al*., 1996).

Upon viral infection, plants give responses such as hypersensitive cell death response, systemic acquired resistance and gene silencing (Baker *et al*., 1997, Waterhouse *et al*., 2001). A microarray research has shown that invasion of different types of viruses induce genes involved in plant defense including the resistance genes, cell rescue, cell death and ageing, signal transduction such as protein kinases and transcription such as DNA-binding proteins and Transcription factors in *Arabidopsis thaliana* (Whitham, 2003).

Virus interactions with plant resistance genes can best explained by Rx-mediated resistance against potato virus X (PVX). Upon recognition of the PVX, coat protein, which is the elicitor by Rx receptor, mechanisms to suppress accumulation of the virus including Hypersensitive Response start (Bendahmane *et al*., 1995).

One of the most important cellular antiviral responses discovered in mammalian systems is the Interferon-Induced Protein Kinase (PKR). PKR is the critical element of IFN-induced cellular antiviral response. RNA-activated Protein Kinase (PKR) is constitutively expressed in all mammalian tissues at low levels and is composed of an NH2-terminal regulatory domain and a COOH-terminal protein kinase catalytic domain (See Fig:2.5). It has been found that PKR is rapidly activated upon binding double-stranded RNA (dsRNA) via its two NH2 regulatory domain-binding motifs (dsRBM). Upon activation, PKR undergoes conformational alteration and dimerization process that triggers its catalytic activities. PKR is found to be involved in growth factor and calcium-mediated signal transduction, regulation of transcription and induction of apoptosis. However, the most important function of PKR that is targeted for regulation by viruses is the control of mRNA translation initiation mediated through the phosphorylation of translation initiation factor eIF-2α. Binding of viral-encoded or cellular dsRNAs to PKR activates PKR and autophosphorylation following dimerization of PKR takes place. Activated PKR phosphorylates eIF-2α. This blocks eIF-2-B by exchanging GDP with GTP and eIF-2 stays inactive in a complex with GDP. As a result, viral replication is blocked at the level of protein synthesis. PKR is also found to contribute to regulation expression of IFN-inducible genes by phosphorylation of IκB (Reviewed by Gale Jr and Katze, 1998) (See Fig: 2.6).

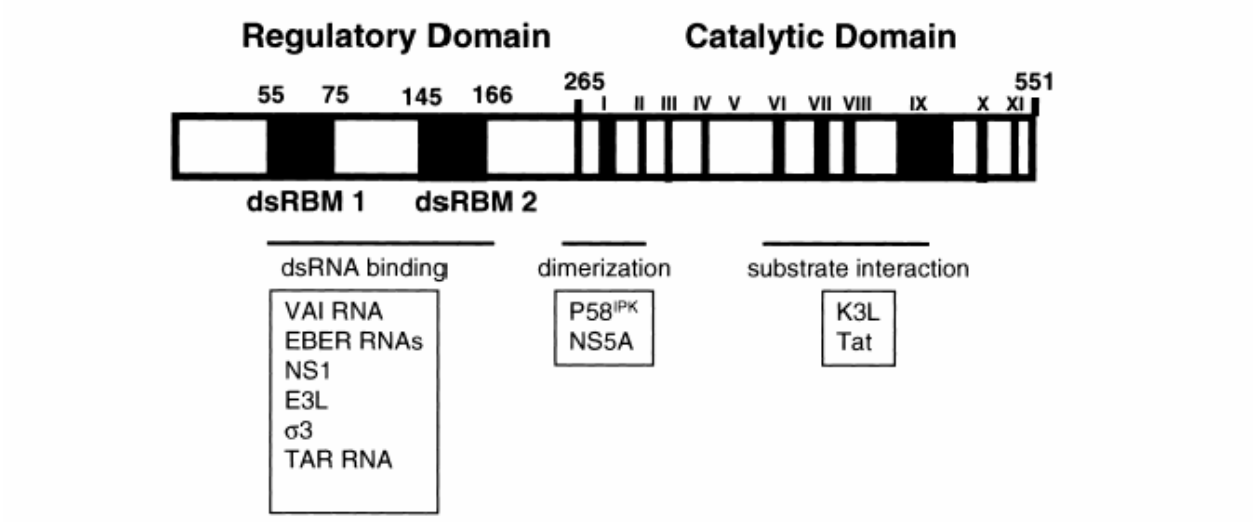


Figure 2.5: Structural representation of mammalian PKR and target sites for viral-directed PKR inhibition (Gale Jr and Katze, 1998)

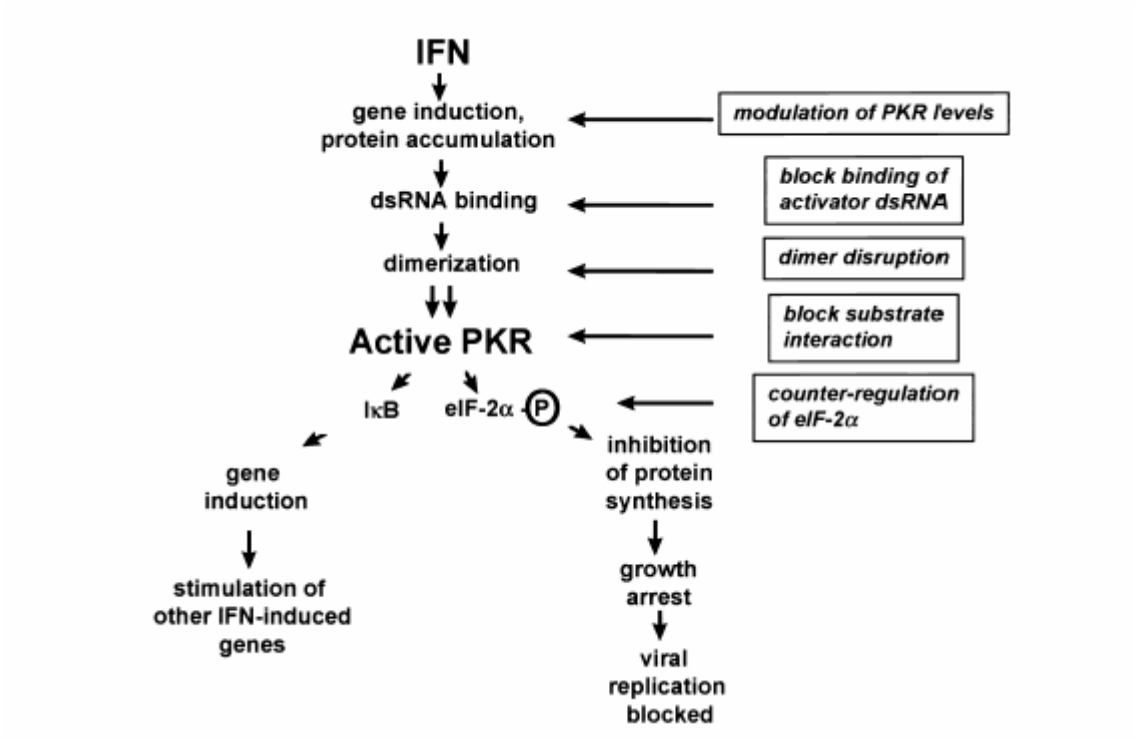


Figure 2.6: Mammalian PKR maturation pathway and sites of viral-directed regulation (Gale Jr and Katze, 1998)

Many viruses have developed strategies to block PKR function in order to avoid deleterious effects upon viral replication due to PKR-mediated eIF-2α phosphorylation. (See Fig: 2.6). These are, inhibitors binding to conserved dsRNA binding domains or sequestering RNA activators and therefore interfering with the dsRNA-mediated activation of PKR; inhibitors interfering with kinase dimerization; inhibitors blocking the kinase catalytic site and PKR-substrate interactions; inhibitors altering the physical levels of PKR and finally inhibitors regulating eIF-2α phosphorylation or components downstream from eIF-2α. Between these blocking strategies, influenza virus that results in the activation of inhibitor of PKR (P58IPK) uses the most interesting one. P58IPK is a member of tetratricopeptide repeat (TPR) family of proteins that possess 9 tandemly arranged TPR motifs known to mediate homotypic and heterotypic protein-protein interactions. P58IPK is found to be constitutively expressed but resides in an inactive complex with specific inhibitory molecules (I-P58IPK) in uninfected mammalian cells. Influenza virus infection disrupts the P58IPK/ I-P58IPK complex and activates P58IPK. Activated P58IPK forms a complex with PKR and results in inhibition of both PKR phosphorylation and activity (Reviewed by Gale Jr and Katze, 1998).

Biochemical and immunological comparisons have shown a cytosolic and ribosome associated protein named as pPKR similar to mammalian PKR is present in plants (Langland *et al*., 1995). Another study showing wheat eIF2α can act as a substrate for PKR and functionally interacts with mammalian eIF2α phosphorylation pathway (Gil *et al*., 2000).

Recently a plant ortholog of P58IPK is found to be functioning in viral pathogenesis in plants. Massive cell death observed in wild-type Nb P58IPK silenced *N.benthamiana* plants when challenged with Tobacco mosaic virus (TMV) and tobacco etch virus(TEV) imply that plant P58IPK protein is required for development of viral symptoms since death has not been induced in TMV and TEV infected wild-type *N. benthamiana* and *Arabidopsis* plants. Increased levels of phosphorylated eIF-2α upon viral infection in the NbP58IPK –silenced plants and rescuing of P58IPK –silenced plants by *Bos taurus* P58IPK Bt P58IPK expression from virus infection shows that PKR might function in virus infection of plants like mammalian systems (Bilgin *et al*., 2003). Yeast-two-hybrid screen has shown that plant P58IPK interacts with a

transcription factor which is thought to be involved in virus susceptibility (Bilgin, unpublished data).

2.4 Transcription Factors

Transcription factors are proteins that modulate the rate of initiation of mRNA synthesis by interacting with the promoter regions of target genes through sequence-specific binding of DNA. Transcription factors function in gene transcription regulation depending on the tissue, cell type in response to internal or external signals. Transcription factor activity can be regulated by cellular concentrations of regulatory proteins transcriptionally or by phosphorylation, acetylation, hydroxylation, nitrosylation, gluthathiolation, intra- and intermolecular S-S bridge formation, myristoylation, farnesylation, ubiquitination or glycosylation post-transcriptionally. By this way protein conformations may be altered, interaction with other regulatory proteins can be allowed or subcellular localization can be affected. These changes then affect DNA binding, affinity, activation potential, nuclear localization and /or protein stability (Reviewed by Vom Endt *et al*., 2002). ORFeome analysis of *Arabidopsis* transcription factors have shown that transcription factor genes are highly expressed in vegetative organs, flower organs and germinating seed showing the importance of transcription factors in developmental and metabolic processes (Gong *et al*., 2004). Overexpression analyses have shown that TFs are involved in many processes such as development, hormone action, biotic stress resistance, abiotic stress resistance and biochemistry of plants (Reviewed by Zhang, 2003).

APETALA 2(AP2) is a member of a complex system of genes that regulate *Arabidopsis* flower initiation and development. It plays a central role in the gene network controlling *Arabidopsis* flower homeotic gene expression, flower development and seed coat development. It has been found that AP2 is expressed in nonfloral and floral tissues and organs in *Arabidopsis* and encodes a polypeptide of 432 amino acids containing a 68-amino acid repeated motif called the AP2 domain. AP2 domain is necessary for AP2 genetic functions such as floral meristem identity establishment,

sepal and petal organ identity specification and the spatial control of AG homeotic gene expression. This domain has been found to contain an 18-amino acid conserved core region, which can theoretically form an amphipathic α-helix. Protein-protein interactions are mediated by these amphipathic α-helices through formation of coiled-coil structures. Due to the interactions of AP2 with AP1 and LFY that function in nucleus and role in regulation of AG gene expression AP2 protein is hypothesized to function as a nuclear transcription factor (Jofuku *et al*., 1994).

Several genes that encode AP2 domain containing proteins are found in *Arabidopsis* and tobacco and characterization of these genes have revealed that AP2 domain has been evolutionarily conserved in both Arabidopsis and tobacco. There has been found to be two subfamilies of AP2 domain containing proteins in Arabidopsis designated as AP2-like and EREBP-like and both proteins have been found bind to DNA in a sequence specific manner. It is also shown that AP2 domain is sufficient for binding of EREB-like proteins to DNA.

Expressions of EREB-like proteins in floral and vegetative tissues, indirect or direct regulation of gene expression by AP2 during reproductive and vegetative development and presence of AP2-domain containing proteins in maize, rice, castor bean and several members of the *Brassicaceae* strongly suggests that AP2 domain is an important and evolutionarily conserved element necessary for the structure and function of EREB-like and AP2 like proteins (Okamuro *et al*., 1997)

Induction of aba (Absisic acid-deficient) and abi (Absisic acid-insensitive Arabidopsis mutants) genes by drought, salt and cold suggests the existence of an Absisic acid (ABA)-independent pathway leading to rapid responses to drought or cold. This pathway functions through members of the AP2/EREBP family of transcription factors that recognize the drought-responsive element (DRE) in their target promoters. Many of the AP2/EREBP transcription factors are found to be functioning in plant development and hormone-dependent gene expression. AP2/EREBP family proteins contain a highly conserved region of about 60-70 amino acids called the AP2 domain which makes them unique to plants. AP2 domain with its 2 regions : a 20 amino acid long N-terminal stretch rich in basic and hydrophilic residues (YRG element) and a C-

terminal sequence that can form an amphipathic α-helix (RAYD element) is a new type of DNA-binding module. YRG element is thought to be the element responsible for DNA binding and RAYD element is believed to contribute to DNA interaction or mediate protein-protein associations (Reviewed by Kizis *et al*., 2001).

A number of common cis-acting elements and their cognate binding factors have been identified by functional dissection of PR (pathogen-responsive) gene promoter regions. Several of the PR genes have been found to contain a GCC box in their promoter regions.

Transcription factors that bind to the GCC box (TAAGAGCCGCC) of PR genes are specifically called as Ethylene-responsive element binding proteins were first isolated from tobacco and later renamed as Ethylene response factors (ERFs). ERF domain was believed to be closely related to AP2 domain. However, in 2000 Ohme-Takagi *et al*. has shown that ERFs possess a highly conserved DNA binding domain with a novel and unique form of DNA recognition. ERFs from different taxa all share common features such as being induced by biotic and abiotic stresses and mediating the expression of GCC box-containing genes. A novel research has shown that activated disease resistance 1 (ADR1) gene that confers broad-spectrum disease resistance is also involved in drought-resistance of *Arabidopsis* plants. This result proposes that there are shared multiple nodes in both biotic and abiotic signaling pathways and the outputs of these pathways may functionally overlap in a significant manner (Chini *et al*., 2004).

ERFs isolated from tomato -Pti 4, Pti 5 and Pti 6- interact with tomato *Pto* resistance gene and bind to the GCC box *cis* element. Moreover, Pti 4/5/6 have been found to be localized to nucleus and *Arabidopsis* cells. ET-regulated PR genes were found to be activated by the expression of Pti 4 in *Arabidopsis* suggesting that Pti 4 might regulate the expression of genes in the ET signaling pathway. Plants overexpressing *Pti4/5* and *6* have been found to show increased resistance to pathogens. Gu *et al*. suggest a model for the role of Pti 4 in PR Gene Expression proposing that *Pti 4* gene activated upon pathogen attack produces the substrate for Pto kinase phosphorylation. The phosphorylated Pti 4 is then localized into nucleus and binds to DNA and/ or interacts with other transcription factors. This activates different subset of

PR genes and provides the plant with control of the defense responses. It is also hypothesized that Pti 4 may play a role in mediating the communication between SA and ET/JA signaling pathways (Gu *et al*., 2000).

2.5 A novel AP2 domain TF from tomato

A novel AP2 domain TF (JERF1) from has been isolated from tomato *Lycopersicon esculentum* and is supposed to be responsive to stress. This particular transcription factor contains an AP2 DNA binding domain, a conserved DNA binding domain, a basic region in its N-terminal region that might function as a nuclear location signal, and an acidic C-terminal region that might act as an activation domain for transcription. It may specifically interact with GCC-box. There has been no published data in literature for the role of JERF1 showing its role in virus susceptibility or resistance. Plant P58IPK like was found to interact with a partial clone of JERF1 in a yeast-two hybrid screen (Bilgin, D.D Unpublished data). Based on the interaction with plant P58IPK and sequence characteristics of JERF1 it is suggested that JERF1 might be involved in plant-virus interaction.

2.6 Aim of the study

The aim of this study is to clone and characterize the *JERF1* gene from tomato *Lycopersicon esculentum*, to analyze its role in tobacco mosaic virus susceptibility of plants and to detect the subcellular localization of the JERF1 protein.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

All chemicals were supplied by Fluka (Switzerland), Merck (Germany), Riedel de Häen (Germany), and SIGMA (USA).

3.1.2 Primers

Primers were designed according to the *Lycopersicon esculentum* transcription factor *JERF1* gene (TIGR; TC 124207) and synthesized by Proligo (France), Iontek (Turkey) and SeqLab (Germany). The sequences of the primers used are given in Appendix A.

3.1.3 Enzymes

3.1.3.1 Restriction enzymes

*Eco*RI, *Not*I, *Sac*I, *Hind* III, *Xba*I, *Bam*HI, *Kpn*I (Promega and Fermentas)

3.1.3.2 Ligase

T4 DNA Ligase (Fermentas)

3.1.3.3 Taq Polymerase

Expand High Fidelity PCR System (Roche), Taq Master mix (QIAGEN)

3.1.3.4 Reverse Transcriptase

Omniscript Reverse Transcriptase (QIAGEN)

3.1.4 Commercial Kits

Qiaquick® Gel Extraction Kit (250) (QIAGEN)

Qiaprep® Spin Miniprep Kit (250) (QIAGEN)

QIAGEN® Plasmid Midi Kit (100) (QIAGEN)

TOPO® TA Cloning Kit (Invitrogen)

3.1.5 Vectors

Maps of all vectors can be found in Appendix B.

pCR®-II-TOPO4® (Invitrogen)

pGR106

pTRV2

pJG4-5

pTBSI

pRTL2-GUS/NIaΔBam

pEGKG

pDONR-221® (Invitrogen)

GFP-N-Bin was kindly donated by Dr. John Doonan (John Innes Centre, Norwich, UK).

GFP-C-Bin was kindly donated by Dr. John Doonan (John Innes Centre, Norwich, UK).

3.1.6 Cells

E.coli strains TOP10, DH5α, *Agrobacterium tumefaciens* LBA4404.pBBR1MCS virGN54D, yeast EGY 48, yeast RFY206, *Arabidopsis thaliana* suspension culture, and BY2 suspension culture.

3.1.7 Buffers and solutions

All buffers and solutions, except those provided with commercial kits, were prepared according to Sambrook *et al.*, 2001. Buffers, their compositions and preparations are presented in Appendix C

3.1.8 Culture medium

3.1.8.1 Liquid medium

LB (Luria-Bertani) Broth from SIGMA was used to prepare liquid culture media for bacterial growth.

3.1.8.2 Solid medium

LB (Luria-Bertani) Agar from SIGMA was used for the preparation of solid culture media for bacterial growth.

3.1.8.3 Yeast SC (Synthetic Complete) Medium

1, 5 g Yeast Nitrogen Base (without ammonium sulfate and amino acids)

5.0 g ultra pure ammonium sulfate

2.15 g complete amino acids stock mixture

20 g dextrose

20 g agar (only for solid medium)

Add ddH_2O to 1 liter

Medium was autoclaved and stored at 4^0C

3.1.8.4 YPD Medium

10 g yeast extract

20 g peptone

20 g glucose

2% agar

Add ddH_2O to 1 liter

Medium was autoclaved and stored at 4^0C

3.1.8.5 AT Medium

4.4 g MS media

30 g sucrose

0.05 mg kinetin

0.5 mg NAA, pH 5.8

Add ddH2O to 1 liter

Medium was autoclaved and stored at 4^0C

3.1.9 Sequencing

Sequencing service was commercially provided by SEQLAB (Germany) and Iontek (Turkey).

3.1.10 Equipments

List of all the equipments used during this study are presented in Appendix E.

3.2 Methods

3.2.1 Plant growth

Lycopersicon esculentum seeds were sprinkled down into wet perlite and left 2 days for vernalization. After 2 days they were incubated at 24^0C, in dim light for germination. After germination the incubation was done at 24^0C for 16 hours light and 8 hours dark. Seedlings emerge within 2 weeks time and then they are transferred into soil which is mixed with perlite in a ratio of 1:2 (1 units of perlite, 2 units of soil). Hormone mixture was added to the soil and plants were watered 3 times a week. Leaf tissues were collected for sampling by cutting them with clean scissors and frozen in liquid nitrogen. Tissues were stored at -80^{o}C until RNA isolation.

3.2.2 RNA isolation from the plant

2 gr of leaf tissue was grinded for RNA isolation. Solution D was added and the tissues were homogenated by baked mortar and pestle. Homogenates were taken into a RNase free falcon. 2ml of 2M sodium acetate (pH 4.0) and 16 ml of water-equilibrated phenol was added onto the homogenates and vortexed. 4 ml of chloroform was added and falcons were vortexed and left on ice for 15 minutes. Samples were then centrifuged at 6000rpm for 30 min at 4^0C. The upper, aqueous phase which contains the RNA was transferred to a new tube by 25 ml RNase free pipet taking care to avoid the precipitated material at the layer between two phases that contains the genomic DNA and proteins.

The RNA from the aqueous phase was precipitated by adding 1 equal volume of isopropanol and stored on ice for 1 hour. Samples were then centrifuged at 7500 rpm for

15 minutes at 4 ^{0}C in order to pellet RNA. The pellet was washed twice with 70% ethanol, each time followed by centrifugation at 7500 rpm for 2 minutes in order to remove salts which would interfere with subsequent CTAB precipitation by forming a chemical precipitate. RNA was then resuspended in 4 ml of water and 1.3 ml of 5M NaCl was added. 16 ml of CTAB/urea was added which selectively precipitates the RNA. Samples were centrifuged for 15 minute at 7500 rpm and aqueous phase was discarded. RNA pellet was resuspended in 4 ml of 7M GuCL. Resuspended RNA was then precipitated by adding 8 ml of cold ethanol and incubated at -20^{0}C for 1-2 hours. Samples were then centrifuged for 15 minutes at 7500 rpm and at the end pellet was washed with 5 ml of 70% ethanol which was then centrifuged again at 7500 rpm for 5 minutes. The supernatant was discarded and RNA was resuspended in 500-1000µl of RNase-free-water. All the equipments for RNA isolation were baked before the procedure at 180^{0}C overnight. In order to calculate the RNA concentration, optical density at 260 nm was measured and calculation was done according to the formula: Conc. (µg/µl) = OD_{260} x 40 x DF

3.2.3 Preparation of cDNA from total RNA

3.2.3.1 GENE RACER™ Invitrogen

GENE RACER™ Invitrogen allows the amplification of only full length transcript ends by eliminating truncated mRNAs. This technique is based on selective ligation of an RNA oligonucleotide to full length 5' ends of decapped mRNA by using T4 RNA ligase. In this method, first, total RNA isolated from tomato was dephosphorylated by mixing with 1µl of calf intestinal phosphatase (CIP), 1µl of CIP buffer and incubated at 50^{0}C for 1 hour. Samples were then centrifuged at 13,000 rpm for 1 minute and placed on ice. 90 µl RNase free H_2O and 100 µl phenol: chloroform was added onto the samples and was centrifuged for 5 minutes at room temperature at 13,000 rpm. Aqueous phase was transferred to a new tube and 2 µl mussel glycogen (10 mg/ml), 10 µl 3M sodium acetate (pH5.2) and 220 µl 95% ethanol was added and vortexed. Samples were frozen on dry ice for 10 minutes and then centrifuged at maximum speed for 20 minutes at 4^{0}C to pellet the RNA. Supernatant was removed and 500 µl 75% ethanol was added and vortexed. Samples were then centrifuged for 2 minutes at 4^{0}C and ethanol was removed. Centrifugation was repeated to remove the

remaining ethanol and samples were air dried for 2 minutes at room temperature. Pellet was resuspended in RNase free water. 1 µl of 10X tobacco acid pyrophosphatase (TAP) and 2 µl TAP was added onto 7 µl of RNA and samples were mixed and centrifuged prior to incubation at 37^0C for 1 hour. Samples were then centrifuged and placed on ice. RNA was precipitated as explained above. After precipitation, 7 µl dephosphorylated, decapped RNA was added to the tube containing lyophilized GENE RACER ™ RNA Oligo (0.25 µg) and sample was pipetted up and down to mix and spinned to be collected at the bottom. Incubation was done at 65^0C for 5 minutes in order to relax the 2^0 structure of RNA and tubes were placed on ice for 2 minutes and then centrifuged. Subsequently 1 µl of 10X Ligase Buffer, 1 µl of 10mM ATP and 1 µl of T4RNA Ligase was added and samples were incubated at 37^0C for 1 hour. RNA was precipitated as done in the first step except that pellet was resuspended in sterile water and run on gel.

3.2.3.2 RT-PCR

Qiagen Omniscript Reverse Transcription enzymes and reagents were used and the concentrations for primers and template RNA were determined according to the available manual.

94^0C, 3 minutes

94^0C, 1 minute

50^0C, 1 minute

72^0C, 45 seconds

72^0C, 12 minutes

4^0C, on hold

A total of 35 cycles

3.2.4 PCR

Recommended reaction volumes and final concentrations of the Qiagen Taq Master Mix PCR components were used for PCR reaction mixture.

94^0C, 2 minutes

94^0C, 30 seconds

53^0C, 45 seconds

72^0C, 90 seconds

72^0C, 7 minutes

4^0C, on hold

a total of 35 cycles

3.2.5 Isolation of DNA fragments from gels

DNA fragments were purified from 1% Agarose Gel with Qiaquick® Gel Extraction Kit (250) (QIAGEN). DNA fragment was excised from agarose with a clean and sharp scalpel and weighed in a sterile eppendorf tube. 3 volumes of QC buffer were added to 1 volume of gel (100 mg ~ 100 µl) and the tube was incubated at 50^oC for 10 minutes until the gel slice completely dissolves. 1 gel volume of isopropanol was added to the sample and mixed. Sample was transferred into QIAquick column and centrifuged for 1 minute. The flow-through was discarded. For washing, 0.75 ml of Buffer PE was added and centrifuged for 1 minute. The flow-through was discarded and samples were then centrifuged for an additional 1 minute at 13,000 rpm. QIAquick column was placed into a clean 1.5 ml microcentrifuge tube and DNA was eluted in EB buffer. Elution was done according to the intensity of the DNA on the gel prior to isolation. For increased DNA concentrations, elution was done at 30 µl of EB and for more dilute concentrations, elution was done at 50 µl of EB.

