SCREENING OF PLANT FACTORS THAT MODULATE THE ACTIVATION OF THE NF-κB TRANSCRIPTION FACTOR IN MAMMALIAN CELLS

by

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ABSTRACT

The signal transduction pathway that leads to the activation of NF-κB is extensively studied because of its importance in immune system activation. Any molecule that interferes with the pathway is therapeutically important because of its potential role in triggering or inhibiting the immune response. To identify proteins that interfere with the activation of NF-κB, we designed and performed a genetic screening system of plant proteins. Our screen consisted of ankyrin repeat plant proteins expressed in mammalian tissue culture cells along with an NF-κB reporter vector that expressed Green Fluorescent Protein (GFP) upon NF-κB activation. As a tissue culture system we used the easily transfected human embryonic kidney cell line HEK293T. To perform our screen, we generated an NF-κB sensitive GFP reporter plasmid by standard molecular biology cloning techniques and we constructed a library of mammalian expression vectors that contained 90 *A. thaliana* cDNAs by Cre-mediated recombination between plasmids. We restricted our analysis to 90 cDNAs of ankyrin repeat domain containing proteins from *A. thaliana* for three reasons: First, ankyrin repeat containing proteins encoded by the mammalian genome have been shown to directly bind to NF-κB, second, proteins encoded by different genomes have the potential to interact with higher affinity than those encoded by the same genome and finally, some *A. thaliana* ankyrin repeat domain proteins have been shown to be involved in plant immune responses. In our screen we found out that three out of 90 plant cDNAs that were expressed in mammalian cells affected TNF-α induced NF-κB activation by changing GFP expression from our reporter vector.

ÖZET

NF-κB aktivasyonuna yol açan sinyal iletim yolu, bağışıklık sistemindeki önemi sebebiyle geniş ölçüde çalışılmaktadır. Bu iletim yolundaki moleküller bağışıklık sistemini harekete geçirme ya da baskılamadaki rolleri nedeniyle insan hastalıklarını tedavi açısından da önemlidirler. NF-κB sinyal iletim yoluyla etkişebilecek bitki proteinlerini bulmak amacıyla bir genetik tarama düzeneği hazırladık. Tarama düzeneğimiz memeli hücre kültüründe ifade edilmiş ankyrin tekrarları içeren bitki proteinleriyle birlikte NF-κB aktivasyonuna bağlı olarak yeşil florasan protein (GFP) ifade eden NF-κB raportör vektöründen meydana geldi. Doku kültürü sistemi olarak kolay transfekte edilebilen insan embryonik böbrek hücresi HEK293T hücrelerini kullandık. Taramayı gerçekleştirmek için, temel moleküler biyoloji klonlama teknikleri kullanarak NF-κB aktivasyonuna duyarlı bir GFP raportör plazmidi inşaa ettik ve The Arabidopsis Information Resource'dan (TAIR) temin ettiğimiz plasmidleri kullarak Cre-enzimi rekombinasyon reaksiyonları ile 90 adet *A. thaliana* cDNAsından oluşan bir memeli ifade vektörü kütüphanesi hazırladık. Bitki ankyrin tekrarlı proteinlerine odaklanmamızın üç nedeni vardır. Öncelikle, memeli ankyrin-tekrarlı proteinlerinin direkt olarak NF-κB'ye bağlandığı gösterilmiştir. İkincil olarak, değişik genomlar tarafından kodlanan proteinlerin birbiriyle etkileşme olasılığı yüksektir, ve son olarak da *A. thaliana* ankyrin-tekrarlı proteinleri bitki bağışıklık sisteminde rol oynamaktadırlar. Bu nedenlerden dolayı taramalarımızı 90 adet ankyrin-repeat *A. thaliana* proteiniyle sınırladık. Bu tarama sonucunda memeli hücrelerde ifade edilmiş 90 bitki cDNAsından üçünün raportör vektörümüzün GFP ifade miktarını degiştirerek, TNF-α'ya bağlı NF-κB aktivasyonunu etkilediğini bulduk.

To my family...

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1 INTRODUCTION

1.1 THE TRANSCRIPTION FACTOR NF-κB

Inducible transcription factors regulate immediate and long-lived cellular responses necessary for an organism to adapt environmental changes. In most cases these kinds of responses are mediated by changes in gene expression. Nuclear factor-κB (NF-κB), an evolutionarily conserved signaling molecule that plays a critical role in many biological processes, is a transcription factor that serves as a key responder to envrionmental change.[1] As such, it is one of the best studied factors that control changes in gene expression.

NF-κB is a eukaryotic transcription factor that was first characterized as a factor binding to a sequence motif (κB) located in the immunoglobulin kappa light chain enhancer gene.[2] NF-κB binding sites serve as inducible transcriptional regulatory elements that respond to immunological stimuli like TNF, IL-1, LPS, or T cell activators.[3] NF-κB is a Rel homology domain containing protein and is found in an inactive state in the cytoplasm as a homo or heterodimer. It can be activated by diverse stimuli to move into nucleus, where it binds to a decameric DNA binding motif, 5'-GGGRNNYYCC.[4] The expression of a wide variety of genes are controlled by NF-κB such as those encoding immuno receptors, cytokines and viral proteins.[2]

The NF-κB family is composed of five subunits, c-Rel, RelA(p65), RelB, NF-κB1, and NF-κB2, each encoded by a different gene locus.[5] These proteins form homo- and heterodimers and share a conserved 300-amino acid Rel-homology domain in their N-terminus. This domain is responsible for NF-κB dimerization, nuclear translocation, DNA binding, and activation of gene transcription.[6] In the canonical NF-κB activation pathway mature NF-κB, the dimers (mainly p65/p50 dimers), reside in the cytoplasm in a latent state bound to an inhibitory protein called IκB and the major pathway leading to their activation is based on inducible IκB degradation.[4,6] When an appropriate signal like proinflammatory cytokines, microbes or viruses is received this canonical pathway is triggered and the IκB

Kinase (IKK) complex is activated.[4] This activated kinase complex phosphorylates IκB leading to its degradation via ubiquitinylation. Upon IκB degradation, free NF-κB can translocate into the nucleus and bind to their specific DNA sequences. DNA binding results in enhancer activity and an upregulation of gene transcription.[6]

On the other hand NF-κB1(p50/p105) and NF-κB2 (p52/p100) precursors contain ankyrin repeats, that are also found in IκBs, in their COOH-termini, so they can function as IκBs.[7,8] Unlike IκB degradation, NF-κB1 processing is a constitutive process.[9,10]And it was found that NF-κB2 is activated by a kinase called NIK. NIK induces ubiquitin-dependent processing of NF-κB2.[11] Unlike Rel proteins NF-κB1 and NF-κB2 (p105 and p100, respectively) require proteolytic processing to produce their respective p50 and p52 NF-κB subunits. The p100 and p105 proteins serve regulatory functions in the cell and should not be considered exclusively as precursor forms. Furthermore, some evidence suggests that alternative splicing of the p105 mRNA leads to the translation of additional forms of this protein.[6,12] The regulation of the transcription factor NF-κB activity occurs at several levels including controlled cytoplasmic-nuclear shuttling and modulation of its transcriptional activity.[6] This tight regulation of NF-κB activity is crucial for an appropriate immune response.

1.2 ACTIVATION OF NF-κB BY MULTIPLE RECEPTORS

In human cells at least three surface receptors activate the transcription factor NF-κB. Canonical NF-κB is a heterodimer composed p50 and p65 subunits. In the absence of signal NF-κB is sequestered in the cytoplasm in an inactive form bound to the inhibitor IκB. Signaling by TCR, TLR or TNFα–R results in the phosphorylation, ubiquitinylation and proteasomal degradation of IκB, releasing and activating NF-κB. Activated NF-κB can migrate into the nucleus and bind to NF-κB binding sites that have been found in the gene regulatory regions of many immediate early genes. Some examples of these genes are TNF-α, IFN-β, IL-2, IL-2R and Igκ. In these enhancers, NF-κB sites are rarely found by themselves. Rather, they tend to be together with DNA binding elements of other

transcription factors. It is thought that the combinatorial use of multiple transcription factors in enhancers results in tissue specific transcriptional activation.

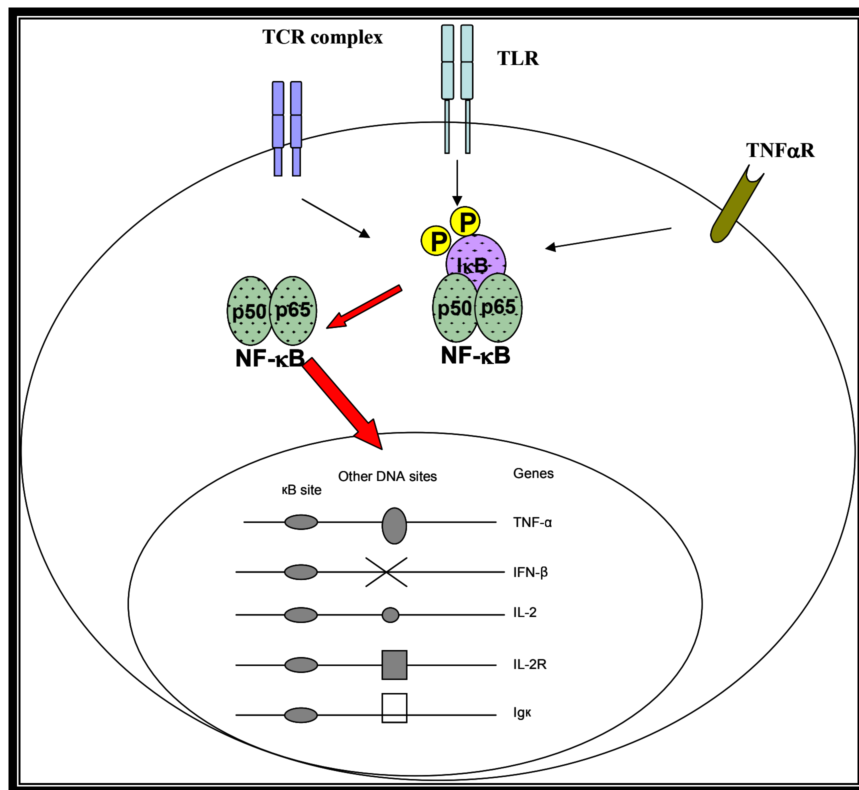

Figure 1.1: Activation of NF-κB through TCR, TLR and TNF Receptors.

1.2.1 T-CELL RECEPTOR STIMULATION ACTIVATES NUCLEAR TRANSLOCATION OF NF-κB

T cell activation has a critical role in the immune response regulation and malfunctioning in this process results in cancer, autoimmunity, and immunodeficiency diseases. The transcription factor NF-κB is a major regulator of lymphocyte survival and activation. That is why controlled activation of NF-κB is crucial for immune and inflammatory response.[5] Following T cell activation, NF-κB activates genes such as interleukin-2 (IL-2), the IL-2 receptor α-chain(CD25) and Bcl-X_L, that are known to regulate cellular proliferation and cell survival.[13]

Coreceptor activation is also required together with T cell receptor (TCR) activation in order to fully activate the T cell. This was proposed by two signal hypothesis.[14] The first signal is initiated upon binding of the TCR to a specific antigenic peptide presented by major histocompatibility complex (MHC) on the surface of an antigen-presenting cell (APC) and the second co-receptor derived signal by costimulation of CD28 by B7 on APC is necessary to boost a productive immune response that leads to cytokine production, increased survival and clonal expansion of naïve T cell.[15,16] Upon T cell activation a contact is formed between T cell and the APC. The contact between T cell and the APC is a highly organized interface, which is referred to as immunological synapse. In this synapse a lipid raft is formed. The lipid rafts are small, cholesterol-rich areas in the cell membrane that are relatively resistant to solubilization by detergents. TCR activation together with costimulatory signals control the amplitude and duration of the NF-κB activation.[17]

T cell receptor (TCR) is a heterodimer and is most commonly composed of α and β chains. The ligand-binding subunits of the receptor, the α and β TCR chains, cannot transduce signals on their own. Rather, this is accomplished by the associated ε/δ/γ chains of CD3 molecules and ζ chains although these proteins don't possess any intrinsic enzymatic activity. The picture that has emerged in recent years is that the TCR complex is coupled to cytoplasmic tyrosine kinases, which in concert with a myriad of adaptor proteins initiate signals that determine the specific outcomes of antigen recognition.(Figure 1.4)[13] T cell activation is induced when TCR is engaged by majorhistocompatibility-complex (MHC) molecules with antigen peptides on antigen-presenting cells (APC).[18]

Antigen receptor-induced NF-κB activation is known to proceed through the classical NF-κB pathway.[19] TCR-induced NF-κB activation depends on proximal signaling events that depends on a cascade of tyrosine phosphorylation events. This phosphorylation cascade controls the localization and/or functional properties of the Src and Syk family kinases Lck and ZAP70(ζ-associated protein of 70 kDa), the adaptor molecules LAT(linker for activation of T cells) and SLP-76 (Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa), as well as the enzymes Vav and phospholipase Cg1 (PLCg1).[13] Upon costimulation series of signal transduction events that lead to activation of Lck, which is a cytosolic Src family tyrosine kinase, are activated leading to phosphorylation of immunoreceptor tyrosine based activation motifs (ITAMs) in the cytoplasmic tail of CD3 and ζ chains. Phosphorylated ITAMs serve as docking sites for SH2 domain of ZAP70 resulting in their activation via

phosphorylation.[20] This signalling cascade results in the recruitment of serine-threonine kinase PKC-θ, which was shown to be the only PKC isoform that has been shown to rapidly translocate to the immunological synapse.[20] Some of the TCR signal transducers, such as Src kinases, linker for T cell activation, and Ras, are constitutiveliy associated with the lipid rafts, [21,22] where as others, including CD3ζ, ZAP70, Vav, phospholipase C-γ1 (PLC-γ1), PKC-θ and CARMA1 are recruited to the lipid rafts upon triggering of the TCR.[23-27] CARMA1 is a member of membrane-associated Guanylate Kinase (MAGUK) protein-1 family containing a CARD domain [27,28] and is a lipid raft-associated protein.[27] MAGUK-family member proteins as CARMA1 contains one to three PDZ domains followed by a SRC homology 3 (SH3) domain and a guanylate kinase (GUK) domain.[29] CARMA1 is an important protein acting in the downstream of TCR signalling pathway and it was shown to recruit BCL10 and MALT1 to the lipid raft upon CD3/CD28 costimulation.[27,30] CARMA1 interacts with BCL10 through their CARD domains.[31] CARMA1, BCL-10 and MALT1 act as crucial signalling compounds downstream of the antigen receptor and upstream of the IKK complex.[32,33](figure 1.4) This complex transmits signals to IKK complex that leads to activation of NF-κB.

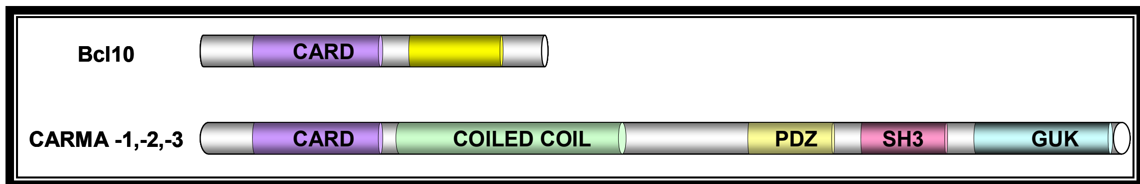

Figure 1.2: The structure of Bcl10 and MAGUK family protein CARMA1.

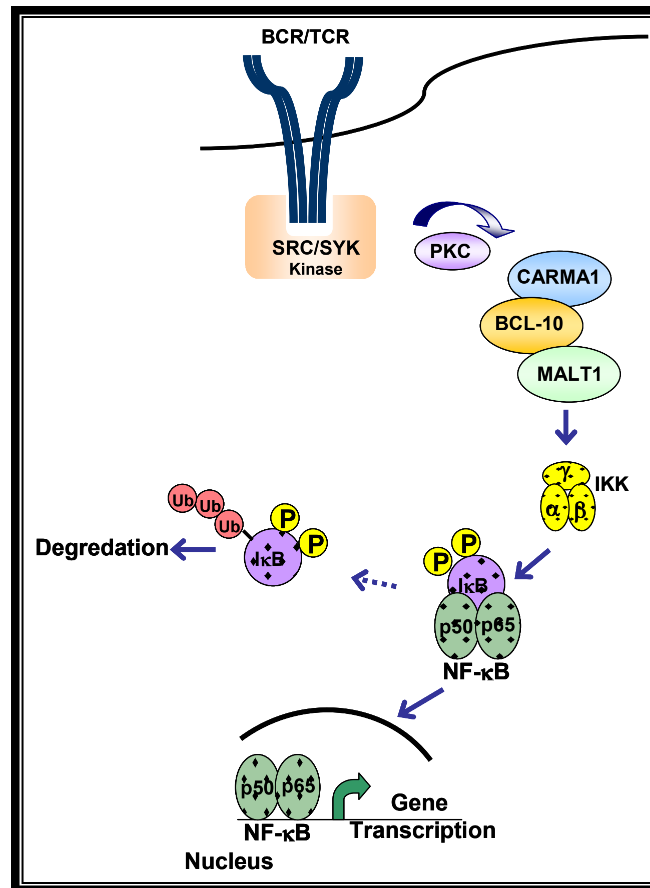

Figure 1.3: Antigen-receptor induced NF-κB activation by CARMA1, Bcl10 and MALT1

1.2.2 TNF-α ACTIVATES NUCLEAR TRANSLOCATION OF NF-κB

NF-κB is stimulated by diverse stimuli including tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) signals during inflammatory responses and regulates the transcription of other proinflammatory genes.[34] TNF-α was characterized about 30 years ago and found to be activated and to exert significant cytotoxicity on tumor cell lines and cause tumor necrosis in many animal models. Subsequently after realizing the structural and functional homology of TNF-α with lymphotoxin (LT) α (TNF-β), it was recognized that TNF-α is a member of large cytokine family, the TNF ligand family.[35] TNF-α is produced as a type II transmembrane protein[36,37] and via proteolytic cleavage by the TNF-α converting enzyme (TACE), a metalloprotease, the soluble TNF (sTNF) is released from the membrane bound form.[38]

When bound to TNF-α, TNF receptor (TNFR) transduces growth regulatory signals into the cells through death domain (DD) and TNF-receptor associated factors (TRAFs). Although TNF-α is mitogenic in normal cells it causes programmed cell death (PCD) in transformed cells. TNF's primary role is immune response regulation and control of TNF-α signalling is important because dysregulation and, in particular, overproduction of TNF-α have been implicated in a variety of human diseases, as well as cancer.[39] Upon stimulation by proinflamatory cytokine, TNF-α, the β subunit of IκB kinase (IKK) complex is activated through TRAF2 and IκB is phosphorylated on the two N-terminal serine residues leading to degradation of IκB(figure 1.2). Alternatively IKKα is activated upon TNF-α ligand binding to the receptor and p100 is phosphorylated. Phosphorylation is a signal for the ubiquitin ligase machinery of the cell, and upon phosphorylation IκB is polyubiquitinated and subsequently degraded. After degradtion of the inhibitory molecule, free NF-κB dimers can enter into the nucleus, where they bind to the specific sequences found in the promoter or enhancer regions of the target genes.[1] TNF-α signalling leading to NF-κB activation is shown in figure 1.4.

