MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF A WRKY TRANSCRIPTION FACTOR FROM CHICKPEA

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CERTIFICATE

The research work embodied in this thesis entitled "**Molecular and functional characterization of a WRKY transcription factor from chickpea**" has been carried out at the National Institute of Plant Genome Research, New Delhi. This work is original and has not been submitted so far in part or in full, for the award of any degree or diploma of any university.

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Dedicated to…..

My Family

&

Norman E. Borlaug

(Nobel Peace Prize, 1970)

"Science will always be a Search, never really a Discovery.
It is a Journey, never really an Arrival"

- Sir Karl Raimund Popper.

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ABBREVIATIONS

β-ME	β-mercaptoethanol
β-gal	β- Galactosidase
$CaCl_2$	Calcium chloride
°C	Degree centigrade
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
g	Gram
hr	Hour
Hcl	Hydrochloric acid
IPTG	Isopropyl-D-thio galactopyranoside
No.	Number
Kb	Kilo bases
kDa	Kilo Dalton
LB	Luria-Bertani
$MgCl_2$	Magnesium chloride
MES	2-(-N-Morpholino)-ethane sulfuric acid
min	Minute
mRNA	Messenger RNA
µM	Micro molar
µg	Micro gram
mM	Milli molar
MQ	MilliQ
MS	Murashige and Skoog
NaOH	Sodium hydroxide
OD	Optical density
PCR	Polymerase chain reaction
PEG	Polyethylene glycol

RNA	Ribonucleic acid
rpm	Rotations per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
Sec	Second
TE	Tris EDTA
Tris-HCl	Tris hydroxymethyl aminomethane hydrochloride
uidA	β- glucouronidase
X-gal	5-Bromo-4-chloro-3-indolyl-D-galactopyranoside
X-gluc	5-Bromo-4-chloro-3-indolyl-D-glucopyranoside
Y2H	Yeast two-hybrid
YPD	Yeast peptone dextrose
YPDA	adenine supplemented YPD

Contents

Chapter 1

Introduction

Plants being the primary producers, because of their capacity to convert inorganic molecules into organic energy through photosynthesis, are always engaged in complex dialogue with other organisms. Plant-Biotic interactions are mostly hostile but some organisms have developed neutral or symbiotic relationships to maintain energy flow towards them. From the start of agricultural practices plant diseases have concerned humans as it limits the food supply to them and with the growing population this concern as elevated. Diseased plants may be toxic for humans and animals, and they reduce food quality and production. Breeding of high-yielding varieties, use of synthetic fertilizers, and improved irrigation has contributed to double the world food production within the last 40 years to match the demands of an increasing human population. Owing to limitations in resources such as yield potential of crops and availability of arable land, water and climate change, sustainability of production at elevated levels is only possible with adequate disease control. Understanding the molecular & biochemical complexities of plant-biotic interactions and applying obtained knowledge for sustainable agriculture is the aim of research projects around the globe.

The resistance against most non-adapted pathogens (non-host resistance) and the ability to reduce the disease severity of an adapted pathogen (basal resistance) lets a plant to complete its life cycle. Preformed structural and biochemical barriers and induced defense both contribute to tolerance or resistance against pathogens. Like animals plants also have the capacity to recognize a potential pathogen and mount defense responses against it. Most of the pathogens are recognized by their conserved structural or chemical components and plants mount defense responses but pathogens have also evolved to suppress this basal defense strategy by their virulence effectors. These effectors are recognized in resistant plant through resistance (R) genes (Flor, 1971). Thus, throughout the co-evolution of plant and microbes this 'tug of war' is going on. Between recognition of a pathogen to the mounting of defenses, various complex-signaling cascades, plant hormones, transcriptional changes are involved. In order to better visualize and understand the complexities of plant-biotic interactions, scientists have classified the interactions and defense mechanisms of plants based upon presence and absence of hypersensitive response (HR) and major hormone pathways involved in defense against a particular pathogen. Pathogens are also classified based upon their life-style inside host plant like biotrophic, hemi-biotrophic and necrotrophic.

Chickpea (*Cicer arietinum* L.) is an important pulse crop, contributing enormously in supplying dietary protein and soil nitrogen fixation. It accounts for about 15% of the

world's total pulse production and India contributes to 64% of the world production of this legume crop (FAO, 2008). The yield potential of this crop is affected by various biotic and abiotic stresses. The necrotrophic foliar fungal disease Ascochyta blight (caused by *Ascochyta rabiei* (Pass.) Labrousse) and the soil-borne necrotrophic fungal disease Fusarium wilt (caused by *Fusarium oxysporum* f. sp. *ciceris*) are considered the most serious biotic stresses. Other diseases are more geographically localized like pod borer (*Helicoverpa armigera*) in Australia and India. In many areas of production, the chickpea crop is affected by the Ascochyta blight (AB) and in case of severe infection 100% crop loss is reported (Singh and Reddy, 1993; Chang *et al.*, 2007). Use of resistant cultivars is considered the most viable option for long-term management of AB. However, only partial resistance is available among the cultivated chickpea germplasm and the improved cultivars are only moderately resistant to AB (Anbessa *et al.*, 2009). Despite many reports on Quantitative Trait Loci (QTLs) for resistance to AB, the use of marker-assisted selection in breeding for resistance to AB in chickpea has been limited. Therefore, the other option is to increase basal resistance against AB for durable tolerance. This could be done by identifying important defense regulators and use them in chickpea through biotechnological tools for improved resistance.

To identify the genes involved in chickpea defense against AB, three forward Suppression Subtractive Hybridization (SSH) cDNA libraries from early stages of chickpea infected tissues were generated and sequenced earlier in the laboratory. Many genes related to transcription factors were present in these libraries. Our interest focuses on regulatory mechanisms controlling the defense response of plants to invading pathogens. Reprogramming of cellular functions in response to external stimuli involves complex changes in gene expression. The activities of a multitude of genes are subject to up- or down regulation and follow defined temporal programmes. The perception of an external stimulus immediately leads to the activation of primary-response genes. Activation of these genes is mediated by pre-existing signaling components including transcription factors. Since, the temporal and spatial expression of genes is controlled by the transcription factors; they may be utilized for the generation of broad-based pathogen tolerant plants. In present study, we aim for the functional characterization of a WRKY transcription factor gene of chickpea. The WRKY family members bind to W-box (TGACC/T) motifs which are present in the promoters of several defense-related genes. This gene also showed induction at mRNA levels shortly after pathogen infection revealed by macroarray experiments.

The objectives selected for this study are:

1. To isolate the full-length gene of a WRKY family transcription factor and its expression analyses.
2. To elucidate its functional role in plant defense by overexpression in transgenic tobacco.
3. To isolate and analyse the 5'-upstream sequences of this gene in transgenic tobacco.
4. To isolate the interacting partners of this protein using yeast two-hybrid or related strategy.

Chapter 2

Review of Literature

Plants have co-evolved with epiphytic, symbiotic and pathogenic organisms and the developmental success of modern day plants depends upon their ability to detect and mount defense against potential pathogens (Chisholm *et al.*, 2006). Disease and susceptibility in plants are governed by the combined genotypes of host and pathogen and depends on a complex exchange of signals and responses occurring under given environmental conditions. Although many times plant immunity is compared with the mammalian in context to evolution of genes for pathogen associated molecular pattern (PAMP) recognition and signaling cascades, the lack of mobile defender cells in plants and presence of a somatic adaptive immune system in mammals presents two major differences. To meet the ever-changing pathogen landscape, plants have relied on a lateral expansion of their innate immune system. This has led to a complex and layered defense system.

Despite constantly exposed to above- and below-ground attack by vast array of potential pathogens with different pathogenic lifestyles and infection strategies, most plants in nature look healthy. Plants are susceptible to a small number of adapted pathogens and within a plant species all plants do not show same level of susceptibility to this pool of pathogens. Though literature is biased towards the host resistance, which is a cultivar or accession specific resistance, the more common form of resistance that exists in nature is Non-Host Resistance (NHR) (Heath, 2000). Both resistance mechanisms have been reviewed separately in this chapter to explain the resistance mechanisms operating in nature. However, these mechanisms show overlap at various points, largely among signaling cascades and transcriptional changes associated with inducible defense responses. While non-host resistance relies equally on preformed and induced defenses mechanisms, host mediated defense mechanisms relies on induced mechanisms. The basal defense mechanisms and components of non-host resistance also work in host-mediated resistance.

2.1 NON-HOST RESISTANCE

A common observation in nature is that most of the plants are resistant to a large fraction of the pathogenic microbes and a pathogen isolated from one plant species in many cases cannot infect, reproduce, and cause disease on other distantly related species. The pathogen incapable of infecting the non-host plants are referred as heterologous pathogens and this basic incompatibility is provided through NHR mechanisms is also referred as heterologous plant-microbe interaction) (Gabriel and Rolfe, 1990; Thordal-

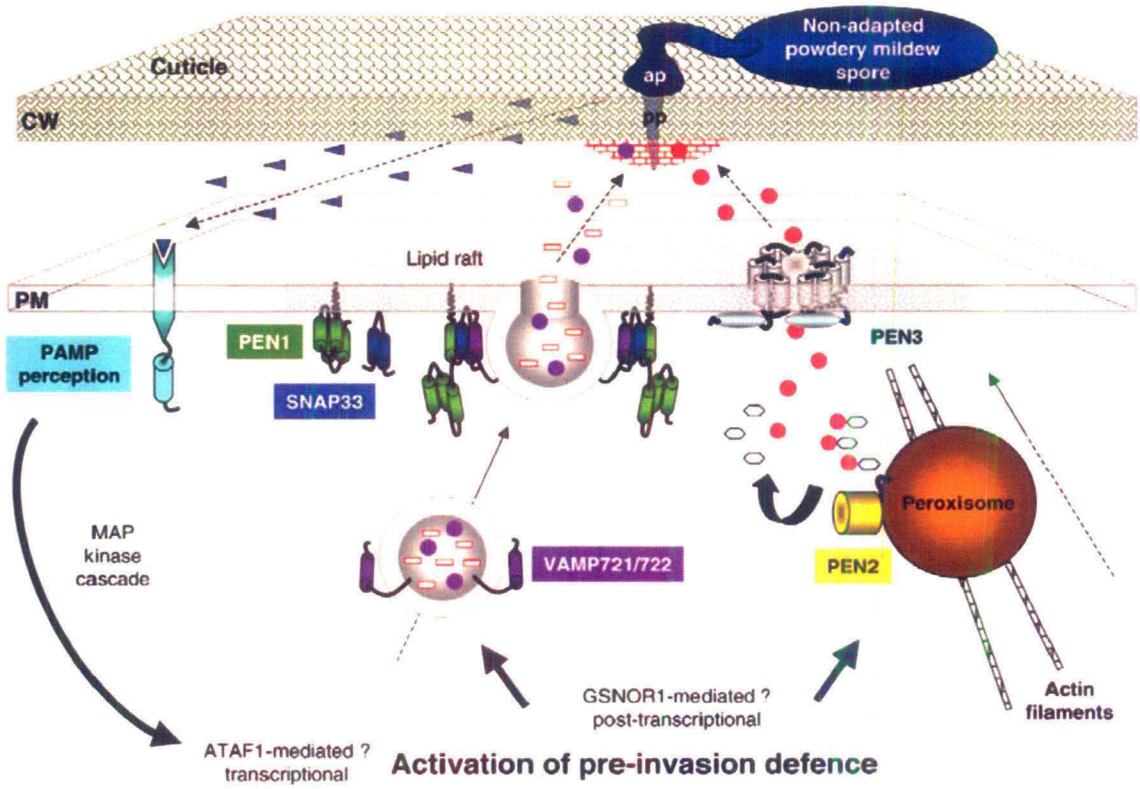
Christensen, 2003). This resistance seems to be highly effective and durable as there is infrequent change in host range of phytopathogenic microorganisms. It is often suggested that this mechanism can be used to generate resistant crop plants. However the understanding of non-host resistance mechanisms is poor as compared to host resistance mechanisms where crosses within the species can help in elucidating the genetics of race-specific host resistance. Some authors also use the term 'qualitative basal defense' for NHR.

With the advancement of modern biological tools, sequencing of plant genomes and selecting model system for NHR like *Arabidopsis*-Powdery mildew species, there is enough scope of dissecting this complex mechanism. There are numerous obstacles that a pathogen must overcome before causing the disease (Thordal-Christensen, 2003). It is believed that resistance relies on both the preformed barriers and inducible reactions (Heath, 2000; Thordal-Christensen, 2003; Mysore and Ryu, 2004; Nurnberger and Lipka, 2005; Lipka *et al.*, 2008). The effect of each component in countering pathogen growth, penetration and survival is governed by the ability to overcome that obstacle by the pathogen.

2.1.1 PREFORMED DEFENSES

Despite the recent focus on inducible defensive responses, there is a considerable evidence that preformed defenses are the major components of NHR. The first line of plant defense that limit the pathogen are wax layers and rigid cell wall. The plant cell wall is a highly integrated and structurally complex network of polysaccharides, including cellulose, hemicellulose and pectin (Hückelhoven, 2007). As pathogen comes in contact with the plant surface, some cues are required from plant to induce hyphal differentiation and express essential pathogenicity genes. This appears true for rust fungi that require hyphal differentiation before penetrating plant surface (Hoch *et al.*, 1987). While working with barley powdery mildew fungus (*Blumeria graminis* f. sp. *Hordei* [*Bgh*]) Tsuba *et al.* (2002) concluded that the appressorial differentiation is possibly controlled by wax composition of non-host as on tomato and tobacco, *Bgh* differentiates poorly as compared to cabbage.

Plants contain preformed peptides like defensins (Thomma *et al.*, 2002), proteins (Filippone *et al.*, 1999; Joshi *et al.*, 1999) and secondary metabolites with antimicrobial and deterrent properties, which act against both adapted and non-adapted pathogens. These compounds may be stored in biologically inactive form or in different



Schematic overview of pre-invasion resistance mechanisms.

Barley powdery mildew spores try to penetrate the cuticle and cell wall (CW) of host and non-host plant by means of appressorium (ap) and penetration peg formation (pp). On the non-host plant *Arabidopsis*, PRR-mediated recognition of fungal PAMPs (blue triangles) is likely to induce MAP-kinase signalling and ATAF1-mediated (?) transcriptional activation of the pre-invasion defence machinery. Post-translational control (e.g. via GSNOR1-mediated S-nitrosylation) represents another regulatory layer. The plasma membrane (PM)-localised syntaxin PEN1 and ABC-transporter PEN3 accumulate in a lipid raft-like microdomain. PEN1 forms a SNARE complex with the membrane-anchored adaptor SNARE SNAP33 and endo-membrane compartment- associated R-SNAREs VAMP721/722. SNARE complex formation drives secretion of cell wall precursors (red rectangles) and/or antimicrobial compounds (purple dots) at sites of attempted fungal invasion. PEN3 discharges potentially toxic aglycons (red dots) that were catalytically released from non-toxic glycosidic (black hexagons) precursors by PEN2 enzyme activity. PEN2 is associated with the periphery of peroxisomes. These are known to shuttle along a focallyreorganised actin cytoskeleton. Together, PEN1/SNAP33/ VAMP721/722- and PEN2/PEN3-mediated defence mechanisms contain the majority offungal invasion attempts. Figure adapted from Current Opinion in Plant Biology 2008, 11, 404–411.

compartments and are released upon pathogen attack or tissue damage. Two well-characterized classes of preformed inhibitors are the saponins (classified as either triterpenoids, steroids or steroidal glycoalkaloids) and the glucosinolates. *Gaeumannomyces graminis* var. *tritici*, a major root pathogen of wheat and barley is highly sensitive to avenacin A-1 (a biologically active triterpenoid saponin). The related root pathogen *Gaeumannomyces graminis* var. *avenae* produces the saponin detoxifying enzyme avenacinase and thus can infect oat roots. Saponin-deficient (*sad*) mutants of a diploid oat species *Avena strigosa* lack or have little amount of avenacin and are compromised against the non-host fungal pathogens *Gaeumannomyces graminis* var. *tritici* and *Fusarium culmorum*. This *sad* mutant does not show any alterations of leaf saponins and hence do not compromise NHR against the leaf-infecting fungus *Stragonospora nodurum* (Papadopoulou *et al.*, 1999). Because saponins are widespread throughout the plant kingdom, this group of secondary metabolites may have general significance as antimicrobial phytoprotectants. This example clearly confirms that antimicrobial compounds certainly play important role in immunity but many more compounds are yet to be discovered.