3.2.6 Sub cloning into pCR-II-TOPO4 vector

TOPO TA Cloning® Kits provide cloning of PCR products directly from PCR reaction in a rapid and efficient manner. pCR® -TOPO vectors included in TOPO TA Cloning® Kits contain covalently bound topoisomarese I and 3'-T overhangs so that

PCR products can directly be ligated into the vector so that the need for restriction enzyme digestion and ligation is skipped.

JERF1 gene was amplified and subcloned into pCR-II-TOPO4 (Invitrogen) vector by mixing 4 µl of insert with 1 µl of vector and 1 µl of salt solution. Mixture was incubated at room temperature for 30 minutes.

3.2.7 Subcloning

Basic procedures were carried out according to Manniatis *et al*., 1989.

3.2.7.1 Subcloning into plant silencing vectors

JERF1 gene was amplified with primers 162 and 163 in order to be cloned into vectors pGR106 and pTRV.

3.2.7.2 Cloning into protein over expression vectors

JERF1 gene was amplified with primers 199 and 200 in order to be cloned into vector pRTL2-GUS/NIaΔBam.

3.2.7.3 Cloning into protein expression vector:

JERF1 gene was amplified with primers 207 and 208 in order to be cloned into vector pEGKG.

3.2.8 Gateway Cloning

Gateway® Technology of Invitrogen was used in order to clone *JERF1*. Gateway® Technology is a rapid method for cloning one or more genes into any protein expression system. Gene or fragment of DNA is amplified with primers containing *att*B Gateway adapter sequences and directly ligated into a donor vector by the enzyme BP clonase without any need for restriction enzyme digestion to produce sticky or blunt ends and conventional ligation procedures. Once the fragment of gene of interest is inserted into donor vector then this construct is named as entry clone and c*cd*B fragment of the

original donor vector is released as a by-product. Entry clones can then be incubated with Gateway® Destination Vectors in order to insert the gene or DNA fragments directly into the destination vector so that an Expression clone is obtained at the end of the reaction which is named as LR reaction. Expression clones can be fitted to any expression needs for further experiments

3.2.8.1 Cloning into Gateway Donor vectors

JERF1 was amplified with gateway primers BD1 and BD5 in order to be cloned into Gateway Donor Vector pDONR^{TM}221 (Invitrogen). Cloning was done via BP reaction by mixing 100ng of DNA with 1.3 µl of BP clonase buffer, 1.3 µl of BP clonase enzyme and 1 µl of vector. Mixture was incubated at room temperature overnight.

3.2.8.2 Cloning into Gateway Destination vectors

In order to ligate *JERF1* into GFP-N-Bin and GFP-C-Bin destination vectors, entry clone which was prepared by BP reaction was first linearized with *Pvu*I restriction endonuclease and LR reaction was carried out. LR reaction mixture includes 100 ng of entry clone, 1 µl of vector, 1.3 µl of LR buffer and 1.3 µl of LR enzyme and was incubated at room temperature overnight.

3.2.9 Ligation

PCR amplified and purified *JERF1* containing different restriction enzyme sites were ligated with pGR106, pTRV, pJG4-5, pTBSI, and pRTL2-GUS/NIaΔBam. DNA fragments to be used in ligation were generally purified from agarose gels. Ligation mixture contained 1 µL (10 units) T4 DNA Ligase (Fermentas), 1X ligation buffer (supplied by the manufacturer), approximately 20 ng/ µL DNA with the inserts to vector molar ratio of 1:1 to 5:1 and distilled water. Reaction mixtures were incubated at 16^0C overnight.

3.2.10 Preparation of *E.coli* competent cells

Frozen stock of one *E.coli* strain called DH5 and TOP10 cells were streaked on an LB plate in sterile conditions, and cultured overnight at 37°C. About ten to twelve large colonies were isolated with a loop, inoculated to 250 ml of SOB medium in a 2-liter flask for efficient aeration. The culture was grown to A_{600} of 0.6 at 18°C, with vigorous shaking (200 - 250 rpm). The flask was removed from the incubator and placed on ice for 10 min. The culture was transferred to two separate sterile centrifuge tubes (250 ml each) and spun at 2500xg for 10 min. at 4 °C. The pellet was suspended in 80 ml of ice-cold TE (Appendix D), incubated in ice bath for 10 min., and centrifuged as done previously. The cell pellet was gently resuspended in 20 ml of TE, and DMSO was added with gentle swirling to a final concentration of 7%. After incubating in an ice bath for 10 min. the cell suspension was dispensed by 0.4l into eppendorf tubes and immediately chilled by immersion in liquid nitrogen. The frozen competent cells were stored at -80°C. At this condition, they can be kept for at least a few months without a detectable loss of competence.

3.2.11 Transformation of competent *E.coli* cells

Ligation mixtures were transformed into endonuclease deficient strains of *E.coli* TOP10. 100 µl of cells was mixed with ligation mixtures and left on ice for 30 minutes. Then the mixtures were incubated at 42^0C for 90 seconds and taken on ice for 2-3 minutes. 900 µl of LB was added onto the mixture and samples are incubated at 37^0C shaking at 300 rpm for 1 hour. 100 µl of mixture was plated onto LB agar containing the proper antibiotics. Remaining 900 µl was centrifuged at 13,000 rpm for 1 minute and 800 µl of supernatant was discarded. Pellet was resuspended in the remaining 100 µl solution and spreaded onto selective LB agar plates. Plates were incubated at 37^0C overnight. Positive colonies were selected and grown on liquid LB culture containing the appropriate antibiotic for both preparing glycerol stocks and plasmid isolation.

3.2.12 Plasmid isolation

Plasmid isolation was done with QIAprep® Spin Miniprep Kit (250) (QIAGEN). Pelleted bacterial cells were resuspended in 250 µl Buffer P1 and transferred to a microcentrifuge tube. 250 µl Buffer P2 was added and the tubes were gently inverted 4-6 times to mix. 350 µl of N3 buffer was added and mixed immediately by inverting the tube. Tubes were left on ice for 10 minutes and then centrifuged for 10 minutes at 13,000 rpm. Supernatants were applied to QIAprep column and centrifuged for 60 seconds. The flow-through was discarded and column was washed with QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 60 seconds. The flow-through was discarded and tubes were centrifuged for 2 minutes to remove the residual wash buffer. QIAprep columns were placed in a clean 1.5 ml centrifuge tube and DNA was eluted with 50 µl of EB buffer.

3.2.13 Restriction Enzyme Digestions

Purified plasmids containing the *JERF1* gene were digested with the appropriate restriction enzymes according to the suppliers' instructions for verification of the presence of the corresponding gene and further cloning experiments.

3.2.14 Agarose gel electrophoresis

Agarose gels were used for DNA analysis at a concentration of 1%. The final concentration of ethidium bromide in the gels was 0.5 µg/mL. The electrophoresis buffer used was 0.5 X TBE. Purified plasmids and digested plasmids were analyzed by agarose gel electrophoresis. Appropriate DNA markers were used for size and concentration determination. In addition, concentration and $OD_{260/280}$ ratio were monitored by absorption measurements.

3.2.15 Preparation of *A.tumefaciens* competent cells

A single colony of *A.tumefaciens* was inoculated in 2 ml of LB containing the proper antibiotic at 28^0C shaking at 250 rpm for overnight. The overnight culture was transferred to 50 ml LB without antibiotic in a sterile flask and incubated at 250 rpm, 28^0C until the optical density is 0.3. Cultures were spinned in sterile 50 ml screw cap tubes at 4^0C 3000 g for 15 minutes. The supernatant was then removed and pellet was resuspended in 1ml ice cold, freshly prepared 10mM $CaCl_2$. The suspension was kept on ice for some time and 100 µl of aliquots were taken into sterile eppendorf tubes and quick-frozen in liquid nitrogen. Prepared competent cells were stored at -80^0C.

3.2.16 Transformation of competent *A.tumefaciens* cells

5 µl of plasmid was layered on 100 µl competent *A.tumefaciens* cells while cells were in frozen form and incubated at 37^0C for 5 minutes. 900 µl LB was added onto the cells and incubated at 28^0C 250 rpm for 4 hours. Cells were centrifuged for 2 minutes at 12000g to pellet the bacteria. 900 µl from the supernatant was removed and pellet was resuspended in the remaining 100 µl. The suspension was spreaded on LB plates containing the proper antibiotic for selection. The plates were incubated at 28^0C for 2 days.

3.2.17 Transformation of Yeast cells

Several yeast colonies, 2-3 mm in diameter were inoculated into 1 ml of YPD medium and vortexed to disperse the clumps. Mixture was then transferred into a flask containing 50 ml of YPD and incubated at 30^0C for 16-18 hours with shaking at 250 rpm until the OD_{600} reached 1.5. 30 ml of overnight culture was transferred to a flask containing 300 ml of YPD so that the OD_{600} of the diluted culture was 0.2-0.3. Culture was incubated at 30^oC for 3 hours with shaking at 230 rpm until the OD_{600} is 0.4-0.6. Cells were placed in 50 ml tubes and centrifuged at 1,000 g for 5 minutes at room temperature. The supernatants were discarded and cells were resuspended in sterile

distilled water. Cells were pooled into one tube which was then centrifuged at 1,000 g for 5 minutes at room temperature. The supernatant was decanted and cell pellet was resuspended in 1.5 ml of freshly prepared, sterile 1X TE/1X LiAc. 0.1 µg of plasmid DNA and 0.1 mg of salmon sperm carrier DNA was added to a fresh 1.5 ml tube and mixed. 0.1 ml of yeast competent cells were added to each tube and mixed by vortexing. 0.6 ml of sterile PEG/LiAc solution was added to each tube and vortexed at high speed for 10 seconds to mix. Mixture was incubated at 30^{o}C for 30 minutes with shaking at 200 rpm. 70 µl of DMSO was added and mixed by gentle inversion. Heat shock was applied for 15 minutes at 42^{o}C and cells were then chilled on ice for 1-2 minutes. Subsequently cells were centrifuged for 5 seconds at 14,000 rpm at room temperature and supernatant was removed. Cells were then resuspended in 0.5 ml of sterile 1X TE buffer and 100 µl of cells were plated onto SC agar plates with the proper selection. Plates were then incubated up-side-down at 30^{o}C until the colonies appear.

3.2.18 Mating Assay

DB112 construct (pJG4-5+*JERF1*) containing EGY48 and RFY 206 yeast cells and bait plasmids containing RFY 206 yeast cells were grown overnight in YPD liquid medium at 30^{0}C with shaking at 250rpm. The next day 10 µl cultures were streaked onto YPD plates in parallel lines and plates were incubated at 30^{0}C until lines of colonies appear. Yeast cells to be mated were transferred to a fresh YPD plate by velvet so that the streaks from the two plates were perpendicular to each other and the plates were incubated at 30^{0}C overnight. Diploid colonies form where the two strains intersect. Replica from the YPD plate is transferred to the proper selection plates and incubated at 30^{0}C until the interactors grow on these plates.

3.2.19 Frozen stocks of cells

Frozen stocks of *E.coli, A.tumefaciens* and yeast cells containing different plasmids with *JERF1* sequence were prepared in 15% glycerol and kept at -80^{0}C.

3.2.20 Sequence verification

QIAprep® Spin Miniprep Kit (250) (QIAGEN) purified corresponding plasmids containing DNA sequences for *JERF1* were sent for sequence analysis.

3.2.21 Surface Sterilization of *N. benthamiana* seeds

¼ of 1.5 ml eppendorf tube was filled with seeds and water was added into the tube and spinned for a short time in order to take away the dust and other stuff by removing the water. 500 µl of 75 % ethanol was added and vortexed for 1.5-2 minutes and spinned down in minispin centrifuge. The ethanol was removed and 500 µl bleach solution (1:1 Bleach-H_2O + 0.05 of Tween 20) was added. Seeds were vortexed for 5 minutes and bleach solution was removed. This step was repeated and subsequently seeds were rinsed with sterile ddH_2O for 5 times. 1 ml of 0.4% agarose was added onto the seeds and they were plated on MS medium with a micropipette. Seeds were left at 4^0C for three days for vernalization and then moved into incubator for germination.

3.2.22 Silencing analysis

3.2.22.1 Virus induced gene silencing

Virus induced gene silencing of *N.benthamiana* was done according to protocols given by Liu *et al*. (2002) and Bendahmane *et al*. (2000). *N. benthamiana* plants were grown in pots at 25^0C under 16 hours light and 8 hours dark. Constructs containing *JERF1* in pTRV2 and pGR106 were transformed into *Agrobacterium tumefaciens* LBA4404.pBBR1MCSvirGN54D chemically. 5 ml cultures were grown overnight at 28^oC in LB broth containing the proper antibiotics. The next day they were inoculated into 50 ml LB media which included the proper antibiotics, 10 mM MES and 20 µM acetosyringone and culture was grown at 28^0C overnight. *Agrobacterium* cells were harvested and resuspended in infiltration media which contained 10 mM $MgCl_2$, 10 mM MES, 200 µM acatosyringone. Optical density was adjusted to 2.0 and left at room temperature for 3 hours. *Agrobacterium* cells were then infiltrated into plant leaves by using needles.

3.2.22.2 Virus challenging

After 2 weeks from silencing lower leaves of *N.benthamiana* plants were rubbed with 100 µl of Tobacco mosaic virus (TMV) with sterile sponges.

3.2.23 Transient transformation of *Arabidopsis thailana*

Arabidopsis thaliana Col0 suspension cultures were subcultured weekly and grown in AT medium.Dilution was done in the ratio of 1:5 in a total of 50 ml (10 ml of suspension culture and 40 ml of AT medium).

Expression vectors containing the *JERF1* gene were transformed into *Agrobacterium tumefaciens* LBA4404.pBBR1MCSvirGN54D chemically. Transformed *Agrobacterium* was grown in 28^{o}C overnight in LB broth containing the proper antibiotics. Upon subculturing of *Arabidopsis thaliana*, 50 µl of *Agrobacterium* was inoculated into suspension cultures of 3 ml and they were incubated for 2 days at 25^{0}C with shaking at 200 rpm.

4 RESULTS

4.1 Total RNA Isolation from Plant

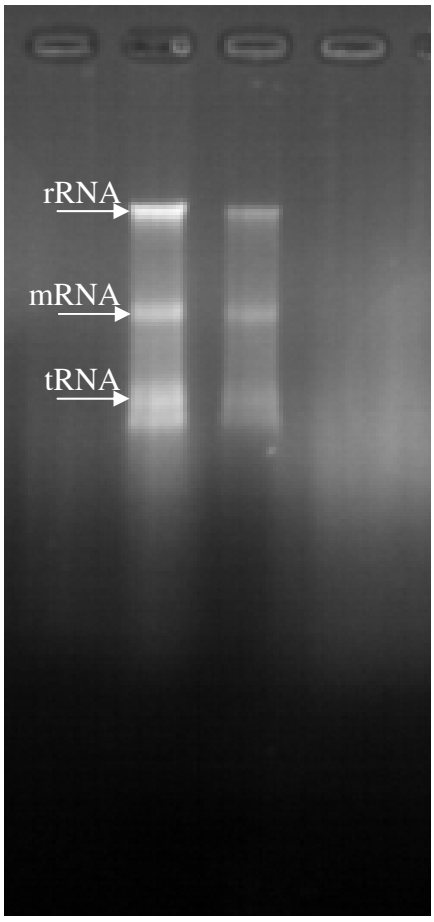


Figure 4.1: 1.2% agarose gel electrophoresis of total RNA isolation from tomato.

Total RNA was isolated from tomato cDNA and ran on 1.2% agarose gel under RNAse-free conditions. Three bands corresponding to rRNA, mRNA and tRNA were easily seen on agarose gel.

4.2 cDNA Preparation

At first Gene Racer™ Kit from Invitrogen was used for amplification of full-length cDNA ends. However, this method did not give any results.

cDNA from total RNA isolated from tomato was then prepared by QUIAGEN Omniscript® Reverse Transcriptase for cloning experiments.

4.3 PCR Amplification of *JERF1* for subcloning

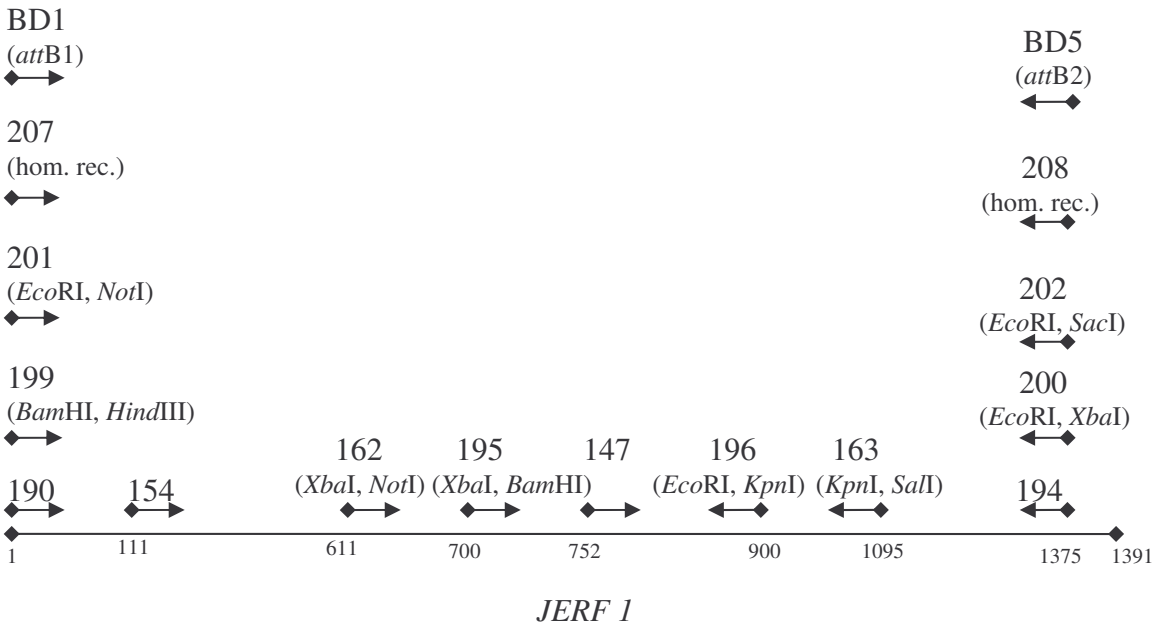


Figure 4.2: Figure showing the primer sites corresponding to *JERF 1*

JERF1 was amplified from tomato cDNA library by using the primers 190 and 194. The sequences of the primers are shown in Appendix A. 190 and 194 were designed according to the sequence of *JERF1* from the 5' end forward and from the

1375th base reverse. Therefore, on 1% agarose gel electrophoresis, a band corresponding to around 1, 4 kb is expected. The band obtained from PCR amplification of *JERF1* is shown in figure 4.3.

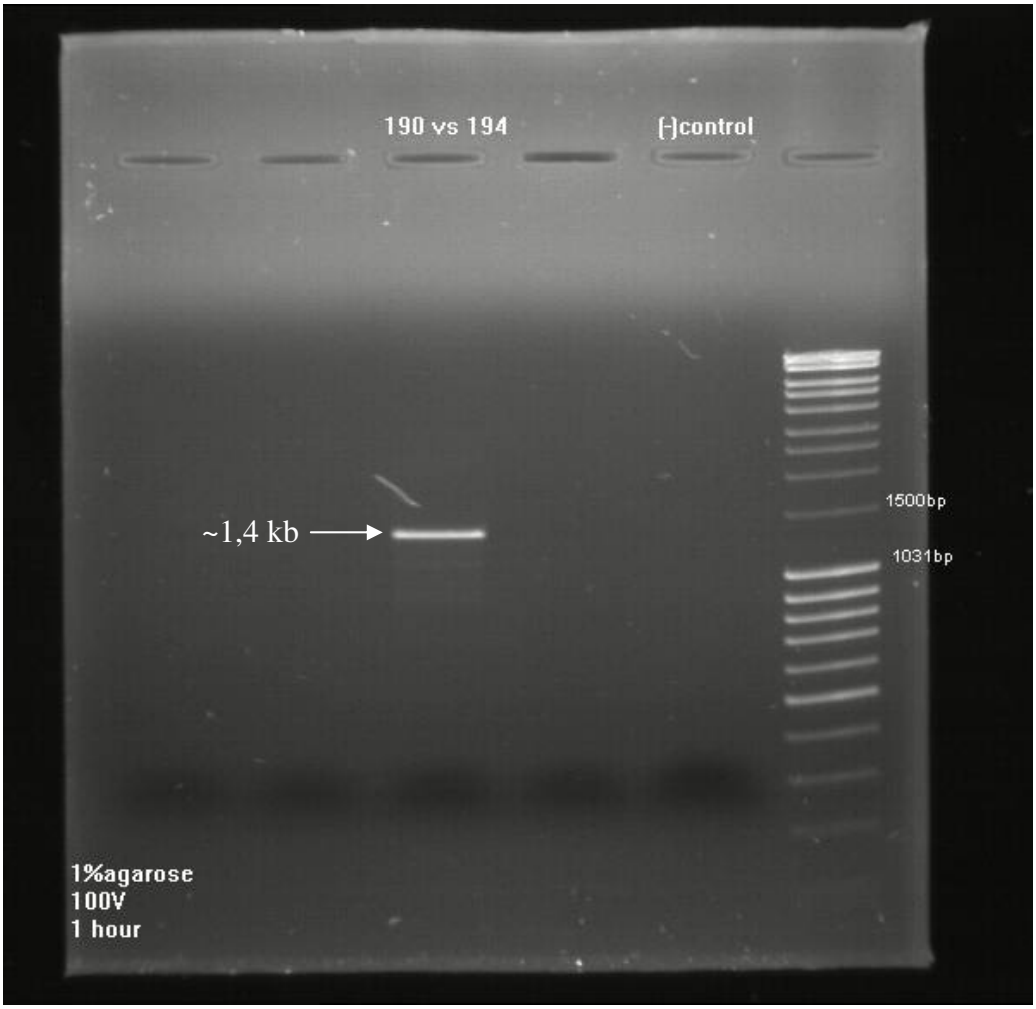


Figure 4.3: Amplification of *JERF1* from cDNA with primers 190 and 194.

4.4 Subcloning of JERF1 into various expression vectors

4.4.1 Subcloning to pCR®4-TOPO for sequencing

TOPO vector is a "TA" vector. PCR products of *JERF1* were ligated into pCR®4-TOPO vector by following the instructions given in the vector manual. pCR®4-TOPO

vector was used for rapid cloning and sequencing of *JERF1*. This vector provides the 3'-T overhangs so that PCR products can directly be ligated. *E.coli* Top 10 cells were then transformed with the ligation mixtures so that the plasmid multiplication was achieved. Transformed cells were selected on Ampicillin LB media which is proper for the selection of pCR®4-TOPO vector. Next day colony PCR with gene specific primers 190 and 194 was done in order to check the insert (See Fig: 4. 4). The construct prepared was named as DB90.

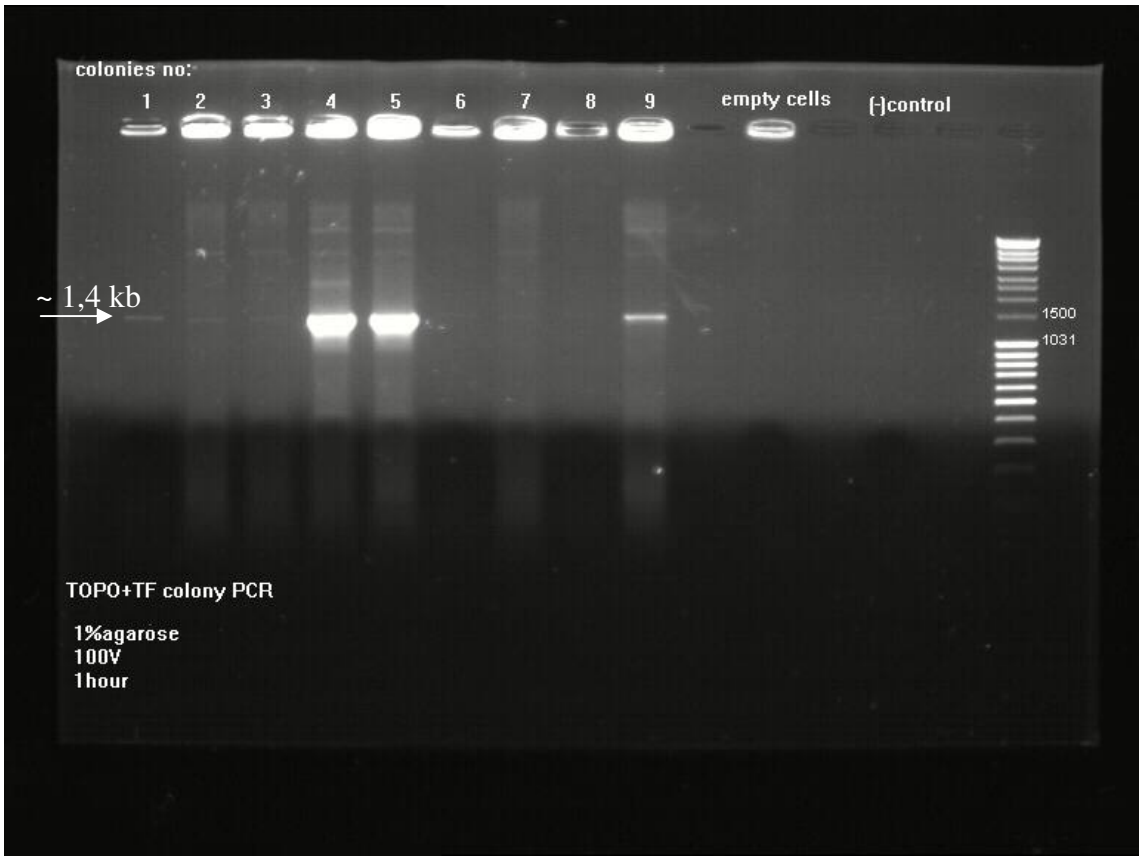


Figure 4.4: Colony PCR of transformed *E.coli* Top 10 cells containing the pCR®4-TOPO vector ligated with *JERF1*(DB90)

4.4.2 Verification of the sub cloning of JERF1 into pCR®4-TOPO in E.coli

pCR®4-TOPO vectors contains *Eco*RI sites flanking the PCR product insertion. Therefore, DB90 was digested with *Eco*RI endonuclease in order to verify the presence of *JERF1* in the construct. *JERF1* does not have any internal *Eco*RI sites. By this

digestion *JERF1* should be cut from the vector pCR®4-TOPO which should give a band of 1,4kb corresponding to *JERF1* and 4.0 kb corresponding to the pCR®4-TOPO vector (See Fig: 4.5).