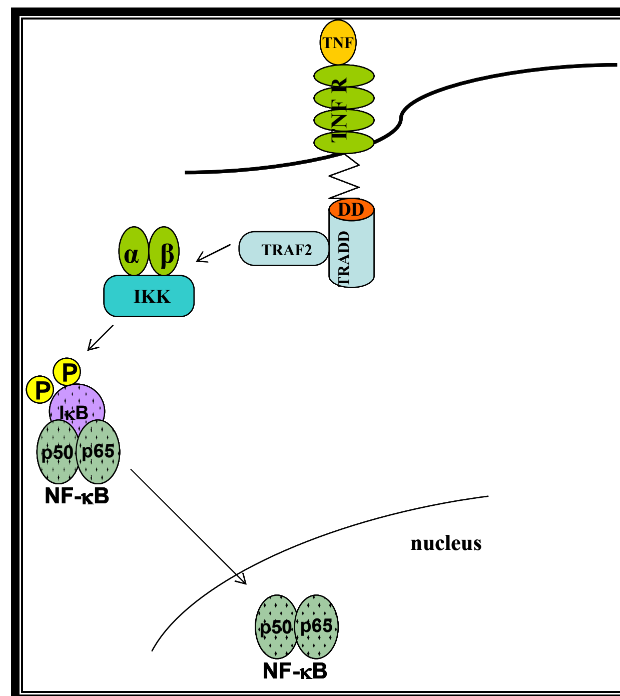

Figure 1.4: TNF-α activates nuclear translocation of NF-κB.

1.3 INNATE IMMUNITY AND TLRs

The innate immune system consists of the cells and mechanisms that defend the host from infection by other organisms, in a non-specific manner. This means that the cells of the innate system recognize, and respond to, pathogens in a generic way, but unlike the adaptive immune system, it does not confer long-lasting or protective immunity to the host.[40]

The induction of the adaptive immune response depends on the expression of co-stimulatory molecules and cytokines by antigen-presenting cells and it was proposed that expression of these co-stimulatory molecules is controlled by the non-clonal, or innate, component of immunity that preceded in evolution the development of an adaptive immune system in vertebrates.[41] Although adaptive immunity is unique to vertebrates, the innate immune response seems to have ancient origins. There are common features of innate immunity in vertebrates, invertebrate animals, and plants, such as; defined receptors for microbe-associated molecules, conserved mitogen-associated protein kinase signalling and the production of antimicrobial peptides. This shows that this immune response is shared by almost all multicellular organisms.[39,42,43]

The primary roles of the vertebrate innate immune system are:

- Recruiting immune cells to the site of infection and inflammation, via the production of chemical factors, like specialized chemical mediator molecules, called cytokines.
- Activation of the complement cascade to identify bacteria, activate cells and to promote clearance of dead cells or antibody complexes.
- Identification and removal of foreign substances present in organs, tissues, the blood and lymph, by specialized white blood cells.
- Activation of the adaptive immune system by antigen presenting.

As mentioned above innate immunity not only provides a first line defense against microbial proteins, but also has an impact on the establishment of adaptive immunity.[39,41] Upon infection, microorganisms are recognized by host cell defense mechanism through some receptors, called pattern-or pathogen-recognition receptors (PRRs), that recognize microbe-

associated molecules.[39,41,43] These PRRs recognize invariant molecular structures called pathogen associated molecular patterns (PAMPs) that are shared by many pathogens but not expressed by hosts.[41] Examples of PAMPs are; bacterial flagellin, a component of the flagellum that binds to TLR5, and DNA rich in CpG dinucleotides which binds to TLR9.

A family of conserved transmembrane Toll-like receptors (TLRs) functions directly or indirectly as PRRs for microbe-associated molecules in insects and mammals.[44] These receptors are characterized by an extracellular leucine-rich repeat (LRR) domain and an intracellular TIR protein-protein interaction domain. LRRs are common in a variety of receptors in plants and animals.[43] Interaction of TLRs with a pathogen elicits a signaling pathway that eventually leads to activation of NF-κB, through which an innate immune response is activated.[45] It has been known that TLR-mediated NF-κB activation is an evolutionarily conserved event that occurs in phylogenetically distinct species ranging from insects to mammals.[6,9] This very conserved receptors are important in first defense, innate immune response, because they recognize unspecifically and respond immediately according to the conserved features, PAMPs, that are found in the structure of pathogens but not in host.

Recognition of PAMPs by PRRs results in the activation of different intracellular signaling cascades that in turn lead to the expression of various effector molecules.[41] These effector molecules further activate a signalling cascade eventually leading to the activation of an immune response. Engagement of TLRs by pathogens leads to the activation of innate immune responses,[45] and a major signaling target of the TLRs is activation of the transcription factor NF-κB, a key regulator of immune and inflammatory responses.

TLR has a leucine-rich extracellular domain, which is required for recognition and an intracellular TIR (Toll/IL-1R) motif. This intracellular motif is significantly homologous to the intracellular domain of IL-1R. Although the IL-1R and TLRs have different extracellular domains, the presence of TIR domain allows both receptors to activate similar intracellular signaling pathways.[46] TLR2 and TLR4 are the most extensively characterized TLR family members.

TLRs are believed to function as dimers. Although most of the TLRs function as homodimers, TLR2 forms heterodimers with TLR1 or TLR6. TLRs may also depend on other co-receptors for full ligand sensitivity, such as in the case of TLR4's recognition of LPS,

which requires MD-2. CD14 and LPS Binding Protein (LBP) are known to facilitate the presentation of LPS to MD-2. When activated TLRs recruit adapter molecules in order to propogate signal. There are four adapter proteins known that are involved in the signalling. These are MyD88, Tirap (also called Mal), Trif, and Tram. NF-κB is activated through a MyD88-dependent payhway. IRF3, a transcription factor is activated in the alternative, MyD88-independent, pathway.[47]

Some of the characterized TLRs and their ligands can be listed as: bacterial flagellin, which is a component of the flagellum binds to TLR5, and DNA rich in CpG dinucleotides binds to TLR9. TLR2 binds to lipoarabinomannan from mycobacteria, zymosan from yeast, LPS from spirochaetes and peptidoglycan and TLR2 forms a heterodimer with either TLR6 or TLRX. TLR4 is one of the best characterized receptors of this family of receptors and binds to LPS, as well as a number of host protein molecules which are released at sites of damage or infection, such as heat-shock protein 60 (HSP60) and an alternative fibronectin produced at sites of inflammation.

1.4 THE PLANT IMMUNE SYSTEM

Plants have evolved an array of rapid and efficient defence responses against a wide variety of pathogens including bacteria, fungi, viruses and nematodes. Receptors and signal transduction components that play role in plant disease resistance have been shown to contain remarkable similar similar conserved domains as in animals and insects.[48] In insects and animals PAMPs are recognized by Toll and Toll-like receptors(TLRs), respectively, that contain extracellular LRR domain.[49,50] In plants this LRR domain is found in the products of disease-resistance genes, which are important in plant immune response.[48] As mentioned previously it has been shown that PAMPs are recognized by a variety of organisms including plants and an immune reaction is triggered in response. Through the response that plants have evolved, plants give a rapid and efficient response against a wide variety of pathogenic agents, including viruses, bacteria, and fungi.[48] One of the most affective weapon against pathogen attack is the hypersensitive response (HR). As a result of this rapid repsonse localised cell death occurs at the site of infection.[51] This prevents pathogen from accessing nutrient and by the way limits pathogen proliferation. There is also evidence that there are

signal molecules that are produced by dying cells and these molecules induce expression of a variety of disease-resistance genes.[51,52] A wide range of physiological changes occur in response to pathogen attack including reactive oxygen species (ROS) production, pH changes due to transient ion-flux, cell wall strengthening near to the infection site, release of secondary signal molecules such as nitric oxide (NO) and the synthesis of antimocrobial products including phytoalexins and pathogenesis-related (PR) proteins. These changes and some additional changes in the cellular structure aims to prevent pathogen attack and provide resistance.[48]

Although plants are prone to pathogens, rarely they have disease. The reasons are; plant is an uncomfortable host because it doesn't support the niche requirements of a pathogen, there are preformed structural barriers and toxins against a pathogen attack, and the systemic resistance elaborated upon pathogen attack prevents invasion of the pathogen to the other parts and localizes the invasion.[51] Prior pathogen attack can result in systemic acquired resistance (SAR) which is a component of the complex system that is used by plants to defend themselves against pathogens. SAR is induced by pathogen attack and it renders a systemic resistance against a broad spectrum of infectious agents, including viruses, bacteria, and fungi. If the SAR signalling pathway is blocked, the plant becomes more susceptible to not only the pathogens that cause disease but also to the ones that normally do not cause disease.[53,54] Experiments done with some mutant plants revealed some important proteins that have roles in plant imunity. Some of these mutants were claimed to modify the SAR signaling such as *lsd* (for lesion simulating disease) and *acd2*(for accelerated cell death).[55] The studies done with *Arabidopsis thaliana* showed that there also some mutants isolated that are blocked in SAR signal transduction pathway one of which is *nim1*. It was found that the protein encoded by this gene has a high homology with human IκB protein. It has several ankyrin repeats that are important in protein protein interaction.[56] This data suggested that in plants there has to be a functionally homologous pahway to NF-κB/IκB pathway in mammals. There are other ankyrin repeat containing proteins in *Arabidopsis* system that are susceptible to function in plant disease resistance.

Salicylic acid (SA) plays an important signalling role in pathogen-induced resistance response in plants. The level of endogenous SA was shown to increase during resistance responses; hypersensitive response (HR) and Systemic Acquired Resistance (SAR).[57] The

studies done with the mutant plants that are defective in accumulating SA showed that these plants are susceptible to pathogens and they can't develop a resistance to the pathogens.[58]

WRKY proteins are important factors in plant immune system. These proteins were identified recently as a class of DNA-binding proteins that recognizes the TGAC containing W-box elements that are found in the promoters of a large number of plant defense-related genes.[57] The first cDNA encoding a WRKY protein, SPF1, was cloned from sweet potato (*Ipomoea batatas*)[59] Since then many WRKY proteins were experimentally identified from different organisms including *Arabidopsis thaliana*.

WRKY family proteins has up to 100 representatives in Arabidopsis. This class of proteins are unique to plants and has a varity of roles in the regulation of physiological programs including pathogen defense, senescence and trichome development. The name WRKY[60] comes from the fact that these transcription factors have a highly conserved DNA-binding (WRKY) domain which is defined by the highly conserved aminoacid sequence, WRKYGQK, in the N-terminal end of the protein. This domain is assumed to be their only conserved domain among all WRKY proteins, as they all bind to the same DNA sequence through this domain.[60] WRKY proteins are divided into two major groups. (Figure 1.5) Group I members have two WRKY domains whereas group II members have only one.[60] Current data point to many WRKY proteins having a regulatory function in the response to pathogen infection and other stresses.[60] WRKY transcription factors control the expression of genes including themselves and many defense-related genes. This shows that they are important factors in immune response in plants.

As in animal immune responses, plant defense responses, when induced, include a network of signal transduction and the rapid activation of gene expression following pathogen infection. Studies revealed some structural and functional conservation of some of their signal transduction processes between animal and plant defense responses. For example, several plant disease resistance (R) genes, which confer recognition and resistance to specific plant pathogens, were shown to encode an amino-terminal domain homologous to the cytoplasmic signaling domain of the mammalian interleukin-1 receptor and *Drosophila* Toll protein.[61,62]

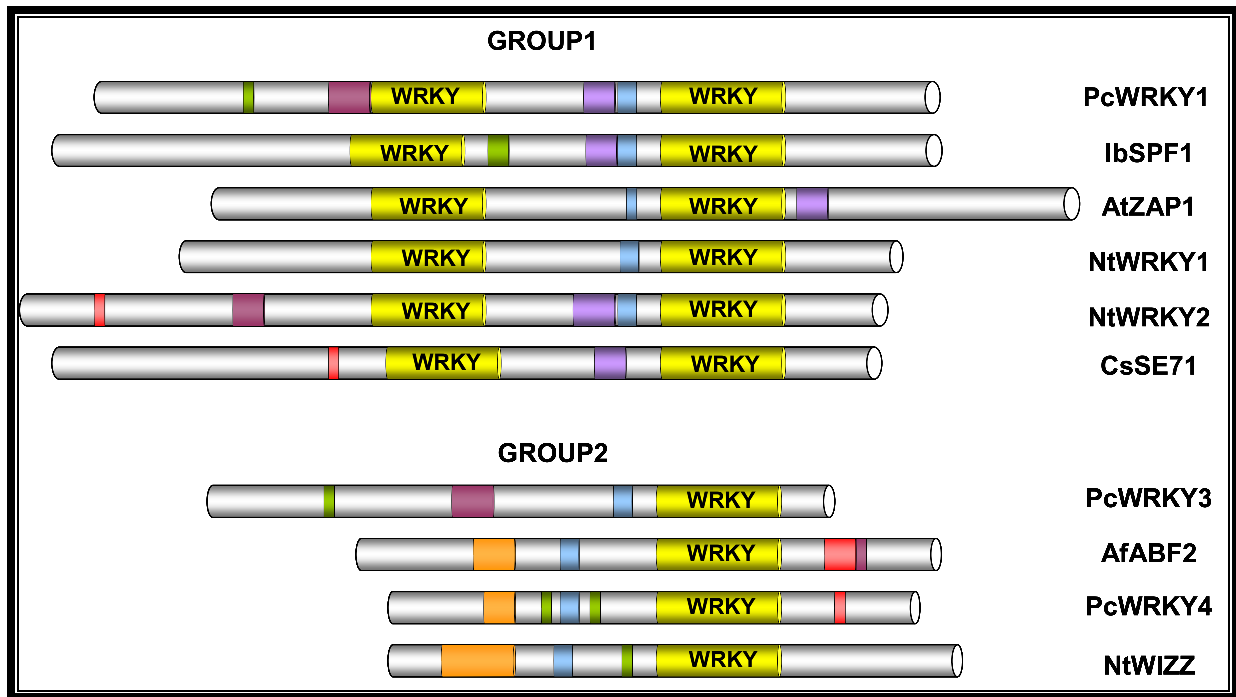

Figure 1.5: The structure of WRKY proteins

1.5 ANKYRIN REPEATs

Ankyrin repeats are found in the structure of a wide range of proteins, they are highly conserved motifs that are shared by the IκB family proteins, including *Drosophila* IκB Cactus, and they are required for association with Rel/NF-κB proteins.[63] The ankyrin-like repeat contains 33 amino acids and was first described in the yeast cell cycle-control proteins[64], which consists of a TPLHLA core sequence (the latter detected first in the *Drosophila* Notch protein [65]) and 8-10 other well-conserved residues. The number and the nature of the conserved amino acids is variable in different proteins. They are known to bind to NF-κB to inhibit it through this domain. The consensus sequence for ankyrin repeats is: ----D--G-TPLH-AA-------V--LL--GA-.[29]

The ankyrin repeats are regions of protein-protein interaction, and the specific interaction between ankyrin repeats and rel-homology domains appears to be a crucial, evolutionarily conserved feature of the regulation of NF-κB proteins. Each IκB differs in the

number of ankyrin repeats, and this number appears to influence the specificity with which IκB pairs with a rel dimer. As mentioned previously, p100 and p105 also contain ankyrin repeats and are sometimes included in the IκB family.[6]

The members of the ankyrin repeat containing family have different functions and are located in different compartments of the cell. This family of proteins include membrane receptors, cytoplasmic control proteins and nuclear transcription factors. That's why it is hard to ascribe a common function to the ankyrin and ankyrin-like repeats in all these proteins except for a very general one like protein-protein interaction.[2]

Recent findings have shown that some proteins in *Arabidopsis* that are important in the expression of pathogenesis related genes (PR), so in the immune response, have these ankyrin repeat domains.[55,66] There is also some other proteins that important in *Arabidopsis* immune system, one of which AKR2, has a high structural homology to mammalian IκB.[29] In the light of these findings, we supposed that there is a high possibility of interaction between ankyrin repeat containing proteins even if they are form different organisms.