Plant actin microfilaments have been implicated in playing role in defense against fungal penetration. Using cytochalasins, specific inhibitor of actin polymerization, it was shown that several non-host plants allow penetration by various non-host fungi after chemical treatment (Kobayashi *et al.*, 1997). Loss of EDS1 (enhanced disease susceptibility) function and cytochalasin E treatment severely compromised NHR in *Arabidopsis* against *B. graminis* f.sp. *tritici* (Yun *et al.*, 2003).

2.1.2 INDUCIBLE DEFENSES

When the earlier mentioned passive defenses are breached by pathogens, plants rely on their innate immune system to prevent disease with active defenses. The ability of potential hosts to discriminate between self and non-self is a key to the activation of innate defense mechanisms in response to attempted microbial infection. Two levels of microbial recognition occur in plant immunity- the first level of microbe recognition is performed mostly by membrane proteins termed 'Pattern Recognition Receptors' (PRRs), which perceive molecular signatures associated with microbes, termed as 'Pathogen-Associated (or Microbe Associated) Molecular Patterns' (PAMPs or MAMPs) (Medzhitov and Janeway, 1997; Nicaise *et al.*, 2009). The second level of a specific type of microbial recognition occurs when resistance proteins (R proteins) recognizes

(directly or indirectly) the effectors secreted by the pathogens to suppress PAMP triggered immunity (PTI). Various PAMP receptor(s) mediated recognition events and their outcomes both in non-host and basal defense is discussed below:

I. PAMPs and Pattern Recognition Receptors (PRRs)

The PAMPs recognized by the plants correspond to molecular components highly conserved within a class of microbes, where they carry out an essential function of fitness or survival but do not necessarily play a role in pathogenicity. The well-known examples are fungal chitin and ergosterol, main structural components of higher fungi cell walls and membranes (Granado *et al.*, 1995); bacterial lipopolysaccharides (LPS), a glycolipid component of gram-negative bacterial outer membranes; and flagellin, the major structural component of the bacterial motility organ. Due to their indispensability for survival, PAMPs have been used as ideal targets by plant surveillance system. A given class of microbe is perceived through several PRRs. For example, *Arabidopsis* can recognize bacteria through perception systems for flagellin, EF-Tu, LPS, peptidoglycan (PGN), and probably other PAMPs. Mostly PRRs consist of an extracellular ligand-binding domain (often comprised of leucine-rich repeats), a single transmembrane domain and an intracellular serine/threonine kinase-signaling domain are referred to as receptor-like kinases (RLKs). Receptor-like proteins (RLPs) are similarly structured, but lack the cytoplasmic kinase domain. In *Arabidopsis*, 610 RLKs and 56 RLPs have been identified while in *Oryza sativa* they are at least 1132 and 90, respectively (Shiu *et al.*, 2004; Fritz-Laylin *et al.*, 2005). Many RLKs and RLPs are transcriptionally induced upon PAMP treatments. These genes play roles ranging from growth regulation to defense response, and the dramatic expansion of this family has been postulated to be crucial for plant-specific adaptations but most of these genes are not well characterized except a few like BRI1, CLV1, FLS2, etc (Lehti-Shiu *et al.*, 2009).

Perception of PAMPs by PRRs is common to all multi-cellular organisms and leads to an array of defense responses and redeployment of cellular energy. Recently, a number of reports have provided a deeper understanding on how perception of PAMPs contributes to basal resistance at both layers of pre-invasive and post-invasive immunity. Comparative profiling of gene expression revealed a large overlap of plant responses towards different PAMPs, indicating common downstream signaling components. Few PAMPs and their receptors are under extensive investigation in various labs around the world like flg22/FLS2, chitin/CEBiP, EF-Tu/EFR, etc.

A. Flagellin and FLS2

Plants have a highly sensitive chemoperception system for eubacterial flagellins. Flagellin and its N-terminus 15-22 peptide as an elicitor was serendipitously discovered while Felix and co-workers (1999) were trying to elucidate the elicitor activity of harpin preparations from *Pseudomonas,* which induces strong medium alkalization in tomato cell cultures. The detection of flagellin, the building block of the motility organ flagellum, seems to be evolutionary ancient as it is shared by members of all major groups of higher plants (Boller and Felix, 2009). The N-terminus 22 amino acid peptide flg22 induces callose deposition, PR1 induction, and strong inhibition of seedling growth. It was growth inhibition assay that led to the identification of FLS1 (FLAGELLIN-SENSING 1) in *Arabidopsis* Ws-0 background based on insensitivity of this accession to flg22 and later FLS2 (FLAGELLIN-SENSING 2) in La-er accession as they screened for flagellin insensitivity mutants in this background (Gomez-Gomez *et al.*, 1999; Gomez-Gomez and Boller, 2000).

FLS2 belongs to subfamily XII of LRR-RK and consists of extracellular domain of 28 LRR motifs. Functionally, FLS2 is similar to animal Toll-like receptor 5 (TLR5) that recognizes bacterial flagellin (Hayashi *et al.*, 2001). Its orthologs have been identified in *Nicotiana benthamiana*, tomato, and Rice (Hann and Rathjen, 2007; Robatzek *et al.*, 2007; Takai *et al.*, 2008). Monocots were believed to be insensitive to flg22 but later it was shown that rice cultured cells recognize flagellin and flg22. The isolation of *OsFLS2* and its over-expression, as well as heterologous complementation in *Arabidopsis fls2* mutant proved that rice does recognize flg22, but it induces weak defense responses (Takai *et al.*, 2008). Rice perceives certain flagellin monomers of *Pseudomonas avenae* in additional ways based on strain-dependent post-translational modifications (Che *et al.*, 2000). To know the binding sites and recognition specificities, structural studies of the FLS2/flg22 complex are needed. Earlier it was believed that PTI doesn't induce cell death but findings with full-length flagellin in tomato, tobacco and rice disprove this while according to recent finding in *Arabidopsis*, flagellin and flg22 peptides induce FLS2-dependent cell death (Naito *et al.*, 2008). In *Arabidopsis*, pretreatment with flg22 restricts the growth of the pathogenic bacterium *Pseudomonas syringae* pv *tomato* DC3000 (*Pto* DC3000), and lack of flagellin perception makes plant more susceptible to this bacterium (Zipfel *et al.*, 2004; Li *et al.*, 2005). Also in *N. benthamiana* down-regulation of *NbFLS2* leads to increased susceptibility to virulent and non-adapted pathogens (Hann and Rathjen, 2007).

Between plants PAMP signaling mechanisms, AtFLS2 mediated flagellin perception is best studied. AtFLS2 is less mobile in the presence of flg22, suggesting its ligand-dependent confinement to microdomains or transient interaction with other less mobile membrane proteins (Ali *et al.*, 2007). FLS2 and SERK3/BAK1 (SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3/ Brassinosteroid-Associated Kinase 1) interact rapidly (less than 2 min) in a ligand-dependent manner (Chinchilla *et al.*, 2007). FLS2 ligand binding may have conformational changes, which lead to trans-phosphorylation between FLS2 and BAK1, and signal is amplified as in brassinosteroid (BR) signaling BRI1-BAK1 complex. Although BAK1 is not required for flg22 binding, early and late flg22 responses are strongly impaired in *bak1* mutants (Heese *et al.*, 2007). In *Arabidopsis* and *N. benthamiana,* BAK1 is also required for responses triggered by the orphan PAMPs like CSP22, HrpZ, PGN, and LPS but not chitin. Being a common partner of various PAMP signaling, it is targeted by bacterial effectors (Heese *et al.*, 2007; Shan *et al.*, 2008). The fact that BAK1 is involved in BR and PTI responses, as well as in cell death control suggests that BAK1 is a general signaling adaptor for receptor kinases. Null *bak1* mutants are not completely insensitive to flg22, suggesting that the receptor complexes might involve additional proteins. Analysis of FLS2-GFP fate using confocal microscopy revealed that FLS2-GFP is rapidly internalized into intracellular vesicles after flg22 treatment. This finding parallels the fact that other plants RLKs are endocytosed. FLS2 endocytosis depends on receptor activation, its PEST motif present in the cytoplasmic domain, the proteasome, cytoskeleton functions and BAK1 (Robatzek *et al.*, 2006; Chinchilla *et al.*, 2007). Whether FLS2 internalization regulates its recycling, degradation, or signaling is still unclear.

B. Elongation Factor Tu (EF-Tu/elf18) and EFR

The elicitor activity of EF-Tu was discovered serendipitously as well, when suspension-cultured cells of *Arabidopsis* were treated with extracts from a flagellin mutant of *E. coli* FliC$^-$ to check for the elicitor activity in the absence flagellin (Kunze *et al.*, 2004). EF-Tu is highly conserved and is among most abundant proteins in all bacteria. *Arabidopsis* plants specifically recognize the N-terminus of the protein, and an N-acetylated peptide comprising the first 18 amino acids, termed elf18, is fully active as inducer of defense responses. Though EF-Tu is mostly intracellular, playing an important role in translation, but it is released during bacterial cell death and its subnanomolar concentration is sufficient to trigger signaling events. It is present in secretome of several bacteria and

serves as an adhesion factor at bacterial surface. Responsiveness to elf18/elf26 was observed in various Brassicaceae species but not in members of other plant families tested, indicating that perception of EF-Tu as a PAMP is an innovation in the Brassicaceae. Using covalent chemical affinity cross-linking technique and elf-^{125}I as ligand, it was shown that the receptor for EF-Tu is at cell surface (~3× 10^4 sites/cell). Various T-DNA mutants of FLS2 like RLKs were checked for their insensitivity for EF-Tu. Among them one of the mutant *efr-1* showed EF-Tu insensitivity but flg22 sensitivity (Zipfel *et al.*, 2006). Transient expression of EFR in *N. benthamiana* imparted elf18/elf26 sensitivity suggesting that the downstream signaling components are conserved in both *Arabidopsis* and *Nicotiana*.

The EFR is the PRR for EF-Tu with a 21-LRR extracellular domain and belongs to RLK family LRR-RK XII. It carries active cytoplasmic Ser/Thr kinase domain like FLS2. The ligand dependent signaling seems to be like FLS2, as *bak1* mutants also show reduced early elf18-triggered responses, although direct interaction between EFR and BAK1 is yet not shown. The importance of EFR for bacterial resistance is evident as *Arabidopsis efr* mutants are more susceptible to *Agrobacterium tumefaciens* and weakly virulent mutant strains of *Pto* DC3000 (Zipfel *et al.*, 2006). Pretreatment of elf26 induces resistance in *Arabidopsis* against *Pto* DC3000 (Kunze *et al.*, 2004).

C. AvrXa21 and Xa21

In rice, the LRR-RK Xa21 confers resistance to *Xanthomonas oryzae* pv. *oryzae* strains carrying the Avr gene *AvrXa21* (Song *et al.*, 1995). It is highly similar to EFR and possesses a non-RD kinase domain. Lee *et al.* (2006) claimed that AvrXa21 is a molecule playing role in bacterial quorum sensing and thus cannot be included in classical PAMP or effector definition but still authors have reviewed it under PAMPs. The analysis of *Xoo Rax* (*required for Avr21 activity*) genes revealed that AvrXa21 is a novel type-1 secreted sulfated peptide that is highly conserved in all *Xanthomonas* strains tested and its production is controlled by dual two-component regulatory systems (Lee *et al.*, 2008).

D. CeBiP & CERK1/ Chitin

Chitin, a β-1,4-linked linear polymer of *N*-acetyl-D-glucosamine, is a major constituent of the cell walls of the higher fungi. *N*-acetylchitooligosaccharides derived from chitin are potent PAMPs on several plant species. Chitin binding site on rice plasma membrane

was isolated by biochemical purification and the corresponding glycoprotein CEBiP (chitin oligosaccharide elicitor binding protein) was a LysM-containing RLP (Kaku *et al.*, 2006). LysM domains are important for chitin and peptidoglycan binding in animals. In *Arabidopsis* CERK1 (chitin elicitor receptor kinase 1), a LysM RLK1, was identified through reverse-genetics by two independent groups as being required for chitin responses. Direct chitin binding to CERK1 could not be assigned as binding experiments failed in *Arabidopsis*, therefore it is tempting to speculate that rice contains a homolog of CERK1 and vice-versa *Arabidopsis* may contain a homolog of CEBiP, and that these two proteins act in concert as a functional receptor (Miya *et al.*, 2007; Wan *et al.*, 2008).

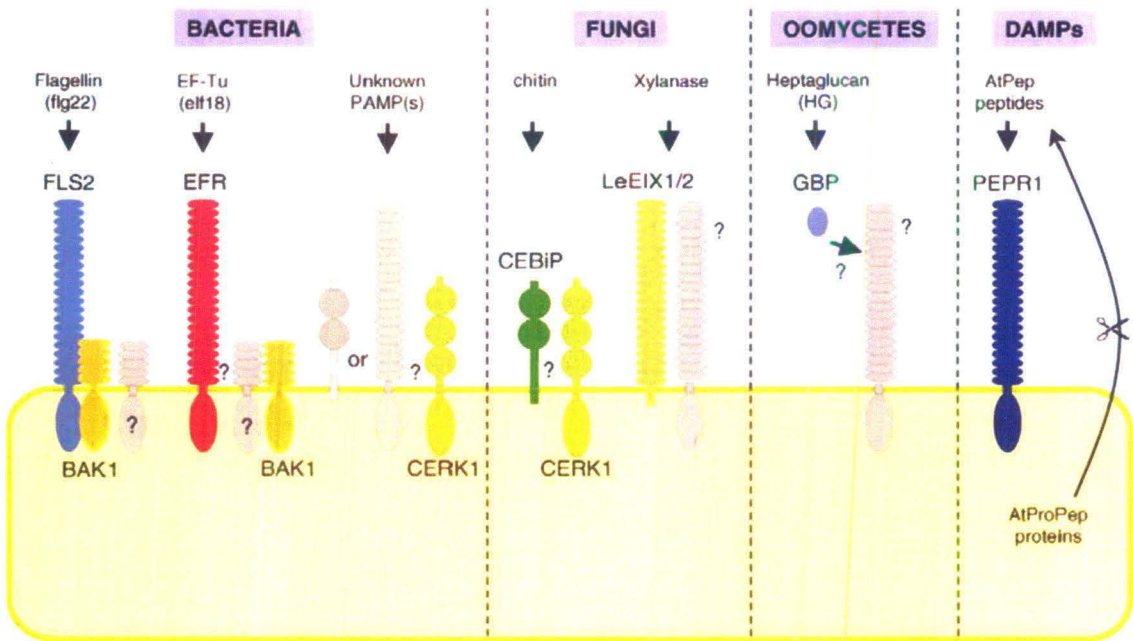
Silencing of *CEBiP* in rice reduces chitin binding and responsiveness. *CERK1* KO mutant lost the ability to respond to chitin as evident from chitooligosaccharide-responsive genes and was partly impaired in resistance against necrotrophic fungus *Alternaria brassicicola*, biotrophic fungus *Erysiphe cichoracearum,* and surprisingly to the adapted bacterium *Pto* DC3000 which suggested that CERK1 is not only restricted to chitin perception. It will be interesting to test if CEBiP and CERK1 interact in a ligand-dependent manner, as demonstrated for FLS2-BAK1, if it does interact then CERK1-CEBiP complex would be reminiscent of the TLR4-CD14 complex involved in LPS perception in mammals (Jerala, 2007).

E. Damage-Associated Molecular Patterns (DAMPs)

Both animal and plant cells can recognize DAMPs that are molecules released from host after damage by microbes and higher order predators (Lotze *et al.*, 2007). Polysaccharides released from the plant cell wall (e.g. oligogalacturonides, or OGs), and some endogenous peptide like AtPep1 are DAMPs. In *Arabidopsis*, the 23-aa peptide AtPep1 is derived from the C-terminus of a 92-aa precursor protein AtproPep1 and a LRR-RK PEPR1 recognizes this peptide. Treatment with AtPep1 peptides induces defense gene expression and over-expression of ProPep1 enhances resistance to the fungal root pathogen *Pythium irregulare* (Huffaker *et al.*, 2006; Yamaguchi *et al.*, 2006; Huffaker and Ryan, 2007).

F. Orphan PAMPs

The above-mentioned PAMPs and their receptors are studied extensively but there are many other PAMPs that are represented less in literature. Table 2.1 summarizes the available information on remaining PAMPs.