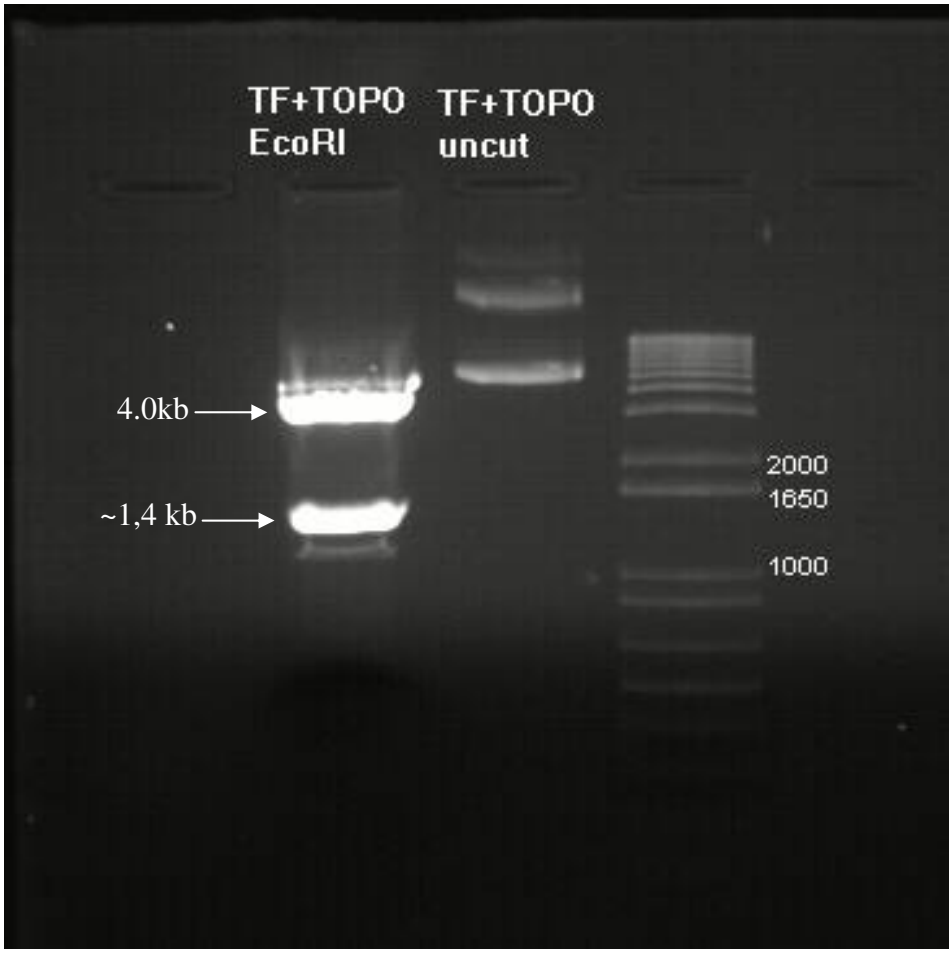


Figure 4.5: Electrophoretic analysis of DB90 construct digested with *Eco*RI and undigested plasmid

4.4.3 Characterization of JERF1 gene

Sequence analysis of the cloned gene was done by using universal M13 primer pair. The sequencing result was blasted in TIGR tomato and NCBI databases. It matched with the *JERF1* sequence of the NCBI accession number AY044235. Sequencing results are shown in Appendix C.

4.4.4 PCR Amplification for cloning into expression vectors

Primers containing the proper restriction endonuclease recognition sites were designed for in frame insertion of *JERF1* into vectors pGR106 and pTRV2 for silencing, pRTL2-GUS/NIaΔBam for over expression, pJG4-5 and pTBSI for yeast mating assay, pDONR™221 for gateway cloning and finally pEGKG for yeast protein expression. Primer sequences, restriction sites and the corresponding expression vector maps are shown in Appendix A and Appendix B respectively. DB90 was used as template for amplification of *JERF1* gene and PCR was carried out by using a proofreading Taq DNA polymerase.

It has been shown that a 200-500 base pair region is enough for virus-induced gene silencing of genes. Therefore, PCR was done with primers 162 and 163 for cloning of *JERF1* into the silencing vectors pGR106 and pTRV2. These primers correspond to around 500 base pair region in the gene (See Fig: 4.6). This region was blasted in TIGR and NCBI databases and found to be unique to *JERF1*.

JERF1 was cloned into pRTL2-GUS/NIaΔBam vector for over-expression *in planta*. Primers 199 and 200 contained the *Bam*HI and *Xba*I recognition sites respectively so that *JERF1* could be cloned between *Bam*HI and *Xba*I sites in the vector. We obtained 1,4kb PCR product with primers 199 and 200, corresponding to full length of *JERF1* (See Fig: 4.6).

In order to clone and express the *JERF1* gene in yeast, pJG4-5 and pTBSI vectors were used. These constructs were then used for yeast mating assay for protein-protein interaction analysis. Primers 201 forward and 202 reverse contain the *Eco*RI, *Not*I and *Sac*I recognition sites in order to clone the *JERF1* gene into pJG4-5 and pTBSI vectors respectively. The forward primer 201 was designed according to 5' and 202 reverse was designed from 1375 th base of the *JERF1* gene (See Fig: 4.6).

JERF1 was amplified with primers containing Gateway adapter sequences, BD1 and BD5 in order to be inserted into the donor vector pDONR™ 221. Primers BD1 and BD5 contained the corresponding *att*B sites for direct insertion into the donor vector. *att*B sites totally are about 60 base pairs long therefore from this amplification a longer fragment than the full length *JERF1* is obtained (See Fig: 4.6).

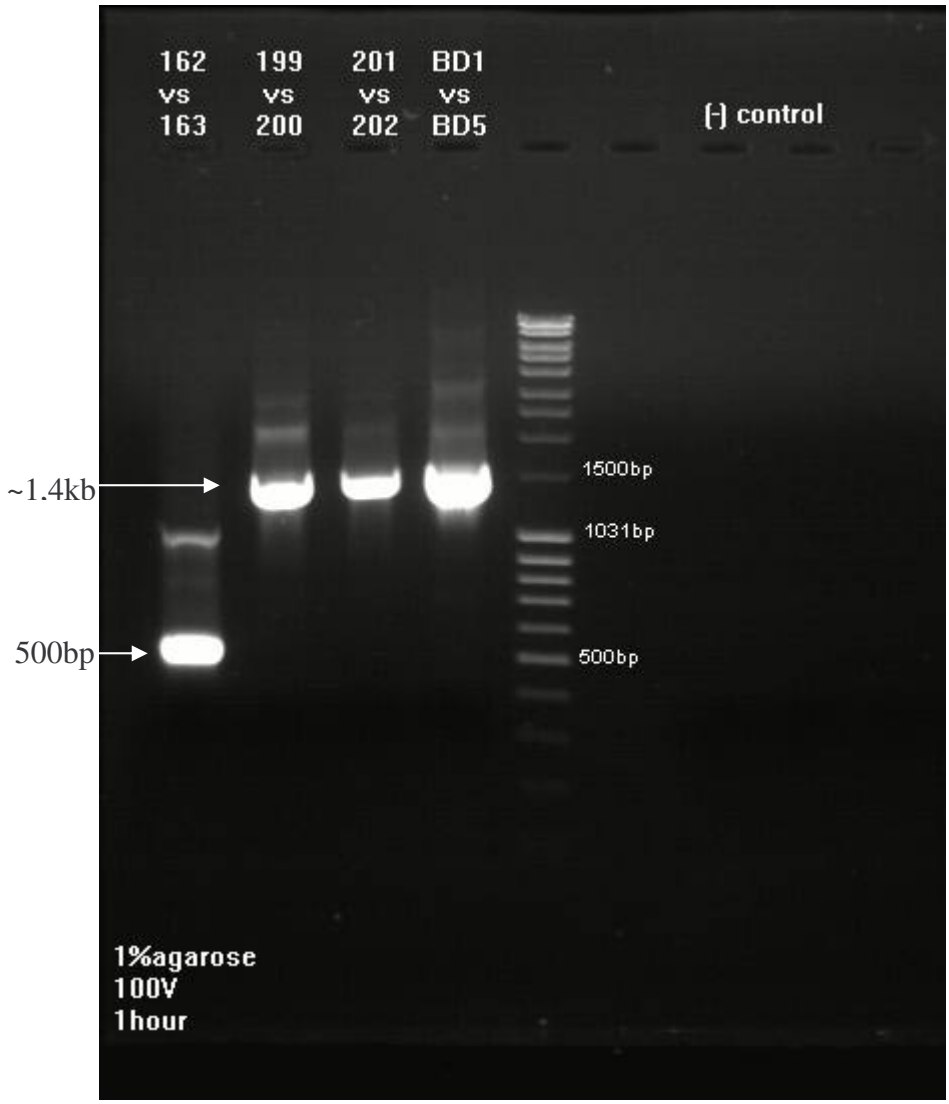


Figure 4.6: 1% Agarose gel electrophoresis result showing the amplification of *JERF 1* with different primers for different expression vectors. DNA molecular weight markers, different samples and electrophoresis conditions are indicated on the gel photograph.

4.4.5 Gel purification of *JERF1*

After PCR amplification whole PCR product was run on gel and isolated from gel in order to clean the contaminants. Gel extraction was performed with Qiaquick® Gel Extraction Kit (250) (QIAGEN). 5 µl of purified sample was loaded on the gel to check whether the PCR product had been purified and its concentration was enough for further cloning experiments (See Fig: 4.7).

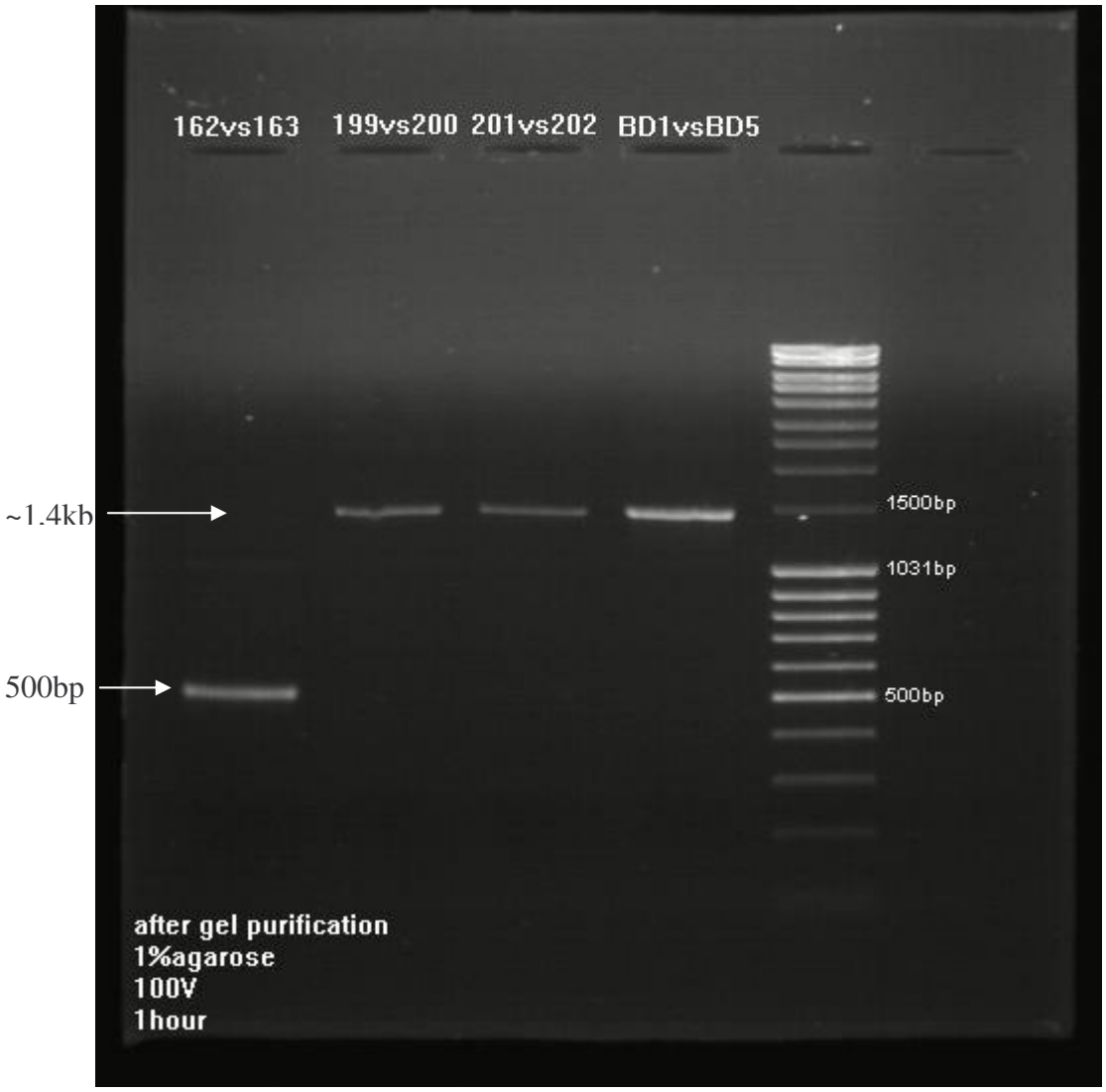


Figure 4.7: 1% Agarose gel electrophoresis result after the purification of PCR products of *JERF1* amplified with different primers.

4.4.6 Digestion of *JERF1* with proper restriction endonucleases

JERF1 amplified with different primers for different expression vectors were digested with the proper restriction endonucleases in order to be inserted into the desired plasmids (See Fig: 4.8).

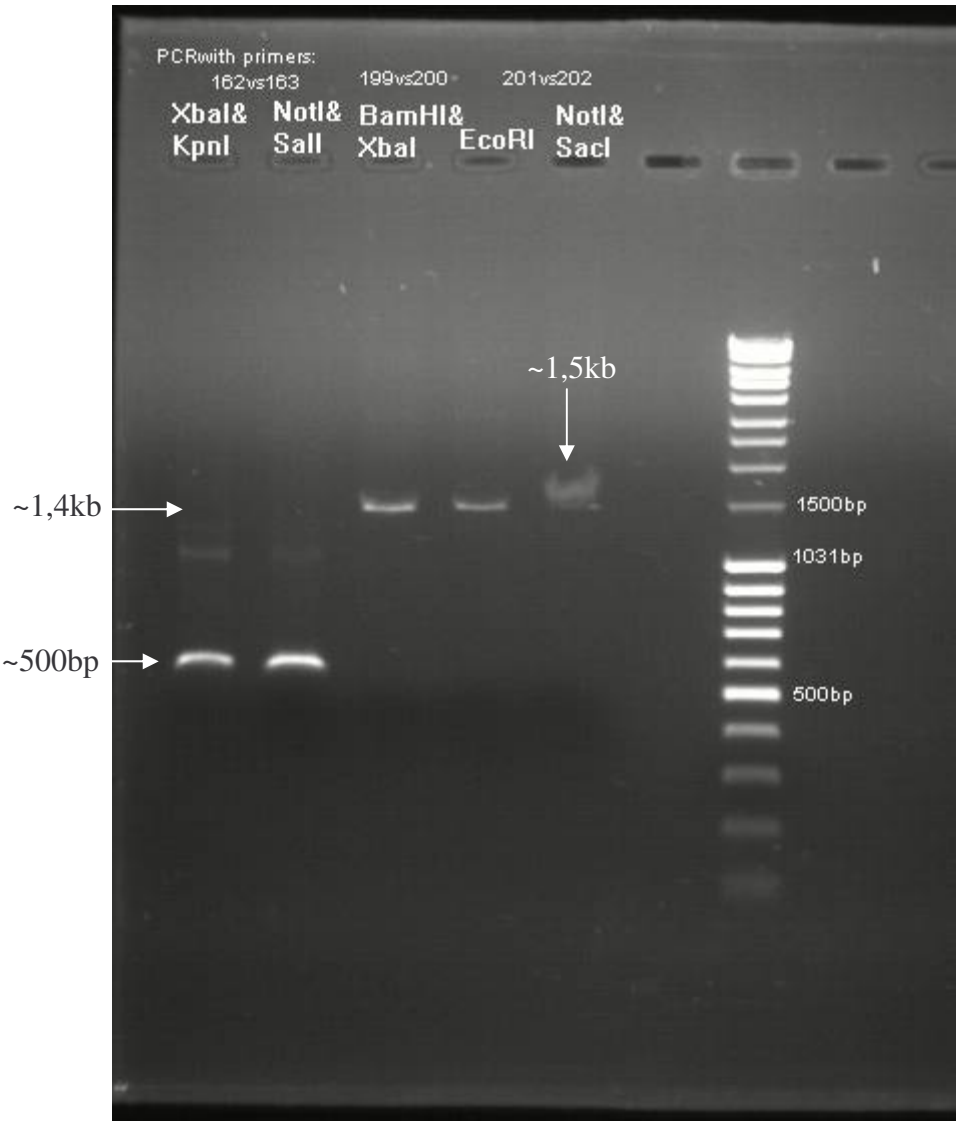


Figure 4.8: 1% Agarose gel electrophoresis result of the digestion of *JERF1* gene with proper restriction endonucleases for expression vectors.

4.4.7 Digestion of different expression vectors for cloning of *JERF1*

pTRV2, pGR106, pRTL2-GUS/NIaΔBam, pJG4-5 and pTBSI were digested with restriction endonucleases *XbaI* and *Kpn*I, *Not*I and *Sal*I, *Bam*HI and *Xba*I, *Eco*RI, *Not*I and *Sac*I respectively and gel purified for ligation process (See Fig: 4.9).

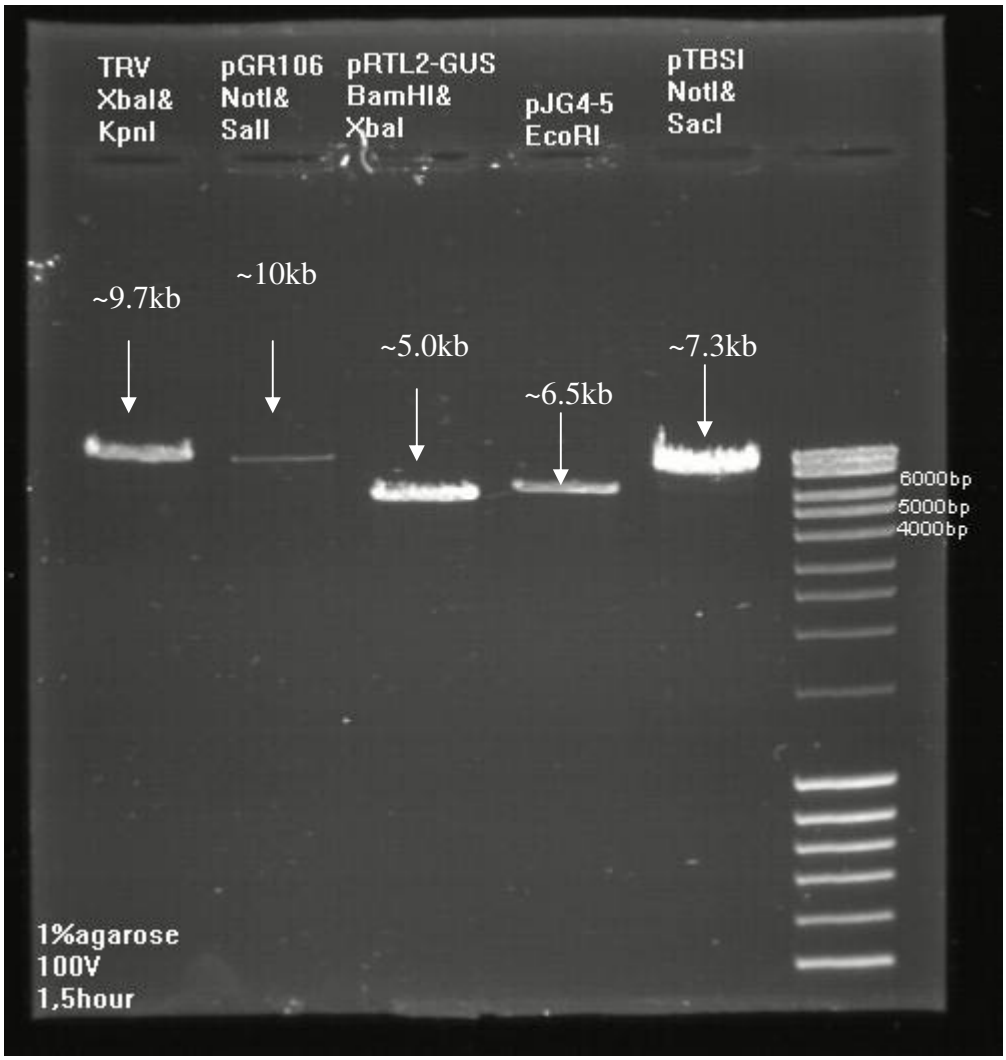


Figure 4.9: Electrophoretic analysis of different vector digestions with different restriction endonucleases.

4.4.8 Ligation

PCR amplified fragments were ligated into corresponding vectors for further analysis of the gene. Ligations were done following precipitating the vector and fragment by Ethanol overnight so that the efficiency was higher.

PCR amplified *JERF1* with primers BD1 and BD 5 was ligated into vector pDONR™ 221 by BP reaction so that the entry clone DB109 (pDONR™ 221+*JERF1*) was obtained. After BP reaction *JERF1* was inserted into destination vectors GFP-N-Bin and GFP-C-Bin by LR reaction so that the expression clones DB110 (GFP-N-Bin + *JERF1*) and DB 111 GFP-C-Bin+ *JERF1*) were obtained.

4.4.9 Cloning of *JERF1* gene

The constructs prepared by ligating *JERF1* fragment into pTRV2 and pGR106 and full length *JERF1* into pJG4-5, pTBSI and pRTL2-GUS/NIaΔBam were transformed into *E.coli* TOP10 cells chemically. DB109, DB110 and DB111 were transformed into *E.coli* DH5 α since this strain shows the highest efficiency of transformation with Gateway vectors. Transformants that were grown on the proper antibiotic selection LB medium were screened by colony PCR with gene specific primers to check whether they contain the insert. Colony PCR results of the different constructs are shown below.

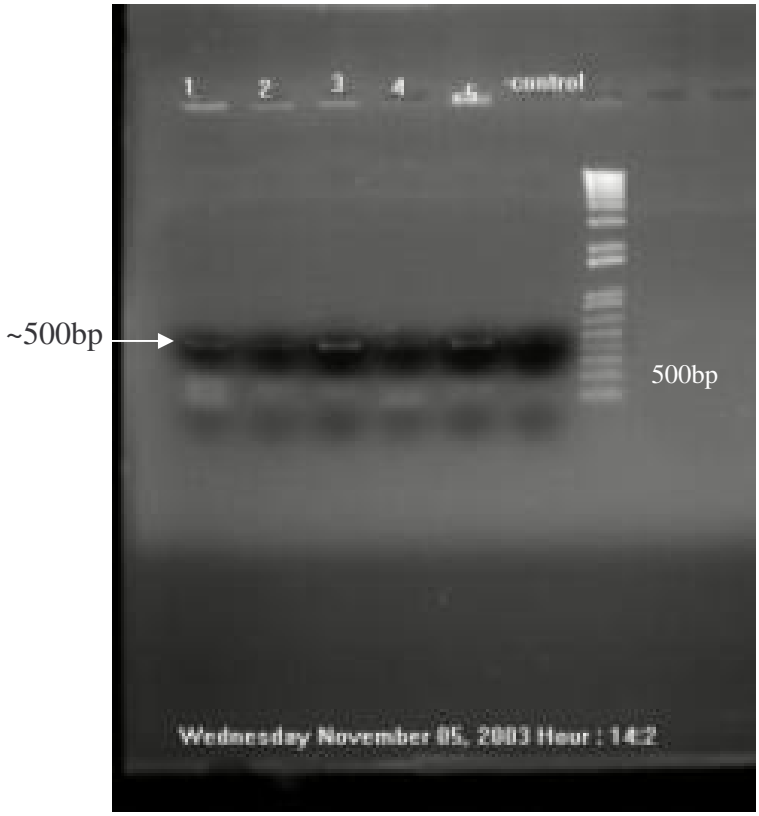


Figure 4.10: 1% Agarose gel electrophoresis showing the colony PCR result of pTRV2+ *JERF1* transformation

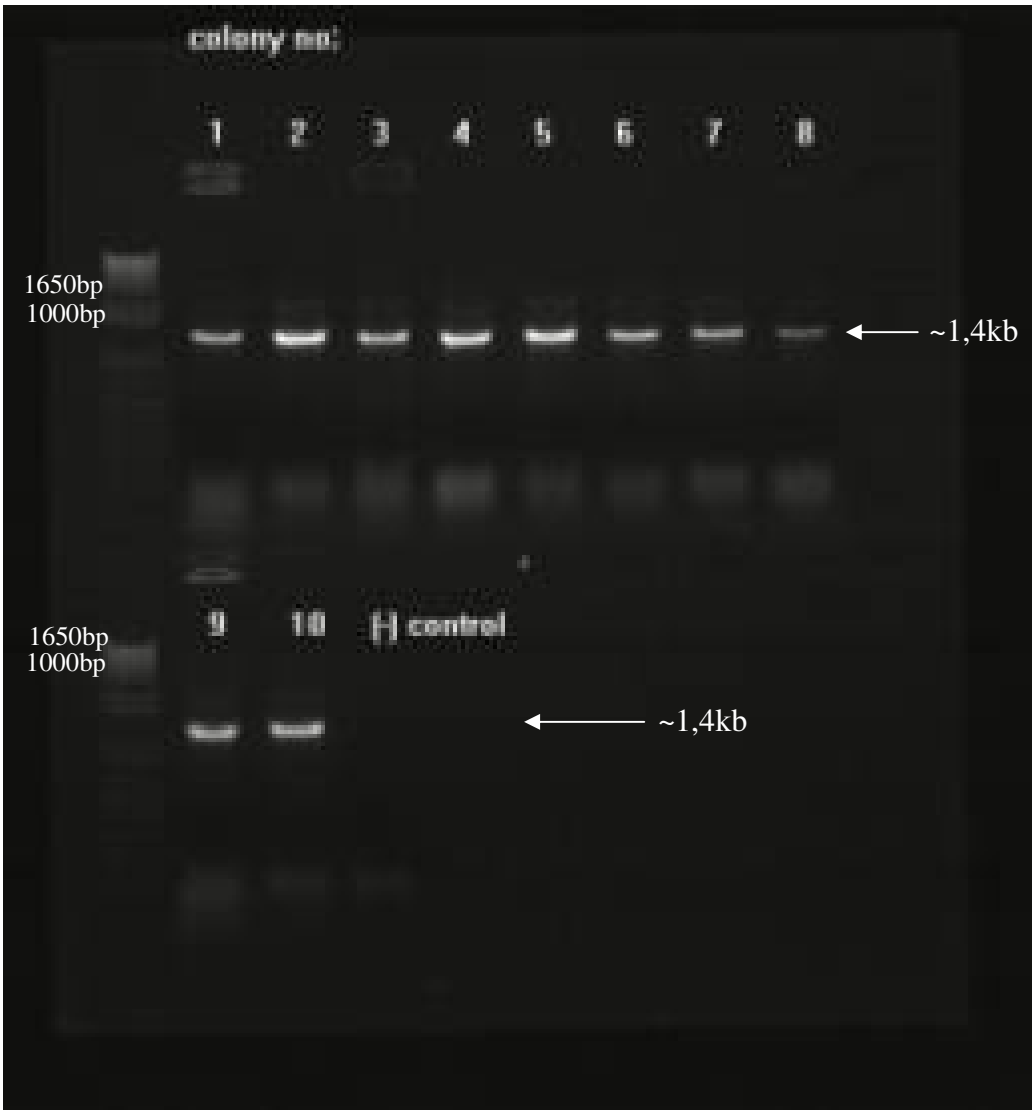


Figure 4.11: 1% Agarose gel electrophoresis photograph showing the colony PCR result of the transformation of pJG4-5+ *JERF1* into *E.coli* TOP10 cells.