1.6 INTER-PROTEOME INTERACTIONS

A major post-genomic scientific and technological approach is to describe the functions performed by the proteins encoded by the genome. One strategy is to identify the protein–protein interactions in a proteome, to determine pathways, structure, and the function of individual proteins.[67] It is also possible to search for interactions between different proteomes. Up to now inter-proteome interactions have led to discovery of many natural products that are used as drugs. For example cyclosporin and FK506 whose original forms were produced in *Tolypocladium inflatum Gams* and *Streptomyces tsukubaensis* respectively are commonly used as immunosuppressive drugs, because of their ability to bind to and inhibit human proteins.[68] Chemical structures which are modified forms of these natural compounds are now used as immunosuppressive drugs in humans.

Rational drug design requires a reduction of the side effects of the drug, which means reducing the possibility of its interaction with pathways other than the target one. In 2003 Zarrinpar *et al* found that the probability of a protein binding to a protein from the proteome of a different organism is rather high, (around 0.5).[69] Recent experiments have probed these interactions by replacing a protein domain involved in the osmoresistance signalling pathway in yeast with domains of the same class of protein from other species. These experiments show that domains from other species interact specifically with their target proteins without interacting with the other pathways.[67] These studies indicate that the study of inter-proteome interactions may yield good candidates for modulators of human proteins that can be used as specific drugs. Our hypothesis is that nature contains in itself the solution to protein-protein interactions, therefore, we designed a screening strategy in which we tried to identify a natural product (a plant protein) that can be used as a therapeutical agent against the signal transduction pathway that activates NF-κB. In our study we preferred to study plant, *A. thaliana*, ankyrin repeat containing proteins due to the evolutionarily conserved nature ankyrin repeats and their previously identified functions as mediators of protein-protein interactions.

2 AIM OF THE STUDY

The regulation of T and B cell development is crucial for proper functioning of the adaptive and the innate immune system. NF-κB is a eukaryotic transcription factor that is known to be activated through T and B cell receptor stimulation and it activates the expression of genes that function in the T and B cell development. For its importance in immune system cells' development, regulated activation of the NF-kB is important.

The aim of the study was to discover protein inhibitors of complex biological processes and the characterization of their molecular targets. We screened plant cDNAs coding for the the proteins that have ankyrin repeats for the presence of encoded polypeptides that inhibit or activate NF-κB signal transduction pathway in mammalian immune system cells. This approach is similar to classical mutagenic genetics screening or the recently popular chemical genetic screening for inhibitors of signal transduction pathways. The information obtained about the mammalian immune system from our studies can, in the future, be confirmed in more complex and physiological (such as transgenic and knockout mice) systems. The plant factor that is found to be interacting with the mammalian system has the potential to be a therapeutical agent and can be used in humans. That's why the result is important therapeutically. The screening was done by using a Green Fluorescent Protein (GFP) reporter system, in which GFP was expressed by NF-κB binding tp the reporter vector enhancer region.

Ankyrin repeat containing 90 *A. thaliana* cDNAs in pUNI51 vectors were inserted into mammalian expression vector, pHM200-Myc3, by using Cre-mediated recombination. After recombination reactions plant cDNAs in mammalian expression vectors were transfected along with the reporter vector to the HEK293T cells by calcium-phosphate mediated transfection. We analysed the transfected cells flow cytometrically instead of microscope imaging to comment quantitatively on the results.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 CHEMICALS

The chemicals used in the study are listed in Appendix A.

3.1.2 EQUIPMENT

The equipment used in the study is listed in Appendix B.

3.1.3 BUFFERS AND SOLUTIONS

Buffers and solutions used in the cloning and expression steps were prepared according to Sambrook *et al*., 2001[70]. Trypan blue dye used for exclusion of dead cells under hematocytometer was prepared at a final concentration of 0.4 %(w/v) by dissolving 40 μg of trypan blue in 10 ml 1X PBS. Calcium Chloride ($CaCl_2$) Solution used for competent cell preparation contained 60mM $CaCl_2$ (diluted from 2.5M stock), 15% Glycerol,10mM PIPES (pH=7) and all the solutions prepared was autoclaved at 121^0 C for 15 min if not stated otherwise and stored at indicated temperatures.

For SDS gel electrophoresis, separating and stacking gels were prepared according to the table 3.1. We used 10% gel in our work.

Separating Gels, in 0.375 M Tris, pH 8.8				
	7 %	10 %	12 %	15 %
distilled H_2O	5.1 ml	4.1 ml	3.4 ml	2.4 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml
20 % (w/v) SDS	0.05 ml	0.05 ml	0.05 ml	0.05 ml
Acrylamide/Bis-acrylamide (30 % / 0.8 % w/v)	2.3 ml	3.3 ml	4.0 ml	5.0 ml
10 % (w/v) ammonium persulfate	0.05 ml	0.05 ml	0.05 ml	0.05 ml
TEMED	0.005 ml	0.005 ml	0.005 ml	0.005 ml
Total monomer	10.005ml	10.005ml	10.005ml	10.005ml

Stacking Gels, 4.0 % gel, 0.125 M Tris, pH 6.8	
distilled H_2O	3.075 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
20 % (w/) SDS	0.025 ml
Acrylamide/ Bis-acrylamide (30 %/ 0.8 % w/v)	0.67 ml
10 % (w/v) ammonium persulfate	0.025 ml
TEMED	0.005 ml
Total monomer	5.05 ml

Table 3.1: SDS Gel components

Blocking solution used for membrane blocking while making western blotting, was prepared by dissolving milk powder in PBS-Tween at a final concentration of 5%.

PBS-tween was prepared by mixing 0.1% (w/w)Tween with 1X PBS.

Anti-c-myc antibodies that are HRP conjugated were diluted 1:1000 in 5% milk powder solution for blotting.

TNF-α stock solution was prepared according to manufacturer's protocol.[71] 100µg/ml TNF alpha aliquots were prepared in sterile H_2O. Further dilutions to make 10µg/ml aliquots were done in 1X PBS supplemented with 0.5 % BSA. The transiently transfected cells were then treated with 10 ng/ml TNF alpha 24 hours after transfection.

Annealing buffer:100 mM KAc + 30mM HEPES + 2mM Magnesium acetate. Prepared buffer was sterilized by autoclaving at 121° for 15 minutes.

10X Recombination Buffer: 0.5 M Tris-HCl (pH 7.5), 0.1 M $MgCl_2$, 0.3 M NaCl, bovine serum albumin (BSA, 1 mg/ml)

2X HeBS: 16.4 g NaCl + 11.9 g Hepes(acid form)+ 0.2 g Na_2HPO_4 were dissolved in 800 ml sterile water, the pH was adjusted to 7.05 with 5N NaOH and the solution was completed to 1 liter with sterile water.

Total Lysis Buffer: 150 mM NaCl+ 1% NP40 (nonionic detergent) + 50 mM Tris(pH:8.0). To this mixture PMSF was freshly added up to the concentration where it is 1 mM. Complete protease inhibitor (Roche) was added to mixture yielding 1 mM EDTA and proteases.

NETN: 0.5 % (v/v) Nonidet P-40 (NP-40) + 1 mM EDTA+ 20 mM Tris-HCl (pH 8)+ 100 mM NaCl NETN Buffer was used to lyse the cells. NP-40 is the detergent used to lyse the cells.

NT: 100 mM Tris-HCl (pH: 8) + 120 mM NaCl

NTG: 100 mM Tris-HCl (pH 8) + 120 mM NaCl+ 20 mM Glutathione. NTG is the elution buffer used in the final step of protein purification.

SDS gel running buffer: 10X running buffer was prepared by dissolving 30.3 g Tris-Base, 144.1g glycine and 10 g SDS in 1 liter deionized water.

Transfer Buffer was prepared by dissolving 1.45 g Tris-Base in 1 liter solution containing 7.2 g Glycine, 200 ml MetOH and deionized water.

10X PBS: 38 mM NaH_2PO_4+ 162 mM Na_2HPO_4+ 43.84 g NaCl and adjust pH to 7.4.

3.1.4 GROWTH MEDIA

Bacterial Growth Media: Luria Broth from Sigma was used for liquid culture of bacteria. 20 g of LB Broth powder was dissolved in 1 L of distilled water and autoclaved at 121^0 C for 15 min. For selection, kanamycin at a final concentration of 50 μg/ml and ampicilin at a final concentration of 100 μg/ml were added to liquid medium after autoclave. Luria Agar from Sigma was used for preparation of solid medium for the growth of bacteria. 40 g of LB Agar was dissolved in 1 L distilled water and autoclaved at 121^0 C for 20 min. Autoclaved medium was poured to sterile Petri plates after cooling down to room

temperature. For selection, antibiotics at the previously indicated concentrations were added to medium prior to pouring the medium.

Mammalian Growth Media: Suspension cell lines (Jurkat) were grown in RPMI 1640 cell culture medium that is supplemented with 10 % fetal bovine serum (FBS), 2mM L-Glutamine, 100unit/ml penicillin and 100unit/ml streptomycin. The cells were frozen in medium containing DMSO added into fetal bovine serum at a final concentration of 10% (v/v) and stored at 4 ^{0}C.

Adherent cell lines (HEK 293 and 293T) were grown in DMEM cell culture medium that is supplemented with 10% FBS, 2mM L-Glutamine, and 100unit/ml penicillin and 100unit/ml streptomycin.

3.1.5 MOLECULAR BIOLOGY KITS

- QIAGEN Plasmid Midi Kit, QIAGEN,Germany
- Qiaprep Spin Miniprep Kit, QIAGEN,Germany
- Qiaquick Gel Extraction Kit, QIAGEN,Germany
- Qiaquick PCR Purification Kit, QIAGEN,Germany

3.1.6 ENZYMES

Enzymes and their corresponding reaction buffers used for cloning and diagnostic digestions are given in table 3.2.

Enzyme	Buffer	Company
BamH1	E	Promega
EcoR1	H	Promega
Hind3	R	Fermentas
Sal1	D	Promega

Taq Polymerase	Taq Buffer	Fermentas
T4 DNA Ligase	T4 DNA Ligase Buffer	Fermentas
Xba1	D	Promega
Xho1	D	Promega

Table 3.2: List of enzymes used.

3.1.7 CELLS, PLASMIDS, OLIGO NUCLEOTIDES

DH-5α competent cells were used for bacterial transformation of plasmids and BL21 strain of *E. coli* was used for expression experiments. For the reporter assays, human endothelial kidney (HEK) 293T cells were used. Plasmids used in the study and basic features of these plasmids are given in Table 3.3. Primers used are listed in Table 2.4.

Plasmid	**Company**	**Feature-Use**	**Selective Marker**
pCDNA3	Invitrogen	Cloning plasmid	Amp
pDSRedExpess-DR	Clontech	DSRed-repoter	Kan
peGFP-N2	Clontech	EGFP	Kan
pTurboGFP-PRL-Dest	Evrogen	GFP- reporter	Kan
pQL123		GST-Cre	Amp

Table 3.3: List of Plasmids

Name	**Location**	**Start**	**Sequence**	**Product length**
PTurboGFPleft	11-362	11	TAGCGCTACCGGACTCAGAT	352
pTurboGFPright		362	GATGCGGCACTCGATCTC	
DsREDleft	11-372	11	TAGCGCTACCGGACTCAGAT	362
DsREDright		372	AAGCGCATGAACTCCTTGAT	

Table 3.4: List of Primers

NF-κB oligo nucleotides(SalI-XhoI)

NF-κB consensus sequence: GGGAATTTCC

5'-TCGAC**GGGAATTTCCGGGAATTTCC**C-3'

5'-TCGAG**GGAAATTCCCGGAAATTCCC**G-3'

3.2 METHODS

3.2.1 CELL CULTURE

Bacterial Culture Growth: Bacterial cells were grown overnight (12-16h) at 37 °C shaking at 270 rpm in Luria Broth. Depending on the application selective antibiotics were added to the media. Bacterial cells either spreaded or streaked on LB Agar Petri plates were grown overnight (12-16h) at 37 °C. To make the glycerol stock of cells, glycerol was added to the overnight grown culture to a final concentration of 15%. Cells were frozen first in liquid nitrogen and then stored at -80°C.

Mammalian Culture Growth: Adherent cell lines (HEK 293 and HEK 293T) were grown in DMEM Medium that is supplemented with 10% fetal bovine serum, 2mM L-Glutamine, 100 unit/ml penicillin and 100 unit/ml streptomycin. Cell cultures were splitted every 2-3 days by 1/10 dilutions. For the preparation of frozen stock of HEK 293 cells, cells at mid to late growth phase were resuspended in freezing medium (10%DMSO in fetal bovine serum) and stored at -80°C for at least 24 hours. Then, the cells were taken to the liquid nitrogen tank and kept there. After thawing the cells were immediately washed with growth medium to get rid of DMSO.

Suspension cell lines (Jurkat T cells) were also grown in a humidified atmosphere of 5%CO_2 at 37 °C in RPMI 1640 Medium supplemented with 10% fetal bovine serum, 2mM L-Glutamine, 100unit/ml penicillin and 100unit/ml streptomycin. Freezing medium was same as the one used for adherent cells.

3.2.2 POLYMERASE CHAIN REACTION (PCR)

PCR Reaction Components	Volume used	Final concentration
Template DNA		
10X Polymerase Buffer	2.5 µl	1X
$MgCl_2$	2 µl	2 mM
dNTP Mix(10mM)	0.5 µl	0.2 mM
Primer forward(10mM)	2 µl	0.8 µM
Primer reverse(10mM)	2 µl	0.8 µM
Taq Polymerase(5u/µl)	0.125 µl	0.025u/µl
ddH_2O	12.875 µl	
Total	25 µl	

Table 3.5: PCR Components

Step	Temperature	Duration	Cycle
for pDsRED			
Initial Denaturation	95°C	5 min	1
Denaturation	95 °C	0.5 min	30
Annealing	55 °C	1 min	
Extension	72 °C	1.5 min	
Final extension	72 °C	5 min	1
Hold	4 °C	∞	1
for pTurboGFP			
Initial Denaturation	95°C	5 min	1
Denaturation	95 °C	0.5 min	26
Annealing	56 °C	1 min	
Extension	72 °C	1.5 min	
Final extension	72 °C	5 min	1
Hold	4 °C	∞	1

Table 3.6: PCR Conditions

3.2.3 VECTOR CONSTRUCTION

DH5α competent cells were prepared starting from a single colony of previously streaked DH5α. Single colony from the plate was grown overnight at 37°C, 270 rpm in 50ml LB. Next day 4 ml from the overnight grown culture was diluted in 400ml LB (w/o antibiotic) and incubated at 37°C, 270 rpm until OD_{590}=0.375 is reached. Then the $CaCl_2$ solution (as in 2.1.3) was used for resuspension in the successive centrifuge steps and the final preparation of competent cells. The cells prepared were frozen immediately in liquid nitrogen and then stored at -80°C. The competency of the prepared cells was tested by transforming 1-2 ng of a known plasmid.

For transformation of competent cells, $CaCl_2$ treated competent cells were taken out of -80°C and the plasmids to be transformed were added (no more than 50ng in a volume of 10 µl or less) to each tube. The tubes were then stored on ice for 20-30 minutes. The cells were then incubated at 42°C for 90 seconds and transferred back to the ice rapidly to chill for 1-2 minutes. 800 µl of autoclaved Luria Broth was added and the cultures were incubated for about 45 minutes in a 37°C water bath to allow the bacteria recover and express the antibiotic resistance marker encoded by the plasmid. Transformed cells were spreaded on Luria Agar plates containing the appropriate antibiotic. The plates were then incubated overnight at 37°C to observe the colonies.

Plasmid DNA isolation was carried out either by the alkaline lysis protocol in Sambrook *et al.*, 2001[70] or by the Qiaprep Spin Miniprep or QIAGEN Plasmid Midi Kits. A single colony of *E coli* from Luria Agar plate or glycerol stock was grown overnight at 37°C, 270 rpm in liquid culture containing selective antibiotics (ampicillin 100 µg/ml or kanamycin 50µg/ml). Cultures grown overnight were used for DNA isolation and for the preparation of glycerol stocks. The concentration of the plasmid DNA isolated was determined by a NanoDrop (UV Spectrophotometer) and quantitatively on an agarose gel. DNA miniprep concentrations were approximately 200 ng/µl, with a total DNA yield of 5 µg.

Restriction Enzyme Digestion: Restriction Enzyme Digestion: Digestion reactions were carried in an eppendorf tube containing the template DNA, the enzyme and the compatible buffer, and incubated at 37°C, for 2.5-3 hrs. Agarose gels to observe the DNA according to

their size were prepared in different concentrations ranging from 0.7% to 2% depending on the size of the fragments to be separated. Agarose gels were prepared by mixing 2gm of agarose powder in 100ml 0.5X TBE (for 2%gels) and boiling for 3-5 minutes in a microwave oven, ethidium bromide was added to a final concentration of 0,001(v/v). 0.5X TBE buffer was also used as the running buffer. DNA samples were mixed with 6X loading dye prior to electrophoresis at 100-135 volts for 40-60 minutes, bands were observed under UV light in a BioRad Quantity One Gel Quantitation Apparatus.

Annealing of oligo nucleotides: Oligo nucleotides with SalI, XhoI overhangs were dissolved in water at a concentration of 3μg/μl DNA concentration. 2 μl of each oligos were mixed in a PCR tube with 48 μl of annealing buffer, incubated at 95°C for 4 minutes, at 70°C for 10 minutes followed by annealing At room temperature for more than 1hr. Before ligation, annealed oligonucleotides were diluted from12 μg/ 50 μl oligo nucleotide to 2ng/ml.

Ligation: The ligation reactions were carried out using T4 Ligase (Fermentas), in a 1:15 molar vector to insert ratio, using 100 ng vectors. Ligation reaction mixtures were kept at room temperature 25° C for 2-3 hours, transformed into chemically competent E. coli, and correct colonies were identified via colony PCR.