Plant PRRs and their signalling adapters

Bacterial flagellin (flg22) and EF-Tu (elf18) are recognised by the *Arabidopsis* LRR-RKs FLS2 and EFR, respectively. The *Arabidopsis* LysM-RK CERK1 mediates recognition of an unknown PAMP in anti-bacterial immunity and is also required for chitin responses. In tomato, xylanase is recognised by the RLPs LeEIX1 and LeEIX2. In legumes, the soluble glucan-binding protein (GBP) directly binds oomycetal heptaglucan. The *Arabidopsis* LRR-RK PEPR1 recognise the endogenous AtPep peptides that act as DAMPs.

(Figure adapted from, Curr. Opin. Plant Biol. Vol. 12, 2009)

Table 2.1 PAMPs and their relevant features

PAMP	Pathogen(s)	Minimal active motif	Biological features	Reference(s)
LPS	Gram-negative bacteria (Xanthomonads, Pseudomonads)	LipidA?	Oxidative burst, production of antimicrobial enzymes in pepper	Zeidler *et al.*, 2004; Newman *et al.*, 2007; Aslam *et al.*, 2008
Harpin (HrpZ)	Gram-negative bacteria (*Erwinia*, Pseudomonads)	Undefined	HR-like cell death	Wei *et al.*, 1992; He *et al.*, 1993; Lee *et al.*, 2001
Cold shock protein	Gram-negative and positive bacteria	RNP-1 motif	Oxidative burst, ET production in Solanaceae	Felix and Boller, 2003
Necrosis-inducing proteins	Bacteria (*Bacillus* spp.), Fungi (*Fusarium* spp.), Oomycetes (*Phytophthora* spp., *Pythium* spp.)	Undefined	HR-like cell death, induction of defense responses in many dicot plants	Bailey, 1995; Veit *et al.*, 2001; Fellbrich *et al.*, 2002; Qutob *et al.*, 2002; Mattinen *et al.*, 2004; Pemberton and Salmond, 2004;
Transglutaminase	Oomycetes (*Phytophthora* spp.)	Pep-13 motif (surface-exposed epitope)	Induction of defense responses in parsley and potato	Nürnberger *et al.*, 1994; Brunner *et al.*, 2002
Lipid-transfer proteins (elicitins)	Oomycetes (*Phytophthora* spp., *Pythium* spp.)	Undefined	HR-like cell death, SAR to microbial infection	Osman *et al.*, 2001
Xylanase	Fungi (*Trichoderma* spp.)	TKLGE pentapeptide	HR-like cell death, ET production in tomato, tobacco	Enkerli *et al.*, 1999; Ron and Avni, 2004
Invertase	Yeast	N-mannosylated peptide	Activation of phenylpropanoid pathway, ethylene production in tomato	Basse *et al.*, 1993
β-glucans	Fungi (*Pyricularia oryzae*), oomycetes (*Phytophthora* spp.), brown algae	Tetraglucosyl glucitol, branched hepta-β-glucoside, linear oligo-β-glucosides	Induction of defense responses in legumes, tobacco, rice	Klarzynski *et al.*, 2000; Yamaguchi *et al.*, 2000; Fliegmann *et al*, 2004
Sulphated fucans	Brown algae	Fucan oligosaccharide	Induction of defense responses in tobacco, systemic resistance to viral infection	Klarzynski *et al.*, 2003
Ergosterol	All fungi		Induction of ion fluxes in tomato	Granado *et al.*, 1995
Cerebrosides A, C	Fungi (*Magnaporthe* spp.)	Sphingoid base	Phytoalexin production in rice	Koga *et al.*, 1998
Arachidonic acid	Oomycetes		Elicitor in potato	Boller, 1995

II. PAMP triggered early signaling events

Typical early PAMP responses are ion fluxes across the plasma membrane, the generation of reactive oxygen species (ROS), NO, ethylene, and later deposition of callose and synthesis of antimicrobial compounds. PAMPs trigger calcium-dependent protein kinases (CDPKs), activation of mitogen activated protein kinase (MAPK) cascades, and leads to changes in the transcription of numerous genes. These transcriptional changes significantly overlap with the ETI induced transcriptome (Navarro *et al.*, 2004).

In mammals, the heterotrimeric G protein complexes (composed of three subunits, α, β, and γ) are associated to the plasma membrane and interact with specific receptors to initiate intracellular signaling cascades (Luttrell, 2006). In plants, heterotrimeric G proteins are involved in many diverse physiological processes. The gene *AGB1*, encoding the β-subunit in *Arabidopsis*, is highly induced after flg22 and elf18 treatment. Ishikawa (2009) studied *agb1* mutants and showed that they are impaired in oxidative burst and seedling growth inhibition by flg22 and elf18. The role of heterotrimeric proteins in plant resistance or susceptibility has been studied (Trusov *et al.*, 2009). Heterotrimeric G proteins regulate innate immunity through Rac1 (Suharsono *et al.*, 2002) and activated Rac1 interacts with RACK1a in rice. RACK1a complex consisting of Rac1, RAR1, SGT1, HSP90, and HSP70 and RACK1a functions as a scaffolding protein for the immune complex (Nakashima *et al.*, 2008). This complex probably regulates NADPH oxidase (RbohD) for ROS production.

The first easily detectable physiological response within 0.5-2 min to PAMPs in plant cell cultures is the alkalization of the growth medium (Nürnberger *et al.*, 2004; Garcia-Brugger *et al.*, 2006). Fluxes of H^+, K^+, Cl^-, and Ca^{2+} have been observed after PAMP treatment. Elevation of cytoplasmic calcium is a critical step in plant innate immunity and is mediated by an increase in Ca^{2+} influx (Ma and Berkowitz, 2007). Changes in $[Ca^{2+}]_{cyt}$ are perceived by calcium-binding proteins such as calmodulin, CDPKs, and calcineurin B-like proteins (Reddy and Reddy, 2004). Some of these have a demonstrated role in plant defense, particularly in the control of reactive oxygen species (ROS), and salicylic acid (SA) production (Du *et al.*, 2009; Wang *et al.*, 2009). MAPKs are involved in various processes in eukaryote cells, including plant defense. In *Arabidopsis*, a complete MAPK cascade including MEKK1-MEKK4/5-MPK3/6 was initially proposed by Asai *et al.* (2002) but later it was demonstrated that flg22 induces

MEKK1 that activates MPK4 but not MPK3/6 (Suarez-Rodriguez *et al.*, 2007; Gao *et al.*, 2008). Antimicrobial compounds like phytoalexins are *de novo* synthesized in response to pathogen attack. Several phytoalexin-deficient (*pad*) mutants have been identified in *Arabidopsis* (Glazebrook, 2005). Mutant *pad3-1* (PAD3 is required for camalexin biosynthesis) is compromised for non-host resistance against *Alternaria brassicicola*. Phytoalexin biosynthesis against pathogens is regulated by MPK3/6 cascade (Ren *et al.*, 2008). Virus- induced gene silencing of *NbSIPK* and *NbWIPK* in *N. benthamiana* resulted in multiplication of *Pseudomonas cichorii* for which this plant is non-host, suggesting importance of MAPKs in non-host resistance. Further, the role of MAPKs in plant immunity is discussed later in this chapter.

The accumulation of callose, a plant β-1,3-glucan polymer synthesized between the cell wall and the plasma membrane, is a classical marker of PTI responses. The callose synthase GSL5/PMR4 is responsible for callose synthesis in response to PAMPs. *pmr4* mutant plants allow 20-fold more growth of *Pto DC3000 hrcC*⁻ than wild-type plants (Kim *et al.*, 2005). Though sequential events in early signaling are not clear, callose deposition may be downstream of ROS production. Callose deposition was recently shown to depend on PAMP-induced glucosinolates (Clay *et al.*, 2009).

Stomata constitute one entry point for bacteria and PAMP treatments induce stomatal closure in a manner dependent on abscisic acid, SA, K^+ fluxes, and heterotrimeric G proteins (Melotto *et al.*, 2006; Zhang *et al.*, 2008). Bacterial phytotoxin coronatine (a mimic of JA-Ile) reverts the effects of PAMP mediated stomatal closure.

III. Broad spectrum disease resistance

Several non-host disease resistance genes have now been identified and they are required for non-host resistance against certain non-host pathogens. *Arabidopsis NHO1* encodes for a glycerol kinase and is required for resistance against *Botrytis cinerea* and *P. syringae* isolates from bean or tobacco (Kang *et al.*, 2003). The expression of *NHO1* is suppressed by *Pst DC3000*, suggesting its importance as a virulence target.

The best example of layered non-host resistance mechanism comes from *Arabidopsis* and its interaction with non-host fungal pathogen *Bgh* that causes powdery mildew in barley. The resistance to non-adapted powdery mildews is both pre-invasive and post-invasive (reviewed by Ellis, 2006). The adapted mildews like *Golovinomyces cichoracearum* (*Gc*) and *G. orontii* (*Go*) can penetrate more and establish themselves in

host tissue but non-host or non-adapted mildews has less percentage of entry at epidermal pavement cells. Entry failure correlates with timely and localized defense responses, dynamic cytoskeletal rearrangements, organelle transport, protein translocation, secretion processes and focal cell wall remodeling (formation of multi-layered, callose containing papilla) at sites of attempted fungal ingress. These pre-invasion defense mechanisms are backed up by post-invasion resistance mechanisms like callose deposition at the site of invasion and hypersensitive response (Fig...) (Lipka *et al.*, 2008). Based on these cytological evidences, chemically induced three *Arabidopsis PENETRATION1* (*PEN1*), *PEN2*, *PEN3* mutants with altered non-host interactions were isolated. These mutants showed enhanced invasion frequency by *Bgh* and *Erysiphe pisi* (*Ep*) but no increase in overall susceptibility owing to hypersensitive response after invasion. Systemic gene interaction analyses suggested that PEN1 and PEN2 act in two distinct entry control mechanisms and that PEN2 cooperates with PEN3 that is epistatic (Stein *et al.*, 2006). PEN1 (Collins *et al.*, 2003) encodes a membrane-associated syntaxin containing a SNARE (for soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) domain and is a member of a large family of proteins involved in membrane fusion and secretion events. GFP-PEN1 fusion is secreted and gets accumulated at papillae that are formed at the site of infection peg formation (Assaad *et al.*, 2004). Though the substrates and products of PEN2 (family1 glycosyl hydrolase) are presently unknown but *pen2* mutants show increased *Bgh* haustoria and additive effects are observed in *pen1pen2* double mutant. The effect of *pen2* is broader than that of *pen1* (which affects only *Bgh* infection) (Lipka *et al.*, 2005). PEN2-GFP functional fusion protein is localized in peroxisomes that move to and accumulate at *Bgh* penetration sites, consistent with the predicted role of this organelle in delivering an antifungal product. PEN3 encodes an ATP binding cassette (ABC) transporter protein (previously annotated as PDR8). The role of R genes, if any, requires further examination for better understanding of non-host resistance to *Bgh* in *Arabidopsis*.

Broad-spectrum resistance to adapted powdery mildews is conferred by loss-of-function mutant alleles of mildew resistance locus *O* (*MLO*). Powdery mildew fungi are believed to require a subset of functional MLO proteins as compatibility factors for successful invasion of host epidermis cells (Consonni *et al.*, 2006; Humphry *et al.*, 2006). This loss of function mutant shows many characteristics of NHR. Screening with the *Bgh* pathogen on host plant barley resulted in isolation of two mutants, *ror1* and *ror2* (required for MLO-specified resistance) that enhances penetration of *Bgh*. Interestingly,

ROR2 gene is a functional homolog of *PEN1* gene. These results provide a link between non-host and basal penetration resistance (Freialdenhoven *et al.*, 1996).

Though *Arabidopsis EDS1* (enhanced disease susceptibility) was initially isolated as an essential component of race-specific disease resistance, *eds1* mutant is partially more susceptible to several isolates of *Peronospora parasitica* and *Albugo candida* for which *Arabidopsis* is a non-host (Wiermer *et al.*, 2005). Post-haustorial NHR requires EDS1, PAD4, and senescence-associated gene 101 (SAG101).

NHR of *Arabidopsis* mesophyll cells to the non-adapted bean pathogen *Ps* pv. *phaseolicola* NPS3121 (*Pph*) shows features of layered basal defenses and is associated with vesicle transport, cell wall remodeling and accumulation of ROS but not HR (Soylu *et al.*, 2005; Ham *et al.*, 2007; Mishina and Zeier, 2007). This pathogen seems to elicit a minimum of three basal defense-signaling pathways in *Arabidopsis*. ARGONAUTE4 (AGO4/OCP11)-mediated DNA methylation is described as a novel NHR mechanism against *Ps* (Agorio and Vera, 2007).

IV. Types of non-host resistance

Hypersensitive response is commonly used as a visual marker for incompatible plant-pathogen interactions. In many instances, depending upon host and pathogen genotype, inoculation of a pathogen on one plant species into another non-host plant species elicits the HR associated with non-host resistance. Interestingly, in some instances, non-host disease resistance is not associated with induction of a HR. Based on these observations, Mysore and Ryu (2004) proposed that non-host resistance against bacteria, fungi and oomycetes can be classified into two types: type I and type II. The type I non-host resistance does not produce any visible symptoms (necrosis) and the type II non-host resistance is always associated with rapid localized necrosis (HR). *Arabidopsis dnd1* (defense no death) is an excellent example of gene-for-gene defense mechanism without HR.

2.2 HOST RESISTANCE

Resistance gene mediated recognition triggers highly effective resistance, stopping pathogen growth (termed an **incompatible interaction**; the plant is resistant and the pathogen is avirulent). Absence of specific recognition allows pathogen growth and spread (termed a **compatible interaction**; the plant is susceptible and the pathogen, virulent). However, even in the absence of specific recognition, the plant defense system

is activated to a certain level (**basal defense**) limiting the extent of disease.

Between plants and pathogens, it is hypothesized that biological 'arms race' is occurring and this situation is explained through 'Zig-Zag model' (Jones and Dangl, 2006). Pathogens have acquired mechanisms to evade PAMP-triggered immunity by evolving effectors that modify the state of the host cell or suppress PTI signaling in host cell, thereby bypassing the first line of induced defense. In the subsequent evolutionary struggle to combat these pathogens, plants have evolved means to recognize the secreted effector proteins and mount a robust amplified defense response. This type of secondary induced defense is referred to as effector-triggered immunity (ETI) and the genes encoding specificity determinants of ETI are known as resistance (R) genes. The 'Zig-Zag model' can be explained using the example of tomato and *Fusarium oxysporum*. The fungus requires the effector AVR3 for full virulence, possibly to suppress PTI. The R protein I-3 that subsequently triggers ETI can recognize AVR3. To counteract this defense response, it has been hypothesized that the fungus evolved a second effector AVR1 that suppresses these I-3 mediated defenses. To thwart the fungus, the plant in turn evolved R protein I-1 that recognizes AVR1 and activates host defenses once more. In broad terms, the defense responses associated with both PTI and ETI are qualitatively similar; however, those associate with ETI are generally faster and stronger (Jones and Dangl, 2006). Since R proteins in a cultivar evolved against particular race of a pathogen, this resistance mechanism is often termed as 'race or cultivar-specific resistance'.

Trigger of local defense responses through R gene mediated signaling also induces systemic defense responses, though in some non-host interactions also systemic resistance is activated. In general, induced plant defenses are of two major types, systemically induced resistance (SIR) and localized innate immunity (LII). SIR refers to resistance that is induced in uninfected parts of the plants by a pathogen infection or chemical treatment. The well-studied SIR includes systemic acquired resistance (SAR, Durrant and Dong, 2004), induced systemic resistance (ISR, van Loon *et al.*, 1998) and wound inducible resistance (WIR, Kessler and Baldwin, 2002).

2.2.1 RESISTANCE GENES

After the rediscovery of Mendel's work many biologists in the early 1900s realized that resistance to plant pathogens was often inherited as a single dominant or semidominant trait. It was Harold H. Flor in 1940s to propose gene-for-gene model based on his work

on Flax and Flax rust pathosystem. This model predicts that plant resistance will only occur when both plant and pathogen possesses dominant resistance (*R*) and avirulence (*Avr*) genes, respectively (Dangl and Jones, 2001). This R-Avr model holds true for most biotrophic pathogens but for necrotrophic pathogens the mechanism is different as necrotrophs secretes host-selective toxins for successful pathogenesis and thus a modified model is presented.