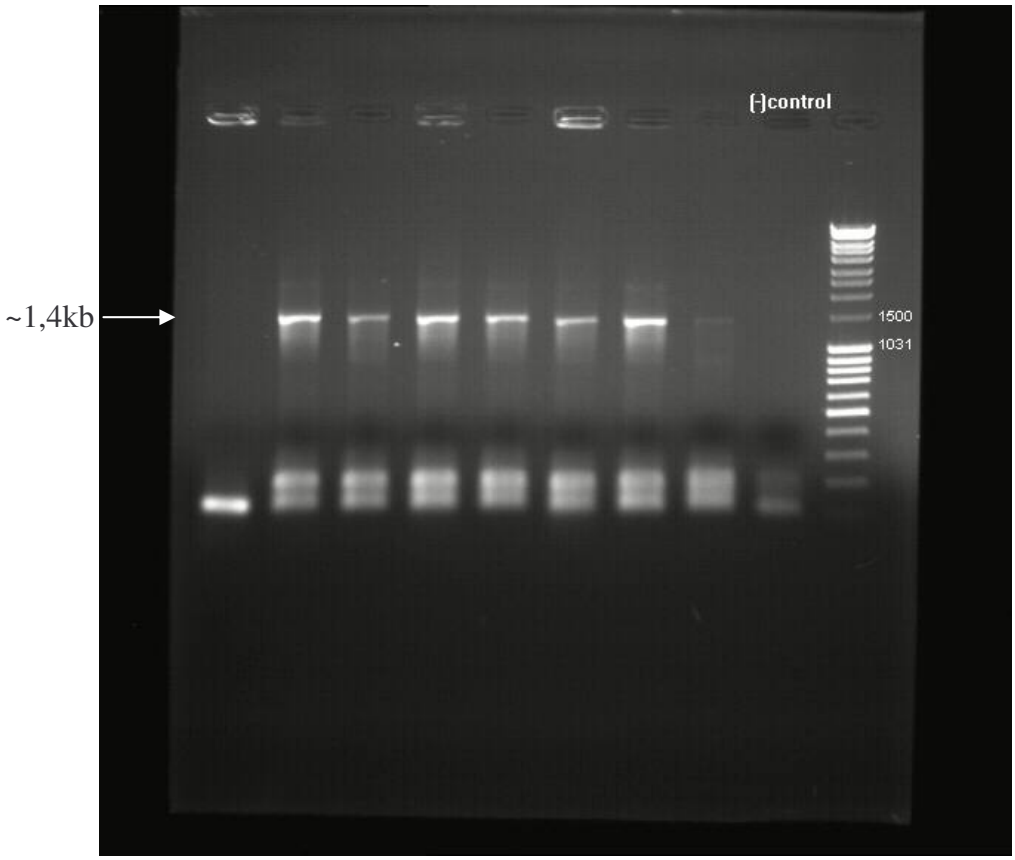


Figure 4.12: 1% Agarose gel electrophoresis photograph showing the colony PCR result of the transformation of pTBSI+ *JERF1* into *E.coli* TOP10 cells.

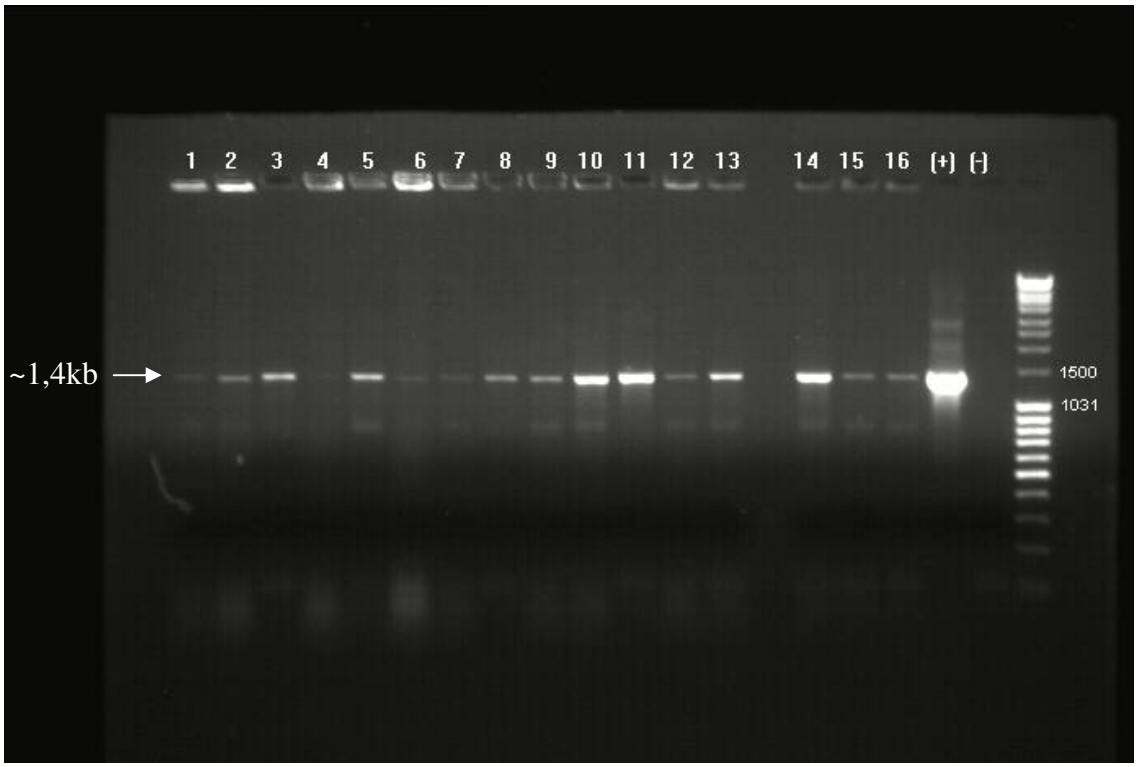


Figure 4.13: 1% % Agarose gel electrophoresis photograph showing the colony PCR result of the transformation of pRTL2-GUS/NIaΔBam + *JERF1* into *E.coli* TOP10 cells. 1-16= PCR of 16 different colonies of pRTL2-GUS/NIaΔBam + *JERF1* transformation with primers 190 and 194. (+)=PCR amplification of *JERF1*+ pCR-4-TOPO with primers 190 and 194 as positive control. (-)= negative control.

Only a single colony grew after transformation of DB 109. Colony PCR was done with different primers to check the presence of the gene in the construct. PCR of 190 and 194 is expected to give the full length *JERF1* which is about 1, 4 kb. PCR analysis with internal primers of 190 and 196 and 195 and 194 is expected to give fragments of 900 bp and 700 bp respectively. DB90 construct was used as positive control for the PCR reaction.

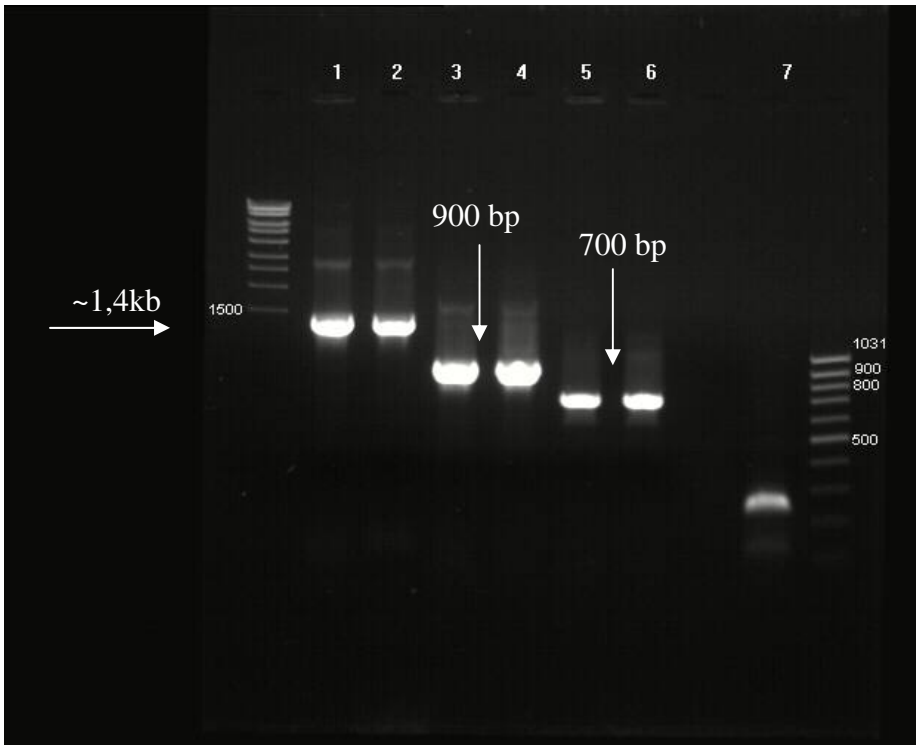


Figure 4.14: 1% Agarose gel electrophoresis of colony PCR of DB109. 1= PCR amplification with primers 190 and 194 of colony grown after BP reaction, 2= PCR amplification of *JERF1*+ pCR-4-TOPO with primers 190 and 194 as positive control. 3= PCR amplification with primers 190 and 196 of colony grown after BP reaction. 4= PCR amplification of *JERF1*+ pCR-4-TOPO with primers 190 and 196 as positive control. 5= PCR amplification with primers 195 and 194 of colony grown after BP reaction. 6= PCR amplification of DB90 with primers 195 and 194 as positive control. 7= negative control.

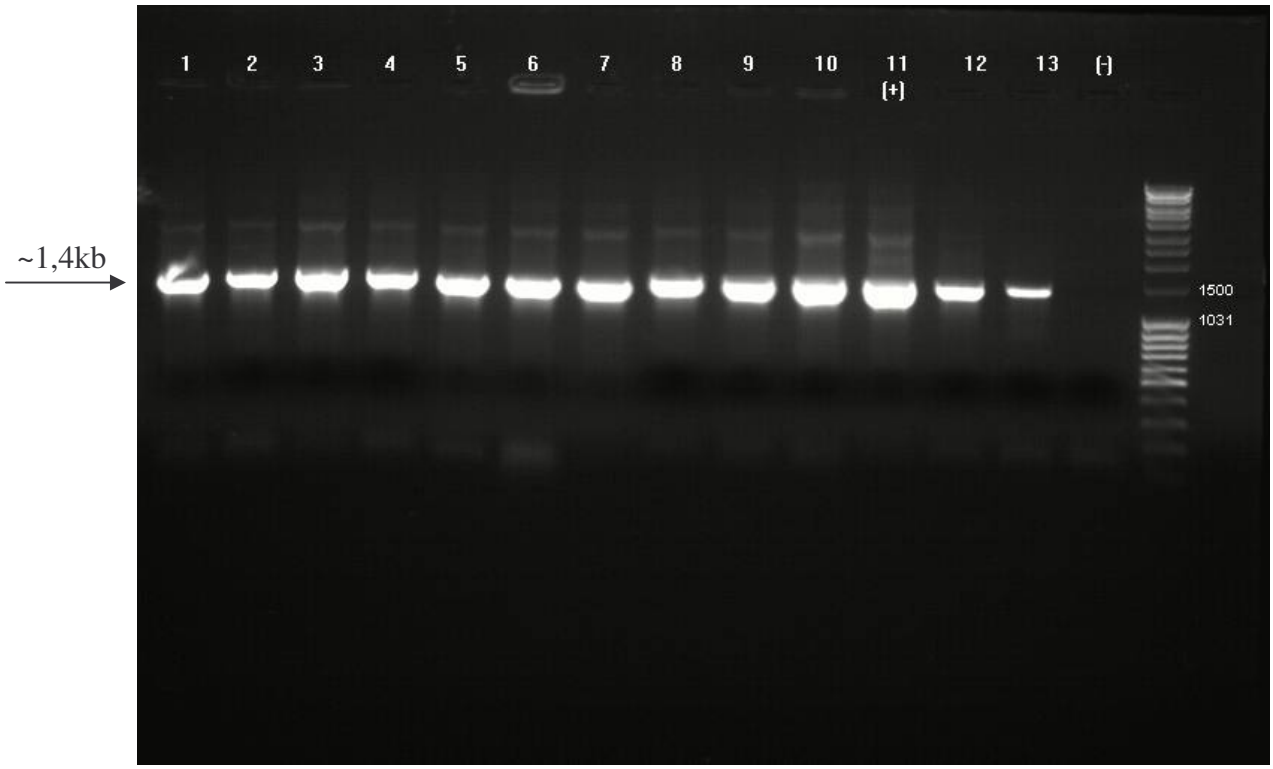


Figure 4.15: 1% Agarose gel electrophoresis of colony PCR with primers 190 and 194 of 12 colonies grown on selective media. 2, 3, 4, 5, 6, 12= colonies grown after transformation of GFP-N- Bin +*JERF1* constructs. 1, 7, 8, 9, 10, 13= colonies grown after transformation of GFP-C- Bin +*JERF1* constructs. 11= PCR amplification of *JERF1*+ pCR-4-TOPO with primers 190 and 194 as positive control. (-)= negative control.

4.4.10 Sequence confirmations of constructs prepared

Sequence alignments of the constructs with *JERF1* (TIGR; TC 124207) have shown that the gene is successfully inserted into vectors pDONR™ 221, GFP-N-Bin and GFP-C-Bin. Colonies were grown on selective medium after transformation and gave the fragment of 1, 4 kb by PCR with primers 190 and 194. Sequence alignments are shown in Appendix C.

4.5 Silencing Analysis of *JERF1*

JERF1 was silenced by virus-induced gene silencing (VIGS) method in *N. benthamiana* plants in order to predict its function in viral pathogenesis. In order to silence the gene constructs TRV2+ *JERF1* (DB226) and pGR106+*JERF1* (DB213) were transformed into *A.tumefaciens* hyper virulent strain LBA4404. pBBR1MCS virGN54D chemically and the transformants were selected on LB agar plates containing the rifampicin (25 µg/ml) and kanamycin (50 µg/ml) antibiotics. Colony PCR was performed with gene specific primers for *JERF1* for checking the presence of the gene before plant infection. Prior to infiltration *Agrobacterium* cultures were grown until the optical density reached 2. After plant growth for 3 weeks *Agrobacterium* containing the constructs were infiltrated into leaves and left for 2 weeks for the silencing. Then the plants were challenged with Tobacco Mosaic Virus (TMV) by infecting the leaves. *JERF1*-silenced *N.benthamiana* plants were observed after 3 weeks following virus infection. Both susceptible (nn) genotype and resistant (NN) genotype plants were used for this experiment. Observation of the virus infected- *JERF1*-silenced wild-type plants has shown a massive death phenotype whereas no massive death was observed in virus infected- *JERF1*-silenced resistant plants.

4.5.1 Plant observation

After 15 days of plant growth, leaves were infiltrated with *Agrobacterium* containing DB226 and DB 213 constructs for silencing which takes about 15 days. Then plants were challenged with TMV and observed daily. Silencing results are shown below. Silencing experiments has been done in duplicates but has to be repeated for confirmation.

Figure 4.16: Control wild-type (nn) plant which is neither silenced nor TMV infected

Figure 4.17: Control nn plant which is not silenced but TMV infected

Figure 4.18: Control NN plant which is not silenced but TMV infected

Figure 4.19: NN plant that is *JERF1* silenced and TMV infected

Figure 4.20: nn plant that is *JERF1* silenced and TMV infected

Figure 4.21: nn plant that is *JERF1* silenced and TMV infected

4.6 GFP-fusion protein Expression and Localization Analysis of *JERF1*

In order to see the sub-cellular localization of JERF1, the gene was inserted into GFP fusion vectors, GFP-N-Bin and GFP-C-Bin. GFP constructs DB110 and DB 111 were inserted into *A.tumefaciens* hyper virulent strain LBA4404.pBBR1MCSvirGN54D for transient expression of the protein in *A.thaliana* Col-O suspension cultures.

4.6.1 Transformations of constructs into *A.tumefaciens*

Constructs DB110 and DB111were transformed into *A.tumefaciens* hyper virulent strain LBA4404.pBBR1MCSvirGN54D chemically. Transformants were selected on LB agar media containing kanamycin (50 µg/ml) and rifampicin (25 µg/ml) antibiotics. Colonies grown were screened by colony PCR with primers 190 and 194 for the presence of the gene. 95% transformation efficiency was achieved.

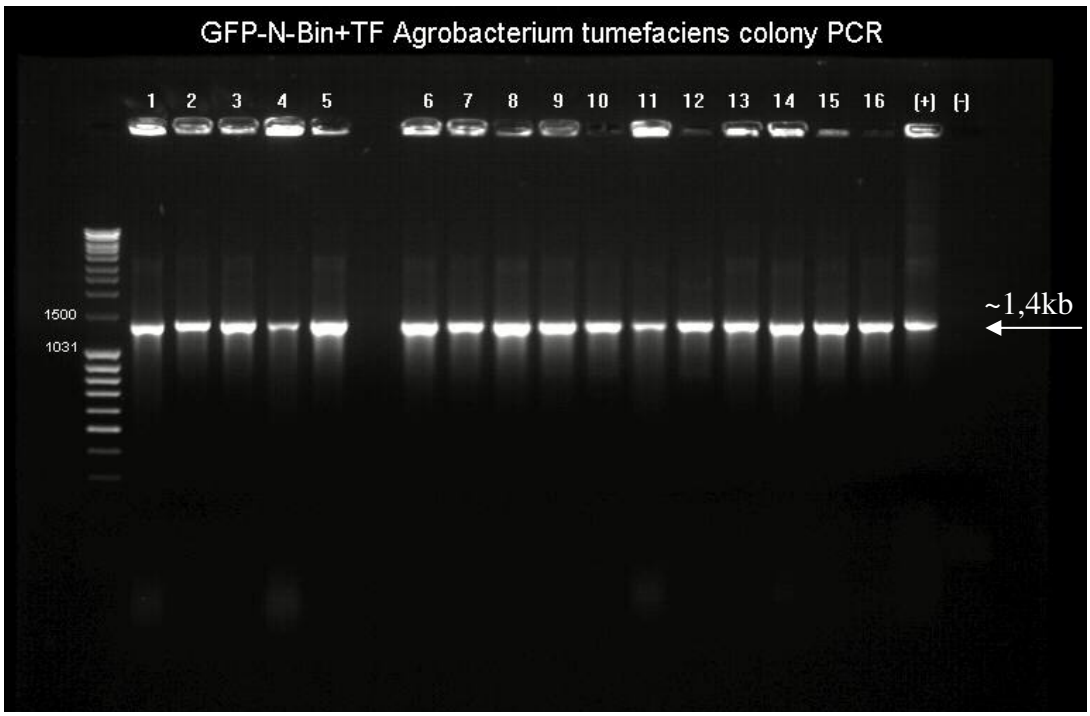


Figure 4.22: 1% Agarose gel electrophoresis of colony PCR with primers 190 and 194 showing the presence of *JERF1* gene in *A.tumefaciens* transformed with GFP-N-Bin+*JERF1* construct. 1-16= colonies grown after transformation of GFP-N- Bin +*JERF1* constructs. (+)= = PCR amplification of *JERF1*+ pCR-4-TOPO with primers 190 and 194 as positive control. (-)= negative control.

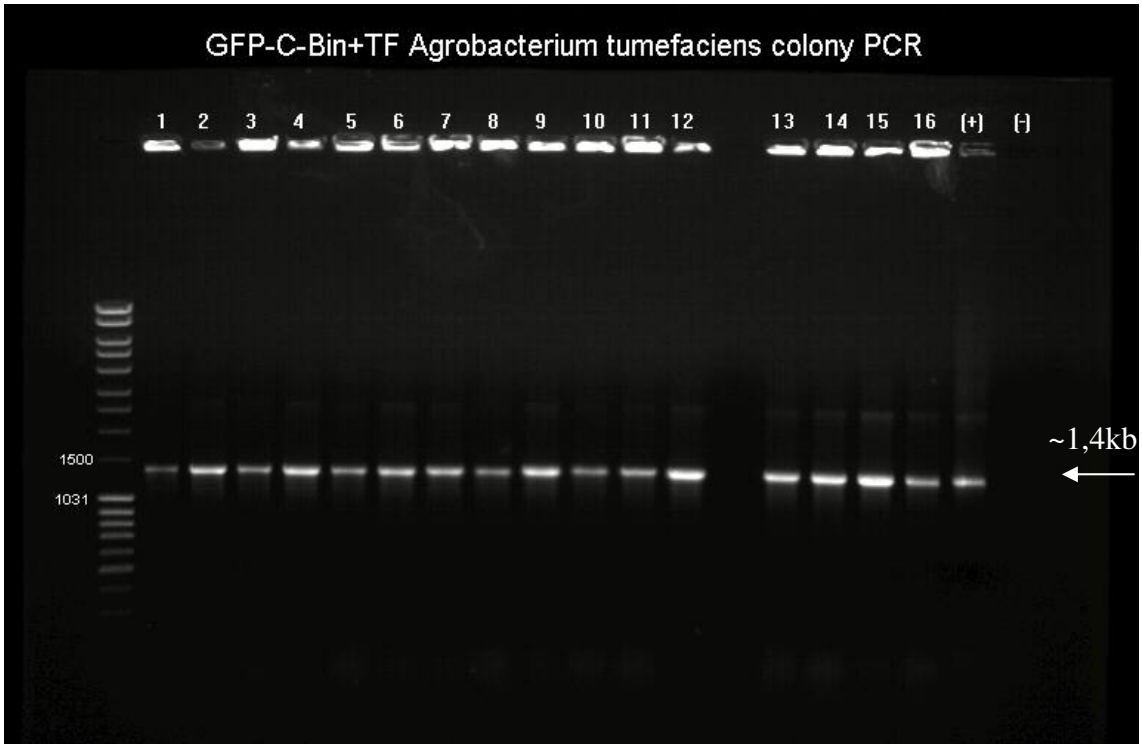


Figure 4.23: 1% Agarose gel electrophoresis of colony PCR with primers 190 and 194 showing the presence of *JERF1* gene in *A.tumefaciens* transformed with GFP-C-Bin+*JERF1* construct. 1-16= colonies grown after transformation of GFP-C- Bin +*JERF1* constructs. (+)= = PCR amplification of *JERF1*+ pCR-4-TOPO with primers 190 and 194 as positive control. (-)= negative control

4.6.2 Transient expression of GFP constructs in *A. thaliana*

A.thaliana Col-O suspension cultures were inoculated with *Agrobacterium* containing the DB110 and DB111 constructs following the subculturing of the suspensions. After incubation of 2 days with *Agrobacterium,* the suspensions were observed under inverted and fluorescent microscope for GFP expressions. (Data not shown)

4.7 Protein-protein interaction analysis of JERF1

In order to observe the interactors of JERF1, yeast-mating assay was performed. For mating-assay, first yeast strains EGY 48 and RFY 206 were transformed with pJG4-5+*JERF1* (DB112) and pTBSI+*JERF1* (DB113) constructs. Then the recombinant yeast were mated with Plant P58^{IPK} containing yeast.

4.7.1 Yeast Transformations

pJG4-5+*JERF1* and pTBSI+*JERF1* constructs were transformed into yeast EGY 48 and RFY 206 by Li-Ac method. The transformants were selected on –trp and – ura media for selection. DB113 transformed yeast did not grow on the selective media whereas DB112 transformations gave several colonies. The yeast colonies grown on selective media after transformation were screened by colony PCR with gene specific primers 190 and 194 (See Fig: 4.24).