Colony PCR: The single colonies from the plates were dissolved in 8.5 μl distilled water in autoclaved PCR tubes by the aid of sterile toothpick, after twirling the toothpick in the distilled water the toothpick was then used to streak the colony just by touching, onto a fresh replicate agar plate using a numbered template. The tubes were then incubated at 94 ^{o} C for 8 minutes and the PCR master mix and the primers were added to the tube and normal PCR reaction as in section 3.2.2 was carried out. The PCR products were then run on agarose gel to see if there was any positive colony with an amplification of the inserted fragment.

Reporter vectors: The GFP reporter vector pTurboGFP. Fos vector was constructed by inserting fos promoter from pGL3.Fos plasmid into pTurboGFP-PRL-dest1 (Evrogen) plasmid. The fos promoter containing pTurboGFP-PRL-dest1 vector(pTurboGFP. Fos) was digested with SalI, upstream of the promoter, and the oligo nucleotides that were annealed previously and that had flanking SalI, XhoI sites were inserted to this site by using the fact that both XhoI and SalI cut DNA leaving compatible ends. This feature was also used to predict the direction of the response element containing oligos. Each oligo contained two NF-

κB binding sites and the oligos were 26 bp in length. Totally 4 oligo nucleotides were ligated one by one which made 8 NF-κB binding sites in total. After each ligation the insertion was first checked by colony PCR and then for further confirmation and direction determination miniprep DNAs of putative positive colonies were digested by using restriction enzymes.

Plasmid pDSRedPro-ESV40 had been constructed previously in our lab and to this vector NF-κB response elements-containing oligos were inserted one by one as in pTurboGFP.Fos vector.

3.2.4 GST-CRE PURIFICATION

BL21 containing pQL123 plasmid was grown overnight in 5ml LB. 0.5 ml of this overnight (o/n) culture was diluted into 50 ml and grown at 37°C in LB/ampicillin medium until OD_{600} of 0.6 was reached. At this concentration IPTG was added to a final concentration of 0.4 mM to the cells. Induction was carried out o/n at room temperature. The next day the cells were pelleted at 4500 rpm for 10 minutes at 4° in a 50-ml conical falcon tube. The cells were then resuspended in 2.8 ml of ice-cold NETN that was freshly supplemented with 1mM phenylmethylsulfonyl fluoride (PMSF), and complete protease inhibitors. The cells were sonicated five times for 8 seconds and incubated on ice for 9 seconds between each sonication in a cold room for 2 minutes. After transferring the cell lysate to 1.5 ml Eppendorf tubes, lysate was cleared by spinning at 13200 rpm for 15 min at 4°C to remove cell debris. The supernatant containing cellular protein extract was poured on to the previously prepared glutathione-sepharose beads in a 100 µl bead volume per 1.5-ml Eppendorf tube. The beads were washed three times with 1 ml of ice-cold NETN and spinned at 3000 rpm for 15 seconds at each time and after the last wash, the supernatant was added to the beads. The lysate was mixed by inverting the tubes several times and by rotating it at 4° for 1 hour on a rotating rod. After 1 hour of incubation the beads were pelleted at 3000 rpm for 15 seconds at room temperature and washed with 1 ml ice-cold NETN this was repeated for four times. Next, the beads were washed with 1 ml of NT and after the last wash excess liquid were removed with thin pipette tips. Elution was done by adding 100 µl of freshly prepared NTG and mixing by rotating the tubes at 4° for 1 hour. Lastly the beads were

pelleted as previous and the supernatant containing the protein was collected. Activity of enzyme was detected by doing a recombination reaction.[72]

3.2.5 CRE-*loxP* RECOMBINATION

In order to recombine the vectors containing *loxP* sites; 0.2- 0.4 µg of each of the plasmid vectors were put in an Eppendorf tube and 3 µl of purified GST-Cre, 1.5 µl of 10X recombination buffer and sterile water upto 15µl were added. The recombination mixture was incubated at 37° water bath for 20 minutes for the recombination reaction to take place and the tubes were kept at 65° for 5 minutes for the inactivation of the enzyme. After the recombination, 10 µl of the recombination mixture was transformed into the chemically competent *E. coli* DH5α cells by heat-shock transformation and the ones that were transformed with the recombined vectors were selected on Kan plate. [72]

3.2.6 TRANSIENT TRANSFECTION OF MAMMALIAN CELLS

Preparation of the cells:

HEK 293 and 293T cells are adherent cells and calcium phosphate transfection was applied to these cells. For this transfection, adherent cells were grown in DMEM medium supplemented with 10 % FBS till 100% confluency on 100 mm tissue culture plate. The transfection efficiency decreases when the cells are not available to $DNA/Ca_3(PO_4)_2$ crystals, therefore the cells should be confluent. The day before transfection, the cells were splitted 1:10. The next day, 2 hours prior to transfection the medium was changed.

Preparation of the plasmid DNA:

Qiagen Plasmid Midi Kit was used to prepare DNA. For each transfection in 100 mm tissue culture flask 10 µg DNA was mixed well with 440 µl sterile H_2O + 50 µl 2.5 M $CaCl_2$ in an Eppendorf tube. To this mixture equal volume (500 µl) of 2X HeBS was added while vortexing the mixture slowly. The final solution was let to stand at room temperature for 10 minutes and then all the mixture was poured drop wise to the cells distributing it around the cells. The cells were incubated in 5 % CO_2 and 37^oC incubator. Between 15 and 24 hours after the transfection we washed the precipitate away and incubated the cells for another 12 to

24 hours in 10 ml medium. The expression of the plasmid gene can be examined well between 24 and 48 hours after transfection. That is why any analysis like western blotting and FACS analysis was done 24 hours after transfection.

Jurkat cells were grown to 1-2 x 10^6cells/ml as explained in section 2.1.5. About 10^7 cells were centrifuged at 1000 rpm for 5 minutes at 25° C. Pelleted cells were then washed with serum-free RPMI 1640 and centrifuged again. Cells were then resuspended in 500 µl serum-free RPMI 1640 and mixed with 20 µg of plasmid DNA, incubated at room temperature for 10 minutes before transferring into 4mm electroporation cuvettes. We electroporated the cells at 290 V, 1400 µF and 100 Ω. After electroporation, cells were transferred to T-25 cell culture flasks and 10 ml of complete RPMI 1640 was added.

3.2.7 TNF-α TREATMENT OF CELLS

Recombinant human TNF-α (17.5 kDa) produced in *E. coli* and purified via sequential chromatography[36] was used for NF-κB activation in 293T cells. Induction was done 24 hours after transfection, and 10 ng/ml TNF-α was used. Flow cytometric analysis was performed 24 hours after TNF-α induction, which was determined by time course experiment done at the 2nd, 6th, 8th, and 24th hours.

3.2.8 WESTERN BLOTTING

Total protein extraction: Plant proteins that were transfected into 293T cells were N-terminal myc tagged proteins. 1-2 days after transfection of the plant protein, transfected cells on plate were washed with 1X PBS and afterwards they were scrapped off with 1 ml PBS and transferred into a 1.5 ml eppendorf. After centrifugation of the cells, total lysis buffer (details in 2.1.5) was added to the mixture according to the concentration of the cells e.g 200 µl of lysis buffer was used for about 10^6 cells. We next wortexed the lysate and then, the lysate was kept on ice for at least 30 minutes and after incubation on ice the tubes were centrifuged for 10 minutes at 13200 rpm at 4°C to pellet the cell debris leaving the cellular proteins in the supernatant.

SDS gel was prepared according to the protocol and the total protein extract was loaded to the wells after mixing the sample with 2X loading buffer and incubating the mixture at 95°C for 3-5 minutes. The gel ran at 120V for about 2 hours. Transfer to the PVDF membrane was done at cold room, at 4°C, for about 2.5 hours at 50V. Before beginning to transfer procedure, membrane was washed for 30 seconds in methanol to be activated. Prestained protein marker used during loading gel made it easier to confirm the transfer. Seeing the marker on the membrane showed that the transfer was successful. I made the detection in dark room. The detection reagent kit was warmed to room temperature before use. The substrate in the detection reagent kit was mixed 1:1 with the enhancer and 1 ml of mixture was poured on the membrane. After waiting a few minutes I put the transparent film on the membrane to complete the detection reaction.

3.2.9 FLOW CYTOMETRIC ANALYSIS

About 10^6 cells were centrifuged at 1000 rpm for 5 minutes at 25° C. Pelleted cells were washed with PBS twice, they were centrifuged and resuspended in 1 ml PBS. Resulting 10^6cells/ml solution was directly used for flow cytometric analysis on a BD FACSCanto flow cytometer. GFP fluorescence emission, excited by the argon laser was detected using a FITC 530/30 nm band pass filter.

4 RESULTS

4.1 NF-κB REPORTER VECTOR CONSRUCTION

NF-κB response element was prepared by annealing oligo nucleotides as described in 3.2.2 and the reporter vectors were constructed by one by one ligating NF-κB binding elements upstream of the turboGFP gene. The backbone of the reporter vector was pTurboGFP-PRL-dest1 (Evrogen), which was a promotorles vector to which the fos promoter, a weak promoter, had been previously inserted upstream of tGFP in our laboratory. After each ligation of the NF-κB response element containing oligo nucleotide and transformation of the ligation product into *E. coli* we did a colony PCR with the related primers (table 3.6). The construction is summarized in figure 4.1 and the agarose gel of the first colony PCR can be seen in figure 4.3.

The related primers amplify a region of 352 bp in an empty vector while this becomes 378 in a vector with an oligo nucleotide inserted. The gel prepared was 2% to be able to differentiate this slight difference. After colony PCR to confirm once more we cut the vector purified from the positive colony with EcoRI and BamHI, which created a fragment of 246 bp in empty vector and 272 bp in vector with the insert. After confirming the insert with both colony PCR and digest we further had to show the direction of it by doing a restriction digestion with SalI and BamHI. SalI site is the site where we inserted the oligo nucleotide having SalI and XhoI sites on the lefthand and righthand sides respectively. When ligation occurs between SalI and XhoI the restriction site diseappears so if the oligo nucleotide is inserted at correct orientation the fragment size increases as 26 bp and if it is inserted at wrong orientation the digest gives a fragment as in the negative control, the one without insert, a fragment of 236 bp.

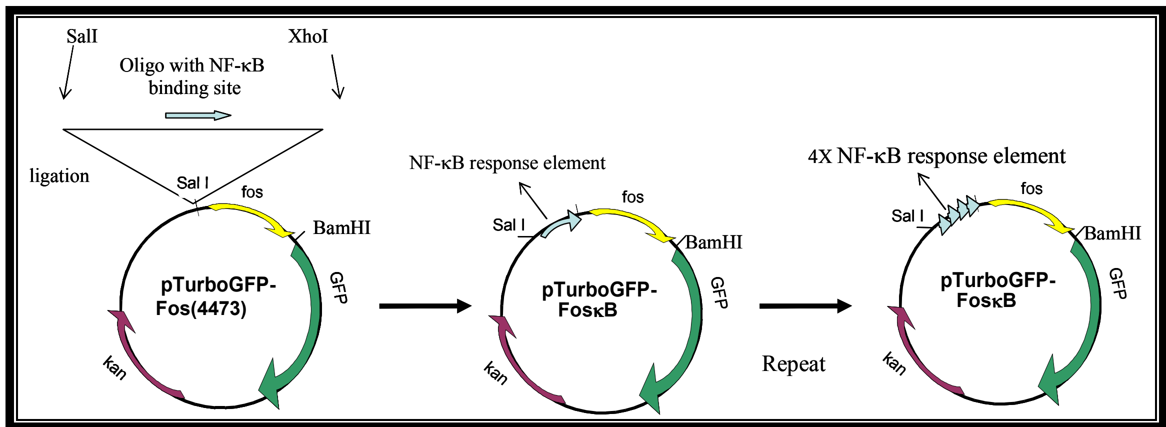

Figure 4.1: Reporter vector construction

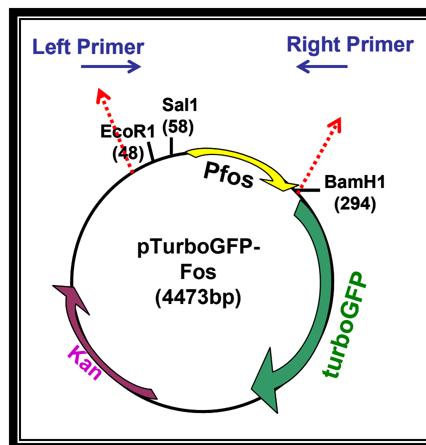

Figure 4.2: Restriction map of pTurboGFP.Fos vector.

SalI, BamHI digestion produces a 236-bp fragment if there is no insert and in case of an insertion the fragment amplified becomes 262 bp. The primers in figure 4.2 amplify a region of 352 bp in case there is no insert and the region becomes 378 when there is an insert.

Figure 4.3 shows the colony PCR agarose gel results after first oligo nucleotide insertion. In the figure the asterisk shows the positive colony which has the 26 bp insert. The difference between the positive one and the others is due to this insertion. The agarose gel was 2 %, which was suitable for short fragment differentiation. In figure control is the empty pTurboGFP.Fos vector.

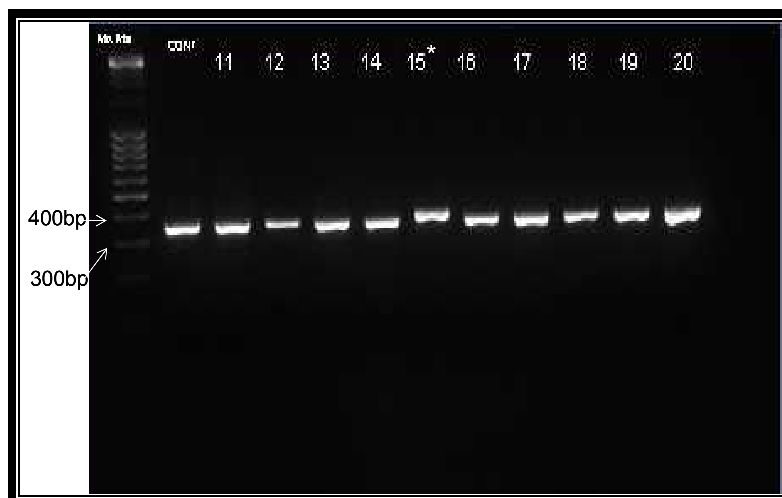

Figure 4.3: Colony PCR of first NF-κB insert in pTurboGFPFos.

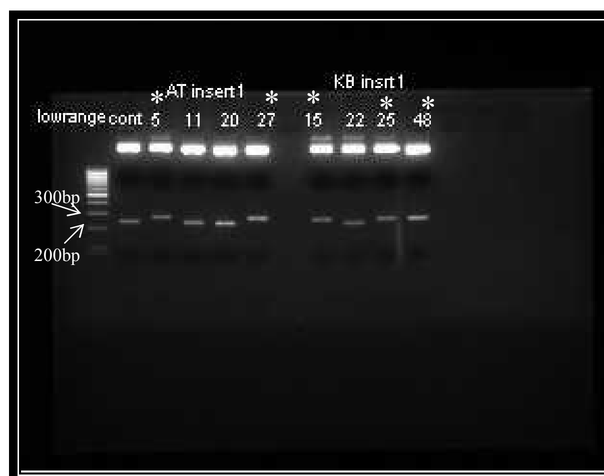

Figure 4.4: Sal1- BamH1 diagnostic digest of first NF-κB insert in pTurboGFPFos.

Diagnostic digests were carried out with Sal1 and BamH1. Sal1 site is at the 58th position and the BamH1 site is at the 294th position. Sal1, BamH1 digest gives a band of 236 bp if there is no insert or insert is in the wrong orientation and the same digest gives a 262-bp fragment when the insert is at the correct orientation. The colonies labeled with asterisks in figure 4.4 have the plasmids with the inserts at correct orientation. We did the further oligo nucleotide insertions to the correctly oriented plasmids as we did previously. Each time first a colony PCR was done to screen the positive ones and then we diagnostically digested the positive ones with Sal1 and BamH1 for orientation determination. Figure 4.5 and 4.6 are the photos of the agarose gel electrophoresis of the colony PCRs for 3rd and 4th inserts.

Figure 4.5: Colony PCR of second NF-κB insert in pTurboGFPFos NF-κB.

The asterisk in figure 4.5 shows the colony with the correct 26 bp insert,indicated by the difference of the bands. There are two negative controls foscon is the empty pTurboGFP.Fos vector and κBcon is the one with 1 oligo nucleotide inserted to pTurboGFP.Fos vector.

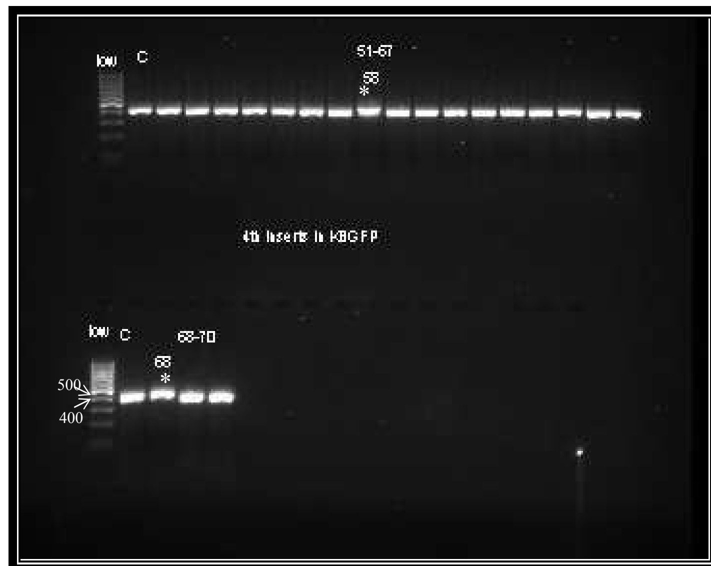

Figure 4.6: The 4th colony PCR gel electrophoresis result.