Despite the wide range of taxa in which *R* genes have been described, they encode few specific class of proteins. Most of the *R* genes isolated till now encodes for proteins that contain leucine-rich repeat (LRR) domain and among them nucleotide-binding site (NBS) containing proteins are more dominant. Less common are serine/threonine kinase domain and extracellular LRR. The ubiquitous use of NBS throughout the plant and animal kingdom probably reflects the biochemical suitability for such a module for coupled ligand recognition and subsequent activation of downstream signal transduction. The NB-LRRs often contain four domains connected by linkers; a variable N-terminus, the NB-ARC domain, the LRR domain and a variable C-terminal extension. The intracellular NB-LRR R proteins are present in large gene families in *Arabidopsis* (~150), rice (~400), and poplar (~400) (Meyers *et al.*, 2003; Monosi *et al.*, 2004; Tuskan *et al.*, 2006). Plant NBS-LRR proteins (also called NB-LRR or NB-ARC-LRR proteins) can be categorized into TIR and non-TIR classes based on the identity of the sequences that precedes the NBS domain. The TIR class of proteins has an amino-terminal domain having homology to the Toll/interleukin-1 receptors. The less well-defined non-TIR class proteins have α-helical coiled-coil-like sequences (CC-NBS-LRRs) or a leucine- zipper (LZ-NBS-LRRs). Members of the CC-NBS-LRR group are further divided based on the presence of additional domains at their amino-terminus (Meyers *et al.*, 1999; Martin *et al.*, 2003). Several distinct *R* gene sequences, like AtRRS1 (with a C-terminal WRKY domain), *Pi-d2* encoding a novel type of receptor-like kinase (Chen *et al.*, 2006), *Xa5* encoding TFIIA transcription factor (Jiang *et al.*, 2006), *Xa13* with homology to nodulin MtN3 (Chu *et al.*, 2006), have extended the repertoire of NBS-LRR proteins diversity. The summary of various cloned gene is illustrated in table 2.2 that is adapted from classifications of Hammond-Kosack and Parker (2003) and Liu *et al.* (2007).

Table 2.2. The plant disease resistance genes according to their classes

Class	Gene	Host	Pathogen	Protein type
1	*Hm1*	Maize	*Helminthosporium maydis* (race 1)	HC toxin reductase
2	*Asc-1*	Tomato	*Alternaria alternata* f. sp. *lycopersici* (AAL toxin)	TM helix-LAG1 motif
3A	*Pto*	Tomato	*P. syringae* pv. tomato	kinase
3B	*PSB1*	*Arabidopsis*	*P. syringae* pv. *phaseolicola* (*avrPphB*)	Different subfamily
4A	*RPS2*	*Arabidopsis*	*P. syringae* pv. *maculicola* (*avrRpt2*)	CC-NB-LRR intracellular
	RPS5	*Arabidopsis*	*P. syringae*	LZ-NB-LRR
	RPM1	*Arabidopsis*	*P. syringae*	CC-NB-LRR
	RPP8/HRT	*Arabidopsis*	*Peronospora parasitica*	CC-NB-LRR
	RPP13	*Arabidopsis*	*Peronospora parasitica*	CC-NB-LRR
	RCY1	*Arabidopsis*	*Cucumber mosaic virus*	CC-NB-LRR
	RPP/HRT	*Arabidopsis*	*Turnip crinkle virus*	CC-NB-LRR
	RPM1	Tomato	*P. syringae*	CC-NB-LRR
	Prf	Tomato	*P. syringae*	CC-NB-LRR
	I2	Tomato	*Fusarium oxysporum*	CC-NB-LRR
	Mi-1	Tomato	*Meloidogyne javanica*	CC-NB-LRR
	Mi-9	Tomato	*Meloidogyne javanica*	CC-NB-LRR
	Sw-5/Mi	Tomato	*Tospovirus*	CC-NB-LRR
	Rx2	Potato	*PVX* (Potato X virus)	CC-NB-LRR
	Gpa2/Rx1	Potato	*Globodera pallida/PVX*	CC-NB-LRR
	R1	Potato	*Phytophthora infestans* race1	CC-NB-LRR
	Mla1	Barley	*Blumeria graminis* f. sp. *hordei* race1	CC-NB-LRR
	Mla6	Barley	*Blumeria graminis*	CC-NB-LRR
	Mla12	Barley	*Blumeria graminis*	CC-NB-LRR
	Mla13	Barley	*Blumeria graminis*	CC-NB-LRR
	Pib	Rice	*Magnaporthe grisea*	CC-NB-LRR
	Pi36	Rice	*Magnaporthe grisea*	CC-NB-LRR
	Xa1	Rice	*Xanthomonas oryzae*	CC-NB-LRR
	Rp1	Maize	*Puccinia sorghi*	CC-NB-LRR
	Pm3b	Wheat	*Blumeria graminis* f. sp. *tritici*	CC-NB-LRR
	Lr10	Wheat	*Puccinia triticina*	CC-NB-LRR
	Pl8	Sunflower	*Plasmopara halstedii*	CC-NB-LRR
4B	*N*	Tobacco	*TMV*	TIR-NB-LRR
	RPP4	*Arabidopsis*	*P. parasitica* isolate Emoy2	TIR-NB-LRR
	RPP5	*Arabidopsis*	*Peronospora parasitica*	TIR-NB-LRR
	RPP1	*Arabidopsis*	*Peronospora parasitica*	TIR-NB-LRR
	Rpp10	*Arabidopsis*	*Peronospora parasitica*	TIR-NB-LRR
	Rpp14	*Arabidopsis*	*Peronospora parasitica*	TIR-NB-LRR
	RPS4	*Arabidopsis*	*P. syringae*	TIR-NB-LRR-NLS
	SSI4	*Arabidopsis*	*P. syringae* pv. *maculicola*	TIR-NB-LRR
	L6	Flax	*Melampsora lini*	TIR-NB-LRR
	L, L1-L11, LH	Flax	*Melampsora lini*	TIR-NB-LRR
	M	Flax	*Melampsora lini*	TIR-NB-LRR
	P	Flax	*Melampsora lini*	TIR-NB-LRR
	P2	Flax	*Melampsora lini*	TIR-NB-LRR
	Bs4	Tomato	*Xanthomonas campestris*	TIR-NB-LRR
4C	*Bs2*	Pepper	*Xanthomonas campestris* pv. *vesicatoria* (*avrBs2*)	NB-LRR
	Dm3	Lettuce	*Bremia lactuca*	NB-LRR

	Pi9	Rice	*Magnaporthe grisea*	NB-LRR
	Pi2	Rice	*Magnaporthe grisea*	NB-LRR
	Piz-t	Rice	*Magnaporthe grisea*	NB-LRR
	Cre3	Wheat	*Heterodera avenuae*	NB-LRR
	Cre1	Wheat	*Heterodera avenuae*	NB-LRR
	I2C	Tomato	*Fusarium oxysporum*	NB-LRR
	Hero	Tomato	*Globodera rostochiensis*	NB-LRR
4D	*RRS-1*	*Arabidopsis*	*Ralstonia solanacearum* strain GMI1000	TIR-NB-LRR-NLS-WRKY
4E	*Pi-ta*	Rice	*Magnaporthe grisea* (*avrPita*)	NB-LRD (Leucine Rich Domain)
5A	*Cf-2*	Tomato	*Cladosporium fulvum* (*Avr2*)	eLRR-TM-sCT
	Cf-4	Tomato	*Cladosporium fulvum* (*Avr4*)	eLRR-TM-sCT
	Cf-5	Tomato	*Cladosporium fulvum* (*Avr5*)	eLRR-TM-sCT
	Cf-9	Tomato	*Cladosporium fulvum* (*Avr9*)	eLRR-TM-sCT
5B	*Ve1*	Tomato	*Verticillium albo-atrum*	CC-eLRR-TM-ECS
	Ve2	Tomato	*Verticillium albo-atrum*	eLRR-TM-PEST-ECS
6	*RPW8.1*	*Arabidopsis*	*Multiple powdery mildew species*	Small, TM-CC
	RPW8.2	*Arabidopsis*		
7	*Rpg1*	Barley	*P. graminis* f.sp. *tritici*	Receptor kinase-like protein with 2 tandem kinase domains
8	*Mlo*	Barley	*Blumeria graminis* f. sp. *hordei*	TM, G-protein coupled receptor
9	$Hs1^{pro-1}$	Sugar beet	*Heterodera schachtii*	Not clear

Seeking the function of each domain in various *R* genes would be interesting, although function of a few domains like NBS, LRR, and CC is being worked out in some genes. LRR domains are involved in the interaction with effectors and are major determinants of resistance specificity (Hulbert *et al.*, 2001). For example, the variation in LRR copy number in tomato LRR-TM genes *Cf-2*, *Cf-4*, *Cf-5*, and *Cf-9* determines resistance specificity (Wulff *et al.*, 2001). In *RPS5* transgenic tobacco, the LRR domain interacts with the NBS domain forming an inactive structure to inhibit its defense signaling (Ade *et al.*, 2007). CC domain of RPM1 has been shown to interact with RIN4 (RPM1 INTERACTING PROTEIN4) and CC domain of RPS5 interacts with PBS1.

The regulation of NBS-LRR protein activation and signaling by them are questions of immense interest among the molecular plant-pathologists. It is suggested that intra- and inter-molecular interactions negatively regulate NBS-LRR signaling and in presence of effectors this negative regulation is disrupted like in Rx protein in presence of coat protein of *PVX* (Moffett *et al.*, 2002). NBS-LRR proteins may be negatively regulated by their *trans* partners like RIN4 which negatively regulates the inappropriate activation of RPS2 and RPM1 (Axtell and Staskawicz, 2003; Mackey *et al.*, 2003); BONZAI1 (BON1) is a negative regulator of temperature-dependent SNC1

(a TIR-NBS-LRR protein) activation (Yang and Hua, 2004). Some examples suggest that *trans* partners might be required to mold and maintain intra-molecular interactions that are required for signal competence and/or to mediate indirect recognition during infection as in case of HSP90s with RPM1, PRS2, tobacco N, and potato Rx proteins (Hubert *et al.*, 2003; Lu *et al.*, 2003; Liu *et al.*, 2004). Though not thoroughly understood, various R protein activation mechanisms are intensively investigated around the globe in various laboratories (Reviewed by Lukasik and Takken, 2009).

At least two signal transduction cascades acting downstream of R genes exist, one is *EDS1*-dependent and the other *NDR1* (NON-RACE SPECIFIC DISEASE RESISTANCE1)-dependent. The other pathways include those that function independently of *NDR1* or *EDS1*. EDS1 along with PAD4 is required genetically by the spectrum of TIR-NB-LRR class of proteins and some other genes like *HRT* and *RPW8.2* (Aarts *et al.*, 1998; Chandra-Shekara *et al.*, 2004). Importance of *EDS1* orthologues from tobacco and tomato was also demonstrated (Peart *et al.*, 2002; Hu *et al.*, 2005). Though absolute discrimination is an over simplification, but the CC-NB-LRR class of proteins require NDR1. EDS1 exerts early activity by acting upstream of the oxidative burst and programmed cell death. EDS1 and PAD4 are required for SA accumulation, processing of ROI-derived signals around infection foci, and for the establishment of SAR. In some cases the functions of EDS1 and PAD4 are uncoupled. *ACD11* (*ACCELERATED-CELL-DEATH11*) encodes for a protein that has *in vitro* sphingosine transfer activity and represses a PCD pathway that relies on EDS1 and PAD4 while LSD1 (lesion simulating disease) behaves as an ROI modulator and holds an EDS1/PAD4-dependent cell-death pathway in check (Brodersen *et al.*, 2002; Mateo *et al.*, 2004). EDS1 also interacts with SAG101 that possesses a defense regulatory function, which is partially redundant with PAD4 in both *R* gene mediated and basal defense. NDR1 interacts with RIN4 and is also required for defense signaling by R protein, like RPS2, RPM1, and RPS5. NDR1 contains a glycosylphosphatidyl-inositol (GPI)-anchor. GPI-anchors localize proteins to cholesterol- and glycosphingolipid-rich domains called lipid rafts that may provide sites for bacterial and host-cell interaction (Shah, 2005).

Signaling components like *RAR1* (*REQUIRED FOR MLA12 RESISTANCE*) and *SGT1* (*SUPPRESSOR OF THE G2 ALLELE OF SKP1*) have further complicated the downstream responses of different *R*-gene mediated signaling pathways, in some cases requiring RAR1 and SGT1 together, singly or neither (Austin *et al.*, 2002; Tor *et al.*, 2002; Muskett and Parker, 2003; Shirasu and Schulze-Lefert, 2003). RAR1 was

originally isolated from barley and its orthologues are isolated from tomato, tobacco, potato, and wheat as the protein is highly conserved in eukaryotes (Top and Jogensen, 1986; Liu *et al.*, 2002; Leister *et al.*, 2005; Bhaskar *et al.*, 2008). AtRAR1 is required for the function of multiple and distinct *R* genes like *RPS5*, *RPP5*, *RPM1*, *RLM1*, and *RLM2*. The resistance provided by RAR1 varies with pathogen-ecotype combination. SGT1 was isolated as an interacting protein of RAR1 like HSP90. Transient silencing of *SGT1* resulted in loss of resistance provided by NBS-LRR and non-NBS-LRR class. SGT1 provides broad range of resistance and is involved in *Arabidopsis* development unlike RAR1 that is involved only in defense. The complex of SGT1-RAR1-HSP90 is required for the stabilization of many NBS-LRR proteins (Shirasu, 2009). Recently, this immune complex is shown to be involved in activation of resistance by activated Rac1 through RACK1a in rice (Nakashima *et al.*, 2008). In *Arabidopsis,* the *pbs3* mutant compromises resistance for all *R*-genes tested (Warren *et al.*, 1999).

2.2.2 PERCEPTION OF AVR EFFECTORS

Except for *Xa27*, most *R* genes rely on their protein products to carry out Avr recognition. Initially, it was thought that products of *R* genes act as receptors that directly interact with the Avr gene products but with time the direct interactions of R-Avr have not been observed and the perception is thought to be indirect. Thus, at present three models exist that explains the *R* gene mediated Avr recognition namely Lignad-Receptor model, Guard model, and a modified model (Decoy model) to explain evolutionary constraints.

A. Ligand-Receptor model

The receptor-ligand model states that R proteins are direct receptors for pathogen avirulence proteins. This model initially gained support, as avirulence products are small and colocalize with R proteins (most of which contain LRR domain) (Keen, 1990). Though direct binding of a few R-Avr was found but the model failed to explain the evolutionary aspects of R gene evolution. According to this model, each *Avr*-gene has a cognate *R*-gene acting as receptors that doesn't seem to be possible as pathogens can evolve at faster rate for which plants also need to develop variable *R*-genes at the same pace.

The few examples of R-Avr interaction are AvrPita and Pi-ta in *Magnaporthe grisea*-rice pathosystem, PopP2 and RRS1-R in *Ralstonia solanacearum-Arabidopsis*

pathosystem, and AvrL5/6/7 and L5/6/7 interactions in *Melampsora lini*-Flax pathosystem (Bryan *et al.*, 2000; Deslandes *et al.*, 2003; Ellis *et al.*, 2007).

B. Guard model

In susceptible plant lacking a functional *R*-gene, its cognate avirulence protein contributes to pathogen virulence so the effector molecules have targets in host plant cell and this is the basis of guard model. Guard model predicts that R proteins act by guarding the effector target (also called the guardee) and that modification of this target by the effector results in the activation of the R protein, which triggers disease resistance in the host plant. This model was originally proposed for Pto and Prf mediated AvrPto perception (Van der Biezen and Jones, 1998; Dangl and Jones, 2001). This indirect effector perception mechanism can explain how a single R protein perceives multiple effectors, thus, enabling a relatively small *R*-gene repertoire to target the broad diversity of pathogens. Over the years support for this model has increased with few classical guardees are *Arabidopsis* RIN4 and PBS1 and tomato RCR3 and Pto.

The RPM1 and RPS2 R proteins in *Arabidopsis* guard RIN4 guardee. *Pseudomonas syringae* effectors, AvrRpm1 and AvrB causes phosphorylation of RIN4 that is sensed by RPM1 while AvrRpt2 effector causes elimination of RIN4 that is sensed by RPS2 (Mackey *et al.*, 2002; Axtell and Staskawicz, 2003; Mackey *et al.*, 2003). Therefore, signaling initiated here results from changes in host targets of the effectors rather than direct binding of effectors to the R proteins.