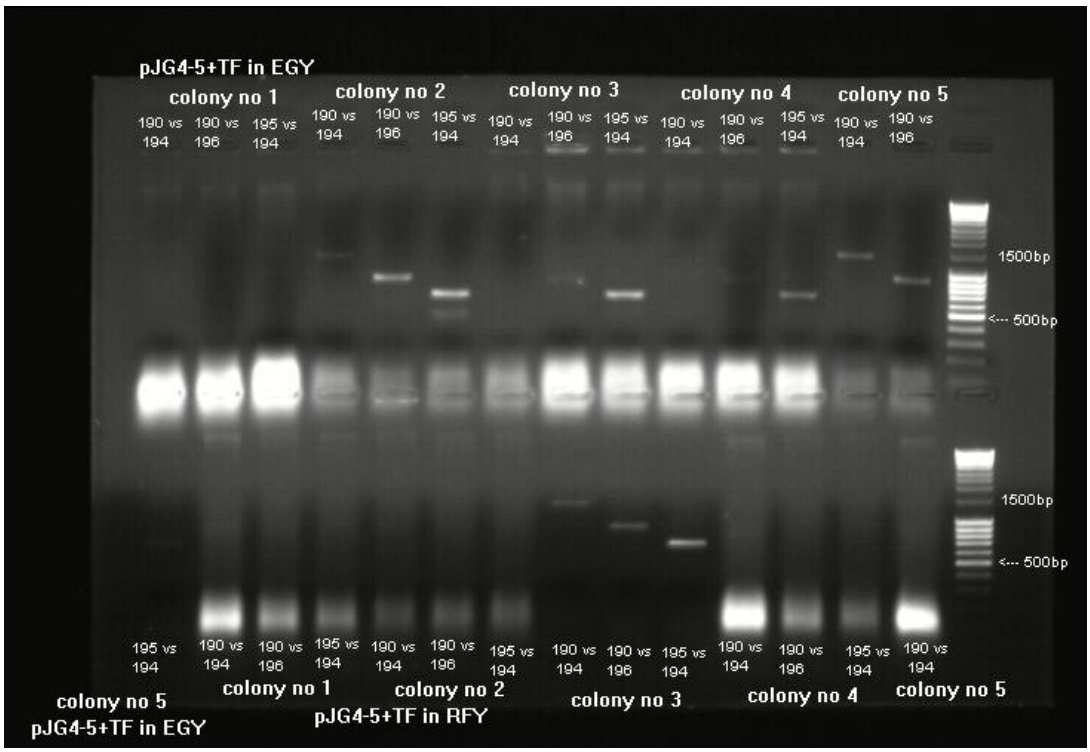


Figure 4.24: Electrophoresis analysis of colony PCR with primers 190 and 194 of 5 yeast EGY48 and 5 yeast RFY206 colonies grown on selective medium after transformation with DB112 construct.

4.7.2 Mating Assay

Plant P58^{IPK} had been ligated into pTBSI vector previously and transformed into RFY 206 yeast cells. DB112 containing EGY48 yeast cells were mated with Plant P58^{IPK}+ pTBSI transformed RFY 206 yeast cells and DB112 containing RFY 206 yeast cells were mated with Plant P58^{IPK}+ pTBSI transformed RFY 206 as negative control. 3 colonies were observed in the plate where *JERF1*+pJG4-5 construct containing EGY yeast cells mated with Plant P58^{IPK}+ pTBSI construct containing RFY yeast cells. In the lower part of the plate, *JERF1*+pJG4-5 construct containing RFY yeast cells were mated with Plant P58^{IPK}+ pTBSI construct containing RFY yeast cells as negative control (See Fig: 4.25).

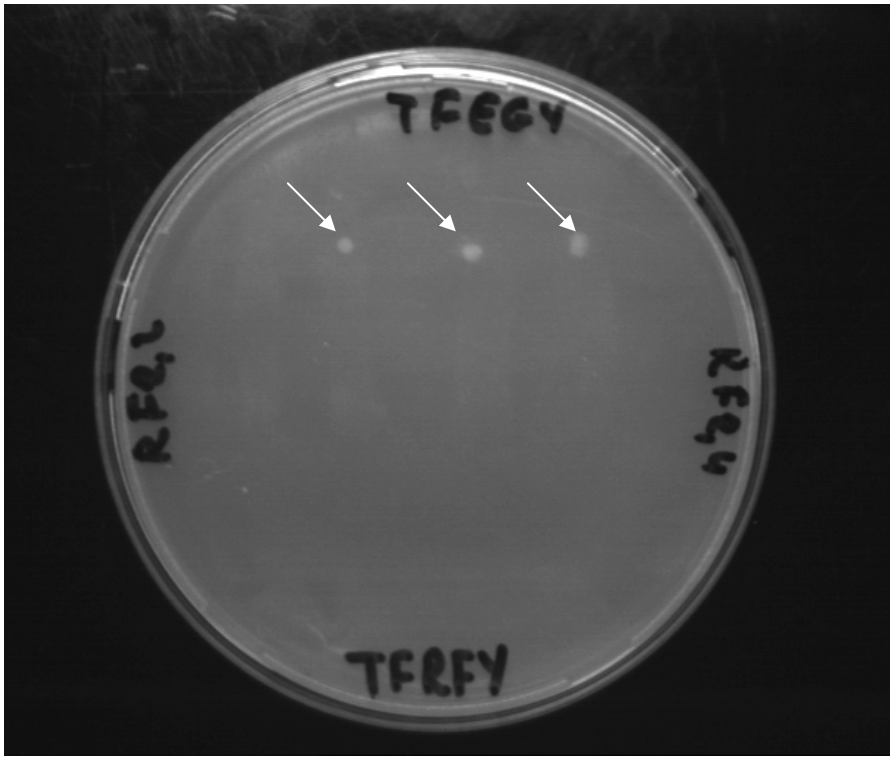


Figure 4.25: Mating Assay Result showing the in vivo interaction of Plant P58IPK with *JERF1*

4.8 Subcloning into Yeast Protein Expression Vector by Homologous Recombination

JERF1 was amplified with primers 207 and 208 which were specifically designed for cloning into expression vector pEGKG by homologous recombination. A full length of *JERF1* which corresponds to about 1, 4 kb on the agarose gel electrophoresis was obtained from this amplification (See Fig: 4.26).

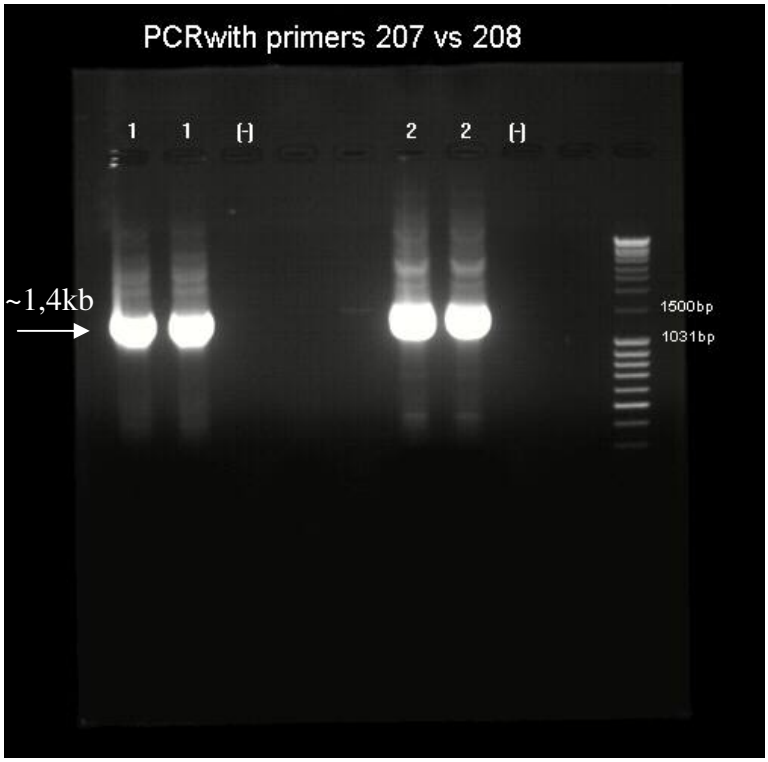


Figure 4.26: 1% Agarose gel electrophoresis result showing the amplification of *JERF1* with primers 207 and 208.

pEGKG was digested with *Hind*III for linearization for cloning of the *JERF1* gene. Total digestion was run on gel for gel purification (See Fig: 4.27).

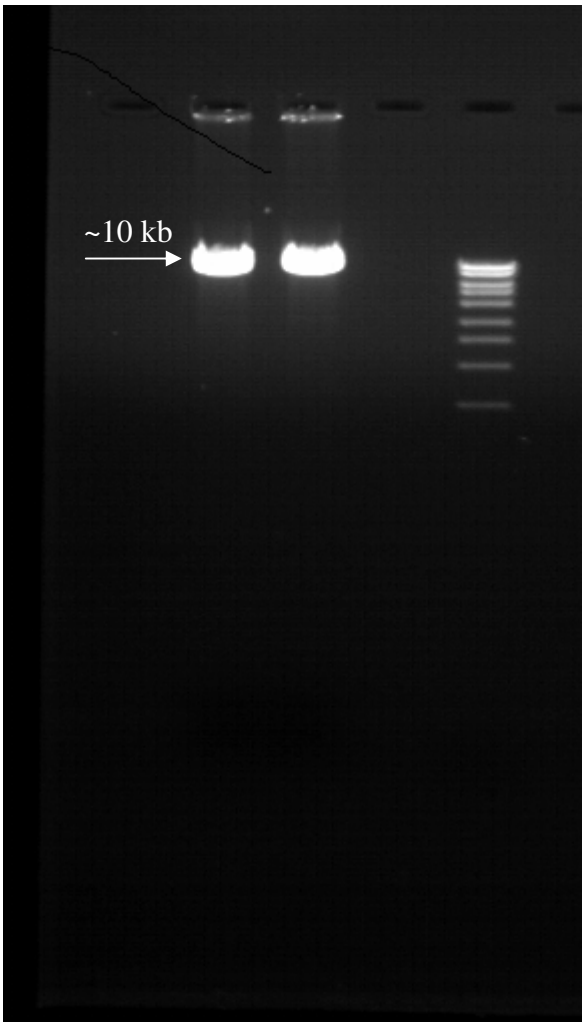


Figure 4.27: 1% agarose gel electrophoresis showing the *Hind*III digestion of the vector pEGKG. Samples were run at 100 V for 1.5 hours

JERF1 amplified with primers 207 and 208 was cloned into pEGKG by homologous recombination in yeast EGY48 strain. 1 µl of PCR product and 1 µl of digested pEGKG were transformed into yeast EGY48 cells by LiAc method. The transformants were selected on the proper selective medium and grown colonies were screened for the presence of *JERF1* with PCR amplification with primers 190 and 194 (See Fig: 4.28).

Figure 4.28: Electrophoresis analysis of colony PCR with primers 190 and 194 of 4 yeast EGY 48 colonies and 4 RFY206 colonies grown on selective medium after transformation with pEGKG and *JERF1*.

Plasmid was isolated from colony 3 and electroporated into *E.coli* TOP10 cells for plasmid rescue. Electroporated *E.coli* samples were grown overnight at 37^0C and the next day PCR was done with primers 190 and 194 to check the insert (See Fig: 4.29).

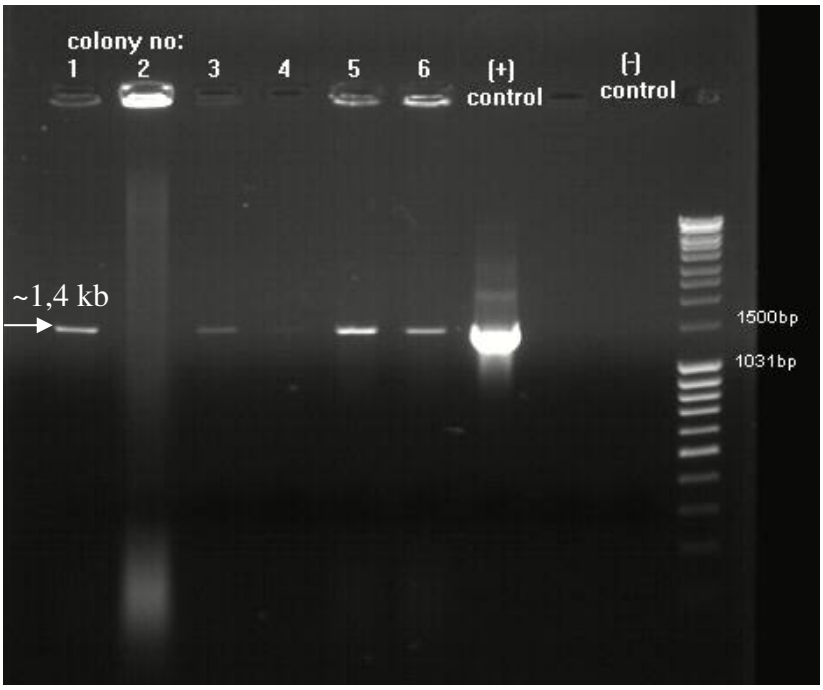


Figure 4.29: Electrophoresis analysis of colony PCR with primers 190 and 194 of 6 *E.coli* colonies grown after electroporation of pEGKG+*JERF1* construct into *E.coli* cells.

5 DISCUSSION

Apetala 2/ethylene responsive factor (AP2/ERF) proteins are AP2 domain containing transcription factors and form the second largest transcription factor family in plants. The AP2 transcription factor family is one of several that are unique to the plant lineage (Reichman *et al*., 2000). AP2 domain was first identified by Jofuku *et al*. 1994. The conserved domain was found in homeotic gene APETALA2 (AP2) and in ethylene-responsive element binding proteins (EREBPs) from tobacco (Ohme-Takagi *et al.,* 1995). AP2 transcription factor family contains the subfamily of ethylene response factor (ERF). 125 genes of *Arabidopsis thaliana* AP2 TFs are categorized into two subfamilies (Sakuma *et al.,* 2002). A subfamily contains those that are similar to the dehydration-responsive element binding (DREB) genes, and the B subfamily is the one similar to AtERF1. Gutterson and Reuber recently (2004 August) reorganized the categories. The gene number increased to 145 and consistent with the previous categorization.

We have identified an AP2 domain containing putative transcription factor in *L. esculentum*. It has been named as *JERF1* because of its putative role as jasmonic acid and ethylene response factor (Huang R.F. *et al.*). JERF1 was identified as an interactor of $p58^{IPK}$ in a yeast two-hybrid analysis (Bilgin D.D unpublished data). Sequence analysis revealed a 594 bp long partial gene sequence that showed high homology to AY044235 in NCBI database. By using gene specific primers 190 forward and 194 reverse we have cloned the full length gene from tomato leaf 1^{st} strand cDNA library and subcloned it into pCR®4-TOPO vector for sequencing. This construct has been given the name DB90. Gene sequence confirms 100% homology to *JERF1* (Appendix C).

Functional analyses of *JERF1* included the silencing, overexpression, protein-protein interaction and localization experiments. For each step, the gene had to be sub-cloned into specific vectors for that function. Different primers containing different restriction enzyme sites compatible with the vector multiple cloning sites were designed according to AY044235 sequence of *JERF1* from the 5' end as forward and 3 ' end as reverse. By using the DB90 construct as template the *JERF1* gene was amplified to be inserted into different expression vectors.

For virus induced gene silencing experiments two different VIGS vectors were used. One of them is potato virus X based pgR106 and the other one is Tobacco Rattle Virus based pTRV2. *N* gene transgenic (NN) and wild type *N. benthamiana* plants (nn) were used for VIGS experiments. A 500 bp long *JERF* 3' was amplified with PCR for cloning. The 500 bp region was selected after aligning *JERF1* with other AP2 domain genes. This region is specific to *JERF1* gene.

When NN *N. benthamiana* plants are infected with TMV, plant develops HR at the site of infection and induces cell death in order to prevent replication and spread of the virus. *JERF1* gene was silenced in NN *N. benthamiana* background by using PVX vector pgR106. After 2 weeks, silenced plants were challenged with TMV. No difference was observed between *JERF1* non-silenced and silenced, TMV challenged NN *N. benthamiana* plants. All induced cell death at the site of infection upon TMV infection. There was no effect on N-mediated resistance to TMV in NN plants.

TMV infection of wild-type (nn) *N. benthamiana* plants does not develop cell death upon infection. However, silencing of *JERF1* caused massive cell death in wild-type *N. benthamiana* plants when challenged with TMV. The experiments were done in duplicates and both plants showed the same response. These results imply that JERF1 protein is required for development of viral symptoms and prevention of nonhost resistance

For timing of the silencing, phytoene desaturase (PDS) gene was used as one of the controls (Ruiz *et al* 1998). PDS gene silenced plants become susceptible to photo

bleaching and the silenced leaves become white. The plants at the same age and conditions are used in parallel for silencing experiments (data not shown).

The silencing experiments that were done with pTRV2 did not show any massive death in wild-type *N. benthamiana* plants. We believe we faced some technical difficulties in the application of this particular silencing experiment series. The silencing experiment is performed by mixing pTRV1 with pTRV2; vectors carry RNA1 and RNA2 of Tobacco Rattle Virus, respectively (Liu *et al.,* 2002). The two vectors have to be mixed in 1:1 ratio OD 600nm 0.5-1, if these conditions cannot be maintained properly the silencing does not work. Northern or RT-PCR analysis experiments of the silenced plants would give more reliable data for silencing of *JERF1*. However, despite we collected and frozen tissue samples due to time limitations experiments could not be performed.

AP2/ERF genes function in a variety of stress regulation. A-subfamily TFs predominantly plays a role in the regulation of abiotic stress responses (C-repeat binding factor CBF and DREB). B-subfamily contains all AP2 genes that are involved in disease resistance responses. ERF genes are regulated by a variety of disease related stimuli, such as infection by virulent and avirulent pathogens; Several ERF TFs are transcriptionally regulated not only by pathogens, but also plant hormones like ethylene, jasmonic acid, salicylic acid which are important signaling components during plant-pathogen interactions (Cheng *et al.,* 2002, Fujimoto *et al.,* 2000, Lorenzo *et al.,* 2003). For example; Pti-4, Pti-5 and Pti-6 interact with tomato disease resistance protein Pto in yeast two-hybrid assays fall into this category. This data was the first to suggest the involvement of this ERF subfamily involvement in the regulation of plant disease resistance pathways (Zhou *et al*., 1997). Especially Pti-4 and AtERF1 are induced not only by pathogen infection but also by salicylic acid as well as jasmonic acid and ethylene. Overexpression of Pti-4 in *Arabidosis thaliana* induces defense genes and produces enhanced resistance to *E. orontii* and increased tolerance to *P. syringae* (Gu *et al.,* 2002). For overexpression analysis of *JERF1* gene in plant, pRTL2-GUS/NIaΔBam vector was used. This vector contains a Cauliflower Mosaic Virus minimal 35S promoter, a multiple cloning site and NOS terminator. However due to time limitations overexpression analysis could not be performed. The future work includes the

production of transgenic plants with high expression levels of *JERF1*, to observe the TMV infection pattern and responses given to different pathogen infection of these transgenic plants.

A partial clone of JERF1 has been found to be an *in vivo* interactor of plant P58^{IPK} (Bilgin D.D., unpublished data). During this study, the interaction of whole length JERF1 and plant P58^{IPK} has been confirmed by yeast mating assay. *JERF1* was inserted into vectors pJG4-5 and pTBSI. Yeast EGY48 and RFY206 cells were then transformed by these constructs. Mating was done with transformed EGY and RFY yeast cells containing the *JERF1*+pJG4-5 construct and *P58^{IPK}* +pTBSI containing RFY206 cells. Colonies grown on selective media after the mating of transformed EGY cells containing *JERF1* with RFY cells containing plant P58^{IPK} indicate that these two proteins interact *in vivo*. The result of mating experiment also puts forward the role of JERF1 in plant-virus interaction.

During this study, *JERF1* was also inserted into yeast vector pEGKG for heterologous protein expression. pEGKG is a GST fusion vector which will provide the purification of JERF1 protein. One of the future works is the isolation of JERF1 protein for in vitro protein-protein interactions. pEGKG-*JERF1* construct will be isolated from *E.coli* and transformed into a yeast strain whose proteases are inhibited so that high levels of protein expression will be obtained.

Finding of subcellular localization of the JERF1 protein was also attempted during this study. To observe where JERF1 is localized before and after virus infection, the gene was subcloned into 2 GFP fusion proteins. GFP-N-Bin vector contains GFP signal at the N terminus whereas GFP-C-Bin vector contains GFP signal at the C terminus of the inserted gene. These two constructs were both prepared in order to minimize the possibility of GFP to obstruct proper folding of the JERF1 protein. Microscopic observations have clearly shown that GFP is expressed in *Arabidopsis thaliana* cell culture. However subcellular localization could not be observed because it needs a confocal microscope. Another reason for not seeing the localization might be the clustering of Arabidopsis cells. Since suspension cultures were used in this

experiment, single cell formation is not possible because the plant cells have the tendency to form calli.

6 CONCLUSION

Although gene sequence of *JERF1* is present in the databases, there has been no functional data about JERF1 in the literature. In this study, we have cloned full length *JERF1* from tomato cDNA. In order to obtain information about the function of JERF1, we have subcloned the gene into several expression vectors including silencing, overexpression, yeast mating assay, yeast protein expression and GFP-fusion vectors.

We have observed a death phenotype in *JERF1*-silenced susceptible (nn) *N. benthamiana* plants upon challenging with TMV. This implies the possible role of JERF1 in virus pathogenicity of tomato plants.

In order to confirm the role of JERF1 in virus pathogenicity, overexpression analysis was aimed however, due to time limitations, this experiment could not be finalized.

GFP-fused JERF1 constructs can be used for analysis of subcellular localization of the protein. Confocal microscopy and cellular fractionation assays will give information about the subcellular localization of the protein.

Yeast mating assay implies that JERF1 interacts with plant $P58^{IPK}$. Other interactors of JERF1 can be found by mating assays with other proteins. Moreover, *in vitro* protein-protein interaction experiments will demonstrate the interactors of JERF1 protein so that the possible role of JERF1 in a specific signaling pathway can be characterized.

Due to time limitations, most of the functional analyses could not be accomplished. Future works include, stable transformant plants carrying silencing constructs, analysis of mutant plants of *JERF1*, *in vitro* and *in vivo* protein-pull down assays to find out the interactors of JERF1; chromatin IP to check the DNA binding sites of JERF1 and northern blotting to see the expression levels of *JERF1* in different tissues and in different plants of normal and stress subjected plants. Combination of these studies will probably give the overall picture of JERF1 functioning in plants

REFERENCES

1. Baker B, Zambryski P, Staskawicz B, Dinesh-Kumar SP, "Signalling in Plant-Microbe Interaction" *Science* 276 (1997) 726-733.

2. Bendahmane A, Köhm BA, Dedi C, Baulcombe DC, "The Coat Protein of Potato Virus X is a Strain-Specific Elicitor of *Rx*1-mediated Virus Resistance in Potato" *Plant J.* 8 (1995) 933-941.

3. Bilgin DD, Liu Y, Schiff M, Dinesh-Kumar SP, "P58^{IPK}, a Plant Ortholog of Double-Stranded RNA-Dependent Protein Kinase PKR Inhibitor, Functions in Viral Pathogenesis" *Developmental Cell* 4 (2003) 651-661.

4. Birch PRJ, Avrova AO, Dellagi A, Lacomme C, Santa Cruz S, Lyon GD. "Programmed Cell Death in Plants in Response to Pathogen Attack" Annual Plant Reviews. 4 (2000) 175-197

5. Botella BA, Parker JE, Frost LN, Bittner-Eddy PD, Beynon JL, Daniels MJ, Holub EB, Jones JDG, "Three Genes of the *Arabidopsis* RPP1 Complex Resistance Locus Recognize distinct *Peronospora Parasitica* Avirulence Determinants" *Plant Cell* 10 (1998) 1847-1860.

6. Buchanan BB, Gruissem W, Jones RL, "Biochemistry & Molecular Biology of Plants" Second Impression, *American Society of Plant Physiologists* 2000.

7. Carrington JC, Kasschau KD, Mahajan SK, Schaad MC, "Cell-to-Cell and Long-Distance Transport of Viruses in Plants" *Plant Cell* 8 (1996) 1669–1681.

8. Chang C, Shockey JA, "The Ethylene-Response Pathway: Signal Perception to Gene Regulation" *Curr. Opin. Plant Biol.* 2 (1999) 352-358.

9. Cheng WH, Endo A, Zhou L, Penney J, Chen HC, Arroyo A, Leon P, Nambara E, Asami T, Seo M, Koshiba T, Sheen J. "A unique short-chain

dehydrogenase/reductase in Arabidopsis glucose signaling and abscisic acid biosynthesis and functions" *Plant Cell*. 11 (2002)2645-26499.

10. Chini A, Grant JJ, Seki M, Shinozaki K, Loake GJ, "Drought Tolerance Established by Enhanced Expression of the CC-NBS-LRR Gene, ADR1, Requires Salicylic Acid, EDS1 and ABI1" *Plant J.* 38 (2004) 810-822.

11. Dangl JL, Jones JDG, "Plant Pathogens and Integrated Defence Responses to Infection" *Nature* 411 (2001) 826-833.

12. Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessmann H, Ward E, Ryals J, "A Central Role of Salicylic Acid in Plant Disease Resistance" *Science* 266 (1994) 1247-1250.

13. Dion M, Chamberland H, St-Michel C, Plante M, Darveau A, Lafontaine JG, Brisson LF, "Detection of a Homologue of bcl-2 in Plant Cells" *Biochem. Cell Biol.* 75 (1997) 457-461.

14. Doke N, Ohashi Y, "Involvement of an O_2 Generating System in the Induction of Necrotic Lesions on Tobacco Leaves Infected with Tobacco Mosaic Virus" *Physiol. Mol. Plant Pathol.* 32 (1988) 163-175.

15. Ellis J, Jones D, "Structure and Function of Proteins Controlling Strain-Specific Pathogen Resistance in Plants" *Curr. Opin. Plant Biol.* 1 (1998) 288-293.

16. Ellis JG, Lawrence GJ, Luck JE, Dodds PN, "Identification of Regions in Alleses of the Flax Rust Resistance Gene *L* That Determine Differences in Gene-for-Gene Specificity" *Plant Cell* 11 (1999) 495-506.

17. Flor HH, "Current Status of the Gene-for-Gene Concept" *Annu. Rev. Phytopathol.* 9 (1971) 275-296.

18. Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M "Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression" *Plant Cell*. 3 (2000):393-404

19. Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J, "Requirement of Salicylic Acid for the Induction of Systemic Acquired Resistance" *Science* 261 (1993) 754-756.

20. Gale Jr. M, Katze MG, "Molecular Mechanisms of Interferon Resistance Mediated by Viral-Directed Inhibition of PKR, the Interferon-Induced Protein Kinase" *Pharmacol. Ther.* 78 (1998) 29-46.

21. Gassmann W, Hinsch ME, Staskawicz BJ, "The Arabidopsis RPS4 bacterial-resistance gene is a member of the TIR-NBS-LRR family of disease-resistance genes" *Plant J*. 3 (1999) 265-77

22. Gil J, Esteban M, Roth D, "In Vivo Regulation of Protein Synthesis by Phosphorylation of the α Subunit of Wheat Eukaryotic Initiation Factor 2" *Biochemistry* 39 (2000) 7521-7530.

23. Gong W, Shen Y-P, Ma L-T, Pan Y, Du Y-L, Wang D-H, Yang J-Y, Hu L-D, Liu X-F, Dong C-X, Ma L, Chen Y-H, Yang X-Y, Gao Y, Zhu D, Tan X, Mu J-Y, Zhang D-B, Liu Y-L, Dinesh-Kumar SP, Li Y, Wang X-P, Gu H-Y, Qu L-J, Bai S-N, Lu Y-T, Li J-Y, Zhao J-D, Zuo J, Huang H, Deng XW, Zhu Y-X, "Genome-Wide ORFeome Cloning and Analysis of Arabidopsis Transcription Factor Genes" *Plant Physiology* 135 (2004) 773-782.