The colonies shown in figure 4.6 with asterisks are positive colonies that are susceptible to contain the 26-bp insert. C is the control with 3 oligo nucleotides inserted to pTurboGFP.Fos vector.

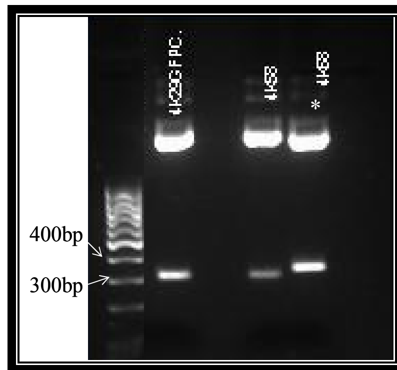

Figure 4.7: SalI-BamHI digestion of positive colonies.

DNAs from colonies that were previously identified by colony PCR were isolated and digested with SalI and BamHI for orientation determination. Sal1 is found on the right sight of the oligo nucleotides and when it is inserted on the right direction the fragment size increases 26 bp compared to the negative one. The gel photo can be seen in figure 4.7. The one with asterisk is the plasmid DNA from the 68th colony that was shown previously in figure 4.6 and it contains the insert at correct orientation. The fist lane after marker contained the the negative control which was the pTurboGFP.Fos vector with 3 oligo nucleotides inserted.

The next step after construction of the reporter vector was to confirm the inducibility of it. The vector constructed was controlled for inducible GFP expression by cotransfecting reporter vector with CARMA1, which is an activator of TCR-induced NF-κB activation. 5 µg CARMA1 and 2µg κB-GFP reporter vector were cotransfected to 293T cells in 6-well plate by calcium-phosphate mediated transfection. The cells were visualised under fluorescence microscope. Microscopy result can be seen in figure 4.8.

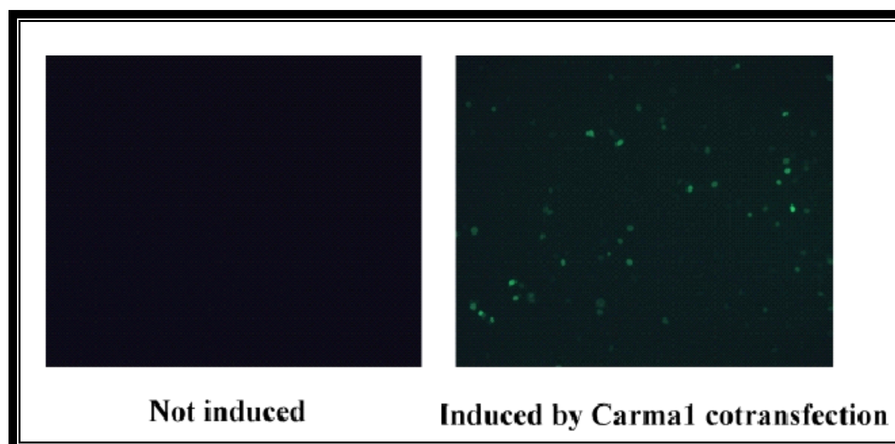

Figure 4.8: CARMA1 κB-GFP cotransfection

4.2 CRE-*loxP* RECOMBINATION

The Cre(Cyclization Recombination) protein is a recombinase encoded by the coliphage P1 and is a 38-kDa protein that efficiently promotes both intra- and intermolecular synapsis and recombination of DNA both in *Escherichia coli* and in vitro.[73] During site-specific DNA recombination, which causes genetic rearrangement in processes such as viral integration and excision and chromosomal segregation, recombinase enzymes recognize specific DNA sequences and catalyze the reciprocal exchange of DNA strands between these sites. The bacteriophage recombinase Cre catalyses site-specific recombination between two 34-base-pair *loxP* (locus of X-over P1) sites.[74] There exists an asymmetric 8 bp sequence in between with two sets of palindromic, 13 bp sequences flanking it. The detailed structure is given below. This 8-bp sequence can change but the flanking regions remain same.

13bp **8bp** **13bp**
ATAACTTCGTATA - GCATACAT -TATACGAAGTTAT

The Cre protein consists of 4 subunits and two domains: The larger carboxyl (C-terminal) domain, and smaller amino (N-terminal) domain. It is a 341- aminoacid long

protein whose catalytic C domain is similar in structure to the domain in the Integrase family of enzymes isolated from Bacteriophage λ. When Cre recognizes the *loxP* site it binds to this site and cuts the double stranded DNA and ligates it back forming a recombined sequence. This kind of recombination technique makes cloning easier by taking place of the cutting and ligating procedure during cloning.

In table 4.1 plant cDNA containing pUNI51 vectors are shown. pUNI vector has a lox*P* site upstream of the plant cDNA. The expression vector also has one *loxP* site and upon Cre recognition of these sites on two vectors homologous recombination occurs and a new vector, which is a combination of two parental vectors, is formed. The recombinant vectors' code and the parental vectors are given in table 4.2. Plant cDNA containing pUNI51 vectors were all recombined with the pHM200-Myc3 (20) called mammalian expression vector.

clone no	ABRC stock no	vector info.	information
27	S63012	PUNI vectors	Ank
28	S63196	PUNI vectors	Ank
29	S67347	PUNI vectors	Ank
30	U09314	PUNI vectors	Ank
31	U09646	PUNI vectors	WRKY
32	U09877	PUNI vectors	Ank
33	U10031	PUNI vectors	WRKY
34	U10244	PUNI vectors	WRKY
35	U10657	PUNI vectors	Ank
36	U10744	PUNI vectors	Ank
37	U11889	PUNI vectors	WRKY
38	U11947	PUNI vectors	Ank
39	U12363	PUNI vectors	Ank
40	U12708	PUNI vectors	Ank
41	U12845	PUNI vectors	Ank
42	U12967	PUNI vectors	Ank
43	U13006	PUNI vectors	WRKY
44	U13262	PUNI vectors	Ank
45	U13350	PUNI vectors	Ank
46	U13356	PUNI vectors	Ank

47	U13446	PUNI vectors	Ank
48	U13690	PUNI vectors	Ank
49	U13826	PUNI vectors	WRKY
50	U13953	PUNI vectors	Ank
51	U14042	PUNI vectors	Ank
52	U14849	PUNI vectors	Ank
53	U14890	PUNI vectors	WRKY18
60	U17040	PUNI vectors	WRKY
61	U17389	PUNI vectors	WRKY25
63	U18005	PUNI vectors	WRKY48
64	U18113	PUNI vectors	WRKY8
65	U18530	PUNI vectors	Ank
66	U18979	PUNI vectors	Protein kinase-like
67	U19945	PUNI vectors	WRKY7
68	U21371	PUNI vectors	WRKY28
69	U21822	PUNI vectors	Ank
71	U22208	PUNI vectors	Ank(NPR3)
72	U22740	PUNI vectors	WRKY24
73	U22831	PUNI vectors	Ank
74	U22836	PUNI vectors	WRKY17
75	U23247	PUNI vectors	WRKY
76	U23493	PUNI vectors	ribosomal binding p.
77	U23639	PUNI vectors	WRKY
81	U51115	PUNI vectors	WRKY
82	U51188	PUNI vectors	Ank
83	U60242	PUNI vectors	Ank
84	U60581	PUNI vectors	WRKY
85	U60582	PUNI vectors	WRKY43
86	U60601	PUNI vectors	WRKY
87	U60629	PUNI vectors	Ank
88	U60815	PUNI vectors	Ank
89	U61624	PUNI vectors	DNA Binding P.
90	U61783	PUNI vectors	WRKY

91	U64945	PUNI vectors	Ank
92	U64966	PUNI vectors	Ank
93	U66236	PUNI vectors	Ank
94	U66403	PUNI vectors	Ank
95	U66414	PUNI vectors	Ank
96	U67756	PUNI vectors	Ank
97	U83592	PUNI vectors	Ank
98	U83707	PUNI vectors	Ank
99	U83738	PUNI vectors	Ank
100	U83871	PUNI vectors	Ank
101	U84176	PUNI vectors	WRKY26
102	U84204	PUNI vectors	WRKY
103	U84218	PUNI vectors	WRKY57
104	U84666	PUNI vectors	WRKY45
105	U84831	PUNI vectors	WRKY
106	U84848	PUNI vectors	WRKY
107	U85182	PUNI vectors	WRKY
108	U85724	PUNI vectors	Ank
109	C103116	PUNI vectors	WRKY
110	C104777	PUNI vectors	WRKY
111	C104778	PUNI vectors	WRKY20
112	C105126	PUNI vectors	WRKY40

Table 4.1: The pUNI vectors that are used in Cre-mediated recombination

Recombinant vector code	Expression vector code	Plant cDNA vector code
20-40		40
20-42		42
20-44		44
20-46		46
20-47		47
20-48		48
20-50		50
20-51		51
20-52		52
20-61	20	61
20-63		63
20-64		64
20-65		65
20-67		67
20-68		68
20-82		82
20-83		83
20-84		84
20-87		87

Table 4.2: The list of vectors generated by Cre-mediated recombination.

Table 4.2 shows the recombined vectors. Out of 90 *A. thaliana* cDNAs these clones are the ones that were recombined with the mammalian expression vector (20). Currently the remaining clones are in the process of being generated.

The Cre-*loxP* recombination depends on the *loxP* sites on DNA. In our study, the two sites were on different plasmid, so after recombination, the vector formed was a combination of two parental vectors. pUNI51 has kanamycin resistance gene and the mammalian expression vector has the ampicillin resistance gene and the selection was done on LB/Kan plates as only the recombinant vector trasformed cells were able to live on this selective

medium. This is because of the fact that pUNI51 vector has an origin of replication, called R6Kγ, that does not let replication in pir$^-$ cells, e.g DH5α, that are used in our lab. So unrecombined vector can not replicate in DH5α. On the other hand the mammalian expression vector had oriColE1 as origin of replication which let the vector replicate in DH5α. After the recombination diagnostic digestions were done to confirm the recombination. HindIII was chosen for this digest because when recombination occured a fragment of about 2500 bp was created between HindIII sites that was not found in the parent vectors

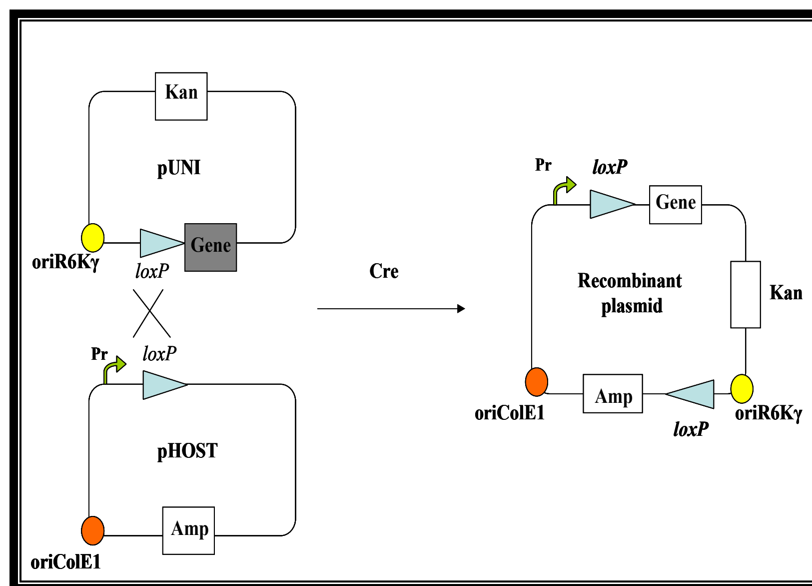

Figure 4.9: Figure for Cre-mediated recombination between loxP containing bacterial plasmids

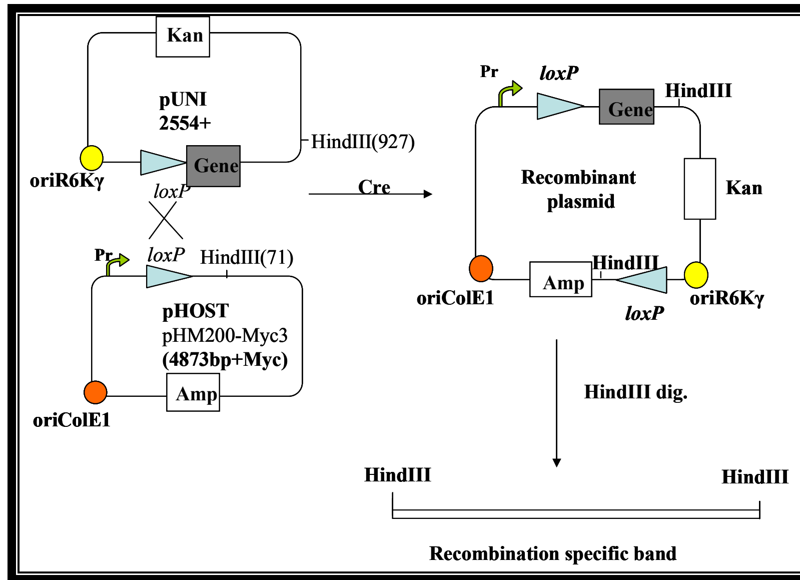

Figure 4.10: HindIII diagnostic digest in the confirmation of recombination

HindIII digestion was used for confirmation of recombination because it was the most suitable and common restriction enzyme that generated a recombination specific band which was about 2500 bp.

Hind III diagnostic digestion of recombinant vectors:

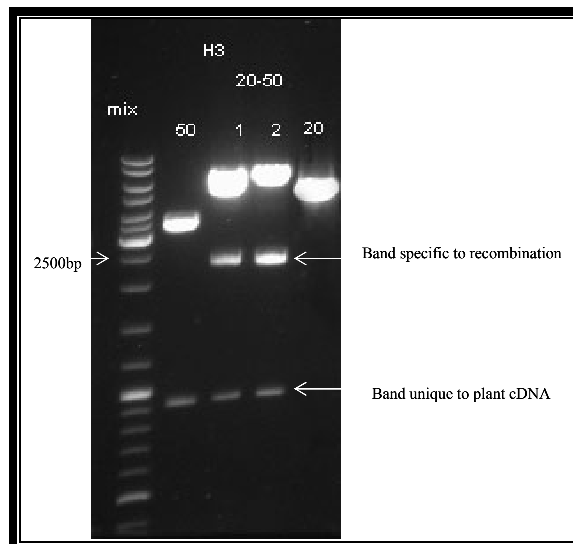

Figure 4.11: HindIII diagnostic digest of 20-50

Demonstration of cre-mediated recombination between loxp containing 20 and 50.

Agarose gel electrophoresis of one of the recombinant plasmids is seen in the figure 4.11. We digested the empty expression vector (20), the carrier vector for plant cDNA(50) and the recombinant plasmids with HindIII. The bands generated upon HindIII digestion at around 2500 bp were the fragments that are specific to recombination. This fragment wasn't found in either pUNI51 containing plant cDNA(50) or the mammalian expression vector(20).

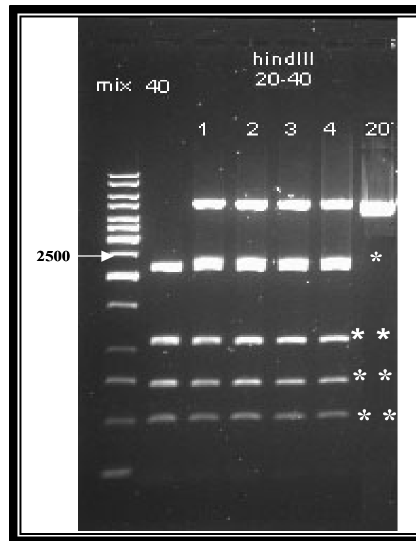

Figure 4.12: HindIII diagnostic digest of 20-40

The agarose gel of other HindIII digested 20-40 recombinant vector is shown in figure 4.12. The bands shown with one asterisk are the bands specific to recombination and the bands shown with two asterisks are the bands unique to the plant cDNA.

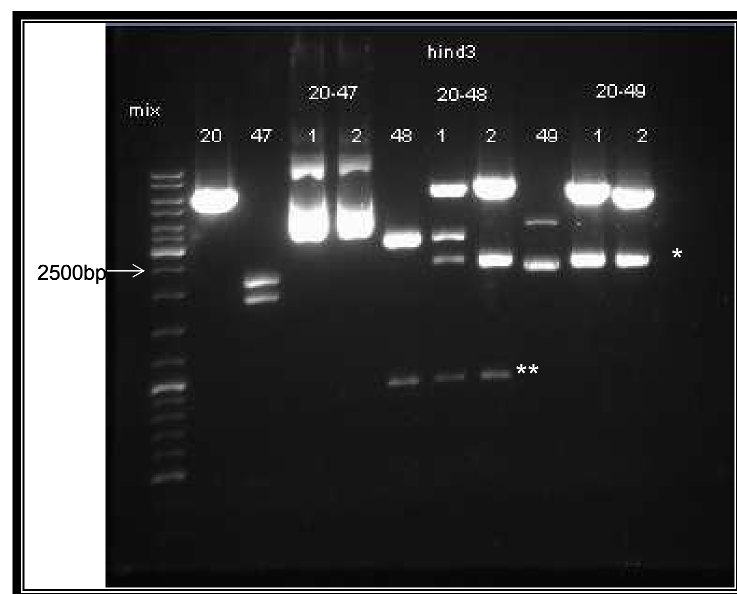

Figure 4.13: HindIII diagnostic digest of recombinant vectors.