3. Decoy model

From the viewpoint of evolution, if we follow guard model the guardee is in an unstable situation. In the absence of a functional *R*-gene, natural selection is expected to drive the guardee to decrease its binding affinity with the effector and thereby evade detection and modification by the effector. However, in the presence of a functional *R*-gene, natural selection is expected to favor guardees with improved interaction with an effector to enhance pathogen perception. These two conflicting selection pressures on the same effector interaction surface results in an evolutionarily unstable situation for guardee.

It was the discovery of new targets of AvrPto and AvrBs3 that prompted proposals of the concept that some host targets of the effectors act as decoys, which mimics effector targets to trap the pathogen into a recognition event (Zhou and Chai, 2008; Zipfel and Rathjen, 2008). The decoy is specialized in perception of the effector by

the R protein but itself has no function either in the development of disease or resistance. This model is different from guard model in context to the guardee, which is useful for pathogen fitness in the absence of functional R protein in guard model. The decoy model is consistent with most of the data described so far and is coherent with the knowledge of evolution in plant-pathogen interactions. This model is supported by four cases of well-studied effector perception mechanisms summarized in table 2.3 (adapted from, van der Hoom and Kamoun, 2008).

Table 2.3 Four cases supporting the decoy model

Case	**1**	**2**	**3**	**4**
Plant	Tomato	Pepper	Tomato	*Arabidopsis*
Pathogen	*P.s.* pv. tomato	*X.c.* pv. *vesicatoria*	*C. fulvum*	*P. syringae*
Site of perception	Cytoplasm	Nucleus	Apoplast	Cytoplasm
R protein	Prf	Bs3	Cf-2	RPS2
R protein class	NB-LRR	Flavin monooxygenase	Receptor-like protein	NB-LRR
Decoy	Pto	*pBs3*	RCR3	RIN4
Function	kinase	*upa* box in promoter of *Bs3* gene	Cys protease	Negative regulator of defense
Operative target	LeFLS2?	*pUpa20*	PIP1	LRR-RKs
Function of operative target	Receptor-like kinase required for basal resistance	*upa* box in the cell size regulator gene *Upa20*	Cys protease	Receptor-kinase
Effector	AvrPto	AvrBs3	Avr2	AvrRpt2
Biochemical function of effector	Kinase inhibitor	Transcripton factor	Protease inhibitor	Cys protease
Reference	Xiang *et al.*, 2008	Kay *et al.*, 2007; Römer *et al.*, 2007	Shabab *et al.*, 2008	Belkhadir *et al.*, 2004

2.2.3. SUPPRESSION OF PTI AND ETI BY PATHOGEN VIRULENCE SYSTEM

To manipulate host defenses, plant pathogens secrete effectors into the host cell. The best-characterized are of the bacterial type III secretion system (TTSS) (da Cunha *et al.*, 2007; Block *et al.*, 2008; Zhou and Chai, 2008), followed by oomycetes RXLR effectors (Birch *et al.*, 2008), nematodes parasitism proteins (Davis *et al.*, 2008), gram-positive bacteria's host immune system suppressors (Hogenhout and Loria, 2008), and cocktail of effectors by aphids and herbivores (Felton and Tumlinson, 2008). These virulence factors behave as enzyme inhibitors, transcription activators, kinase inhibitors, proteases, cellulases. Many of the herbivore effectors mask the perception of herbivore associated molecular patterns (HAMPs) and can mimic plant hormones to modulate host

biochemical pathways. Presence of '*Molecular mimicry*' has recently gained notice in plant-pathogen interaction and seems to be a common theme in various phytopathogens as well as animal pathogens. Identifying specific pathways targeted by these effectors will shed light on new resistance strategies to control disease in plants.

2.3 Chickpea-*Ascochyta rabiei* interactions

Chickpea (*Cicer arietinum* L.), third most important food legume of the world, is a major source of high quality protein. Among temperate pulses, it is the most tolerant crop to heat and drought and is suitable for production in low fertility soils (Pande *et al.*, 2005). Despite its economic importance, chickpea productivity has been low because of yield losses due to devastating foliar and soil-borne fungal diseases like Ascochyta Blight (AB), Fusarium wilt and Botrytis Grey mould, and insect pests like pod borer. Among these AB caused by the ascomycete fungus *Ascochyta rabiei* (Pass.) Labrousse (teleomorph *Didymella rabiei* (Kovachevski) v. Arx) is the most important biotic constraint for chickpea production (Gaur and Singh, 1996). AB fungus rapidly spreads, affecting all aerial parts of the plant at any crop growth stage, resulting in a rapid collapse of tissue and spread of necrotic lesions. Various studies related with the biochemical and genetic basis of Chickpea-Ascochyta interaction has been carried out. The Ascochyta spore germination, hyphal elongation, appressorium formation, and penetration through cuticle, are essentially identical on both resistant and susceptible cultivars (Ilarslan and Dolar 2002). This necrotrophic pathogen produces cell wall degrading enzymes (cutinase, xylanase, and pectinase) and toxins like Solanapyrones (A, B, and C), cytochalasin D and a proteinaceous phytotoxin to kill host tissue (Jayakumar *et al.*, 2005). *Ascochyta rabiei* can degrade antimicrobial isoflavones and pterocarpan phytoalexins and can suppress their production in chickpea (Kessmann and Barz, 1986). In contrast to this, chickpea has preformed structural barriers, which limit fungal growth, and chemical barriers like glandular trichome secreted acidic fluid, antifungal isoflavones (Biochanin A and Formononetin), and negative isomers of pterocarpan phytoalexins, namely (-) medicarpin and (-) maackiain, which are produced rapidly and in higher quantities in resistant genotypes as compared to susceptible genotypes (Khirbat and Jalali, 1998). Studies on RILs, derived from intraspecific and interspecific crosses of AB resistant and susceptible genotypes, has elucidated that there is multigenic inheritance for the blight resistance and quantitative trait loci (QTLs) of which are mainly identified on the linkage groups (LGs) 1,2,3,4, and 6 (Santra *et al.*, 2000;

Flandez-Galvez *et al.*, 2003; Iruela *et al.*, 2006). These differences in genetic basis of AB resistance are probably due to the use of different fungal isolates, host genotypes and environment of study.

Chapter 3

Materials & Methods

Materials

3.1 Plant materials

Cicer arietinum: - Pusa 362 variety, kind contribution of Dr. N.S. Yadav, Dept. of Genetics, IARI, New Delhi, India.
- FLIP84-92C (2) variety, was kind contribution of Fred J. Muehlbauer, Washington State University, USA.

Nicotiana tabacum cv. xanthi

3.2 Fungal strain used

Ascochyta rabiei isolates were kind contribution of Prof. K.D. Srivastava and Dr. Virendra Singh, Division of Plant Pathology, IARI.

3.3 Bacterial strains used

Strain	Genotype
Escherichia coli DH5α	Φ8dlacZ Δ M15, recA1, endA1, gyr A96, thi-1, hsd17 (r_k^-, m_k^-) supE44, relA1,deoR, (LacZYA-argF)U19
Escherichia coli BL21(DE3)	F^- *ompT hsd*S_B (r_B- m_B-) *gal dcm* (DE3)
Agrobacterium tumefaciens (LBA4404)	carry pAL4404 Ti-plasmid with streptomycin selection and rifampicin chromosomal selection

3.4 *S. cerevisiae* strains used

Strain	Genotype
AH109 (Clontech)	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3, GAL2*$_{UAS}$*-GAL2*$_{TATA}$*-ADE2, URA3::MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-LacZ*, MEL1
L40	*MATa, trp1-901, leu2-3, 112, his3-200, ade2 LYS2::(lexAop)*$_4$*-HIS3, URA3::(lexAop)*$_8$*-lacZ*
NMY51 (Dualsystems Biotech)	*MATa, trp1-901, leu2-3, 112, ura3-52, his3Δ200, ade2 LYS2::(lexAop)*$_4$ *–HIS3, URA3::(lexAop)*$_8$ *–LacZ, ADE2::(lexAop)*$_8$ *–ADE2* GAL4

3.5 Plasmid vectors used

Vector	Source	Purpose
pDrive U/A vector	Qiagen	PCR product cloning
pGEM-T easy	Promega	PCR product cloning
pDHB1	DualsystemsBiotech	Yeast two-hybrid bait cloning vector
pPR3-N	DualsystemsBiotech	Yeast two-hybrid prey cloning vector
pBI101.2 vector	Clontech	Binary vector with *uidA* gene for promoter activity studies
pBI121 vector	Clontech	Binary vector with *CaMV35S* promoter
pGBKT7	Clontech	GAL4 BD vector for Y2H
pGADT7	Clontech	GAL4 AD vector for Y2H
pNIA	Purchased from Vitaly Citovsky	'Nuclear import assay' in yeast
pSUPF1, 2, 3	In-house modified from pPR3-N in	Yeast two-hybrid prey vector for

	this study	recombination mediated cloning of cDNAs
pAI-Alg5	DualsystemsBiotech	Bait expression control from DUALhunter kit
PDL2-Alg5	DualsystemsBiotech	Bait expression control from DUALhunter kit
pDHB1-largeT	DualsystemsBiotech	Positive control for DUALhunter kit
pDSL-Δp53	DualsystemsBiotech	Positive control for DUALhunter kit

3.6 Chemicals and Materials used

Type	Material	Source
Molecular weight Markers	50 bp DNA ladder 1 Kb DNA ladder	MBI Fermentas MBI Fermentas
X-ray film	Hyperfilm^{TM} MP	Amersham, Kodak
Nylon Membrane	Hybond N^{+}	Amersham
Antibiotics	Ampicillin, Kanamycin, Cefotaxime, Rifampicin, Streptomycin, Spectinomycin	Sigma
Radioisotopes	α^{32}P dCTP	Amersham
Disposable filters	PVDF 0.22 μm and 0.45 μm filter unit	Millipore
Enzymes	Commonly used restriction enzymes *Taq* DNA Polymerase T4 DNA Ligase RNase	NEB, MBI Fermentas Clontech, Bangalore Genei Fermentas, NEB BioBasic, Amersham
Dyes	Ethidium Bromide, Xylene cyanol Methylene Blue, Coomasie Brilliant Blue	Amersham
Culture media components	Tryptone, Yeast Extract, Agar, MS salts, BAP, NAA, PDA, Synthetic dropt out for yeast	Difco, Sigma, Clontech
Common reagents	Isopropanol, iso-amyl alcohol, $CaCl_2$, NaCl, NaOH, Glucose, Methanol, $MgCl_2$, KOH, Potassium acetate, Chloroform, Glycerol, Acetic acid, NaH_2PO_4, Na_2HPO_4, $MgSO_4$, HCl, H_2SO_4, Glycine, KCl, Sucrose, Pot. Dichromate, Sodium hypochlorite, Mercuric chloride, tri-Sodium citrate, Formaldehyde, etc	Qualigens, HiMedia and Merck
Other reagents used	RNaseZap, DEPC, HEPES, IPTG, MOPS, Sephadex G-50, EDTA, Acrylamide, Bis-Acrylamide, TEMED, Triton-X-100, X-gal, X-gluc, 3-AT, adenine hemisulphate	Amersham, Sigma, Ambion, BioBasic In.

- Other kits or reagents used are mentioned in the text with respective description.

3.7 Oligonucleotides used

Name	Sequence 5′ ⟶ 3′	Purpose
WRKYRTF	GTCACTGAGCCACGTAAGTCAC	RT PCR of CarWRKY1
WRKYRTR	GTCGGATTCTGAACAAGGCTTG	RT PCR of CarWRKY1
WEXF	CGGGATCCATGCAATACAAAATGGAG	For cloning of CarWRKY1
WEXR	GCGTCGACATTCTCAAGTACGATTAGGAG	Cloning in pQE30, pGEX4T-2
WRKYP1	GTGTCCTCAACTCCTCCTTCAATCTC	Promoter isolation
WRKYP2	ATAACGATTCAGGATGTTGGTGCTTG	Promoter isolation

CaWRdoF	AACTGGAGAAAATATGGTCAG	WRKY family member isolation from chickpea
CaWRdoR	CTTTCTGACCATATTTTCTCC	WRKY family member isolation from chickpea
pGEX5SP	CATGTTGTATGACGCTCTTGATG	pGEX sequencing
pGEX5SP	AGAATTATACACTCCGCTATCGC	pGEX sequencing
pQE305SP	GATTCAATTGTGAGCGGATAAC	pQE30 sequencing
pQE305SP	TACGATGCCATTGGGATATATC	pQE30 sequencing
NPTII-F	TGCTCGACGTTGTCACTGAAG	*nptII* gene pBI121 vector
NPTII-R	GTCAAGAAGGCGATAGAAGGC	*nptII* gene pBI121 vector
SP6Pro	CATTTAGGTGACACTATAG	Colony PCR of pDRIVE clones
T7Pro	GTAATACGACTCACTATAG	Colony PCR of pDRIVE clones
W3RE1	CCTGTATGGAGACACGATCC	5′-RACE of *CarWRKY3*
W2RE-1	CAACCAAGTCATCCTTAATGG	5′-RACE of *CarWRKY2*
W1PR1	CGGGATCCTTGAGAGATAAATTCCCTCC	Promoter cloning in pBI101.2
W1PR2	CGGGATCCATAACGATTCAGGATGTTGG	Promoter cloning in pBI101.2
W1PF1	CCCAAGCTTGATCCAGAATTCGTGATTAC	Promoter cloning in pBI101.2
W1PF2	CCCAAGCTTGGATTAATTTTCTTTTTATCTG	Promoter cloning in pBI101.2
W1PF3	CCCAAGCTTGTGGACTTAAGTGATCATATGTG	Promoter cloning in pBI101.2
W1PF4	CCCAAGCTTCATCGTACTTGCTCTACGTC	Promoter cloning in pBI101.2
W1PF5	CCCAAGCTTTAAGCATCAATGATGACTTG	Promoter cloning in pBI101.2
W1PF6	CCCAAGCTTACTCGGTTTCTCAGTCAAAC	Promoter cloning in pBI101.2
WRKY51	GGAAGGTGCACCGATAGTAACTTC	5′-RACE of *CarWRKY1*
WRKY52	CATCTTCATGTGGTCCTTCAAGGC	5′-RACE of *CarWRKY1*
3WRKY1	CTTCATTCGGATACACGACG	3′-RACE of *CarWRKY1*
3WRKY2	GATTCCTTCTTGAGCAGCCT	3′-RACE of *CarWRKY1*
20EW1R	CCGCTCGAGAGTACGATTAGGAGAAAAAGCC	Cloning in pET20b vector
KT7EW1F	CATGCCATGGATATGCAATACAAAATGGAG	Cloning in pGBKT7 vector
KT7EW1R	CGGGATCCCAGTACGATTAGGAGAAAAAGCC	Cloning in pGBKT7 vector
W1ADR1	CGGGATCCAATGTCGGATTCTGAACAAG	CarWRKY1 in pGADT7
W1ADR2	CGGGATCCGCTGCTCAAGAAGGAATC	CarWRKY1 in pGADT7
W1ADR3	CGGGATCCATTATCCGTTTTGACAGTCAAG	CarWRKY1 in pGADT7
W1ADR4	CGGGATCCATTGTTTCCTTGAGAACAGG	CarWRKY1 in pGADT7