24. Gu Y-Q, Wildermuth MC, Chakravarthy S, Loh Y-T, Yang C, He X, Han Y, Martin GB, "Tomato Transcription Factors Pti4, Pti5 and Pti6 Activate Defense Responses when Expressed in Arabidopsis" *Plant Cell* 14 (2002) 817-831.

25. Gu Y-Q, Yang C, Thara VK, Zhou J, Martin GB, "Pti4 is Induced by Ethylene and Salicylic Acid and its Product is Phosphorylated by the Pto Kinase" *Plant Cell* 12 (2000) 771-786.

26. Gutterson N, Reuber TL, "Regulation of disease resistance pathways by AP2/ERF transcription factors" *Curr. Opin. Plant Biol*. 7 (2004) 465–471

27. Hammond-Kosack KE, Jones DJG, "Resistance Gene-Dependent Plant Defense Responses" *Plant Cell* 8 (1996) 1773-1791.

28. Jarpe MB, Widmann C, Knall C, Schlesinger TK, Gibson S, Yujiri T, Fanger GR, Gelfand EW, Johnson GL, "Anti-Apoptotic versus Pro-Apoptotic Signal Transduction: Check-Points and Stop Signs along the Road to Death" *Oncogene* 17 (1998) 1475-1482.

29. Jofuku KD, den Boer BGW, Montagu MV, Okamuro JK, "Control of Arabidopsis Flower and Seed Development by the Homeotic Gene *APETHALA2*" *Plant Cell* 6 (1994) 1211-1225.

30. Jones CG, Tucker GA, Lycett GW, "Pattern of Expression and Characteristics of a Cysteine Proteinase cDNA from Germinating Seeds of Pea (*Pisum sativum* L.)" *Biochim. Biophys. Acta.* 129 (1996) 613-615.

31. Kiziz D, Lumbreras V, Pagès M, "Role of AP2/EREBP Transcription Factors in Gene Regulation During Abiotic Stress" *FEBS Letters* 498 (2001) 187-189.

32. Kobe B, Deisenhofer J, "The Leucine-Rich Repeat: A Versatile Binding Motif" *Trans Biochem. Sci.* 19 (1994) 415-421.

33. Krebs J, "The Role of Calcium in Apoptosis" *Biometals* 11 (1998) 375-382.

34. Langland JO, Langland LA, Browning KS, Roth DA, "Phosphorylation of Plant Eukaryotic Initiation Factor-2 by the Plant-encoded Double-stranded RNA-dependent Protein Kinase, pPKR, and Inhibition of Protein Synthesis *in Vitro*" *Journal of Biol. Chem.* 271 (1996) 4539-4544.

35. Lawrence GJ, Finnegan EJ, Ayliffe MA, Ellis JG, "The *L6* Gene for Flax Rust Resistance is Related to the Arabidopsis Bacterial Resistance Gene *RPS2* and the Tobacco Viral Resistance Gene *N*" *Plant Cell* 7 (1995) 1195-1206.

36. Lawton KA, Potter SL, Uknes S, Ryals J, "Acquired Resistance Signal Transduction in Arabidopsis is Ethylene Independent" *Plant Cell* 6 (1994) 581-588.

37. Lemaitre B, Nicolas E, Michaut L, Reichhart J-M, Hoffmann JA, "The Dorsoventral Regulatory Gene Cassette *spätzle/Toll/cactus* Controls the Potent Antifungal Response in Drosophila Adults" *Cell* 86 (1996) 973-983.

38. Levine A, Pennell RI, Alvarez ME, Palmer R, Lamb C, "Calcium-Mediated Apoptosis in Plant Hypersensitive Disease Resistance Response" *Curr. Biol.* 6 (1996) 427-437.

39. Liu Y, Schiff M, Dinesh-Kumar SP, "Virus-induced gene silencing in tomato" Plant J. 6 (2002) 777-786.

40. Lorenzo O, Piqueras R, Sanchez-Serrano JJ, Solano R, "ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense" *Plant Cell* 1(2003) 165-178.

41. Luck JE, Lawrence GJ, Dodds PN, Shepherd KW, Ellis JG, "Regions outside of the Leucine-Rich Repeats of Flax Rust Resistance Proteins Play a Role in Specificity Determination" *Plant Cell* 12 (2000) 1367-1377.

42. Martin GB, Bogdanove AJ, Sessa G, "Understanding the Functions of Plant Disease Resistance Proteins" *Annu. Rev. Plant Biol.* 54 (2003) 23-61.

43. Medzhitov R, Preston-Hurlburt P, Janeway CA, "A Human Homologue of the Drosophila Toll Protein Signals Activation of Adaptive Immunity" *Nature* 338 (1997) 394-397.

44. Mittler R, Del Pozo O, Meisel L, Lam E, "Pathogen-Induced Programmed Cell Death in Plants, a Possible Defense Mechanism" *Developmental Genetics* 21 (1997) 279-289.

45. Moffett P, Farnham G, Peart J, Baulcombe DC, "Interaction between Domains of a Plant NBS-LRR Protein in Disease Resistance-Related Cell Death" *EMBO J.* 21 (2002) 4511- 4519.

46. Ohme-Takagi M, Shinshi H, "Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element" *Plant Cell*. 2 (1995) 173-82

47. Okamuro JK, Caster B, Villarroel R, Montagu MV, Jofuku KD, "The AP2 Domain of *APETALA2* Defines a Large New Family of DNA Binding Proteins in Arabidopsis" *Proc. Natl. Acad. Sci.* USA 94 (1997) 7076-7081.

48. Parker JE, Coleman MJ, Szabo V, Frost LN, Schmidt R, van der Biezen EA, Moores T, Dean C, Daniels MJ, Jones JD, "The Arabidopsis downy mildew resistance gene RPP5 shares similarity to the toll and interleukin-1 receptors with N and L6" *Plant Cell*. 6 (1997) 879-894

49. Pedley KF, Martin GB, "Molecular Basis of Pto-mediated Resistance to Bacterial Speck Disease in Tomato" *Annu. Rev. Phytopathol.* 41 (2003) 215-243.

50. Prescott LM, Harley JP, Klein DA, “Microbiology” Fifth Edition, *McGraw Hill* 2002

51. Reymond P, Weber H, Dammond M, Farmer EE, “Differential Gene Expression in Response to Mechanical Wounding and Insect Feeding in *Arabidopsis*” *Plant Cell* 12 (2000) 707-719.

52. Riechmann JL, Heard J, Martin G, Reuber L, Jiang C-Z, Keddie J, Adam L, Pineda O, Ratcliffe OJ, Samaha RR, Creelman R, Pilgrim M, Broun P, Zhang JZ, Ghandehari D, Sherman BK, Yu G-L, “Arabidopsis Transcription Factors: Genome-Wide Comparative Analysis Among Eukaryotes” *Science* 290 (2000) 2105-2110.

53. Ruiz MT, Voinnet O, Baulcombe DC “Initiation and maintenance of virus-induced gene silencing” Plant Cell. 6 (1998) 937-946

54. Sakuma Y, Liu Q,Dubouzet JG, Abe H, Shinozaki K, Yamaguchi-Shinozaki K, “DNA-Binding Specificity of the ERF/AP2 Domain of Arabidopsis DREBs, Transcription Factors Involved in Dehydration- and Cold-Inducible Gene Expression” *Biochem. Biophy.Res. Commun* 290(2002) 998-1009.

55. Sessa G, D’Ascenzo M, Martin GB, “Thr38 Ser198 are Pto Autophosphorylation Sites Required for the AvrPto-Pto-mediated Hypersensitive Response” *EMBO J.* 19 (2000) 2257-2269.

56. Shinozaki K, Dennis ES, “Cell Signaling and Gene Regulation Global Analysis of Signal Transduction and Gene Expression Profiles” *Curr. Opin. Plant Biol.* 6 (2003) 405-409.

57. Song W-Y, Wang G-L, Chen L-L, Kim H-S, Pi L-Y, Holsten T, Gardner J, Wang B, Zhai W-X, Zhu L-H, Fauquet C, Ronald P, “A Receptor Kinase-like Protein Encoded by the Rice Disease Resistance Gene, *Xa21*” *Science* 270 (1995) 1804-1806.

58. Sticher L, Mauch-Mani B, Metraux JP, “Systemic acquired resistance” *Annu. Rev. Phytopathol*. 35 (1997) 235-70

59. Suzuki K, Fukuda Y, Shinshi H, "Studies on Elicitor-Signal Transduction leading to Differential Expression of Defense Genes in Cultured Tobacco Cells" *Plant Cell Physiol.* 36 (1995) 281-289.

60. van der Biezen EA, Jones JDG, "The NB-ARC Domain: A Novel Signalling Motif shared by Plant Resistance Gene Products and Regulators of Cell Death in Animals" *Curr. Biol.* 8 (1998) R226-R227.

61. Volpe F, Clatworthy J, Kaptein A, Maschera B, Griffin A-M, Ray K, "The IL-1 Receptor Accessory Protein is Responsible for the Recruitment of the Interleukin-1 Receptor Associated Kinase to the IL-1/IL-1 Receptor I Complex" *FEBS Letters* 419 (1997) 41-44.

62. Vom Endt D, Kijne JW, Memelink J, "Transcription Factors Controlling Plant Secondary Metabolism: What Regulates the Regulators?" *Phytochemistry* 61 (2002) 107-114.

63. Waterhouse PM, Wang M-B, Lough T, "Gene Silencing as an Adaptive Defence Against Viruses" *Nature* 411 (2001) 834-841.

64. Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B, "The Product of the Tobacco Mosaic Virus Resistance Gene *N*: Similarity to Toll and the Interleukin-1 Receptor" *Cell* 78 (1994) 1101-1115.

65. Xie DX, Feys BF, James S, Nieto-Rostro M, Turner JG, "An Arabidopsis Gene Required for Jasmonate-Regulated Defense and Fertility" *Science* 280 (1998) 1091-1094.

66. Xu Y, Chang P F L, Liu D, Narasimhan M L, Raghothama K G, Hasegawa P M, Bressan R.A "Plant defense genes are synergistically induced by ethylene and methyl jsamonate" Plant Cell. 6 (1994) 1077–1085

67. Yang J, Steward R, "A Multimeric Complex and the Nuclear Targeting of the Drosophila reI Protein Dorsal" *Proc. Natl. Acad. Sci. USA* 94 (1997) 14524-14529.

68. Yang Y, Shah J, Klessig DF, "Signal Perception and Transduction in Plant Defense Responses" *Genes and Development* 11 (1997) 1621-1639.

69. Zhang JZ, “Overexpression Analysis of Plant Transcription Factors” *Curr. Opin. Plant Biol.* 6 (2003) 430-440.

70. Zhang S, Klessig DF, “Resistance Gene *N*-mediated *de novo* Synthesis and Activation of a Tobacco Mitogen-Activated Protein Kinase by Tobacco Mosaic Virus Infection” *Proc. Natl. Acad. Sci. USA* 95 (1998) 7433-7438.

71. Zhou J, Tang X, Martin GB, “The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a *cis*-element of pathogenesis-related genes” EMBO J. 11 (1997) 3207-3218.

72. Zhu T, “Global Analysis of Gene Expression Using GeneChip Microarrays” *Curr. Opin. Plant Biol.* 6 (2003) 418-425.

APPENDIX A

Primer Sequences:

A1) 190 forward primer

5’ TTCAAATTG AGCTTTTTCTCCATTAAAATT 3’

A2) 194 reverse primer

5’ TTATGAAAACAAGGATACATACTTACATAT 3’

A3) 195 forward primer

5’ GGTCTAGAGGATCCGTCAGTTTTTTCGAAGAGAAACCAG 3’

*Xba*I *Bam*HI

A4) 196 reverse primer

5’ GGGAATTCGGGGTACCGGATGAGATCTCTGGAGTCCTTGCAC 3’

*Eco*RI *Kpn*I

A5) 199 forward primer

5’ GGGGATCCAAGCTTGATGTTCAAATTGAGCTTTTTCTCCATT 3’

*Bam*HI *Hind*III

A6) 200 reverse primer

5’ GGGAATTCTCTAGAGTTATGAAAACAAGGATACATACTT 3’

*Eco*RI *Xba*I

A7) 201 forward primer

5’ GGGAATTCCCGCGGCCGCATGTTCAAATTGAGCTTTTTCTCC 3’

*Eco*RI *Not*I

A8) 202 reverse primer

5’ GGGAATTCCCGAGCTCGGTTATGAAAACAAGGATACATACTTTAT 3’

*Eco*RI *Sac*I

A9) 207 forward primer

5’ GGGGGTCTAGACTCCATGGGTCGACTCGAGATGTTCAAATTGAGCTTTTTCTCCA

Homologous recombination site

TAAA 3’

A10) 208 reverse primer

5’ GCGAGGCAGATCGTCAGTCAGTCACGATGATTATGAAAACAAGGATACATACTT

Homologous recombination site

ACATAT 3’

A11) BD1 forward primer

5’ GGGACAAGTTTGTACAAAAAAGCAGGCTTTTTCAAATTGAGCTTTTTCTCC 3’

*att*B1

A12) BD5 reverse primer

5’ GGGGACCACTTTGTACAAGAAAGCTGGGTTTTATGAAAACAAGGATACATA 3’

*att*B2

A13) 162 forward primer

5’ CCCTCTAGACGCGGCCGCCAAAAGGCACTTCGTGAGGAAACC 3’

*Xba*I *Not*I

A14) 163 reverse primer

5’ CCCGGTACCGTCGACCCAATTTCCCTCGAGATATGGGAT 3’

*Kpn*I *Sal*I

A15) 154 forward primer

5’ GATTTGGTACCTCCTAGCCGGATTTCTCGCCGG 3’

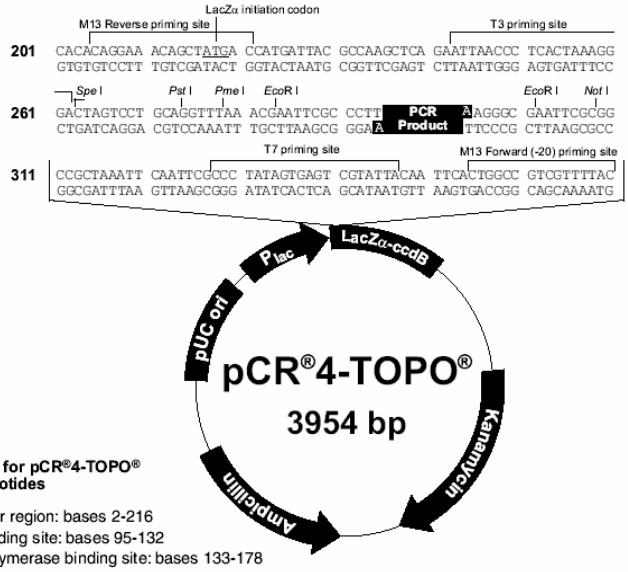
A16) 147 reverse primer

5’ CACATTCTCGAAGCCGTACGTCTTGGTTGC 3’

APPENDIX B

Vector Maps

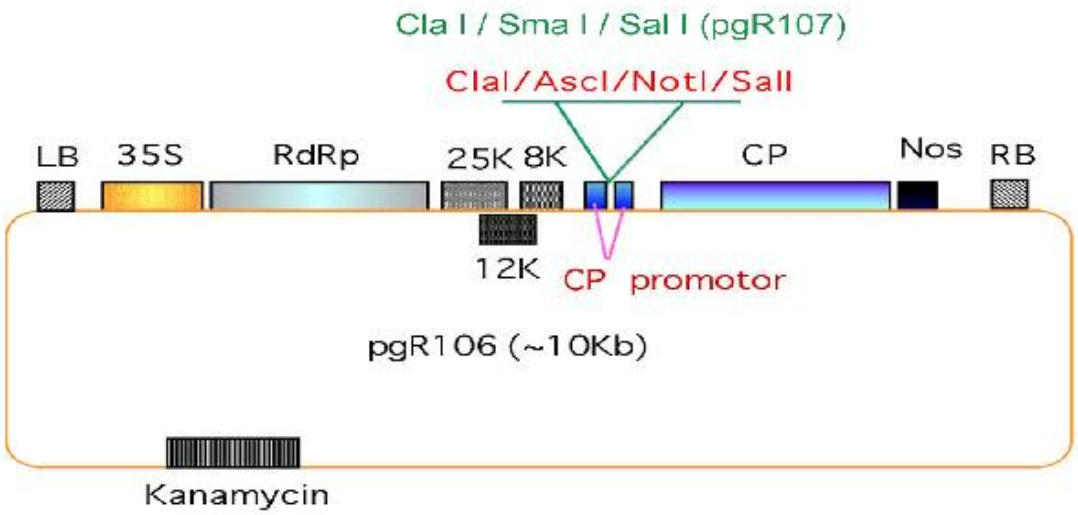
B1) pCR®-II-TOPO4® (Invitrogen)



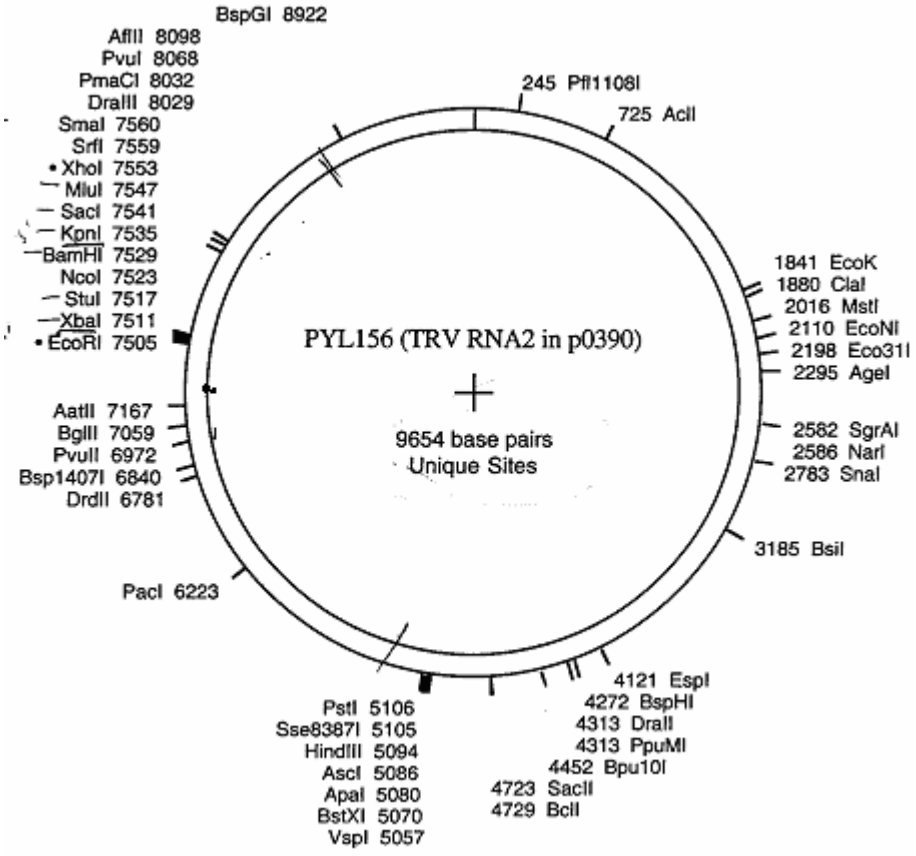
Comments for pCR®4-TOPO®
3954 nucleotides

lac promoter region: bases 2-216
CAP binding site: bases 95-132
RNA polymerase binding site: bases 133-178
Lac repressor binding site: bases 179-199
Start of transcription: base 179
M13 Reverse priming site: bases 205-221
LacZα-*ccdB* gene fusion: bases 217-810
LacZα portion of fusion: bases 217-497
*ccd*B portion of fusion: bases 508-810
T3 priming site: bases 243-262
TOPO® Cloning site: bases 294-295
T7 priming site: bases 328-347
M13 Forward (-20) priming site: bases 355-370
Kanamycin promoter: bases 1021-1070
Kanamycin resistance gene: bases 1159-1953
Ampicillin (*bla*) resistance gene: bases 2309-3061 (c)
Ampicillin (*bla*) promoter: bases 3062-3158 (c)
pUC origin: bases 3159-3832
(c) = complementary strand

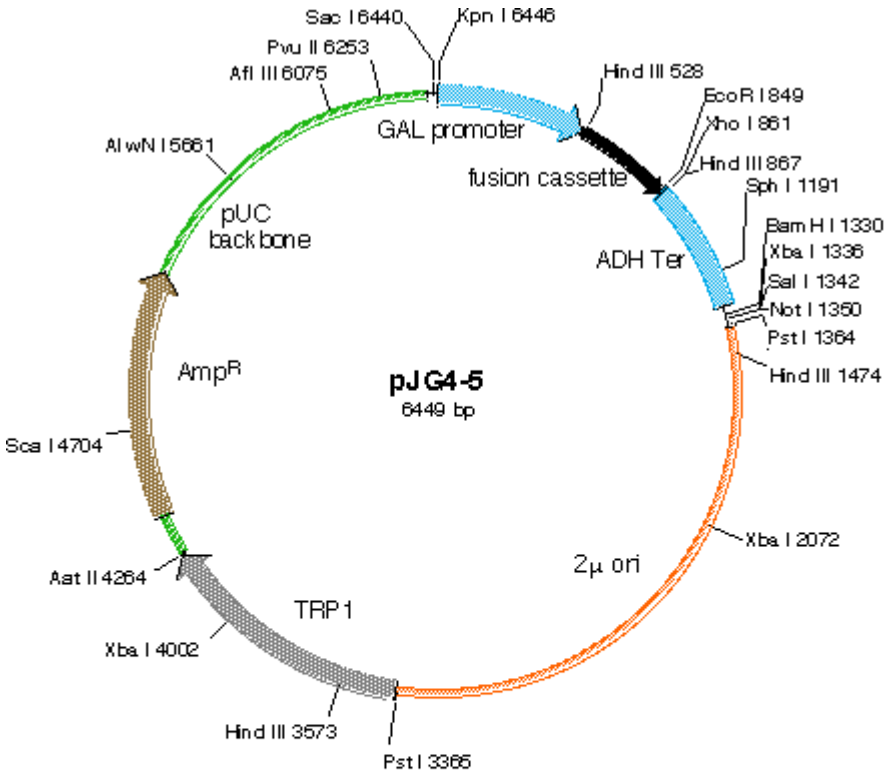
B2) pGR106



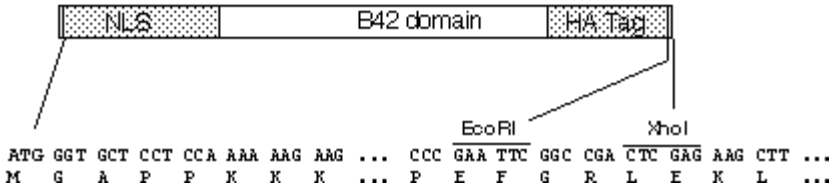
B3) pTRV2



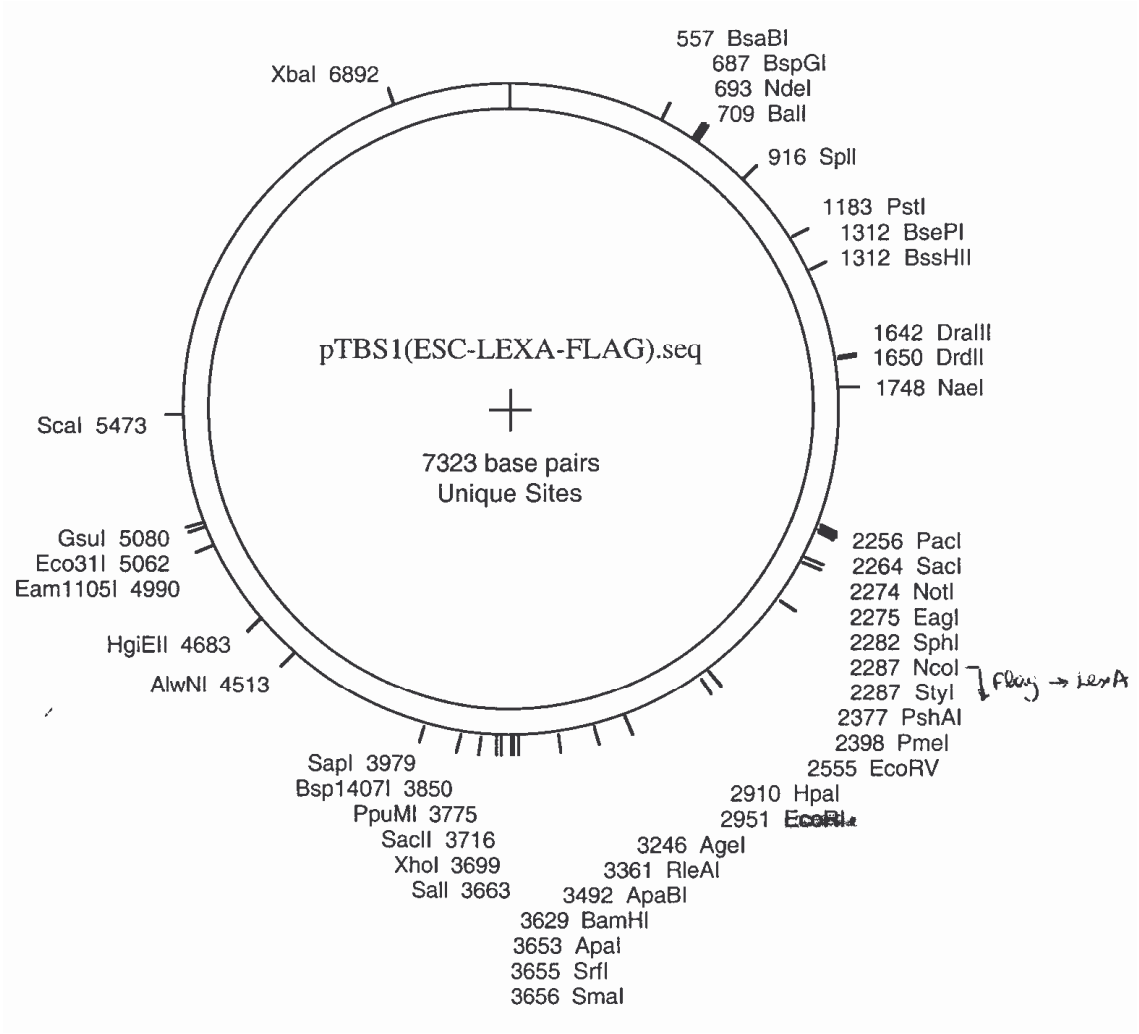
B4) pJG4-5



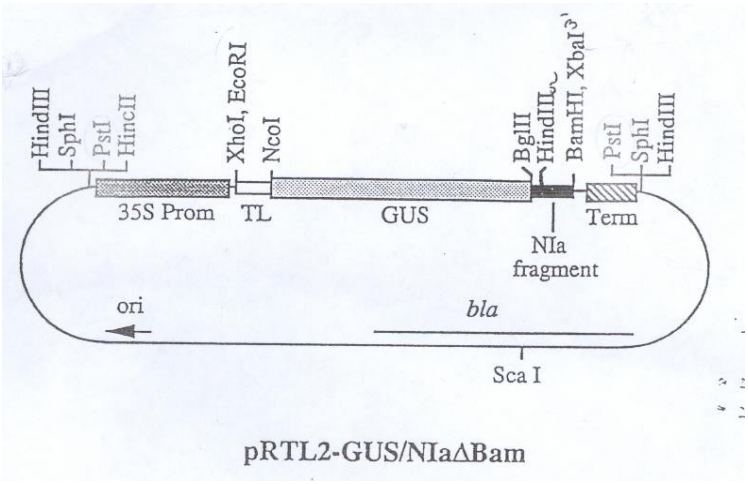
fusion cassette:



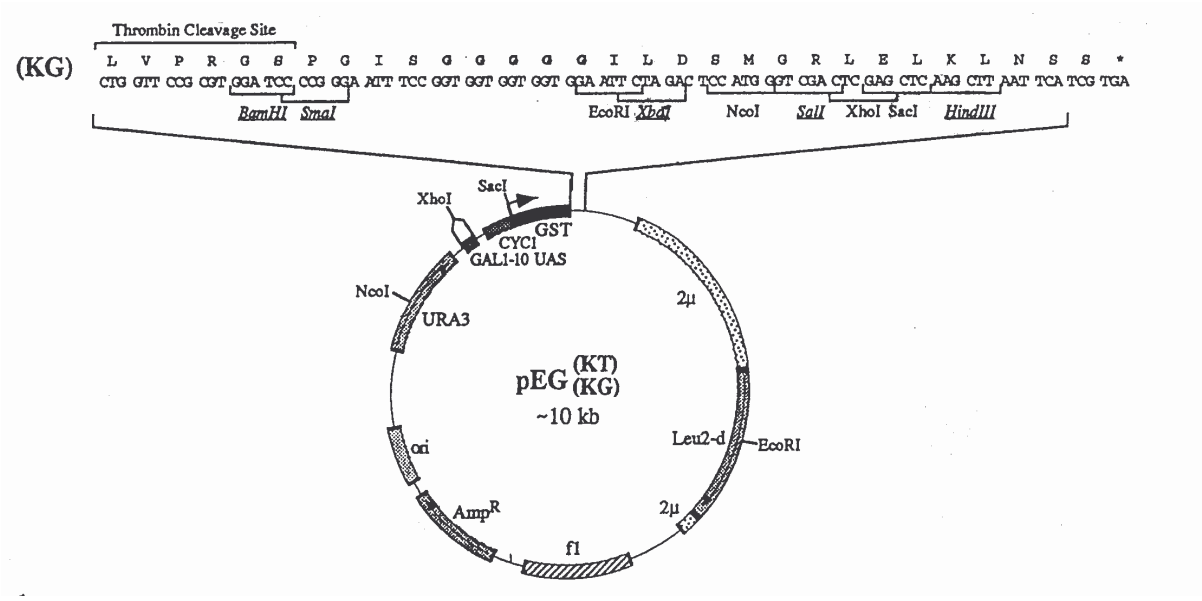
B5) pTBSI


pTBS1(ESC-LEXA-FLAG).seq
7323 base pairs
Unique Sites
557 BsaBI
687 BspGI
693 NdeI
709 BalI
916 SplI
1183 PstI
1312 BsePI
1312 BssHII
1642 DraIII
1650 DrdII
1748 NaeI
2256 PacI
2264 SacI
2274 NotI
2275 EagI
2282 SphI
2287 NcoI
2287 StyI
2377 PshAI
2398 PmeI
2555 EcoRV
2910 HpaI
2951 EcoRI
3246 AgeI
3361 RleAI
3492 ApaBI
3629 BamHI
3653 ApaI
3655 SrfI
3656 SmaI
SapI 3979
Bsp1407I 3850
PpuMI 3775
SacII 3716
XhoI 3699
SalI 3663
AlwNI 4513
HgiEII 4683
Eco31I 5062
Eam1105I 4990
GsuI 5080
ScaI 5473
XbaI 6892

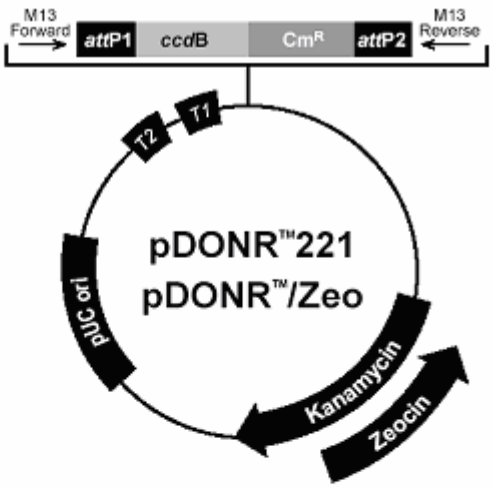
B6) pRTL2-GUS/NIaΔBam



B7) pEGKG



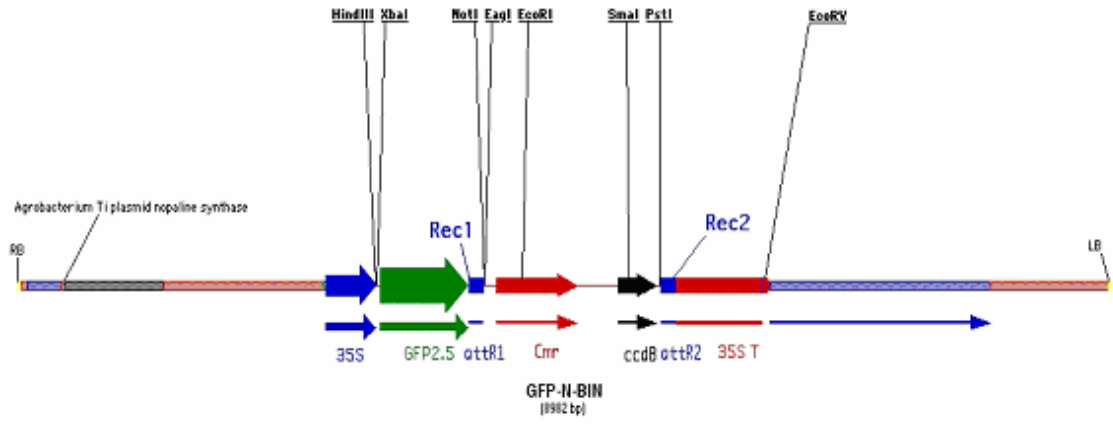
B8) pDONR-221® (Invitrogen)



Comments for:	pDONR™221 4762 nucleotides	pDONR™/Zeo 4291 nucleotides
*rrn*B T2 transcription termination sequence (c):	268-295	268-295
rrnB T1 transcription termination sequence (c):	427-470	427-470
M13 Forward (-20) priming site:	537-552	537-552
attP1:	570-801	570-801
ccdB gene (c):	1197-1502	1197-1502
Chloramphenicol resistance gene (c):	1847-2506	1847-2506
attP2 (c):	2754-2985	2754-2985
M13 Reverse priming site:	3027-3043	3027-3043
Kanamycin resistance gene:	3156-3965	---
EM7 promoter (c):	---	3486-3552
Zeocin resistance gene (c):	---	3111-3485
pUC origin:	4086-4759	3615-4288

(c) = complementary strand

B9) GFP-N-Bin



APPENDIX C

All the sequence alignments in the Appendix C were done on the SDSC Biology Workbench

C1) Sequence alignment of DB90 with JERF1

```
Name: DB90         Len: 1391  Check:  9060    Weight: 1.00
Name: JerfOriseq   Len: 1391  Check:  8138    Weight: 1.00
//
            1                                                          60
       DB90  ..CAAATTGAGCTTNTTCTCCATTAAAATTCTCTCTGCAAATTTATAGTTNTTCTTTTTT
JERF1Oriseq  TTCAAATTGAGCTTTTTCTCCATTAAAATTCTCTCTGCAAATTTATAGTTTTTCTTTTTT
            1                                                          60

            61                                                         120
       DB90  CACTTTTTGAGAAGAAATCAAAAGCTATGTGTGGTGGTGCAATTATCTCCGATTTGGTAC
JERF1Oriseq  CACTTTTTGAGAAGAAATCAAAAGCTATGTGTGGTGGTGCAATTATCTCCGATTTGGTAC

            121                                                        180
       DB90  CTCCTAGCCGGATTTCTCGCCGGTTAACCGCTGATTTTCTATGGGGTACATCCGATCTGA
JERF1Oriseq  CTCCTAGCCGGATTTCTCGCCGGTTAACCGCTGATTTTCTATGGGGTACATCCGATCTGA

            181                                                        240
       DB90  ACAAGAAGAAGAAGAAC......AATTACCACTCAAAGCCCTTGAGGTNTAAGTTTATTG
JERF1Oriseq  ACAAGAAGAAGAAGAACCCTAGTAATTACCACTCAAAGCCCTTGAGGTCTAAGTTTATTG

            241                                                        300
       DB90  ACCNTGAAGATGAATTTGAAGCTGACTTTCAGCACTTCAAGGATAATTCTGATGATGATG
JERF1Oriseq  ACCTTGAAGATGAATTTGAAGCTGACTTTCAGCACTTCAAGGATAATTCTGATGATGATG

            301                                                        360
       DB90  ATGATGTGAAGGCATTTGGCCCCAAATCCGTGAGATCTGGTGATTCAAACTGCGAAGCTG
JERF1Oriseq  ATGATGTGAAGGCATTTGGCCCCAAATCCGTGAGATCTGGTGATTCAAACTGCGAAGCTG

            361                                                        420
       DB90  ACAGATCCTCCAAGAGAAAGAGGAAGAATCAGTACCGGGGGGATCAGACAGCGTCCTTGGG
JERF1Oriseq  ACAGATCCTCCAAGAGAAAGAGGAAGAATCAGTACCGGGGGGATCAGACAGCGTCCTTGGG

            421                                                        480
       DB90  GTAAGTGGGCAGCTGAAATACGTGATCCAAGGAAAGGTATTCGAGTCTGGCTTGGTACTT
JERF1Oriseq  GTAAGTGGGCAGCTGAAATACGTGATCCAAGGAAAGGTATTCGAGTCTGGCTTGGTACTT

            481                                                        540
       DB90  TCAATTCAGCCGAAGAGGCAGCCAGAGCTTATGATGCTGAGGCGCGAAGGATCAGAGGCA
JERF1Oriseq  TCAATTCAGCCGAAGAGGCAGCCAGAGCTTATGATGCTGAGGCGCGAAGGATCAGAGGCA

            541                                                        600
       DB90  AGAAAGCTAAGGTGAACTTTCCTGATGAAGCTCCAGTGTCTGTTTCAAGACGTGCTATTA
JERF1Oriseq  AGAAAGCTAAGGTGAACTTTCCTGATGAAGCTCCAGTGTCTGTTTCAAGACGTGCTATTA

            601                                                        660
       DB90  AGCAAAATCCCCAAAAGGCACTTCGTGAGGAAACCCTGAACACAGTTCAGCCCAACATGA
JERF1Oriseq  AGCAAAATCCCCAAAAGGCACTTCGTGAGGAAACCCTGAACACAGTTCAGCCCAACATGA

            661                                                        720
       DB90  CTTATATTAGTAACTTGGATGGTGGATCTGATGATTCGTTCAGTTTTTTCGAAGAGAAAC
JERF1Oriseq  CTTATATTAGTAACTTGGATGGTGGATCTGATGATTCGTTCAGTTTTTTCGAAGAGAAAC
```

```
            721                                                         780
       DB90  CAGCAACCAAGCAGTACGGCTTCGAGAATGTGTCTTTTACTGCTGTAGATATGGGACTGG
JERF1Oriseq  CAGCAACCAAGCAGTACGGCTTCGAGAATGTGTCTTTTACTGCTGTAGATATGGGACTGG

            781                                                         840
       DB90  GCTCAGTTTCCCCTTCAGCCGGTACAAATGTTTACTTCAGCTCTGATGAAGCAAGTAACA
JERF1Oriseq  GCTCAGTTTCCCCTTCAGCTGGTACAAATGTTTACTTCAGCTCTGATGAAGCAAGTAACA

            841                                                         900
       DB90  CTTTTGACTGCTCTGATTTCGGTTGGGCTGAACCGTGTGCAAGGACTCCAGAGATCTCAT
JERF1Oriseq  CTTTTGACTGCTCTGATTTCGGTTGGGCTGAACCGTGTGCAAGGACTCCAGAGATCTCAT

            901                                                         960
       DB90  CTGTTCTGTCGGAAGTTCTGGAAACCAATGAGACTCATTTTGATGATGATTCCAGACCGG
JERF1Oriseq  CTGTTCTGTCGGAAGTTCTGGAAACCAATGAGACTCATTTTGATGATGATTCCAGACCAG

            961                                                        1020
       DB90  AGAAAAAACTGAAGTCCTGTTCCAGCACTTCATTGACAGTTGACGGTAACACTGTGAACA
JERF1Oriseq  AGAAAAAACTGAAGTCCTGTTCCAGCACTTCATTGACAGTTGACGGTAACACTGTGAACA

            1021                                                        1080
       DB90  CGCTATCTGAAGAGCTATCGGCTTTTGAATCCCAGATGAAGTTCTTGCAGATCCCATATC
JERF1Oriseq  CGCTATCTGAAGAGCTATCGGCTTTTGAATCCCAGATGAAGTTCTTGCAGATCCCATATC

            1081                                                        1140
       DB90  TCGAGGGAAATTGGGATGCATCGGTTGATGCCTTCCTCAATACAAGTGCAATTCAGGATG
JERF1Oriseq  TCGAGGGAAATTGGGATGCATCGGTTGATGCCTTCCTCAATACAAGTGCAATTCAGGATG

            1141                                                        1200
       DB90  GTGGAAACGCCATGGACCTTTGGTCCTTCGATGATGTACCTTCTTTAATGGGAGGTGCCT
JERF1Oriseq  GTGGAAACGCCATGGACCTTTGGTCCTTCGATGATGTACCTTCTTTAATGGGAGGTGCCT

            1201                                                        1260
       DB90  ACTAAGCTGCATACACATCTTCCCTTGCTAAGTTTTGTAAATAACGCTTCATTTGAGTGA
JERF1Oriseq  ACTAAGCTGCATACACATCTTCCCCTGCTAAGTTTTGTAAATAACGCTTCATTTGAGTGA

            1261                                                        1320
       DB90  AGTTTGCGCCTGCGTTTACGTTTATCACCAAACTAAAAGACTATATATGTGTTGTATTAA
JERF1Oriseq  AGTTTGCGCCTGCGTTTACGTTTATCACCAAACTAAAAGACTATATATGTGTTGTATTAA

            1321                                                        1380
       DB90  TTTATTCAAAATTTACTCGTTTGATATATGTAAGTATGTATCCTTGTTTTCATAAAA...
JERF1Oriseq  TTTATTCAAAATTTACTCGTTTGATATATGTAAGTATGTATCCTTGTTTTCATAAAAAAA

            1381   1391
       DB90  ..........
JERF1Oriseq  AAAAAAAAAA
```

C2) Sequence alignment of DB 109 with JERF1

```
            114                                                         173
      DB109 TTCAAATTGAGCTTTTTCTCCATTAAAATTCTCTCTGTAAATTTATAGTTTTTCTTTTTT
JERF1Oriseq TTCAAATTGAGCTTTTTCTCCATTAAAATTCTCTCTGCAAATTTATAGTTTTTCTTTTTT
            1

            174                                                         233
      DB109 TTCACTTTTTGAGAAGAAATCAAAAGCTATGTGTGGTGGTGCAATTATCTCCGATTTGGT
JERF1Oriseq TTCACTTTTTGAGAAGAAATCAAAAGCTATGTGTGGTGGTGCAATTATCTCCGATTTGGT

            234                                                         293
      DB109 ACCTCCTAGCCGGATTTCTCGCCGGTTAACCGCTGATTTTCTATGGGGTACATCCGATCT
JERF1Oriseq ACCTCCTAGCCGGATTTCTCGCCGGTTAACCGCTGATTTTCTATGGGGTACATCCGATCT

            294                                                         353
      DB109 GAACAAGAAGAAGAAGAACCCTAGTAATTACCACTCAAAGCCCTTGAGGTCTAAGTTTAT
JERF1Oriseq GAACAAGAAGAAGAAGAACCCTAGTAATTACCACTCAAAGCCCTTGAGGTCTAAGTTTAT

            354                                                         413
      DB109 TGACCTTGAAGATGAATTTGAAGCTGACTTTCAGCACTTCAAGGATAATTCTGATGATGA
JERF1Oriseq TGACCTTGAAGATGAATTTGAAGCTGACTTTCAGCACTTCAAGGATAATTCTGATGATGA
```

```
           414                                                        473
      DB109 TGATGATGGTGAAGGCATTTGGCCCCAAATCCGTGAGATCTGGTGATTCAAACTGCGAAG
JERF1Oriseq TGATGATGGTGAAGGCATTTGGCCCCAAATCCGTGAGATCTGGTGATTCAAACTGCGAAG

           474                                                        533
      DB109 CTGACAGATCCTCCAAGAGAAAGAGGAAGAATCAGTACCGGGGGATCAGACAGCGTCCTT
JERF1Oriseq CTGACAGATCCTCCAAGAGAAAGAGGAAGAATCAGTACCGGGGGATCAGACAGCGTCCTT

           534                                                        593
      DB109 GGGGTAAGTGGGCAGCTGAAATACGTGATCCAAGGAAAGGTATTCGAGTCTGGCTTGGTA
JERF1Oriseq GGGGTAAGTGGGCAGCTGAAATACGTGATCCAAGGAAAGGTATTCGAGTCTGGCTTGGTA

           594                                                        653
      DB109 CTTTCAATTCAGCCGAAGAGGCAGCCAGAGCTTATGATGCTGAGGCGCGAAGGATCAGAG
JERF1Oriseq CTTTCAATTCAGCCGAAGAGGCAGCCAGAGCTTATGATGCTGAGGCGCGAAGGATCAGAG

           654                                                        713
      DB109 GCAAGAAAGCTAAGGTGAACTTTCCTGATGAAGCTCCAGTGTCTGTTTCAAGACGTGCTA
JERF1Oriseq GCAAGAAAGCTAAGGTGAACTTTCCTGATGAAGCTCCAGTGTCTGTTTCAAGACGTGCTA

           714                                                        773
      DB109 TTAAGCAAAATCCCCAAAAGGCACTTCGTGAGGAAACCCTGAACACAGTTCAGCCCAACA
JERF1Oriseq TTAAGCAAAATCCCCAAAAGGCACTTCGTGAGGAAACCCTGAACACAGTTCAGCCCAACA

           774                                                        833
      DB109 TGACTTATATTAGTAACTTGGATGGTGGATCTGATGATTCGTTCAGTTTTTTCGAAGAGA
JERF1Oriseq TGACTTATATTAGTAACTTGGATGGTGGATCTGATGATTCGTTCAGTTTTTTCGAAGAGA

           834                                                        893
      DB109 AACCAGCAACCAAGCAGTACGGCTTCGAGAATGTGTCTTTTACTGCTGTAGATAT.GGAC
JERF1Oriseq AACCAGCAACCAAGCAGTACGGCTTCGAGAATGTGTCTTTTACTGCTGTAGATATGGGAC

           894                                                        953
      DB109 TGGGCTCAGTTTCCCCTTCAGCCGGGACAAATGTTTACTTCAGCCTCTGATGAAGCAAGT
JERF1Oriseq TGGGCTCAGTTTCCCCTTCAGCTGGTACAAATGTTTACTTCAG.CTCTGATGAAGCAAGT

           954                                                       1013
      DB109 .ACACTTTTGACTGCTCTGATTTCGGTTGGGCTGAACCGTGTGCAAAGG.CTCCCAGAGA
JERF1Oriseq AACACTTTTGACTGCTCTGATTTCGGTTGGGCTGAACCGTGTGC.AAGGACT.CCAGAGA

           1014                                                      1073
      DB109 TCTCATCTG.TCTGGCCGGAGTTCT.GAAACCAATGAGACTCATTTTGGTGATGATTCCC
JERF1Oriseq TCTCATCTGTTCTGTCGGAAGTTCTGGAAACCAATGAGACTCATTTTGATGATGATTCCA

           1074                    1097
      DB109 GACCCGGAGAAAAAACCTGAAGTCCTGTTC
JERF1Oriseq GA.CCAGAGAAAAAA.CTGAAGTCCTGTTC
```

C3) Sequence alignment of DB 110 with JERF1

```
           29                                                          88
      DB110 ACTTTTTGAGAAGAAATCAAAAGCTATGTGTGGTGGTGCAATTATCTCCGATTTGGTACC
JERF1Oriseq ACTTTTTGAGAAGAAATCAAAAGCTATGTGTGGTGGTGCAATTATCTCCGATTTGGTACC
           62

           89                                                         148
      DB110 CCTCCTAGCCGGATTTCTCGCCGGTTAACCGCTGATTTTCTATGGGGTACATCCGATCTG
JERF1Oriseq CCTCCTAGCCGGATTTCTCGCCGGTTAACCGCTGATTTTCTATGGGGTACATCCGATCTG

           149                                                        208
      DB110 AACAAGAAGAAGAAGAACCCTAGTAATTACCACTCAAAGCCCTTGAGGTCTAAGTTTATT
JERF1Oriseq AACAAGAAGAAGAAGAACCCTAGTAATTACCACTCAAAGCCCTTGAGGTCTAAGTTTATT

           209                                                        268
      DB110 GACCTTGAAGATGAATTTGAAGCTGACTTTCAGCACTTCAAGGATAATTCTGATGATGAT
JERF1Oriseq GACCTTGAAGATGAATTTGAAGCTGACTTTCAGCACTTCAAGGATAATTCTGATGATGAT

           269                                                        328
      DB110 GATGATGTGAAGGCATTTGGCCCCAAATCCGTGAGATCTGGTGATTCAAACTGCGAAGCT
JERF1Oriseq GATGATGTGAAGGCATTTGGCCCCAAATCCGTGAGATCTGGTGATTCAAACTGCGAAGCT
```

```
            329                                                         388
      DB110 GACAGATCCTCCAAGAGAAAGAGGAAGAATCAGTACCGGGGGATCAGACAGCGTCCTTGG
JERF1Oriseq GACAGATCCTCCAAGAGAAAGAGGAAGAATCAGTACCGGGGGATCAGACAGCGTCCTTGG

            389                                                         448
      DB110 GGTAAGTGGGCAGCTGAAATACGTGATCCAAGGAAAGGTATTCGAGTCTGGCTTGGTACT
JERF1Oriseq GGTAAGTGGGCAGCTGAAATACGTGATCCAAGGAAAGGTATTCGAGTCTGGCTTGGTACT

            449                                                         508
      DB110 TTCAATTCAGCCGAAGAGGCAGCCAGAGCTTATGATGCTGAGGCGCGAAGGATCAGAGGC
JERF1Oriseq TTCAATTCAGCCGAAGAGGCAGCCAGAGCTTATGATGCTGAGGCGCGAAGGATCAGAGGC

            509                                                         568
      DB110 AAGAAAGCTAAGGTGAACTTTCCTGATGAAGCTCCAGTGTCTGTTTCAAGACGTGCTATT
JERF1Oriseq AAGAAAGCTAAGGTGAACTTTCCTGATGAAGCTCCAGTGTCTGTTTCAAGACGTGCTATT

            569                                                         628
      DB110 AAGCAAAATCCCCAAAAGGCACTTCGTGAGGAAACCCTGAACACAGTTCAGCCCAACATG
JERF1Oriseq AAGCAAAATCCCCAAAAGGCACTTCGTGAGGAAACCCTGAACACAGTTCAGCCCAACATG

            629                                                         688
      DB110 ACTTATATTAGTAACTTGGATGGTGGATCTGATGATTCGTTCAGTTTTTTCGAAGAGAAA
JERF1Oriseq ACTTATATTAGTAACTTGGATGGTGGATCTGATGATTCGTTCAGTTTTTTCGAAGAGAAA

            689                                                         748
      DB110 CCAGCAACCAAGCAGTACGGCTTCGAGAATGTGTCTTTTACTGCTGTAGATATGGGACTG
JERF1Oriseq CCAGCAACCAAGCAGTACGGCTTCGAGAATGTGTCTTTTACTGCTGTAGATATGGGACTG

            749                                                         808
      DB110 GGCTCAGTTTCCCCTTCAGCCGGTACAAATGTTTACTTCAGCTCTGATGAAGCAAGTAAC
JERF1Oriseq GGCTCAGTTTCCCCTTCAGCTGGTACAAATGTTTACTTCAGCTCTGATGAAGCAAGTAAC

            809                                                         868
      DB110 ACTTTTGACTGCTCTGATTTCGGTTGGGCTGAACCGTGTGCAAGGACTCCAGAGATCTCA
JERF1Oriseq ACTTTTGACTGCTCTGATTTCGGTTGGGCTGAACCGTGTGCAAGGACTCCAGAGATCTCA

            869                                                         928
      DB110 TCTGTTCTGTCGGAAGTTCTGGAAACCAATGAGACTCATTTTGATGATGATTCCAGACCG
JERF1Oriseq TCTGTTCTGTCGGAAGTTCTGGAAACCAATGAGACTCATTTTGATGATGATTCCAGACCA

            929                                                         988
      DB110 GAGAAAAAACTGAAGTCCTGTTCCAGCACTTCATTGACAGTTGACGGT.ACACTGTG.AC
JERF1Oriseq GAGAAAAAACTGAAGTCCTGTTCCAGCACTTCATTGACAGTTGACGGTAACACTGTGAAC

            989                                                        1048
      DB110 ACGCTATCT.AAGAAGC.ATCGGCTTTTGAATCCCAGA.GAAGTTCTTGCA.ATCCCATA
JERF1Oriseq ACGCTATCTGAAG.AGCTATCGGCTTTTGAATCCCAGATGAAGTTCTTGCAGATCCCATA

            1049                                                       1100
      DB110 TCTCG.GGGAAA.TGGG.ATGCATCGGTTGAT.CCTTCCTCAATACAAGTGCAATT
JERF1Oriseq TCTCGAGGGAAATTGGG.ATGCATCGGTTGATGCCTTCCTCAATACAAGTGCAATT
                                                                    1133
```