The band shown in figure 4.13 with two asterisks is unique to plant cDNA and the band shown with one asterisk is the recombination specific band.

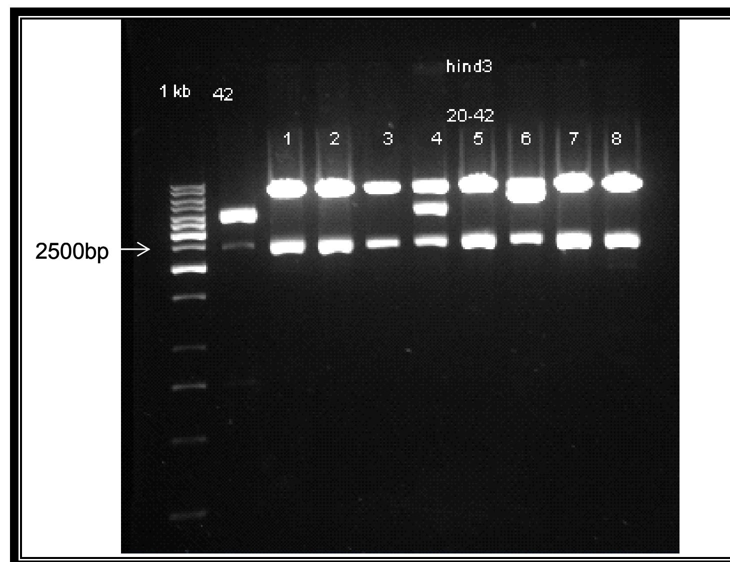

Figure 4.14: HindIII diagnostic digest of 20-42.

The bands around 2500 bp are recombinant specific bands. The ones with two asterisks in figure 4.14 are the bands unique to the plant cDNA. The recombination generates a 2500-bp fragment that is not found in the expression vector (pHM200-Myc3) or pUNI51 containing the plant cDNA.

4.3 TRANSFECTION AND DETECTION OF PROTEIN EXPRESSION BY WESTERN BLOTTING

After recombination of the vectors, we next wanted to detect the expression of the *Arabidopsis thaliana* proteins in mammalian cells. In order to do this plant cDNAs in mammalian expression vectors were transfected into mammalian HEK293T cells. This cell line was used for its ease of handling and efficiency of transfection of this cell line by calcium-phosphate mediated transfection. We did the transfections in 6-well plates with 2 µg of recombinant vector and protein extraction was done according to the procedure in 3.2.8. The protein expression was detected by using the Myc tag at the N-terminal of the protein. Cre-*loxP* recombination was used to insert the plant cDNA downstream of the CMV

promoter. Recombinant Cre enzyme was purified by affinity purification. The recombinant Cre had a GST tag that was used in purification. For western blotting anti-(c-myc) antibodies that recognize at the N-terminal Myc tag of the expressed plant protein and that are HRP-conjugated were used.

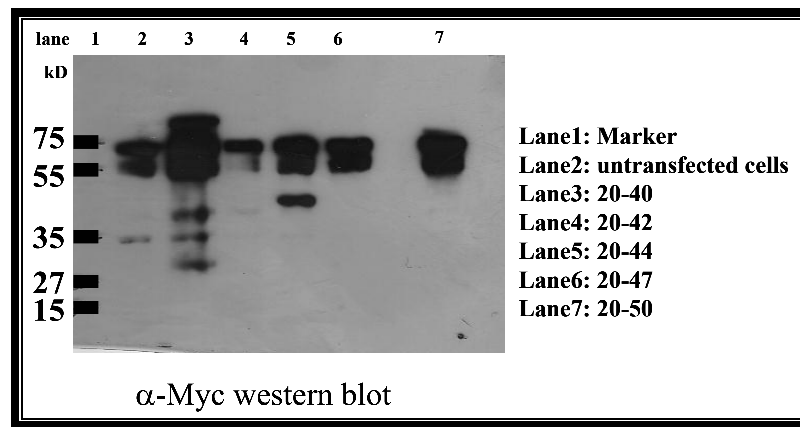

Figure 4.15: Western Blot of the proteins extracted from 293T cells transfected with recombinant plant cDNAs.

Protein extracted from the 293T cells that were transfected with the shown recombinant plant cDNAs in figure 4.15 were run on denaturing gel and after transferring to the PVDF membrane they were blotted by Anti-(c-myc) antibodies. Lane 1 is the marker, lane 2 is the cytoplasmic protein extract of untransfected cells, lane 3 is from 20-40, lane 4 from 20-42, lane 5 from 20-44, lane 6 from 20-47, and lane 7 is the protein extract of the 293T cells transfected with 20-50.

It can be deduced from the figure 4.15 that plant proteins were expressed in mammalian cells at different levels. After showing that the plant proteins can be expressed the flow cytometric analysis was done with transfected cells to see the difference in GFP levels between transfected and untransfected cells.

4.4 FLOW CYTOMETRY RESULTS

Flow cytometry is a technique to count, sort and examine microscopic particles suspended in a stream of fluid. Over the past 30 years, fluorescence activated cell sorting (FACS) machines have evolved to become powerful tools for analyzing and isolating single cells at very high rates.[75] It does not only quantify the fluorescent signal but also separates the cells according to preselected characters such as fluorescence intensity, size and viability from a mixed population. This system is sensitive enough to use for reporter assays even under low transfection efficiencies.

A flow cytometer consists of different systems working together to generate the final data product. There is a fluidics system that provides a suspension of single cells and passes the cells in single file through the flow cell where they produce the optical signals. There is an optical system with a light source, a laser usually, focusing lenses, colorselective (dichroic) mirrors, and filters. A signal detection and evaluation module with photomultiplier tubes or photodiodes is required next to detect the optical signal followed by analog and/or digital electronics to process and evaluate the signals. Finally, there is a computer with software to receive, store, further process, and display the resulting data.[76]

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is comprised of 238 amino acids (26,9 kDa). The GFP fluoresces green when exposed to blue light.[77,78] The fluorescence of GFP is dependent on the key sequence Ser-Tyr-Gly that undergoes spontaneous oxidation to form a cyclized chromophore .[79,80] Due to its widespread use, many different mutants of GFP have been engineered one of which had a single point mutation at Ser^{65} (S65T).[81] This mutation dramatically improved the spectral characteristics of GFP, resulting in increased fluorescence, photostablility and a shift of the major excitation peak to 488nm with the peak emission at 509 nm. This matched the spectral characteristics of commonly used FITC filter sets, increasing the practicality of use by the researchers. In our case we preferred flow cytometric analysis instead of microscopic imaging as it is quantitatively more reliable.

4.4.1 TNF-α TITRATION

Before beginning transfection of plant cDNAs for flow cytometric analysis we had to optimize the flow cytometry conditions. For this purpose we first did a TNF-α titration to find out the optimal TNF-α concentration. For this purpose the HEK 293T cells were transfected with reporter vector and TNF-α was added at different concentrations 24 hours after transfection and flow cytometry was done 24 hours after TNF-α treatment.

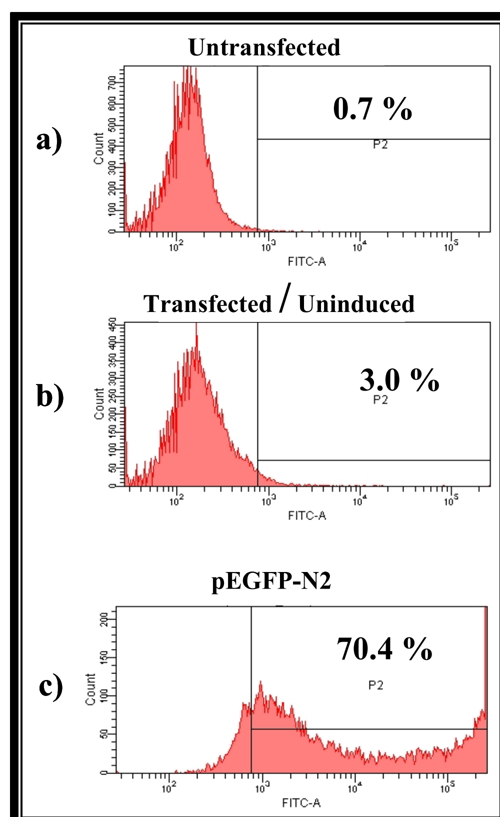

Figure 4.16: Detection of green fluorescence in 293 T cells

$5x10^5$ cells were transfected by using calcium-phosphate-mediated transfection. Transfection was done in 6-well plate. In figure 4.16; **a)** Untransfected cells are shown. **b)** These cells were transfected with 2 µg of κB-GFP vector and were not stimulated. **c)** pEGFP-N2 was transfected to the cells as a control of transfection efficiency. According to the figure P2 showing the percentage of GFP expressing cells is 0.7 % in untransfected cells **(a)**, 3.0% in κB-GFPtransfected but uninduced cells**(b)** and P2 is 70.4 % in pEGFP-N2 transfected cells showing the transfection efficiency**(c)**. These percentages were determined by the percentage of the cells falling into the P2 gate.

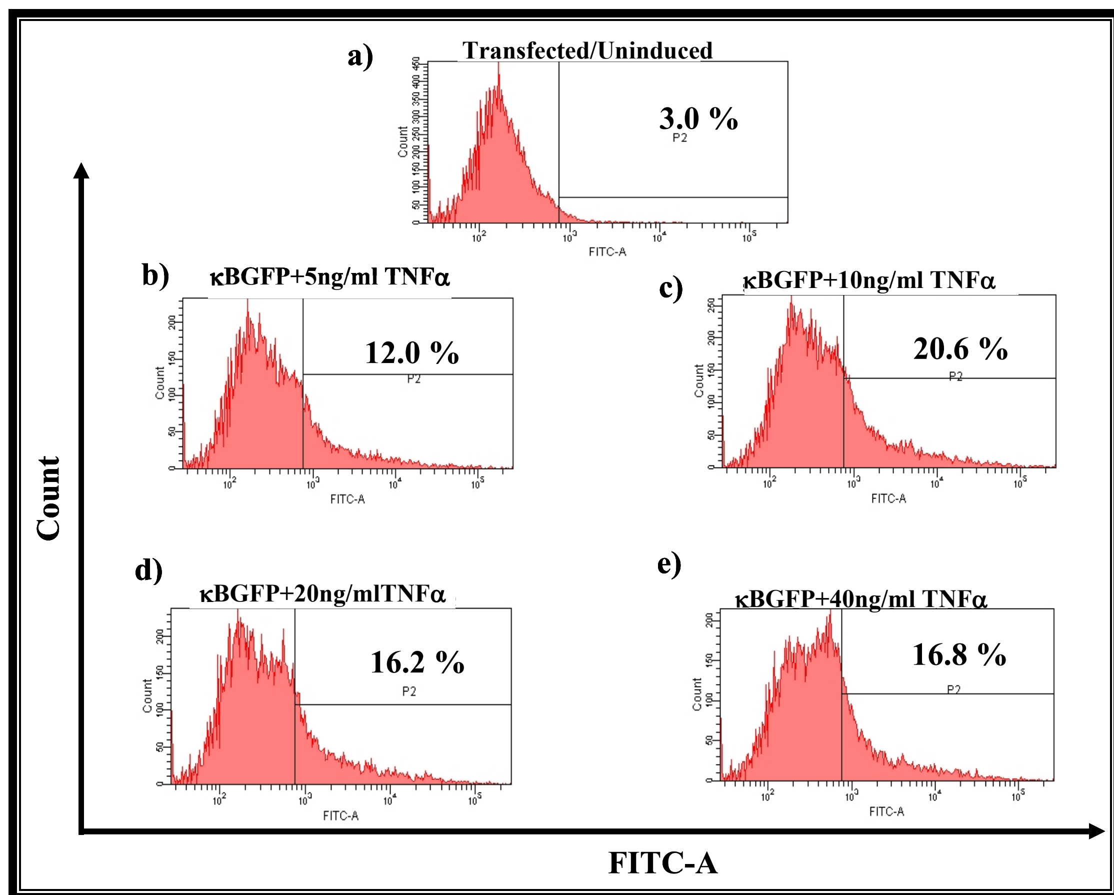

Figure 4.17: tGFP induction change in different TNF-α concentrations.

Figure 4.17 shows the flow cytometric results of TNF-α titration upon reporter vector transfection. 24 hours after transfection cells were treated with **b)** 5, **c)** 10, **d)** 20, and **e)** 40 ng/ml of TNF-α.and flow cytometry was done 24 hours after treatment.

In figure 4.17 the results of TNF-α titration is shown. The percentage of GFP expressing cells, P2 population, are shown on each histogramme. In **(a)** 3.0 % of the cells that were transfected with the κB-GFP reporter vector but not induced are shown. In **(b)** transfected cells were treated with 5 ng/ml TNF-α and the P2 population inscreased to 12 %, in **c** 10 ng/ml TNF-α was added and the P2 population was 20.6%, which was the highest among the others, in **(d)** the percentage of the cells falling into the P2 gate was 16.2 % in the 20 ng/ml TNF-α treated cells and in **(e)** the percentage of the cells that were transfected with the κB-GFP reporter vector and treated with 40 ng/ml TNF-α was 16.8%. These percentages were determined by the percentage of the cells falling into P2 gate. The graphical explanation of the results are shown in figure 3.18.

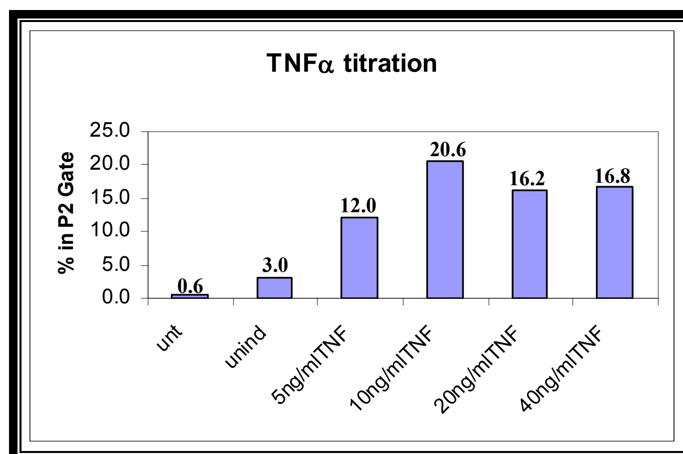

Figure 4.18: Graph showing dose-dependent change in P2 population.

4.4.2 TNF-α TIME COURSE

For detection of at which point the flow cytometric analysis should be done we did a time course experiment. The transfection was done with 2 μg of reporter vector and TNF-α was added 24 hours after transfection. Flow cytometry was performed at different time points as shown figure 4.19.

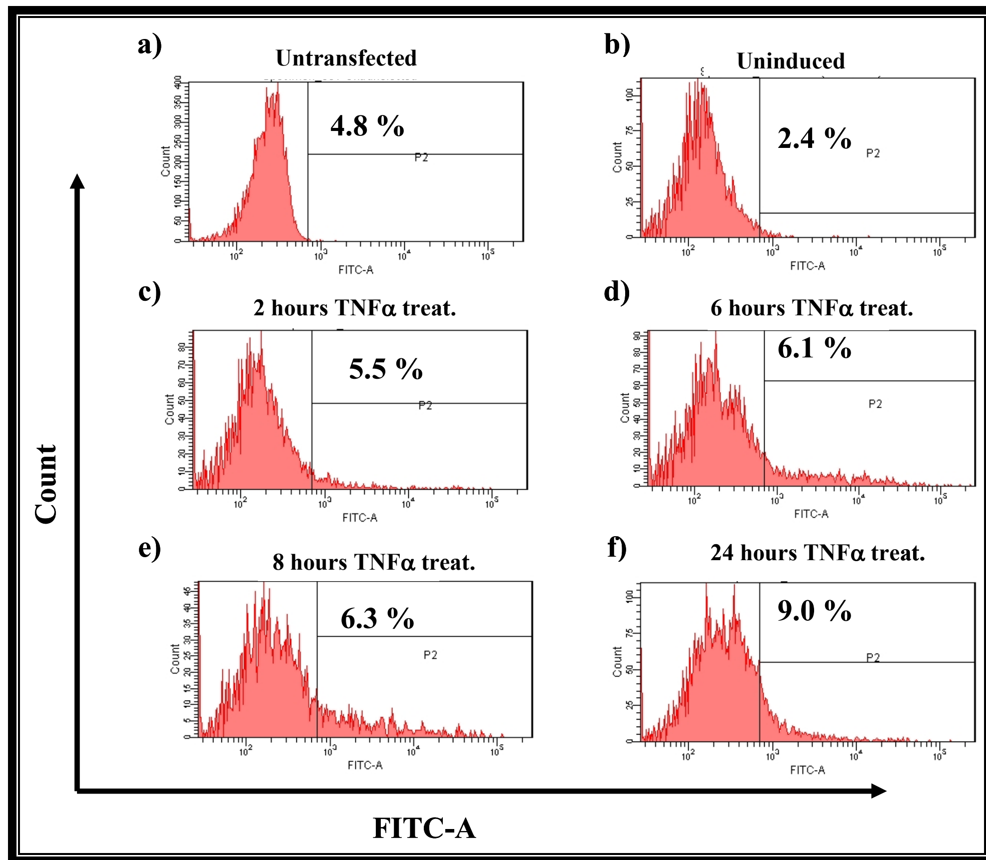

Figure 4.19: Change in tGFP expression at different time points.

The 293 T cells were either untransfected **(a)** or transfected with 2µg of KB-GFP reporter vectors **(b, c, d, e, f)** they were either left unstimulated **(b)** or stimulated by using 10ng/ml TNF-α **(c, d, e, f)**. Flow cytometric analysis was done at 2nd **(c)**, 4th**(d)**, 8th **(e)** and 24th **(f)**hours. Percent of P2 population is given on each histogram indicating that the best induction was at the 24th hour with an increase in P2 population from 2.4% in transfected but uniduced cells to 9.0%.