W1ADR5	CGGGATCCAGGAACCCAGCTGTGGT	CarWRKY1 in pGADT7
W1ADR6	CGGGATCCAGTGGAAGGAAAGGTGAAAG	CarWRKY1 in pGADT7
W1pNIAF	CGGGATCCATATGCAATACAAAATGGAG	Cloning in pNIA vector
W1pNIAR	AACTGCAGTCAAGTACGATTAGGAGAAAAAG	Cloning in pNIA vector
W1ADF2	CATGCCATGGATCAGATAAGAGTGAGC	CarWRKY1 in pGADT7
OE2W1F	TCTAGATAGATACCAAGCACCAACATC	Cloning in PBI121 vector
OE2W1R1	GAGCTCCCTCACTATCATTGCTGATTC	Cloning in PBI121 vector
Cam35SeqF	ATCGTTGAAGATGCCTCTGC	CaMV35S promoter primer
GFPSeqR	AAGTGTTGGCCACGGAAC	GFP primer
Cam35F2	ACACGGGGGACTCTAGA	CaMV35S UTR primer
RNAiW1SF	CCGCTCGAGTCTAGATGAATGAGTTTGACAGGGTC	Sense strand for RNAi
RNAiW1SR	GGGGTACCAATTCCTCACTATCATTGCTG	Sense strand for RNAi
RNAiW1AnF	CCCAAGCTTAATTCCTCACTATCATTGCTG	Antisense strand for RNAi
RNAiW1AnR	TGCTCTAGAGGATCCTGAATGAGTTTGACAGGGTC	Antisense strand for RNAi
pBI121R2	TGCCAAATGTTTGAACGATC	pBI121 sequencing
pBIMW1F2	CGGGATCCAGATACCAAGCACCAACATC	Cloning in pBI121 at *Bam*HI
W1Nde1F	GGAATTCCATATGCAATACAAAATGGAGAATGAATG	*CarWRKY1* primers with *Nde*I
pDRIVE F	GACGGCCAGTGAATTGTG	pDRIVE vector sequencing
pDRIVE R	ACCATGATTACGCCAAGC	pDRIVE vector sequencing
AdLdF	CTATTCGATGATGAAGATACCCCACCAAACC	Primers for pGADT7
AdLdR	GTGAACTTGCGGGGTTTTTCAGTATCTACGATTC	Primers for pGADT7
SCarW1F	CTAAGAACGCGGCCATTACGGCCATGCAATACAAA ATGGAGAATGAATG	In-Fusion based cloning in pDHB1 vector
SCarW1R	CCCCCGACATGGCCGAGGCGGCCAAGGAGAAAAAG CCAAGATTATTGAAAG	In-Fusion based cloning in pDHB1 vector
DHB1CaW1F	CATGCCATGGATGCAATACAAAATGGAGAATGAATG	Cloning in pDHB1 at *Nco*I
DHB1CaW1R	CATGCCATGGGGAGAAAAAGCCAAGATTATTGAAAG	Cloning in pDHB1 at *Nco*I
KKF1	CGGGATCCAAGCAGTGGTATCAACGCAGACATATG GCCATGGAGGCCAGTGAATTC	Amplification of MCS from pGADT7-Rec
KKR1	ACGCGTCGACCTGCAGGAGCTCAGATCTATCGATG CCCACCCTCTAGAG	Amplification of MCS from pGADT7-Rec
pPR3-NSeqF	CTTATACATTAGGACCTTTGCAG	Sequencing of pPR3-N
pPR3-NSeqR	AATAAATAGGGACCTAGACTTCAG	Sequencing of pPR3-N
pDHB1SeqF	CAAGCATACAATCAACTCCAAG	Sequencing of pDHB1
pDHB1SeqR	GCTGATGTGATCACGGATGAG	Sequencing of pDHB1
CarRACK1R1	CTTCTTGGTAGTTTCACTACCAGAC	5'RACE of CarRACK1A
CarRACK1R2	AGCATCGCCGTCTTGAATAG	5' RACE of CarRACK1A

LargeTSfiIR1	GGCCGAGGCGGCCTCATGTTTCAGGTTCAGGGGGAG	Cloning in pPR3-N
mLargeTF1	CTACTCCTCCAAAAGCGAAGAGAAAG	For mutation in NLS
mLargeTR1	CTTTCTCTTCGCTTTTGGAGGAGTAG	For mutation in NLS
CarRACK1F1	GGCCATTACGGCCATGGCTGAGGGGCTCGTTC	CarRACK1A in pPR3-N
CarRACK1R3	GGCCGAGGCGGCCAAATAACGCCCAATACCCCAAAC	CarRACK1A in pDHB1
CarRACK1R4	GGCCGAGGCGGCCCTAATAACGCCCAATACCCCA	CarRACK1A in pPR3-N
CarCysPF1	GGCCATTACGGCCATGGACCGCAGTTTCCTCTT	CarCysProt in pPR3-N
CarCysPR1	GGCCGAGGCGGCCTTAATTGTTGGATGCATGAACTG	CarCysProt in pPR3-N
p53SfiIR2	GGCCGAGGCGGCCAAGTCTGAGTCAGGCCCTTCTG	p53 in pDBH1 vector
PKINESF	GATCCGGTGGTTCTGGTGGATCAGGTACTGGAAATG AATTAGCCTTGAAATTAGCAGGTCTTGATATCAACAA GGGTGGAGGTTCTAAGCAGTGGTATCAACGCAGACA TATGGC	For incorporation of NES in pPR3MK vector
PKINESR	CATGGCCATATGTCTGCGTTGATACCACTGCTTAGAA CCTCCACCCTTGTTGATATCAAGACCTGCTAATTTCA AGGCTAATTCATTTCCAGTACCTGATCCACCAGAACC ACCG	For incorporation of NES in pPR3MK vector
Frame1	CATGGAGGCAGAATTCAGTTTCCACCCAAGCAGTGG TATCAACGCAGAGTGGCCATTATGGC	Creation of pSUPF1 from pNESPK
cFrame1	CCGGGCCATAATGGCCACTCTGCGTTGATACCACTG CTTGGGTGGAAACTGAATTCTGCCTC	Creation of pSUPF1 from pNESPK
Frame2	CATGGAGGCAGAATTCAGTATTCCACCCAAGCAGTG GTATCAACGCAGAGTGGCCATTATGGC	Creation of pSUPF2 from pNESPK
cFrame2	CCGGGCCATAATGGCCACTCTGCGTTGATACCACTG CTTGGGTGGAATACTGAATTCTGCCTC	Creation of pSUPF2 from pNESPK
Frame3	CATGGAGGCAGAATTCAGTAATTCCACCCAAGCAGT GGTATCAACGCAGAGTGGCCATTATGGC	Creation of pSUPF3 from pNESPK
cFrame3	CCGGGCCATAATGGCCACTCTGCGTTGATACCACTG CTTGGGTGGAATTACTGAATTCTGCCTC	Creation of pSUPF3 from pNESPK
LargeTF1	TCCCCCCGGGCCAACCTATGGAACTGATGAATG	Cloning at *Sma*I in pSUPF
LargeTR1	TCCCCCCGGGTTATGTTTCAGGTTCAGGGGGAGGTG	Cloning at *Sma*I in pSUPF
ENH151	GATATACAAGCTCCACACAAGATTGAG	5'RACE of ENH1
ENH152	ACAGCACCTAAACCAGCGAC	5'RACE of ENH1
PPI51	CATGAGCAAAAAGCACAACATAGTATG	5'RACE of PPI1
PPI52	TCACAAACCTAGCCACCCAATTAG	5'RACE of PPI1

* underlined are the restriction sites incorporated in primers.

General sterilization procedures used

All the glassware, tissue culture materials and culture media were sterilized by autoclaving at 121.6°C under 15 lb psi pressure for 15 minutes. The antibiotics and other heat labile components were filter sterilized with disposable syringe driven PVDF filter unit of 0.22μm pore size (Millex^{TM}, Millipore, USA).

Methods

3.8 Plant growth conditions, maintenance and fungal/chemical treatment procedures

Plant growth conditions

All the Chickpea (*Cicer arietinum* L.) varieties used were grown under similar conditions. Seeds were soaked overnight in tap water and sown in soil (3-4 seeds/ pot) in growth chamber at 25±4°C.

Fungal growth conditions

Ascochyta rabiei isolates were grown on sterilized potato dextrose agar (PDA) media (supplemented with crushed chickpea seed) in culture tubes and plates at room temperature and 12 hours photoperiod. To maintain its virulence the chickpea plants were infected with the fungus and once the disease symptoms become visible, the infected samples were inoculated on PDA to facilitate fungal growth. The culture is subsequently sub-cultured before using it for fresh infection.

Fungal inoculum preparation and inoculation

For spore collection, PDA tubes with fungus grown on the media were filled with sterile tap water and left for 10 min. The surface was rubbed with a sterile loop to suspend the spores in water. The suspension was filtered through muslin cloth. The concentration of spores was determined using haemocytometer and dilutions were made in sterilized tap water to obtain 10^6 and 10^8 spores/ ml. Inoculum was sprayed on 3 weeks old chickpea plants until the leaves were completely covered with the suspension. To maintain high humidity conditions, pots were covered with a transparent plastic sheet. The control plants were sprayed with sterile tap water and grown under similar conditions. Following inoculation, samples were harvested after required time intervals, snap frozen in liquid nitrogen and stored at -80°C. The control samples were also similarly harvested. To rule out any kind of discrepancy on account of variation in infection, the samples were randomly collected in triplicates and mixed. RNA/protein were later isolated from randomly mixed samples. Symptoms of fungal growth were regularly monitored. Estimation of disease severity was recorded by a visual assessment of disease symptoms. The 10^8 spores/ml inoculated plants were severely infected and appeared almost bleached after 10 days and were not able complete their life cycle. On the other hand the plants inoculated with 10^6 spores/ml, survived infection to complete their life cycle.

Treatment of signaling molecules and wounding

Aerial parts of 3-weeks old chickpea (Pusa 362) plants, grown in pots were dipped for 30 seconds in the solution of required chemical. The concentrations used were 100µM jasmonic acid (JA), 5mM salicylic acid (SA). The control plants were dipped in sterile MQ water. The wounding treatment was done by cutting half leaf with scissors from upper and lower part of the plant. The samples were collected after appropriate time intervals.

3.9 General cloning procedures

3.9.1 Elution of DNA from agarose gel

The PCR product was fractionated on 1% agarose/EtBr gel. The band was cut by using sterile blade and collected in a 1.5 ml sterile micro-centrifuge tube. The gel elution was done using MinElute gel extraction kit (Qiagen, Germany). The elution was done according to the manufacturers instructions with minor modifications. Three volumes (one volume of gel, 100 mg ~ 100 µl) of buffer QG was added to the eppendorf containing the gel slice and incubated at 40°C for 30 min to dissolve the agarose. After the gel slice has dissolved completely, one gel volume of isopropanol was added and mixed by inverting the tubes 4-5 times. This sample was loaded into the MinElute column that was kept on a 2ml collection tube and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and the column was again placed in the same collection tube. Further, 500 µl of QG buffer was loaded to the column and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and column was again placed in the same collection tube. To wash the column, 750 µl buffer PE was loaded into the column and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and column was again placed in the same collection tube and centrifuged for an additional 1 min to remove the residual ethanol. The MinElute column was then placed in clean 1.5 ml micro-centrifuge tube. To elute the DNA, 10 µl of elution buffer (100mM TrisCl, pH 8.0) or sterile nuclease free water was loaded directly on the matrix. The column was left as such for 5 min and then centrifuged at 13,000 rpm for 2 min. DNA was obtained as flow through. The eluted DNA was stored at -20°C till further use.

3.9.2 Purification of PCR products

The PCR products were purified by using MinElute™ PCR purification kit (Qiagen, Germany). Purification was done according to manufacturer's instructions with minor modifications. Five volumes of PB buffer was added to one volume of the PCR reaction product and mixed. The mixture was then applied to the MinElute column that was kept in 2 ml collection tube and centrifuged at 13,000 rpm for 1 min to bind the DNA to the membrane, flow-through was discarded and column was again placed in same collection tube. To wash the column, 750 µl PE buffer was applied to the column and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and column was again placed in the same collection tube and centrifuged for an additional 1 min to remove the residual ethanol. Now, the MinElute column was placed in a clean 1.5 ml microcentrifuge tube. To elute the DNA, 10 µl of elution buffer or sterile nuclease free water was loaded directly on the matrix. The column was left for 5 min and then centrifuged at 13,000 rpm for 2 min. The DNA was obtained as flow-through. The eluted DNA was stored at -20°C till further use.

3.9.3 Preparation of Competent Bacterial Cells

For cloning purpose, *E. coli* DH5α and related strains were made competent by the following methods and used for transformation.

Calcium Chloride Method

The $CaCl_2$ method was adopted from Sambrook and Russell (2001) with some minor modifications. From the overnight grown pre-culture of bacterial cells, 1ml of inoculum was used to inoculate 100 ml LB medium in a culture flask. This culture was grown at 37°C with vigorous shaking (200-250 rpm) to an A_{600} of 0.3-0.4. The culture was chilled on ice for 15-20 min, transferred to 50 ml round-bottom polypropylene tubes and centrifuged at 5000 rpm for 5 min at 4°C in Sorvall® RC5C plus centrifuge (Kendro Lab., USA) with SA-600 rotor. The pellet in each tube was gently suspended in 0.5 volumes (of original culture) of ice-cold 100mM $CaCl_2$ by gently swirling the tubes and incubated on ice for 30 min. The cells were collected by centrifugation as above and resuspended in 0.1 volumes ice-cold 100mM $CaCl_2$ by gently swirling the tube.

Preparation of ultra-competent bacterial cells

HEPES Transformation Buffer (HTB)-200ml

10 mM HEPES

15 mM $CaCl_2$

25 mM Kcl

Dissolve in about 150 ml autoclaved MQ. Adjust pH to 6-7 with 4M KOH. Add 55 mM $MnCl_2.4H_2O$. Adjust the volume to 200 ml and filter sterilize with 0.45μm filter unit.

The competent cells were prepared as described by Inoue *et al.* (1990) with few modifications. From the frozen culture, DH5α bacterial cells were streaked on LB agar plate and were grown overnight at 37°C. Approximately 5-10 large colonies were inoculated in 200 ml SOB media with a sterile loop and grown at 22°C with vigorous shaking at 200-250 rpm till the OD_{600} reaches to 0.45. The culture flask was removed from the incubator and placed on ice for 10 min. The culture was transferred to sterile round-bottom polypropylene tubes, 50 ml each, and centrifuged at 2500x g for 10 min at 4°C. The pellet obtained was resuspended in 16 ml of ice-cold HTB, incubated on ice for 10 min and centrifuged at 2500x g for 10 min at 4°C. The pellet obtained was gently resuspended in 4 ml of HTB and DMSO was added to a final concentration of 7% with gentle swirling. Cells were kept on ice bath for 10 min. One hundred microlitres of the cell suspension was dispensed in 1.5 ml micro-centrifuge tubes and snap-frozen in liquid nitrogen. The frozen competent cells were stored at - 80°C for further use.

3.9.4 Transformation

Competent *E. coli* cells were transformed according to the standard protocol given by Hanahan, (1983). A vial of competent cells, stored at - 80°C was carefully thawed on ice avoiding any heat shock. The ligated product or plasmid was directly added to 100 μl competent cell suspension, mixed by gentle tapping and subsequently kept on ice for 30 min. All the steps of transformation were carried out in laminar hood under sterile conditions. The cells were then given a heat shock at 42°C for 90 sec and quick chilled on ice for 5 min. This was followed by addition of 0.9 ml of LB and the cells were allowed to grow at 37°C for 45 min with gentle shaking. The transformed competent cells were plated on LB plate containing appropriate antibiotic. Blue-white selection, if

needed, was carried out by plating the cells on X-gal/IPTG plate. The plates were then incubated at 37°C overnight.

3.9.5 Confirmation for the presence of insert

The presence of the insert in the clone was confirmed by the colony PCR by using either gene specific primers or primers compatible with cloning vector. Individual colonies were picked from overnight grown plate and mixed in 20 µl sterile water in a 0.5 ml micro-centrifuge tubes. The cells were lysed by boiling for 2 min and centrifuged at 13,000 rpm for 30 sec. Eight microlitre of the supernatant was taken as template for PCR. The master mix was prepared according to the number of the PCR reactions and distributed in thin-walled PCR tubes. Number of PCR cycles and cycling conditions were adjusted according to the Tm of primers used for amplification.

3.9.6 Isolation of plasmid DNA by Alkaline lysis method

A single colony of bacterial cell containing the desired clone was inoculated to the 100ml of LB medium containing the appropriate antibiotic and allowed to grow overnight at 37°C. The bacterial cells were harvested by centrifugation at 5000 rpm for 10 min at 4°C. The pellet was resuspended in 5ml of ice-cold solution I (50mM glucose, 10mM EDTA, 25mM Tris-Cl). Then 5 ml of freshly prepared solution II (0.2N NaOH, 1%SDS) was added and mixed gently by inversion, and incubated for 5 min at room temperature followed by addition of 5ml of ice cold solution III (3M potassium acetate, pH 4.8) and the mixture was incubated on ice for 15 min. This mixture was then centrifuged at 14,000 rpm for 30 min at 4°C and the supernatant was transferred to a fresh round-bottom polypropylene tube. The supernatant was subjected to RNase treatment 20 µg/ml at 37°C for 45 min. The supernatant was extracted twice with phenol: chloroform: isoamyl alcohol (25:24:1) and followed by separation of upper aqueous phase containing the plasmid in a fresh round-bottom polypropylene tube. Equal volume of isopropanol was added to precipitate the DNA by centrifugation at 10,000 rpm for 30 min at room temperature. This was followed by washing with 70% alcohol. The pellet was dried at 37°C and dissolved in 100 µl of sterile water.