C4) Sequence alignment of DB 111 with JERF1

```
            26                                                          85
      DB111 .......GAGAAGAAATCAAAAGCTATGTGTGGTGGTGCAATTATCTCCGATTTGGTACC
JERF1Oriseq ACTTTTTGAGAAGAAATCAAAAGCTATGTGTGGTGGTGCAATTATCTCCGATTTGGTACC
             62

            86                                                          145
      DB111 CCTCCTAGCCGGATTTCTCGCCGGTTAACCGCTGATTTTCTATGGGGTACATCCGATCTG
JERF1Oriseq CCTCCTAGCCGGATTTCTCGCCGGTTAACCGCTGATTTTCTATGGGGTACATCCGATCTG

            146                                                         205
      DB111 AACAAGAAGAAGAAGAACCCTAGTAATTACCACTCAAAGCCCTTGAGGTCTAAGTTTATT
JERF1Oriseq AACAAGAAGAAGAAGAACCCTAGTAATTACCACTCAAAGCCCTTGAGGTCTAAGTTTATT

            206                                                         265
      DB111 GACCTTGAAGATGAATTTGAAGCTGACTTTCAGCACTTCAAGGATAATTCTGATGATGAT
JERF1Oriseq GACCTTGAAGATGAATTTGAAGCTGACTTTCAGCACTTCAAGGATAATTCTGATGATGAT
```

```
            266                                                         325
      DB111 GATGATGTGAAGGCATTTGGCCCCAAATCCGTGAGATCTGGTGATTCAAACTGCGAAGCT
JERF1Oriseq GATGATGTGAAGGCATTTGGCCCCAAATCCGTGAGATCTGGTGATTCAAACTGCGAAGCT

            326                                                         385
      DB111 GACAGATCCTCCAAGAGAAAGAGGAAGAATCAGTACCGGGGGATCAGACAGCGTCCTTGG
JERF1Oriseq GACAGATCCTCCAAGAGAAAGAGGAAGAATCAGTACCGGGGGATCAGACAGCGTCCTTGG

            386                                                         445
      DB111 GGTAAGTGGGCAGCTGAAATACGTGATCCAAGGAAAGGTATTCGAGTCTGGCTTGGTACT
JERF1Oriseq GGTAAGTGGGCAGCTGAAATACGTGATCCAAGGAAAGGTATTCGAGTCTGGCTTGGTACT

            446                                                         505
      DB111 TTCAATTCAGCCGAAGAGGCAGCCAGAGCTTATGATGCTGAGGCGCGAAGGATCAGAGGC
JERF1Oriseq TTCAATTCAGCCGAAGAGGCAGCCAGAGCTTATGATGCTGAGGCGCGAAGGATCAGAGGC

            506                                                         565
      DB111 AAGAAAGCTAAGGTGAACTTTCCTGATGAAGCTCCAGTGTCTGTTTCAAGACGTGCTATT
JERF1Oriseq AAGAAAGCTAAGGTGAACTTTCCTGATGAAGCTCCAGTGTCTGTTTCAAGACGTGCTATT

            566                                                         625
      DB111 AAGCAAAATCCCCAAAAGGCACTTCGTGAGGAAACCCTGAACACAGTTCAGCCCAACATG
JERF1Oriseq AAGCAAAATCCCCAAAAGGCACTTCGTGAGGAAACCCTGAACACAGTTCAGCCCAACATG

            626                                                         685
      DB111 ACTTATATTAGTAACTTGGATGGTGGATCTGATGATTCGTTCAGTTTTTTCGAAGAGAAA
JERF1Oriseq ACTTATATTAGTAACTTGGATGGTGGATCTGATGATTCGTTCAGTTTTTTCGAAGAGAAA

            686                                                         745
      DB111 CCAGCAACCAAGCAGTACGGCTTCGAGAATGTGTCTTTTACTGCTGTAGATATGGGACTG
JERF1Oriseq CCAGCAACCAAGCAGTACGGCTTCGAGAATGTGTCTTTTACTGCTGTAGATATGGGACTG

            746                                                         805
      DB111 GGCTCAGTTTCCCCTTCAGCCGGTACAAATGTTTACTTCAGCTCTGATGAAGCAAGTAAC
JERF1Oriseq GGCTCAGTTTCCCCTTCAGCTGGTACAAATGTTTACTTCAGCTCTGATGAAGCAAGTAAC

            806                                                         865
      DB111 ACTTTTGACTGCTCTGATTTCGGTTGGGCTGAACCGTGTGCAAGGACTCCAGAGATCTCA
JERF1Oriseq ACTTTTGACTGCTCTGATTTCGGTTGGGCTGAACCGTGTGCAAGGACTCCAGAGATCTCA

            866                                                         925
      DB111 TCTGTTCTGTCGGAAGTTCTGGAAACCAATGAGACTCATTTTGATGATGATTCCAGACCG
JERF1Oriseq TCTGTTCTGTCGGAAGTTCTGGAAACCAATGAGACTCATTTTGATGATGATTCCAGACCA

            926                                                         985
      DB111 GAGAAAAAACTGAAGTCCTGTTCCAGCCCTTCATTGACAGTTTGACGGTAA.CCTGTGAA
JERF1Oriseq GAGAAAAAACTGAAGTCCTGTTCCAGCACTTCATTGACAG.TTGACGGTAACACTGTGAA

            986                                                        1045
      DB111 CACGCTATCTGAAAGAGCTATCGGCTTTTTGAATCCCA.ATGAAAGTTCTTGCAGATCCC
JERF1Oriseq CACGCTATCTG.AAGAGCTATCGGC.TTTTGAATCCCAGATG.AAGTTCTTGCAGATCCC

            1046                                                       1105
      DB111 ATCTCGAGGGGAAATTGGGGATGGCATCGGTTGATGCCCTTCCTCAAA.AC.AAGTGCCA
JERF1Oriseq ATCTCGA.GGGAAATT.GGGAT.GCATCGGTTGATG.CCTTCCTC.AATAC.AAGTGC..

            1106                                        1151
      DB111 ATTTCA..GGATGG.GGAAACCCCATGGGCCTTTGGTCCTTCGATG
JERF1Oriseq AATTCA..GGATGGTGGAAACGCCATGGACCTTTGGTCCTTCGATG
                                                        1173
```

APPENDIX D

BUFFERS AND SOLUTIONS

Tris Borate EDTA Buffer (TBE) (5X):

Tris base: 54 g

Boric Acid: 27.5 g

EDTA (0.5M pH8): 20 ml

Completed to 1L

PEG/LiAc Solution (Polyethylene Glycol / Lithium Acetate):

PEG 4000: 8 ml of 50% PEG

TE Buffer: 1 ml of 10X TE

LiAc: 1ml of 10X LiAC

Completed to 10 ml

10 X TE Buffer:

0.1M Tris-Hcl

10mM EDTA

pH adjusted to 7.5

10X LiAc:

1M Lithium Acetate pH adjusted to 7.5 with dilute acetic acid and autoclaved

APPENDIX E

Equipments

Autoclave:	Hirayama, Hiclave HV-110, JAPAN
	Certoclav, Table Top Autoclave CV-EL-12L, AUSTRIA
Balance:	Sartorius, BP211D, GERMANY
	Sartorius, BP221S, GERMANY
	Sartorius, BP610, GERMANY
	Schimadzu, Libror EB-3200 HU, JAPAN
Centrifuge:	Eppendorf, 5415C, GERMANY
	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
	Kendro Lab. Prod., Heraeus Multifuge 3L, GERMANY
	Hitachi, Sorvall RC5C Plus, USA
	Hitachi, Sorvall Discovery 100 SE, USA
Deepfreeze:	-70^{o} C, Kendro Lab. Prod., Heraeus Hfu486 Basic, GERMANY
	-20^{o} C, Bosch, TÜRKİYE
Distilled Water:	Millipore, Elix-S, FRANCE
	Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Biogen Inc., USA
	Biorad Inc., USA

Gel Documentation:	UVITEC, UVIdoc Gel Documentation System, UK
	Biorad, UV-Transilluminator 2000, USA
Ice Machine:	Scotsman Inc., AF20, USA
Incubator:	Memmert, Modell 300, GERMANY
	Memmert, Modell 600, GERMANY
Laminar Flow:	Kendro Lab. Prod., Heraeus, HeraSafe HS12, GERMANY
Magnetic Stirrer:	VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY
	VELP Scientifica, Microstirrer, ITALY
Microliter Pipette:	Gilson, Pipetman, FRANCE
	Mettler Toledo, Volumate, USA
Microwave Oven:	Bosch, TÜRKİYE
pH meter:	WTW, pH540 GLP MultiCal®, GERMANY
Power Supply:	Biorad, PowerPac 300, USA
	Wealtec, Elite 300, USA
Refrigerator:	+4^{o} C, Bosch, TÜRKİYE
Shaker:	Forma Scientific, Orbital Shaker 4520, USA
	GFL, Shaker 3011, USA
	New Brunswick Sci., Innova™ 4330, USA
Spectrophotometer:	Schimadzu, UV-1208, JAPAN
	Schimadzu, UV-3150, JAPAN
	Secoman, Anthelie Advanced, ITALY
Speed Vacuum:	Savant, Speed Vac® Plus Sc100A, USA
	Savant, Refrigerated Vapor Trap RVT 400, USA

Thermocycler:	Eppendorf, Mastercycler Gradient, GERMANY
Vacuum:	Heto, MasterJet Sue 300Q, DENMARK
Water bath:	Huber, Polystat cc1, GERMANY

REFERENCES

1. Baker B, Zambryski P, Staskawicz B, Dinesh-Kumar SP, "Signalling in Plant-Microbe Interaction" *Science* 276 (1997) 726-733.

2. Bendahmane A, Köhm BA, Dedi C, Baulcombe DC, "The Coat Protein of Potato Virus X is a Strain-Specific Elicitor of *Rx*1-mediated Virus Resistance in Potato" *Plant J.* 8 (1995) 933-941.

3. Bilgin DD, Liu Y, Schiff M, Dinesh-Kumar SP, "P58^{IPK}, a Plant Ortholog of Double-Stranded RNA-Dependent Protein Kinase PKR Inhibitor, Functions in Viral Pathogenesis" *Developmental Cell* 4 (2003) 651-661.

4. Birch PRJ, Avrova AO, Dellagi A, Lacomme C, Santa Cruz S, Lyon GD. "Programmed Cell Death in Plants in Response to Pathogen Attack" Annual Plant Reviews. 4 (2000) 175-197

5. Botella BA, Parker JE, Frost LN, Bittner-Eddy PD, Beynon JL, Daniels MJ, Holub EB, Jones JDG, "Three Genes of the *Arabidopsis* RPP1 Complex Resistance Locus Recognize distinct *Peronospora Parasitica* Avirulence Determinants" *Plant Cell* 10 (1998) 1847-1860.

6. Buchanan BB, Gruissem W, Jones RL, "Biochemistry & Molecular Biology of Plants" Second Impression, *American Society of Plant Physiologists* 2000.

7. Carrington JC, Kasschau KD, Mahajan SK, Schaad MC, "Cell-to-Cell and Long-Distance Transport of Viruses in Plants" *Plant Cell* 8 (1996) 1669–1681.

8. Chang C, Shockey JA, "The Ethylene-Response Pathway: Signal Perception to Gene Regulation" *Curr. Opin. Plant Biol.* 2 (1999) 352-358.

9. Cheng WH, Endo A, Zhou L, Penney J, Chen HC, Arroyo A, Leon P, Nambara E, Asami T, Seo M, Koshiba T, Sheen J. "A unique short-chain

dehydrogenase/reductase in Arabidopsis glucose signaling and abscisic acid biosynthesis and functions" *Plant Cell*. 11 (2002)2645-26499.

10. Chini A, Grant JJ, Seki M, Shinozaki K, Loake GJ, "Drought Tolerance Established by Enhanced Expression of the CC-NBS-LRR Gene, ADR1, Requires Salicylic Acid, EDS1 and ABI1" *Plant J.* 38 (2004) 810-822.

11. Dangl JL, Jones JDG, "Plant Pathogens and Integrated Defence Responses to Infection" *Nature* 411 (2001) 826-833.

12. Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessmann H, Ward E, Ryals J, "A Central Role of Salicylic Acid in Plant Disease Resistance" *Science* 266 (1994) 1247-1250.

13. Dion M, Chamberland H, St-Michel C, Plante M, Darveau A, Lafontaine JG, Brisson LF, "Detection of a Homologue of bcl-2 in Plant Cells" *Biochem. Cell Biol.* 75 (1997) 457-461.

14. Doke N, Ohashi Y, "Involvement of an O_2 Generating System in the Induction of Necrotic Lesions on Tobacco Leaves Infected with Tobacco Mosaic Virus" *Physiol. Mol. Plant Pathol.* 32 (1988) 163-175.

15. Ellis J, Jones D, "Structure and Function of Proteins Controlling Strain-Specific Pathogen Resistance in Plants" *Curr. Opin. Plant Biol.* 1 (1998) 288-293.

16. Ellis JG, Lawrence GJ, Luck JE, Dodds PN, "Identification of Regions in Alleses of the Flax Rust Resistance Gene *L* That Determine Differences in Gene-for-Gene Specificity" *Plant Cell* 11 (1999) 495-506.

17. Flor HH, "Current Status of the Gene-for-Gene Concept" *Annu. Rev. Phytopathol.* 9 (1971) 275-296.

18. Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M "Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression" *Plant Cell*. 3 (2000):393-404

19. Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J, "Requirement of Salicylic Acid for the Induction of Systemic Acquired Resistance" *Science* 261 (1993) 754-756.

20. Gale Jr. M, Katze MG, "Molecular Mechanisms of Interferon Resistance Mediated by Viral-Directed Inhibition of PKR, the Interferon-Induced Protein Kinase" *Pharmacol. Ther.* 78 (1998) 29-46.

21. Gassmann W, Hinsch ME, Staskawicz BJ, "The Arabidopsis RPS4 bacterial-resistance gene is a member of the TIR-NBS-LRR family of disease-resistance genes" *Plant J*. 3 (1999) 265-77

22. Gil J, Esteban M, Roth D, "In Vivo Regulation of Protein Synthesis by Phosphorylation of the α Subunit of Wheat Eukaryotic Initiation Factor 2" *Biochemistry* 39 (2000) 7521-7530.

23. Gong W, Shen Y-P, Ma L-T, Pan Y, Du Y-L, Wang D-H, Yang J-Y, Hu L-D, Liu X-F, Dong C-X, Ma L, Chen Y-H, Yang X-Y, Gao Y, Zhu D, Tan X, Mu J-Y, Zhang D-B, Liu Y-L, Dinesh-Kumar SP, Li Y, Wang X-P, Gu H-Y, Qu L-J, Bai S-N, Lu Y-T, Li J-Y, Zhao J-D, Zuo J, Huang H, Deng XW, Zhu Y-X, "Genome-Wide ORFeome Cloning and Analysis of Arabidopsis Transcription Factor Genes" *Plant Physiology* 135 (2004) 773-782.

24. Gu Y-Q, Wildermuth MC, Chakravarthy S, Loh Y-T, Yang C, He X, Han Y, Martin GB, "Tomato Transcription Factors Pti4, Pti5 and Pti6 Activate Defense Responses when Expressed in Arabidopsis" *Plant Cell* 14 (2002) 817-831.

25. Gu Y-Q, Yang C, Thara VK, Zhou J, Martin GB, "Pti4 is Induced by Ethylene and Salicylic Acid and its Product is Phosphorylated by the Pto Kinase" *Plant Cell* 12 (2000) 771-786.

26. Gutterson N, Reuber TL, "Regulation of disease resistance pathways by AP2/ERF transcription factors" *Curr. Opin. Plant Biol*. 7 (2004) 465–471

27. Hammond-Kosack KE, Jones DJG, "Resistance Gene-Dependent Plant Defense Responses" *Plant Cell* 8 (1996) 1773-1791.

28. Jarpe MB, Widmann C, Knall C, Schlesinger TK, Gibson S, Yujiri T, Fanger GR, Gelfand EW, Johnson GL, "Anti-Apoptotic versus Pro-Apoptotic Signal Transduction: Check-Points and Stop Signs along the Road to Death" *Oncogene* 17 (1998) 1475-1482.

29. Jofuku KD, den Boer BGW, Montagu MV, Okamuro JK, "Control of Arabidopsis Flower and Seed Development by the Homeotic Gene *APETHALA2*" *Plant Cell* 6 (1994) 1211-1225.

30. Jones CG, Tucker GA, Lycett GW, "Pattern of Expression and Characteristics of a Cysteine Proteinase cDNA from Germinating Seeds of Pea (*Pisum sativum* L.)" *Biochim. Biophys. Acta.* 129 (1996) 613-615.

31. Kiziz D, Lumbreras V, Pagès M, "Role of AP2/EREBP Transcription Factors in Gene Regulation During Abiotic Stress" *FEBS Letters* 498 (2001) 187-189.

32. Kobe B, Deisenhofer J, "The Leucine-Rich Repeat: A Versatile Binding Motif" *Trans Biochem. Sci.* 19 (1994) 415-421.

33. Krebs J, "The Role of Calcium in Apoptosis" *Biometals* 11 (1998) 375-382.

34. Langland JO, Langland LA, Browning KS, Roth DA, "Phosphorylation of Plant Eukaryotic Initiation Factor-2 by the Plant-encoded Double-stranded RNA-dependent Protein Kinase, pPKR, and Inhibition of Protein Synthesis *in Vitro*" *Journal of Biol. Chem.* 271 (1996) 4539-4544.

35. Lawrence GJ, Finnegan EJ, Ayliffe MA, Ellis JG, "The *L6* Gene for Flax Rust Resistance is Related to the Arabidopsis Bacterial Resistance Gene *RPS2* and the Tobacco Viral Resistance Gene *N*" *Plant Cell* 7 (1995) 1195-1206.

36. Lawton KA, Potter SL, Uknes S, Ryals J, "Acquired Resistance Signal Transduction in Arabidopsis is Ethylene Independent" *Plant Cell* 6 (1994) 581-588.

37. Lemaitre B, Nicolas E, Michaut L, Reichhart J-M, Hoffmann JA, "The Dorsoventral Regulatory Gene Cassette *spätzle/Toll/cactus* Controls the Potent Antifungal Response in Drosophila Adults" *Cell* 86 (1996) 973-983.

38. Levine A, Pennell RI, Alvarez ME, Palmer R, Lamb C, "Calcium-Mediated Apoptosis in Plant Hypersensitive Disease Resistance Response" *Curr. Biol.* 6 (1996) 427-437.

39. Liu Y, Schiff M, Dinesh-Kumar SP, "Virus-induced gene silencing in tomato" Plant J. 6 (2002) 777-786.

40. Lorenzo O, Piqueras R, Sanchez-Serrano JJ, Solano R, "ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense" *Plant Cell* 1(2003) 165-178.

41. Luck JE, Lawrence GJ, Dodds PN, Shepherd KW, Ellis JG, "Regions outside of the Leucine-Rich Repeats of Flax Rust Resistance Proteins Play a Role in Specificity Determination" *Plant Cell* 12 (2000) 1367-1377.

42. Martin GB, Bogdanove AJ, Sessa G, "Understanding the Functions of Plant Disease Resistance Proteins" *Annu. Rev. Plant Biol.* 54 (2003) 23-61.

43. Medzhitov R, Preston-Hurlburt P, Janeway CA, "A Human Homologue of the Drosophila Toll Protein Signals Activation of Adaptive Immunity" *Nature* 338 (1997) 394-397.

44. Mittler R, Del Pozo O, Meisel L, Lam E, "Pathogen-Induced Programmed Cell Death in Plants, a Possible Defense Mechanism" *Developmental Genetics* 21 (1997) 279-289.

45. Moffett P, Farnham G, Peart J, Baulcombe DC, "Interaction between Domains of a Plant NBS-LRR Protein in Disease Resistance-Related Cell Death" *EMBO J.* 21 (2002) 4511- 4519.

46. Ohme-Takagi M, Shinshi H, "Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element" *Plant Cell*. 2 (1995) 173-82

47. Okamuro JK, Caster B, Villarroel R, Montagu MV, Jofuku KD, "The AP2 Domain of *APETALA2* Defines a Large New Family of DNA Binding Proteins in Arabidopsis" *Proc. Natl. Acad. Sci.* USA 94 (1997) 7076-7081.

48. Parker JE, Coleman MJ, Szabo V, Frost LN, Schmidt R, van der Biezen EA, Moores T, Dean C, Daniels MJ, Jones JD, "The Arabidopsis downy mildew resistance gene RPP5 shares similarity to the toll and interleukin-1 receptors with N and L6" *Plant Cell*. 6 (1997) 879-894

49. Pedley KF, Martin GB, "Molecular Basis of Pto-mediated Resistance to Bacterial Speck Disease in Tomato" *Annu. Rev. Phytopathol.* 41 (2003) 215-243.

50. Prescott LM, Harley JP, Klein DA, “Microbiology” Fifth Edition, *McGraw Hill* 2002

51. Reymond P, Weber H, Dammond M, Farmer EE, “Differential Gene Expression in Response to Mechanical Wounding and Insect Feeding in *Arabidopsis*” *Plant Cell* 12 (2000) 707-719.

52. Riechmann JL, Heard J, Martin G, Reuber L, Jiang C-Z, Keddie J, Adam L, Pineda O, Ratcliffe OJ, Samaha RR, Creelman R, Pilgrim M, Broun P, Zhang JZ, Ghandehari D, Sherman BK, Yu G-L, “Arabidopsis Transcription Factors: Genome-Wide Comparative Analysis Among Eukaryotes” *Science* 290 (2000) 2105-2110.

53. Ruiz MT, Voinnet O, Baulcombe DC “Initiation and maintenance of virus-induced gene silencing” Plant Cell. 6 (1998) 937-946

54. Sakuma Y, Liu Q,Dubouzet JG, Abe H, Shinozaki K, Yamaguchi-Shinozaki K, “DNA-Binding Specificity of the ERF/AP2 Domain of Arabidopsis DREBs, Transcription Factors Involved in Dehydration- and Cold-Inducible Gene Expression” *Biochem. Biophy.Res. Commun* 290(2002) 998-1009.

55. Sessa G, D’Ascenzo M, Martin GB, “Thr38 Ser198 are Pto Autophosphorylation Sites Required for the AvrPto-Pto-mediated Hypersensitive Response” *EMBO J.* 19 (2000) 2257-2269.

56. Shinozaki K, Dennis ES, “Cell Signaling and Gene Regulation Global Analysis of Signal Transduction and Gene Expression Profiles” *Curr. Opin. Plant Biol.* 6 (2003) 405-409.

57. Song W-Y, Wang G-L, Chen L-L, Kim H-S, Pi L-Y, Holsten T, Gardner J, Wang B, Zhai W-X, Zhu L-H, Fauquet C, Ronald P, “A Receptor Kinase-like Protein Encoded by the Rice Disease Resistance Gene, *Xa21*” *Science* 270 (1995) 1804-1806.

58. Sticher L, Mauch-Mani B, Metraux JP, “Systemic acquired resistance” *Annu. Rev. Phytopathol*. 35 (1997) 235-70

59. Suzuki K, Fukuda Y, Shinshi H, "Studies on Elicitor-Signal Transduction leading to Differential Expression of Defense Genes in Cultured Tobacco Cells" *Plant Cell Physiol.* 36 (1995) 281-289.

60. van der Biezen EA, Jones JDG, "The NB-ARC Domain: A Novel Signalling Motif shared by Plant Resistance Gene Products and Regulators of Cell Death in Animals" *Curr. Biol.* 8 (1998) R226-R227.

61. Volpe F, Clatworthy J, Kaptein A, Maschera B, Griffin A-M, Ray K, "The IL-1 Receptor Accessory Protein is Responsible for the Recruitment of the Interleukin-1 Receptor Associated Kinase to the IL-1/IL-1 Receptor I Complex" *FEBS Letters* 419 (1997) 41-44.

62. Vom Endt D, Kijne JW, Memelink J, "Transcription Factors Controlling Plant Secondary Metabolism: What Regulates the Regulators?" *Phytochemistry* 61 (2002) 107-114.

63. Waterhouse PM, Wang M-B, Lough T, "Gene Silencing as an Adaptive Defence Against Viruses" *Nature* 411 (2001) 834-841.

64. Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B, "The Product of the Tobacco Mosaic Virus Resistance Gene *N*: Similarity to Toll and the Interleukin-1 Receptor" *Cell* 78 (1994) 1101-1115.

65. Xie DX, Feys BF, James S, Nieto-Rostro M, Turner JG, "An Arabidopsis Gene Required for Jasmonate-Regulated Defense and Fertility" *Science* 280 (1998) 1091-1094.

66. Xu Y, Chang P F L, Liu D, Narasimhan M L, Raghothama K G, Hasegawa P M, Bressan R.A "Plant defense genes are synergistically induced by ethylene and methyl jsamonate" Plant Cell. 6 (1994) 1077–1085

67. Yang J, Steward R, "A Multimeric Complex and the Nuclear Targeting of the Drosophila reI Protein Dorsal" *Proc. Natl. Acad. Sci. USA* 94 (1997) 14524-14529.

68. Yang Y, Shah J, Klessig DF, "Signal Perception and Transduction in Plant Defense Responses" *Genes and Development* 11 (1997) 1621-1639.

69. Zhang JZ, “Overexpression Analysis of Plant Transcription Factors” *Curr. Opin. Plant Biol.* 6 (2003) 430-440.

70. Zhang S, Klessig DF, “Resistance Gene *N*-mediated *de novo* Synthesis and Activation of a Tobacco Mitogen-Activated Protein Kinase by Tobacco Mosaic Virus Infection” *Proc. Natl. Acad. Sci. USA* 95 (1998) 7433-7438.

71. Zhou J, Tang X, Martin GB, “The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a *cis*-element of pathogenesis-related genes” EMBO J. 11 (1997) 3207-3218.

72. Zhu T, “Global Analysis of Gene Expression Using GeneChip Microarrays” *Curr. Opin. Plant Biol.* 6 (2003) 418-425.