4.4.3 FLOW CYTOMETRIC ANALYSIS OF THE ACTIVITY OF CLONES 20-40, 20-42 AND 20-50

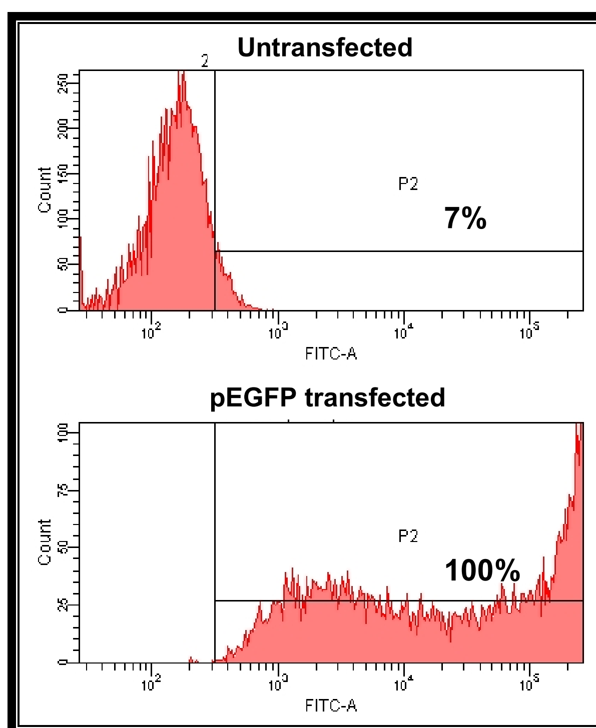

Figure 4.20: Transfection efficiency

Figure 4.20 shows the transfection effieciency for 20-40, 20-42 transfection. pEGFP-N2 was used as effieciency control plasmid. According to the figure almost 100% of the cells fall into the P2 gate in pEGFP-N2-transfected cells while it is the percentage of the cellls in P2 is 7 % in untransfected cells. This result shows that the transfection effieciency is almost 100% for this transfection.

Plant cDNAs in mammalian expression vectors were cotransfected into HEK 293T cells with reporter vector by calcium phospate-mediated transfection. Transfections were done in 6-well plates. We transfected 2 μg of reporter vector and 2 μg of recombinant plant protein containing mammalian expression vectors in each transfection. As a mock vector empty mammalian expression vector was used. The transfected cells were either stimulated 24 hours after transfection or left unstimulated. pEGFP-N2 was transfected to determine the transfection efficiency approximately. Fifteen plant cDNAs (20-40, 20-42, 20-44, 20-46, 20-47, 20-48, 20-50, 20-51, 20-60, 20-61, 20-67, 20-68, 20-82, 20-83, 20-87)were transfected

totally and these transfected cells were flow cytometrically analysed (data not shown). Out of these 15 cDNAs we found out three of them affecting GFP intensity in transfected cells. These are 20-40, 20-42, and 20-50.

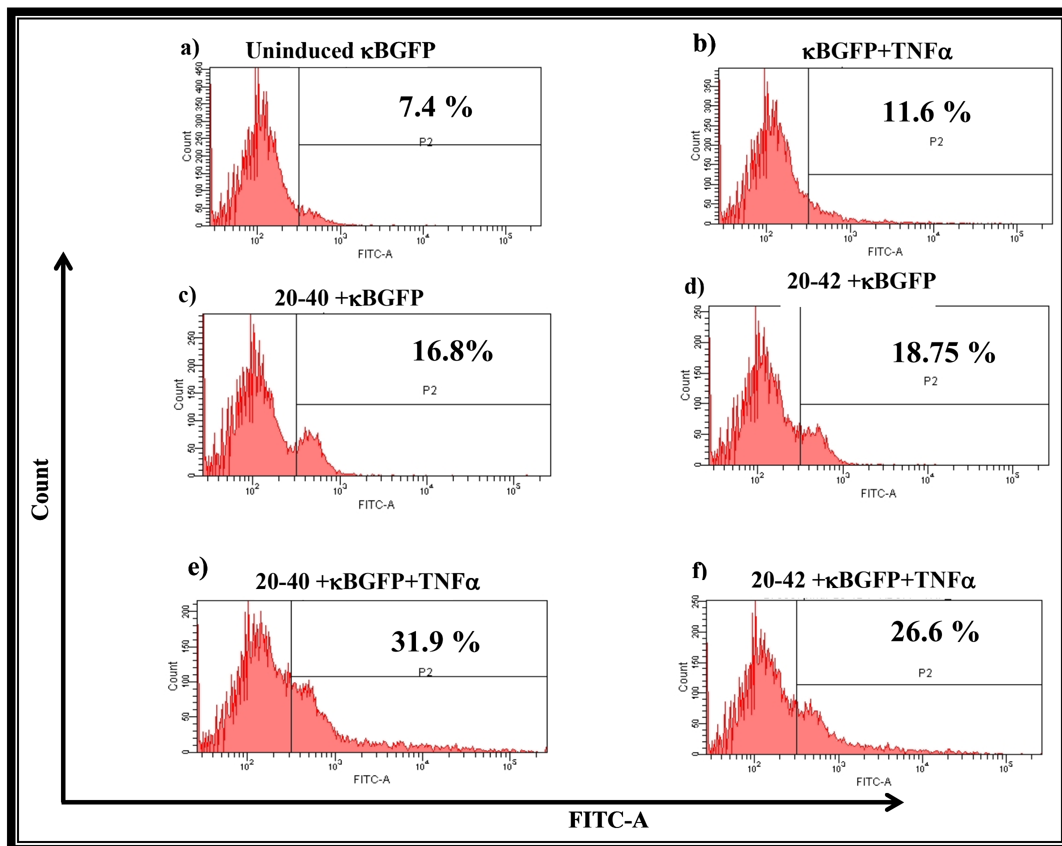

Figure 4.21: GFP change in 20-40 and 20-42 transfected cells.

The results flow cytometry of the transfections done with 20-40 and 20-42 are shown in figure 4.21. The 293T cells were cotransfected with 20-40 and κB-GFP **(c,e)** and 20-42+κB-GFP**(d, f)**. After transfection cells were stimulated with 10 ng/ml TNF-α after 24 hours. The κB-GFP vector alone was transfected as a control**(a).** The P2 population showing the percentage of GFP expressing cells was 7.4 % in uninduced population**(a)**, this went up to 11.6% when 10 ng/ml TNF-α was added **(b)**, when 20-40 and 20-42 recombinant vectors were cotransfected with κB-GFP reporter vector the P2 became 16.8 % and 18.75 % respectively **(c,d)** and when the cotransfected cells were stimulated with 10 ng/ml TNF-α this percenage was 31.9 % in 20-40 cotransfected cells and 26.6 % in 20-42 cotransfected cells**(e,f).**

The graphical results of this flow cytometric analysis are shown in figure 4.22. Due to the affects of these proteins to the GFP expression we made a conserved domain search in NCBI and found out that in 20-40 there are two ankyrin repeats in addition to a zinc-finger domain and in 20-42 there are four ankyrin repeats in addition to a RING-finger domain. The conserved domains of these two proteins are shown in figure 4.23.

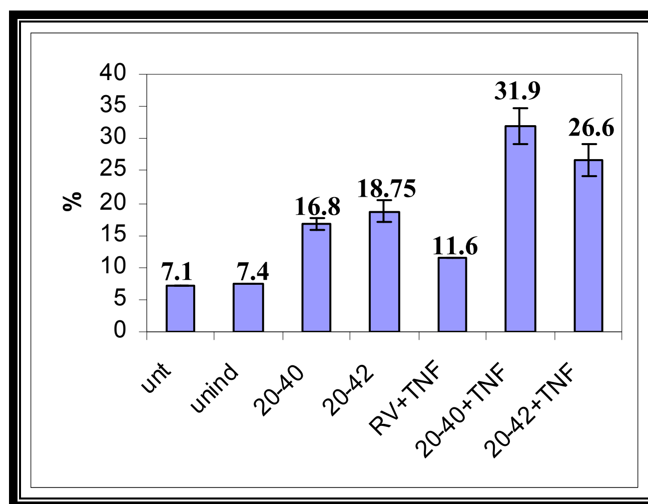

Figure 4.22: Column graph of plant recombinant protein transfection flow cytometry results

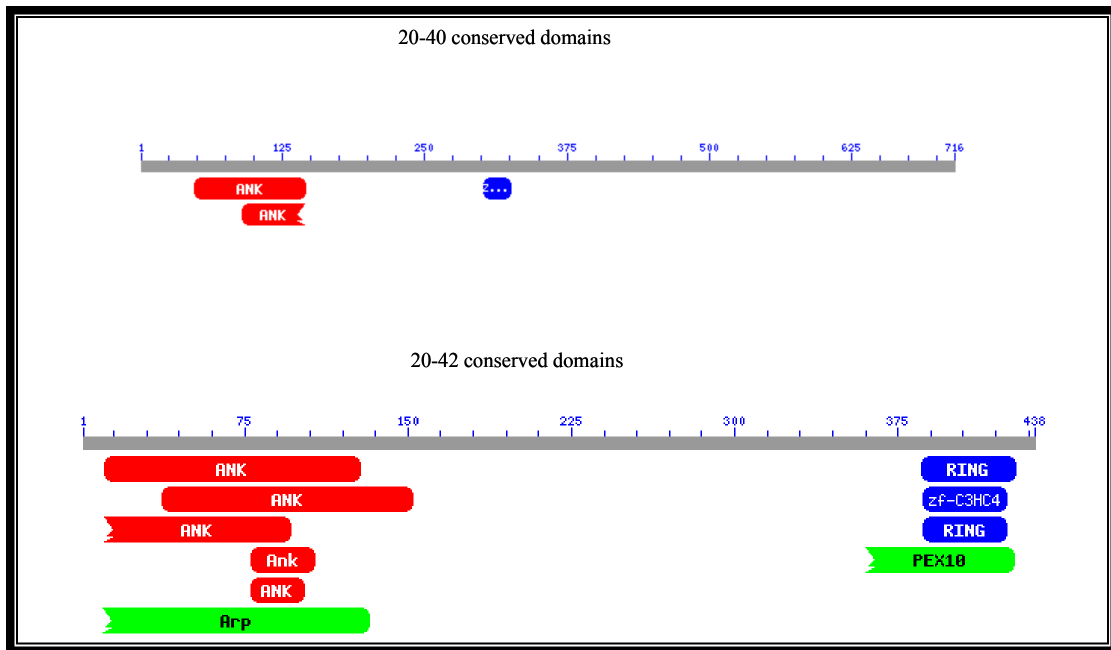

Figure 4.23: Conserved domains of 20-40 and 20-42.

Possible conserved domains were analysed in NCBI. Here I show the output of the conserved domain program that shows that the 716-amino acid protein encoded by the 20-40 construct contains an Ank domain in its N-terminus and a zinc finger domain(z) in its middle. The 438-amino acid protein encoded by 20-42 contains an Ank domain in its N-terminus and a ring finger domain in its C-terminus.

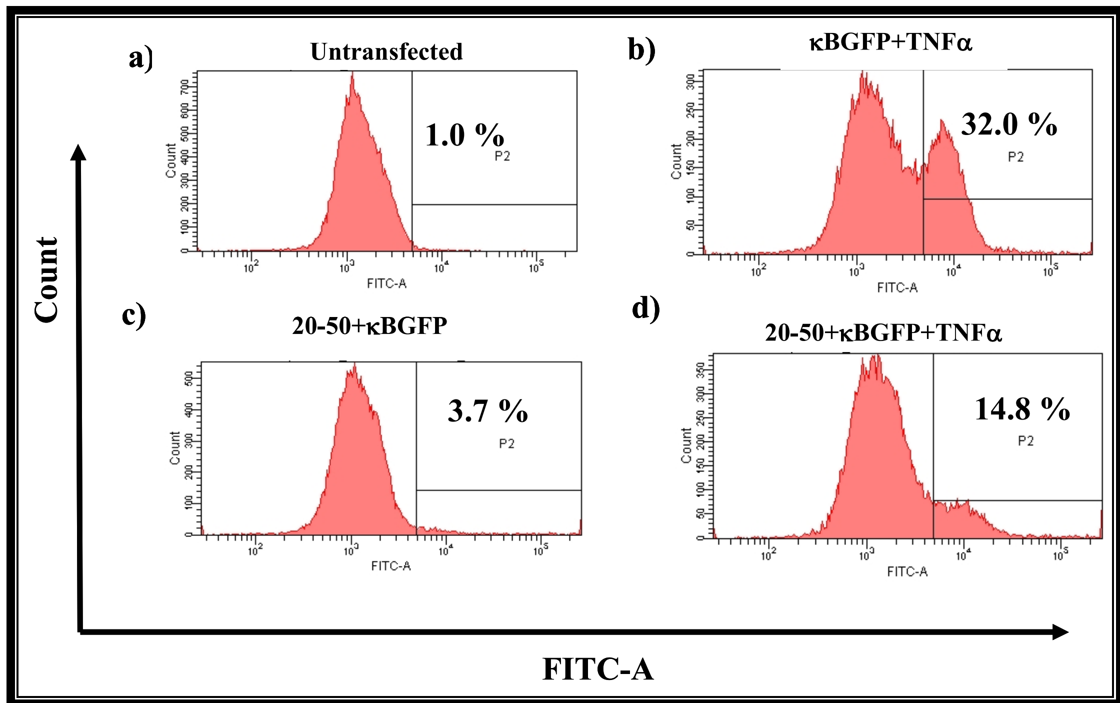

Figure 4.24: The effect of 20-50 on GFP intensity.

The cells in figure 4.24 were transfected with 2μg κB-GFP reporter vector alone **(b)** or cotransfected with 2 μg 20-50 recombinant vector **(c, d)**. The tranfected cells were either stimulated with 10 ng/ml TNF-α **(b, d)** or left unstimulated **(c)**. Flow cytometric analysis was done 24 hours after TNF-α stimulation. The percentage of GFP expressing cell population is seen in each histogram showing a decrease from 32% to 14.8 % in TNF-α induced NF-κB activation in case of 20-50 cotransfection with the κB-GFP reporter vector.

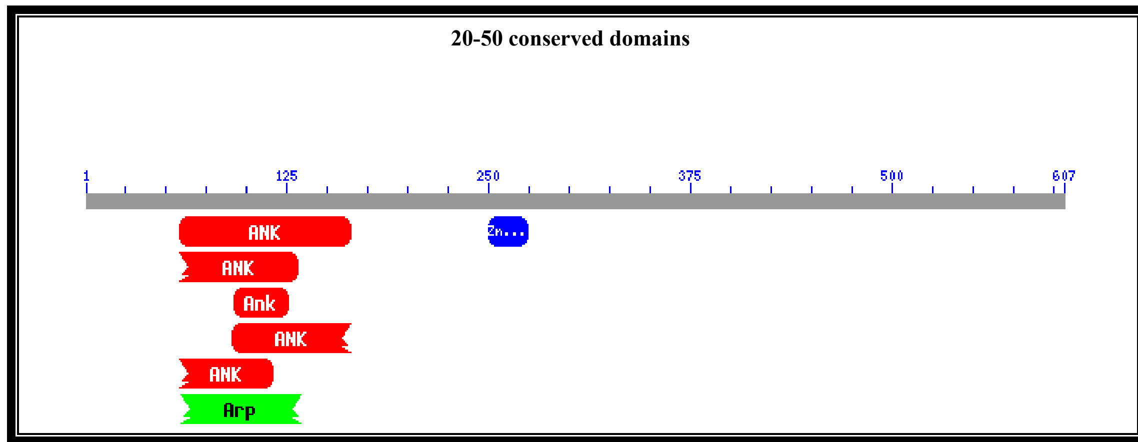

Figure 4.25: Conserved domains of 20-50.

Possible conserved domains were analysed with the NCBI conserved domain programme. Here I show the output of the conserved domain program that shows that the 607-amino acid protein encoded by the 20-50 construct contains an Ank domain in its N-terminus and a zinc finger domain in its middle.

5 DISCUSSION

NF-κB is a key factor which regulates genes responsible for both the innate and the adaptive immune responses. Both the acquired immune system's T- and B-cell receptors, and the innate immune system's Toll-like receptors activate NF-κB after signaling. The activation of NF-κB is critical for TCR and BCR signaling and therefore important for proper functioning of T and B lymphocytes of the immune system. For this reason, NF-κB has served as a therapeutical target.[72]

Plants and animals have an innate immune system that recognizes and responds to pathogenic microorganisms' attacks.[43] While the acquired immune system is unique to mammals, plants have receptors that are homologous to the Toll-like receptors of the mammalian innate immune system. Both plant an mammalian cells recognize pathogen associated molecular patterns (PAMPs) in molecules derived from pathogens using these innate receptors. Surprisingly, the arabidopsis genome was shown to contain genes that encode proteins with highly homology to mammalian IκB.[54,55,66] Moreover this protein called AKR2 was shown to have a role in the plant immune response and protein-protein interactions.[82]

The conservation of the ankyrin repeat domain throughout different species was the starting point that led us to search for an interaction between ankyrin-repeat containing plant proteins and mammalian proteins. In order to study functional interactions between the plant ankyrin domain proteins and components of the mammalian NF-κB signal transduction pathway, we used an NF-κB reporter in screeing experiments. An NF-κB reporter vector is a vector that contains NF-κB response elements upstream of the reporter gene. This reporter gene can be GFP, RFP, luciferase in mammalian cells.