3.9.7 Purification of Plasmid by PEG Precipitation for Sequencing

Eight microlitre of 4M NaCl and 40 µl of 13% polyethylene glycol (PEG 8000) was added to the plasmid dissolved in 32 µl sterile water and the mixture was incubated on

ice for 30 min. DNA was pelleted by spinning at 12,000x g for 20 min at 4°C. The pellet obtained was washed twice with 70% alcohol, dried and dissolved in sterile water. Visual quantification of DNA was done and 150 ng of plasmid in 2 µl was used for automated sequencing with 96 capillary-based DNA analyzer (Hitachi and ABI PRISM, Applied Biosystems).

3.9.8 Maintaining bacterial strain/clones stocks for long-term storage

The bacterial strains or clones were inoculated in their respective media keeping on antibiotic selection, if any, and grown to mid-log phase. To 1 ml of this culture, 430 µl of sterile glycerol was added to get the final concentration of 15% (v/v). The cryo-vials were vortexed and kept at -70°C.

3.10 Gene expression analysis by Northern Hybridization

Before starting RNA work, mortar, pestle, glassware, spatula and other required materials were baked at 180°C for 5-6 hrs. Gel electrophoresis assembly and other plastic wares were treated with 3% H_2O_2 overnight.

3.10.1 Isolation of RNA from Chickpea

Total RNA was isolated from Chickpea using TRIZOL Reagent according to the manufacturer's instructions (Invitrogen, USA) with few modifications. About 0.8g plants tissue was crushed to fine powder with mortar and pestle in liquid nitrogen without letting it to thaw. The powdered material was transferred to a 2 ml eppendorf tube, immediately 1 ml TRIZOL Reagent was added to the tube and it was vigorously shaked in order to homogenize the sample quickly. The homogenized samples were incubated for 15 min at room temperature for complete dissociation of nucleoprotein complexes. Two hundred microlitre of chloroform was added per ml of TRIZOL reagent used and tube was vigorously shaked for 30 sec with tube capped tightly, incubated at RT for 10 min and centrifuged at 12,000x g for 15 min at 4°C. Following centrifugation, the upper aqueous phase was aliquoted into three tubes (kept on ice) equally without disturbing the lower whitish layer. The RNA from the aqueous layer was precipitated by mixing with 0.7 volumes of isopropyl alcohol in each tube, according to the volume of supernatant aliquoted in each tube earlier, incubated for 10 min at RT and centrifuged at 12,000x g for 10 min at 4°C. The supernatant was discarded by inverting the tubes on tissue paper and RNA pellet was washed two times with 75% ethanol by dislodging the

pellet from the surface of tube with vigorous shaking and centrifuging at 7,500x g for 5 min at 4°C. At the end of the procedure, RNA pellet was briefly dried for 10 min and dissolved in adequate volume of DEPC-treated water or for long-term storage, the ethanol washed pellet was left in 75% ethanol and kept at -80°C.

3.10.2 RNA quantification

The water dissolved RNA was incubated at 55°C for 10 min and quickly chilled on ice. After brief centrifugation, it was collected at the bottom of tube and tapped gently to mix. Two microlitre of the RNA was diluted 500 times by adding 1 ml of DEPC-treated water and mixed thoroughly. The O.D of this diluted RNA was taken on 260 nm spectrophotometer (U-2010, HITACHI) against DEPC-treated water as blank. Concentration of the RNA was calculated according to the following formula-

$$\text{RNA conc. } (\mu g / \mu l): \frac{O.D_{260} \times 40 \times \text{Dilution factor}}{1000}$$

Purity of the RNA was checked by taking O.D at 230, 260, and 280 nm wavelengths. The RNA was indicated as pure if the ratio of O.D (260/280) falls between1.7 - 2.0 (<1.7 is typically protein contamination) and O.D (260/230) is >2.0 (<2.0 is due to guanidinium isothiocyanate).

3.10.3 Denaturing formaldehyde gel for RNA electrophoresis

Total RNA was run in 1.2 % denaturing formaldehyde gel. For preparation of gel, 1.2 g agarose was added to 64 ml DEPC treated water and boiled for 1.5 min. Once the temperature comes down to 60°C, 16.4 ml formaldehyde and 20 ml 5X MOPS buffer was added. The contents were mixed by swirling. Formaldehyde is harmful for eyes, hence adequate precautions were taken. The molten gel was poured in casting tray with combs already fitted into it. Meanwhile, RNA samples were prepared by mixing eight microgram of total RNA and RNA loading dye (1 ml contains 500 µl formamide, 166 µl formaldehyde, 200 µl 5X MOPS and 134 µl DEPC water) in 1:3 (v/v) ratio. The samples were heat denatured at 65-67°C for 10 min and immediately chilled. The samples were run at 20-30 Volts for 5-6 hours in 1X MOPS buffer.

3.10.4 Transfer of total RNA on Nylon Membrane

The gel was rinsed with DEPC treated water for 30 min to remove formaldehyde and it was equilibrated with 20X SSC for 30 min. The RNA was transferred to Hybond–N^+

Nylon membrane (Amersham, UK) by vertical capillary action using 20X SSC for 16 h. After that the RNA was cross-linked to the nylon membrane in UV crosslinker (Stratagene, USA) at 1200kJ/cm^2 and this RNA cross-linked membrane was treated with 5% glacial acetic acid for 15 min. To check the RNA transfer on the membrane, it was stained with 0.04% methylene blue (Solution prepared in 0.5 M Na-acetate, pH 5.2). Excess of the stain on the membrane was removed by washing with sterile MQ water. Image of ribosomal RNA was captured on Fluor-S^{TM} MultiImager (Bio-Rad, USA) at highest resolution available to show equal loading of RNA. The hybridized nylon membrane was wrapped in a saran wrap to avoid it from drying.

3.10.5 Radioactive probe preparation, purification and hybridization

For probe preparation radiolabel was used, hence all steps were performed in radioactive room taking adequate safety measures. In a hybridization incubator, the RNA cross-linked nylon membranes were incubated at 60°C with 10 ml of pre-hybridization solution (0.5M Phosphate buffer, pH 7.2, 7% SDS, and 1mM EDTA, pH 8.0) in hybridization bottles for 4 hrs. In the meantime the probe was prepared using random primers labeling NEBlot® kit (NEB Inc., U.K). For probe preparation, in 1.5 ml micro-centrifuge tube 50 ng of DNA (fragment to be used as probe) was taken in final volume of 10 µl. The dsDNA was denatured for 5 min in boiling water bath and quickly chilled on ice. For 50 µl reaction, the following components were added in the following order- 26 µl of MQ H_2O, 5.0 µl of 10X labeling Buffer, 2.0 µl of dATP, 2.0 µl of dGTP, 2.0 µl of dTTP, 2.0 µl of radioactive α^{32}P-dCTP (3000 Ci/mmole, Amersham Biosciences) and 5 units of Klenow polymerase enzyme. The final mixture was incubated at 37°C for one hour in water bath. For purification of free radioactive dNTPs from the mixture, Sephadex G-50 column was prepared as described. One ml fresh disposable syringe was packed at the bottom with the glasswool sterile TE (pH 8.0). This column was packed with sephadex G-50 (soaked in TE, pH 8.0) up to appropriate volume by centrifugation in a 15 ml falcon tube and was equilibrated thrice with TE, pH 8.0. Prior to purification it was centrifuged again, to remove excess TE, at 2,300 rpm for 4 min. The volume of the reaction mix was made upto 100 µl with TE, pH 8.0. The reaction mix was loaded on the packed column and centrifuged at 2,300 rpm for 3-5 min. Purified probe was collected as flowthrough in a decapped eppendorf and transfered to fresh eppendorf. It was subsequently denatured for 5 min in boiling water bath and quick chilled for 5 min. After a brief spin, the probe was added directly to the pre-hybridization solution kept in

hybridization bottle. The probe was left for hybridization for 14-16 hr at 60°C in hybridization incubator.

3.10.6 Washing and Autoradiography

Filters (Hybridized nylon membrane) were washed thrice for 5 min at room temperature in low stringency solution (2X SSC and 1% SDS). Filters were then checked for the count by the radiation monitor. This was followed by washing at 60°C in medium stringency washing solution (0.4X SSC and 0.1% SDS) for 10 minutes or more depending upon the background count. The filters were then wrapped in saran wrap to avoid drying and the X-ray film was exposed to the membrane in the Hypercassette™ (Amersham Pharmacia biotech, U.K) for the time period depending upon the signal intensity. Subsequently, the X-ray film was developed using Developer and Fixer solutions (Kodak Affiliate Products, India). The autoradiograms obtained were scanned in Fluor-S™ MultiImager (Bio-Rad, USA).

3.11 Full-length gene isolation

3.11.1 5′-RACE

Using the SMARTer™ RACE cDNA Amplification kit (Clontech) the ends of the cDNAs were amplified according to the manufacturer's instructions with minor modifications.

3.11.1.1 First Strand cDNA Synthesis

A sterile 0.2 ml microcentrifuge tube was marked 'A' and in this tube a reaction mix of 2 µl 5X first-strand buffer, 1 µl DTT (20 mM), and 1 µl dNTPmix (10mM) was prepared. To another fresh tube marked 'B', 1 µl total RNA (1 µg/µl), 1 µl 5′-CDS Primer A, and 1.75 µl of MQ was added and mixed along with a quick spin. This tube was incubated at 72°C for 3 min and then at 42°C for 2 min in a thermal cycler. A brief spin was given and 1 µl of the SMARTer IIA oligo was added in this mix. For the reaction of first strand cDNA synthesis in a fresh tube marked 'C', 4 µl of buffer mix from tube 'A', 0.25 µl RNase inhibitor (40U/µl), and 1 µl of SMARTScribe™ Reverse Transcriptase (100 U) were mixed. The content of tube 'B' and 'C' were mixed and after gentle spin incubated at 42°C for 90 min in an air incubator, which was pre-set at 42°C. After the reaction was complete, tubes were incubated at 70°C for 10 min. Out of this

first strand cDNA, 5 µl was taken in a separate tube and diluted with 50 µl of Tricine-EDTA buffer for further use.

3.11.1.2 Rapid amplification of cDNA ends

The ends of cDNA were PCR amplified using the first strand cDNA, synthesized in earlier step, as template. A PCR master mix was prepared according to the number of reactions required, for a single reaction of 50 µl; 34.5 µl of PCR-grade water, 5 µl of 10X Advantage 2 PCR buffer, 1 µl of dNTP mix (10 mM), 2.5 µl of first strand-cDNA, 5 µl of 10X universal primer A mix, 1 µl of gene specific reverse primer, and 1 µl of 50X Advantage polymerase mix were added sequentially. The contents were mixed and a quick spin was given. In a thermal cycler (MJ Research), following reaction was set up and to amplify cDNA end;

Denaturation	94 °C for 30 sec	5 cycles
Annealing and extension	72 °C for 3 min	
Denaturation	94 °C for 30 sec	5 cycles
Annealing	70 °C for 30 sec	
Extension	72 °C for 3 min	
Denaturation	94 °C for 30 sec	25 cycles
Annealing	68 °C for 30 sec	
Denaturation	72 °C for 3 min	

4°C, until samples were removed

Five microlitre of the obtained PCR product was diluted with 250 µl Tricine-EDTA buffer. The diluted primary PCR product, 1 µl of nested universal primer A, and 1 µl of nested gene specific primer were mixed with required PCR reagents for amplification using the above mentioned reaction conditions. The obtained PCR product was resolved on 1% agarose gel. The band of expected size, if many, was eluted from the gel and cloned in pDRIVE U/A cloning vector. After sequencing, the 5'end of cDNA was overlapped with the ends of cloned product to confirm the cloning of cDNA end of the desired gene.

3.11.2 3′ RACE

The 3' cDNA ends of genes were amplified using the Adaptor Primer and AUAP primer (3' RACE system, Invitrogen, USA), BD Powerscript™ Reverse Transcriptase Kit, and BD TITANIUM™ *Taq* DNA Polymerase Kit (Clontech, USA).

3.11.2.1 First Strand cDNA Synthesis

In a 0.2 ml thin walled PCR tube following components were added: 1.5 µg of RNA (from the time period where highest mRNA transcript is expected), 1 µl of 10µM adaptor primer [have $(dT)_{20}$ bases] and fresh sterilized MQ to make volume up to 11 µl. These components were mixed and incubated on thermal cycler at 70°C for 10 min. The tube was then kept on ice and sample was collected at the bottom of tube by brief spin. Four microlitres of first-strand buffer, 2 µl of 10mM dNTP mix, and 2 µl of 100mM DTT were added to the tube. The contents of the tube were mixed by tapping and quick spin. After a brief spin, 1 µl of Powerscript™ reverse transcriptase was added in the PCR tube. This tube was incubated in a thermal cycler at 42°C for 90 min and then at 70°C for 15 min to terminate the reaction. The tube was then kept on ice and the reaction mixture was collected at the bottom of tube by a brief spin. For RNA removal, 1 µl of RNase H was added and the tube was incubated at 37°C for 30 min. This cDNA was used further to isolate the specific genes.

3.11.2.2 Amplification of the target cDNA

The target 3′cDNA from the pool of the first strand cDNAs formed in the above procedure was amplified using forward gene specific primer 1 (GSP1), AUAP or UAP primer's and 2 µl of first strand cDNA. PCR reaction was set up and the probable band was amplified. The primary PCR product was diluted 500 times and nested PCR was done using nested forward GSP2 primer. In nested PCR a single specific band was obtained.

3.12 Promoter isolation by Genome Walking

The promoter was isolated using Universal Genomewalker™ Kit (Clontech, USA). From this kit a pool of uncloned, adaptor-ligated genomic DNA fragments were obtained, which were later used for isolation of gene specific promoter. Basically five steps were performed to make genomic library.

3.12.1 Isolation of Genomic DNA from Chickpea

Genomic DNA was isolated as mentioned by Murray and Thompson, 1980 with some modifications. Five gm tissue was frozen in liquid nitrogen, crushed using pestle and mortar, transferred to fresh round-bottom polypropylene tube and 5-8 ml extraction buffer (2% CTAB, 1.4M NaCl, 20mM EDTA, pH 8.0, 100mM Tris-HCl, pH 8.0,

100mM β-ME) was added to the ground tissue. Subsequently, the tubes were transferred to 60^0C and left for 1 hr. To this, 5-8 ml of chloroform: isoamyl alcohol (24:1) was added and mixed gently for 2-3 hrs followed by centrifugation at 10,000x g for 10 min at room temperature. The upper aqueous phase was transferred to another vial, and once again DNA was extracted with 5-8 ml of chloroform: isoamyl alcohol (24:1). To the final aqueous phase 0.6 volume of isopropanol was added for precipitating the genomic DNA that was then spooled out. The genomic DNA was then washed thrice with 70% ethanol, dried in vacuum, dissolved in TE containing 10 mg/ml RNase and incubated at 37^0C for 30 min. This was followed by extraction with phenol: chloroform: isoamyl alcohol (25:24:1) and the aqueous phase was transferred to a fresh tube. Thereafter the genomic DNA was precipitated by adding equal volume of isopropanol. The pellet was obtained by centrifugation at 10,000x g for 20 min at 4^0C and washed with 70% ethanol, air dried and dissolved in TE.

3.12.2 Determination of quantity and purity of genomic DNA

The quality of genomic DNA was checked after running it on agarose/EtBr gel along with control genomic DNA on the gel. 0.1 µg of each genomic DNA was loaded. The DNA obtain was intact as no smear was observed.

3.12.3 Digestion of genomic DNA

In six different 1.5 ml sterile tubes, six digestion reactions were set up using the enzymes *Dra*I, *Eco*RV, *Pvu*II, *Hinc*II, *Sma*I and *Stu*I. All these enzymes produce blunt ends. In each reaction following components were combined:

Genomic DNA (0.1 µg/µl)	25 µl
Restriction enzyme	8 µl
Restriction enzyme buffer	10 µl
Deionized H_2O	57 µl
Total volume	100 µl

Mixed gently and incubated at 37^0C for 2 hr. The tubes were tapped gently and again kept for 16-18 hr. From each tube 5 µl reaction mix was checked for digestion on 0.5% agarose/EtBr.