Applications using GFP reporters have expanded due to the availability of variable mutants with altered spectral properties. pTurboGFP-PRL-dest1 was generated by the addition of PEST sequence, from the C terminus of mouse ornithine decarboxylase -MODC- , correlated with protein degradation, to the C terminus of tGFP. This sequence is a signal for

degradation so addition of it decreased half life of tGFP leading to a destabilized version of GFP and made it more suitable for genetic reporter assays.[83]

The aim of the project is to find plant proteins that modulate mammalian NF-κB signalling pathway. For this purpose a reporter assay was performed by using an NF-κB reporter vector. This reporter vector contains 8 NF-κB response elements residing upstream of a weak promoter, fos promoter, that controls the expression of tGFP gene. The control of inducibility of vector was done by cotransfecting the reporter vector with CARMA1, a protein that is activated during TCR-induced NF-κB activation. It is shown in the figure 4.8 that induction was working.

The next step in the project was to insert plant cDNAs into a mammalian expression vector. The mammalian expression vector used was pHM200-myc3, which had a CMV promoter upstream of the myc tag. The protein expression was detected by using this tag at the N-terminal of the protein. Cre-*loxP* recombination was used to insert the plant cDNA downstream of the CMV promoter. Recombinant Cre enzyme was purified by affinity purification. Confirmation of recombination was done by using HindIII enzyme. After confirming the recombination by diagnostic digestions, further confirmation was done to show that the recombinant plant protein was expressed in mammalian cells. For this purpose 293T cells were transfected with the plant proteins and a Western Blot analysis was done. As the recombinant proteins had an N-terminal myc tag anti-myc antibodies were used. These antibodies are conjugated to Horse Redish Peroxidase (HRP) so I didn't have to blot the membrane with a secondary antibody. One of the results is seen in figure 3.14. This result seemed to be confirming the expression of the plant proteins in mammalian cells. According to the figure the although plant cDNAs were expressed the expression level seemed to be changing among the *Arabidopsis* cDNAs, which can be related with the difference in the transcription, and translation mechanisms between the species.

The strategy of the project was to express a plant protein in human cells that contained a reporter vector by cotransfection. The transfected cells were either stimulated with TNF-α or left unstimulated. A titration of TNF-α indicated that 10ng/ml was the optimal concentration when the cells were analyzed by flow cytometry after 24 hours of stimulation. Based on the titration results, TNF-α at 10 ng/ml concentration was used to stimulate HEK293T cells and stimulated cells were flow cytometrically analysed 24 hours after stimulation. In these

transfection experiments pEGFP-N2 was used as control of transfection efficiency. 90 plant cDNAs were purchased from The Arabidopsis Information Resource (TAIR). These cDNAs encode proteins that contain ankyrin-repeats. They didn't have any common property other than these repeats. Among the ones that were recombined by Cre-mediated recombination, two of the plant proteins(20-40, 20-42) that are known to contain ankyrin repeats seemed to induce NF-κB activation both in unstimulated and stimulated cells and one (20-50) seemed to inhibit NF-κB activation in TNF-α stimulated cells. These two activator proteins can be interacting with IκB through ankyrin repeat domain or there may be some other ankyrin repeat containing proteins upstream that can be activated by the plant protein. To make a decisive conclusion further experiments should be performed including yeast-two hybrid or coimmunoprecipitation to see which mammalian protein interacts, if there is a real interaction, with this plant protein.

A conserved domain search was performed among these three proteins and interestingly one of the interacting plant protein (20-42) was found to contain a RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc; defined by the \'cross-brace\' motif C-X2-C-X(9-39)-C-X(1-3)- H-X(2-3)-(N/C/H)-X2-C-X(4-48)C-X2-C; probably involved in mediating protein-protein interactions; identified in proteins with a wide range of functions such as viral replication, signal transduction, and development; has two variants, the C3HC4-type and a C3H2C3-type (RING-H2 finger), which have different cysteine/histidine pattern; a subset of RINGs are associated with B-Boxes (C-X2-H-X7-C-X7-C-X2-C-H-X2-H). It was previously shown that a lily protein lily ankyrin-repeat containing protein LIANK has a RING-finger domain and ubiquitin ligase activity.[82] The Arabidopsis protein encoded by clone 20-42 also has four consecutive Ank repeats and a RING-finger domain. Both ankyrin repeat and RING-finger are common protein-protein interaction motifs. Since ankyrin repeat appears conserved in structure rather than in function[84] correlating function among the ankyrin repeat is difficult. The idea for using ankyrin repeat-containg proteins was related not with their function mainly but the repeats' role in protein-protein interaction. However, there are some common functions as ubiquitin ligase attributed to RING-finger domain.[85] Therefore the plant recombinant protein (20-42) having this RING-finger domain could have functioned as a ubiquitin ligase and it could have had a function in IκB ubiquitinylation. This can explain the increase in GFP expression seen in figures 4.21.

Although there are some insufficiencies in Flow cytometric analysis, like normalization, the results can serve as starting point and can give an idea about the activity of candidate plant proteins. To be more confident on the results normalization should be done or reporter vectors should be stably transfected to the mammalian cells to normalize the GFP intensity in each transfection. The role of plant protiens in human cells can be identified definitively by identifying the interacting mammalian partner.

6 CONCLUSION

The data presented in this study is the first step in determining plant factors that can interfere with the mammalian NF-κB signalling pathways. The *Arabidopsis thaliana* proteins containing ankyrin repeats were used in the study due to both the role of ankyrin repeats in protein-protein interactions, and the previous findings about the *Arabidopsis thaliana* ankyrin-repeat containing proteins' roles in plant innate immunity. Furthermore these repeats are conserved among different species and although the function of the protein changes the fact that this domain is important in interaction remains clear.

In order to screen plant factors an NF-κB reporter system, which reports NF-κB activation by GFP expression, was used. After screening the activity of plant ankyrin-repeat containing proteins, on the activation of human NF-κB in our reporter system we identified three plant proteins that affect NF-κB nuclear translocation, by the change in expression of our GFP reporter. Two of the plant proteins were found to activate NF-κB nuclear translocation while one was found to inhibit this activity. One of the two activating proteins has a RING-finger domain which is known to be conserved in proteins having ubiquitin ligase activity. As ubiquitinylation is required for the degradation of IκB, this plant protein may increase the nuclear translocation of NF-κB by promoting the degradation of its inhibitor. On the other hand, the plant protein that has inhibitory activity on NF-κB has four ankyrin repeats which may be interact with NF-κB as a dominant inhibitor of TNF-α induced NF-κB nuclear translocation.

The study provides the first step in the identification of a plant-mammalian protein-protein interactions that result in alteration of signal dependent activation of NF-κB. We identified three putative plant factors that may have such activity. Further studies will aim to identify the mechanism of how these plant proteins interact with mammalian signal transduciton pathways. Such studies will include interaction trap assays such as co-immunoprecipitation or yeast-two hybrid experiments.

7 FUTURE STUDIES

Our screen of NF-κB activation modifiers has identified at least three plant proteins that modify the activation of NF-κB. With which part of the NF-κB signal transduction pathway these plant proteins interact is not know. These plant proteins may bind to and inhibit a protein involved in the signal transduction anywhere from the plasma membrane to the nucleus. Inhibiting plasma membrane receptor phosphorylation, activating IκB degradation, inhibiting NF-κB nuclear translocation, inhibition of NF-κB sequence specific DNA binding are all likely possibilities.

A secondary screen can be done to find out the target of the interacting plant proteins by using yeast two hybrid system in which the plant protein is the bait and the mammalian proteins that are playing roles in TNF-α induced NF-κB activation are prey to comment on its therapeutical importance in mammalian systems. Alternative reporter assays with alternative reporter vectors in which other transcription factors' response elements, such as NF-AT, AP-1, or E2F, are used can be done to see the affect of this interacting plant proteins on these transcription factors. We can do an alternative screening in plant cells by using reporter vectors that are activated upon plant transcription factor binding to find an interacting mammalian protein.

Therapeutic implications of the identified inhibitor/activator can be worked out in detail. NF-κB plays a role in autoimmune disorders, for this reason inhibiting it may cause a regression of the autoimmune phenotype, and due to its role in cancer and cell survival, inhibiting NF-κB may retard cancer growth. With these aims, this study searched to find a modifier of this important signal transduction pathway and found at least three plant proteins that may be therapeutically significant.

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APPENDIX A: CHEMICALS

Chemicals used in the study:

6X Loading Dye	Fermentas, Lithuania
2X SDS Loading Buffer	Quality Biological, Inc., USA
Acetic Acid	Riedel-de Haen,Germany
Agarose	AppliChem,Germany
Ampicillin	Sigma,Germany
Ammonium persulfate	Sigma, Germany
Anti-[c-myc] peroxidase	Roche, Switzerland
Boric Acid	Riedel-de Haen,Germany
$CaCl_2$	Merck,Germany
Complete mini, protease inhibitor	Roche, Switzerland
DMEM	Sigma,Germany
DMSO	Sigma,Germany
EDTA	Riedel-de Haen,Germany
Ethanol	Riedel-de Haen,Germany
Ethidium Bromide	Merck,Germany
FBS	Biological Industries,Israel
Glutathione-sepharose beads	Amersham, Sweeden
Reduced glutathione	Merck KGaA, Germany
Glycerol	Riedel-de Haen,Germany
HEPES	AppliChem,Germany
HCl	Merck,Germany
Isopropanol	Riedel-de Haen,Germany
Isopropyl-β-D-thiogalactoside (IPTG)	Fermentas, Lithuania
Kanamycin	Sigma,Germany
L-glutamine	Merck,Germany
Liquid Nitrogen	Karbogaz,Turkey
Lumi-light Western Blotting Substrate	Roche, Switzerland
Luria agar	Sigma,Germany

Luria Broth	Sigma,Germany
$MgCl_2$	Sigma,Germany
Na_2HPO_4	AppliChem, Germany
NaH_2PO_4	Merck, Germany
Penicillin-Streptomycin	Biological Industries,Israel
PIPES	Sigma,Germany
KCl	Fluka,Switzerland
D-MEM	Invitrogen, USA
RPMI 1640	Biological Industries,Israel
NaCl	Riedel-de Haen,Germany
NP-40	AppliChem, Germany
NaOH	Merck,Germany
TNFalpha (Human recombinant)	Sigma, Germany
TNFalpha (Human recombinant)	Biosource, USA
Tris Base	Fluka,Switzerland
Trypan Blue	Sigma,Germany
Trypsin-EDTA	Biological Industries,Israel

APPENDIX B: EQUIPMENT

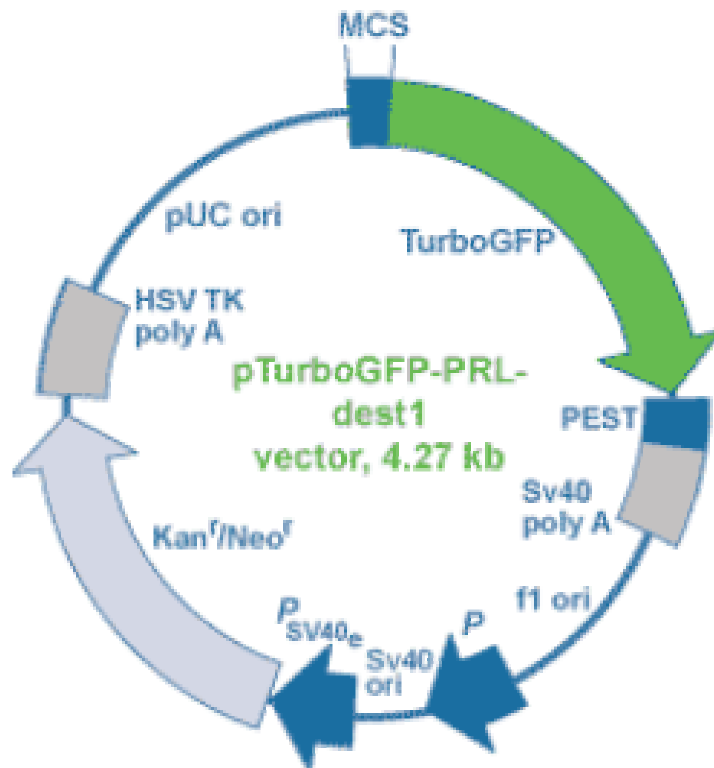
Equipment used in the study

Autoclave	Hirayama,Hiclave HV-110,Japan Certoclav, Table Top Autoclave CV-EL-12L, Austria
Balance	Sartorius, BP221S, Germany Schimadzu, Libror EB-3200 HU, Japan
Cell Counter	Cole Parmer, USA
Centrifuge	Eppendorf, 5415D, Germany Hitachi, Sorvall RC5C Plus, USA Heraeus, Multifuge 3 S-R, Germany Heraeus, Multifuge 3 L, Germany
CO_2 Incubator	Binder,Germany
Deepfreeze	-80^{0}C, Forma,Thermo ElectronCorp.,USA -20^{0}C,Bosch,Turkey
Distilled Water	Millipore, Elix-S, France
Electrophoresis Apparatus	Biogen Inc., USA Biorad Inc., USA
Elecroporation Cuvettes	Eppendorf, Germany
Electroporator	BTX-ECM630, Division of Genetronics, Inc, USA
Falcon tubes	TPP, Switzerland
Filter Membranes	Millipore,USA
Flow Cytometer	BDFACSCanto,USA
Gel Documentation	UVITEC,UVIdocGel Documentation System, UK Biorad, UV-Transilluminator 2000, USA
Heater	Thermomixer Comfort,Eppendorf,Germany
Hematocytometer	Hausser Scientific,Blue Bell Pa.,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

Liquid Nitrogen Tank	Taylor-Wharton,3000RS,USA
Magnetic Stirrer	VELP Scientifica, ARE Heating Magnetic Stirrer, Italy
Microliter Pipettes	Gilson, Pipetman, France Eppendorf, Germany
Microscope	Olympus CK40,Japan Olympus CH20,Japan Olympus IX70,Japan
Microwave Oven	Bosch,Turkey
NanoDrop	NanoDrop Technologies, Inc., USA
pH meter	WTW, pH540 GLP MultiCal, Germany
Power Supply	Biorad, PowerPac 300, USA
Immobilon-P transfer membranes	Millipore, USA
Refrigerator	Bosch,Turkey
Shaker Incubator	New Brunswick Sci., Innova 4330, USA
Spectrophotometer	Schimadzu, UV-1208, Japan Schimadzu, UV-3150, Japan
Thermocycler	Eppendorf, Mastercycler Gradient, Germany
Tissue culture flasks, plates	TPP, Switzerland
Vortex	Velp Scientifica,Italy

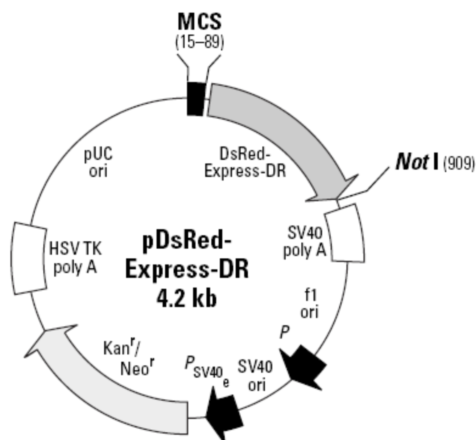
APPENDIX C: PLASMID MAPS

pTurboGFP-PRL-DEST



Features	Location (bases)
MCS	12-89
TurboGFP	90-930
MODC PEST	808-930
SV40 early poly A	1085-1119
F1 Origin of Replication	1182-1637
SV40 Origin of replication	1978-2113
Kan/Neo Resistance gene	2162-2956
HSV poly A	3166-3184
pUC plasmid replication origin	3541-4184

pDsRed-Express-DR Vector Information



11 21 31 41 51 61 71 81 91 DsRed-Express-DR

TA GCG CTA CCG GAC TCA GAT CTC GAG CTC AAG CTT CGA ATT CTG CAG TCG ACG GTA CCG CGG GCC CGG GAT CCA CCG GTC GCC ACC **ATG GCC**

*Eco*47 III *Bgl* II *Xho* I *Sac* I *Hind* III *Eco*R I *Sal* I *Kpn* I *Sac* II *Apa* I *Xma* I *Bam*H I *Age* I

Restriction Map and Multiple Cloning Site (MCS) of pDsRed-Express-DR. All sites shown are unique. The *Not* I site follows the DsRed-Express-DR stop codon.

Features	Location
MCS	12-89
DsRED	90-906
MOD PEST sequence	784-906
SV40 early poly A signals	1059-1064 &1088-1093
F1 Origin of Replication	1156-1611
SV40 Origin of replication	1952-2087
Kan/Neo Resistance gene	2136-2930
HSV poly A signals	3166-3171 & 3179-3184
pUC plasmid replication origin	3515-4158

pUNI 51 Cloning Vector

KpnI
EcoRI
SfiI-A
BamHI
SfiI-B
SalI
NotI
Fwd
Lox P
Kan
Rvs
pUNI 51
(2554 bp)
R6Kγ
MluI
HindIII
SpeI

KpnI T3 Promoter Lox P
GGTACC AATTAACCCTCACTAAAGGG ATA ACT TCG TAT AGC
157

EcoRI *SfiI-A*
ATA CAT TAT ACG AAG TTA TCT GGA ATT CGG CCG TCA AGG

BamHI *SfiI-B* *SalI*
CCA CAG GAT CCC TAG AGG GCC TCA TGG GCC GTC GAC TAG

LacO *NotI*
AAT TGT GAG CGC TCA CAA TTC TAG CGG CCG C

Cloning is done at *SfiI-A/SfiI-B*

DNA and Protein Molecular Weight Markers:

Mass Ruler DNA Ladder, Mix Range	Fermentas, Germany
Gene Ruler DNA Ladder, Mix Range	Fermentas, Germany
Mass Ruler DNA Ladder, Low range	Fermentas, Germany
PageRuler, prestained protein ladder, plus	Fermentas, Germany