3.12.4 Purification of DNA

To each of the reaction tube, an equal amount (95 µl) of phenol was added and slowly vortexed for 10 sec. After brief spin, aqueous layer was transferred to a new tube and

again the above step was repeated to isolate protein contamination. After second extraction, 2 volumes (190 µl) of ice cold 95% ethanol, 1/10 volumes (9.5 µl) of 3M NaOAc, and 20 µg of glycogen was added and vortexed slowly for 10 sec. To pellet the digested DNA, the tubes were centrifuged at 15,000 rpm for 10 min and the supernatant was decanted. The pellets obtained were washed with in 100 µl of ice cold 80% ethanol and centrifuged at 15,000rpm for 5 min. The supernatant was decanted, pellet was air dried and dissolved in 20 µl of TE (10/0.1, pH 7.5). After a slow speed vortex for 5 sec, 1 µl of the digested DNA quality & quantity was checked on a 0.5% agarose/ EtBr gel.

3.12.5 Ligation of Genomic DNA to Genome-walker adaptors

For ligation, 4 µl of each digested and purified DNA was taken in 0.5 ml tubes and to each of the four tubes was added the following- 1.9 µl Genomewalker adaptor (25µM), 1.6 µl 10x Ligation buffer and 0.5 µl T4 DNA ligase (6 units/µl). Incubated overnight at 16^0C. To stop the reaction, the tubes were incubated at 70^0C for 5 min. The ligated DNA was diluted with 72 µl of TE (pH 7.4).

The target promoter regions of the *CarWRKY1* gene were amplified from the five adaptor ligated DNA library created by using *Sma*I, *Hinc*II, *Pvu*II, *Stu*I, and *Eco*RI. PCR was done separately for each library with AP1 primer, WRKYP1 primer and 1 µl of genomic DNA library. Nested amplification of the primary PCR product was done using 100 times diluted primary PCR sample, AP2 and WRKYP2 primer. The amplified product was eluted after resolving on 0.8% agarose gel and cloning was done. The confirmation of the desired sequence was done by sequencing of the clones and aligning with the known sequence.

3.13 Transformation of Tobacco (*Nicotiana tabacum cv. xanthi)*

3.13.1 Transformation of *Agrobacterium tumefaciens* cells

YEP media: 10g/litre of Yeast extract
10g/litre of Peptone
5g/litre of NaCl,
pH adjusted to 7.0

The *Agrobacterium* cells were transformed by the freeze-thaw method described by Gelvin and Schilperoort (1990). The strain LBA4404 was inoculated into 5 ml of YEP media and allowed to grow for 2 days at 28°C with vigorous shaking. Two milliliters of

this culture was inoculated into 50 ml of YEP medium with vigorous shaking till the OD_{600} reached 0.4 to 0.6. The culture was chilled on ice and centrifuged at 4,000 rpm for 10 min at 4°C. The cells were suspended in 1 ml of filter sterilized and ice-cold 20mM $CaCl_2$ solution. One hundred microlitres of suspended cells were aliquoted into pre-chilled eppendorf tubes and frozen at -70°C till further use.

The frozen competent cells were thawed on ice and 200ng of recombinant plasmid DNA was added to the cells and later frozen in liquid nitrogen. The cells were thawed immediately at 37°C in water bath for 5 min. This step was repeated once. One ml of YEP media was added to the cells and the tube was incubated at 28°C for 4 hrs with gentle shaking. This period allowed bacteria to express the antibiotic resistance genes and to overcome the shock. The tubes were centrifuged in a microcentrifuge for 30 sec and the supernatant was discarded. The cells were suspended in 100 µl of YEP media and spread on YEP agar plates containing appropriate antibiotics for transformed plasmid and *Agrobacterium*. The plates were incubated at 28°C for 2 to 3 days before the colonies appeared.

3.13.2 Screening of recombinant colonies

The transformed cells were patched on the YEP plates containing appropriate antibiotics. After two days of growth the bacterial cells were checked for the presence of inert by colony PCR as described in section 3.9.5.

3.13.3 *Agrobacterium* mediated leaf disc transformation

MS salts were purchased from HiMedia, Sigma, and prepared in laboratory.

Murashige and Skoog basal salts:

Macro-nutrients (50 ml/L), Micro-nutrients (1 ml/L), KI (1ml/L), Fe-EDTA (5ml/L), and Glycine (1ml/L).

KI stock	830 mg/L in MQ		
Glycine stock	2 mg/ml in MQ		
Fe-EDTA stock	7.45 gm/L $Na_2EDTA.2H_2O$ and 5.57 gm/L $FeSO_4.7H_2O$ in MQ		
Macro-nutrients:	$NH_4.NO_3$	-	16.5 g/L
	KNO_3	-	19 g/L
	$CaCl_2.2H_2O$	-	4.4 g/L
	KH_2PO_4	-	1.7 g/L
	$MgSO_4.7H_2O$	-	3.7 g/L

Micro-nutrients:	H_3BO_3	-	6.2 g/L
	$Na_2MoO_4.2H_2O$	-	250 mg/L
	$CoCl_2.6H_2O$	-	25 mg/L
	$CuSO_4.5H_2O$	-	25 mg/L
	$ZnSO_4.7H_2O$	-	8.6 g/L
	$MnSO_4.4H_2O$	-	22.3 g/L

Six-week-old axenically grown seedlings were used for transformation as described by Horsch *et al.* (1990). Unblemished healthy leaves from these seedlings were used. Leaf sections were cut with a sterile scalpel and immersed in overnight grown *Agrobacterium* culture [diluted 10 times before use in MSO (MS basal salts 4.3 g/L, B5 vitamins, 1 ml/L, sucrose, 30 g/L] liquid medium for 5 min. The explants were blotted dry on a sterile tissue paper and transferred to MS104 (MSO medium, BAP 1.0 µg/ml and NAA, 0.1 µg/ml, agar 0.8%) for a period of 2 days. The explants were subsequently transferred to the same media containing 100 µg/ml kanamycin and 500 µg/ml cefatoxime. Well-developed shoots on the selection medium were excised and placed upright in the MS rooting medium (MSO medium with 0.6% agar). Rooted plantlets were transferred to agropeat and vermiculite and supplemented with 0.5X Hoagland's solution twice a week. Once the plants were hardened on vermiculite, they were transferred to soil and maintained in green house.

3.13.4 GUS histochemical assays

GUS activity staining was performed for verification of expression of the *uidA* gene (Jefferson, 1987). Plant material to be GUS-stained was immersed in GUS-staining solution (1.5 mM X-gluc, 50 mM phosphate buffer, pH, 7.0 and 0.1% Triton X-100). Tubes were placed in an desiccator and vacuum was applied for 3-5 min. Vacuum was released and this procedure was repeated. Tubes were closed and incubated over night at 37°C. After incubation of the leaves, the GUS staining solution was discarded. Plant material was rinsed with deionised water and tissues were cleared from chlorophyll by putting into 70 % ethanol. The ethanol was replaced several times until tissues were completely cleared and GUS-staining was clearly visible. Tissues were stored in 70 % ethanol until examined by microscopy.

3.13.5 *In vivo* detection of H_2O_2 by 'DAB uptake' method

H_2O_2 accumulation *in planta* was visualized by DAB (3-3'-Diaminobenzidine) staining. Plant tissues to be examined for hydrogen peroxide were vacuum infiltrated for 3 min

with DAB-HCl solution (1 mg/ml, pH 3.8) and incubated 1 hr at RT. The stain was poured off and chlorophyll was removed by incubating in 96% (v/v) boiling ethanol for 10 min. DAB is polymerized locally in the presence of H_2O_2 and peroxidase activity giving a visible reddish-brown staining.

3.14 Yeast Protocols

Plates to be used for yeast growth: 20g/L Difco Agar.

YPD medium: 20g/L Difco peptone
10g/L Yeast extract
pH adjusted to 6.5
100 ml of 20% Glucose per litre of YPD after autoclave

YPDA medium: YPD medium + 15 ml of 0.2% adenine hemisulphate

SD medium: Minimal SD base with dextrose (glucose) and specific dropout (DO) supplements were purchased from Clontech. SD medium with appropriate DO were prepared according to manufacturers instructions. The pH of medium was adjusted to 5.8.

1M 3-amino-1,2,4-trizole (3-AT, Sigma): Prepared in MQ and filter sterilized.

Z buffer:

$Na_2HPO_4.7H_2O$	- 16.1 g/L
$NaH_2PO_4.H_2O$	- 5.50 g/L
KCl	- 0.75 g/L
$MgSO_4.7H_2O$	- 0.246 g/L

pH adjusted to 7.0 and autoclaved.

Z buffer/X-gal: 100 ml of Z buffer, 0.27 ml of β-mercaptoethanol, and 1.67 ml X-gal solution (from 20 mg/ml in DMF)

ONPG: A fresh solution of 4 mg/ml of ONPG (o-nitroohenyl-β-D-galactopyranosidase) in Z buffer was prepared each time. pH adjusted to 7.0.

Z buffer with β-mercaptoethanol: 0.27 ml of β-ME/100 ml of Z buffer.

1.1X TE/LiAc solution: 1.1 ml of 10X TE buffer, 1.1 ml of 10X LiAc (1 M), and 7.8 ml sterile MQ

PEG/LiAc solution: 8 ml 50% PEG 3350, 1 ml 10X TE buffer, 1 ml of 10X LiAc (1 M)

0.9% (w/v) NaCl solution: Prepared and autoclaved/ filter-sterilize

Yeast storage: Glycerol stocks of yeast clones were stored at - 70°C in a final concentration of 25% glycerol. Liquid nitrogen was avoided to snap freeze cells.

3.14.1 Preparation of yeast competent cells

The desired yeast strain was streaked on YPDA plate and incubated upside down at 30°C for 3 days. In a 15 ml falcon tube, a fresh colony was inoculated in 3 ml YPDA medium and kept at 30°C for 8-12 hr at 250 rpm. This culture was then transferred to 50 ml YPDA in a 250 ml flask and kept for growth at 30°C (250 rpm) until OD_{600} reached 0.15-0.3. In swinging bucket centrifuge set at RT, 50 ml of this culture was centrifuged at 700g for 5 min. The supernatant was discarded and cells were resuspended in 100 ml of YPDA. The culture in YPDA was grown until OD_{600} reached 0.4-0.5. At this OD culture was divided into two 50 ml falcon tubes and centrifuged at 700g for 5 min. The supernatant was discarded and each pellet of yeast cells was resuspended in 30 ml of sterile MQ. The yeast cells were pellet down at 700g for 5 min. Each pellet was resuspended in 1.5 ml of 1.1X TE/LiAc and the transferred to 1.5 ml eppendorf tubes. The cells in eppendorf tube were centrifuged at high speed for 15 sec and the supernatant was discarded. In each tube, the yeast pellet was resuspended in 600 µl 1.1X TE/LiAc solution. These cells were used for library and small-scale transformation within one hour of their preparation.

3.14.2 Transformation of yeast competent cells

Yeastmaker™ Yeast Transformation System 2 (Clontech) was both in small-scale and library scale transformation of yeast cells.

3.14.2.1 Small-scale transformation

In a pre-chilled eppendorf tube of 1.5 ml, 100 ng of plasmid DNA and 5 µl of denatured Herring testes carrier DNA (two times denatured at 95-100°C for 5 min) were mixed. To this mix 50 µl of yeast competent cells was added and mixed. Then to the yeast and DNA mix, 500 µl of PEG/LiAc solution was added and mixed. The tubes were incubated at 30°C for 30 min with mixing of cells every 10 min. To this incubated mix, 20 µl of DMSO was added and heat-shock was given for 15 min at 42°C with mixing of contents every 5 min. After heat-shock, the eppendorf tubes were centrifuged at high speed for 15 sec. The supernatant was discarded and cells were resuspended by vortex in 1 ml of

specially formulated YPD plus medium (supplied with kit). After incubation for 15-20 min at 30°C with shaking, cells were pellet down at high speed for 15 sec and suspended in 1 ml of 0.9% NaCl solution. This mix was plated on the selection plates of particular auxotrophy for plasmid selection.

3.14.2.2 Library-scale transformation

In a pre-chilled sterile 15 ml falcon tube; 20 µl of ds cDNA (using SMART technology), 6 µl of linearized prey plasmid (0.5 µg/µl) and 20 µl of denatured Herring testes carrier DNA (two times denatured at 95-100°C for 5 min) were mixed. To this mix 600 µl of yeast competent cells were added and mixed by gentle vortexing. Then to the yeast and DNA mix, 2.5 ml of PEG/LiAc solution was added and mixed. The tubes were incubated at 30°C for 45 min with mixing of cells every 15 min. To this incubated mix, 160 µl of DMSO was added, mixed and heat-shock was given for 20 min at 42°C with mixing of contents every 10 min. After heat-shock, the falcon tubes were centrifuged at 700g for 5 min. The supernatant was discarded and cells were resuspended in 3 ml of YPD plus medium (supplied with kit) by vortexing. After incubation for 90 min at 30°C with shaking, cells were pellet down at 700g for 5 min and suspended in 6 ml of 0.9% NaCl solution. This mix was plated on the selection plates of particular auxotrophy for plasmid selection.

3.14.3 cDNA library construction for yeast two-hybrid

The cDNA library for interacting partner isolation was prepared using Matchmaker™ library construction and screening kit (Clontech).

First-strand cDNA synthesis:

Following reagents were combined in a sterile 0.2 ml microcentrifuge tube: 1–3 µl total RNA (1µg), 1 µl CDS III and deionized sterile MQ was added to make the volume up to 4µl. Contents were mixed and tube was centrifuged briefly in a microcentrifuge. The tube was incubated at 72°C for 2 min, cooled on ice and spun briefly to collect the contents at the bottom. Following reagents were then added to the reaction tube: 2.0 µl 5X First-Strand Buffer, 1.0 µl DTT (20 mM), 1.0 µl dNTP Mix (10 mM) and 1.0 µl MMLV Reverse Transcriptase. Contents were mixed by gentle tapping and tube was given a quick spin. The reaction was incubated at 42°C for 10 min and then 1 µl SMART III oligonucleotide was added to it. The mix was incubated at 42°C for 1 hr in

an air incubator. After incubation, the reaction was terminated by incubating tube at 75°C for 10 min. The tube was then kept at RT and 2 units of RNase H was added to it followed by incubation of 37°C for 20 min. This first-strand cDNA was used as template in long-distance PCR of the next step mentioned.

Amplification of ds cDNA by Long Distance PCR (LD-PCR)

Two microlitres of first strand cDNA was aliquoted and placed in a clean, prechilled 0.2 ml eppendorf tube. Following components were added: 70 µl deionized water, 10 µl 10X advantage 2PCR buffer, 2 µl 50X dNTP mix, 2 µl 5′ PCR primer, 2 µl 3′ PCR primer, 10 µl of 10X GC-melt solution and 2 µl 50X advantage 2 polymerase mix to make the total volume of 100 µl. Contents were mixed, centrifuged briefly and 2 drops of mineral oil was added. Cycling conditions were as follows.

Denaturation	95°C for 30 sec	20 cycles
Annealing of primers	95°C for 10 sec	
Primer extension	68°C for 6 min	
Final extension	68°C for 5 min	

Seven microlitre of this PCR product was analyzed and it appeared as a smear between 500 bp to 2 kb on 1.2% agarose/EtBr gel.

Purification of ds cDNA with CHROMA SPIN^{TM} TE-400 column

The purification columns matrix was resuspended by inverting several times. The lower end of the columns was cut and the columns were placed in 15 ml falcon tubes with a cap-free 1.5 ml eppendorf tube to collect the flow-through. The falcon tubes with columns were centrifuged in a swinging bucket centrifuge at 700g for 5 min. The flow-through was discarded and the columns were again placed in falcon tubes. The earlier step amplified ds cDNA was applied directly at the centre of matrix in the columns and centrifuged at 700g for 5 min. The flow through was collected and mixed in a single tube. To this cDNA, 1/10 volumes of Sodium Acetate and 2.5 volumes of 95% ethanol (chilled at -20°C) was added, mixed and the tube was kept in -20°C freezer for overnight. Next day, the tubes were centrifuged at 14,000 rpm for 20 min at RT. The supernatant was carefully removed and ds cDNA was air dried for 10-15 min at RT. The cDNA was resuspended in sterile MQ and kept at -20°C until use. This ds cDNA was used for yeast two-hybrid library screening.

3.14.4 β-Galactosidase activity assay

The purpose of this experiment was to measure the relative transcription of a *LacZ* (β-Galactosidase) reporter gene. The β-galactosidase activity was assayed by measuring hydrolysis of the chromogenic substrate ONPG (Miller, 1972). The amount of o-nitrophenol formed was measured by determining the absorbance at 420 nm. When excess of ONPG was added, the amount of o-nitrophenol produced is proportional to the amount of β-galactosidase at the time of the reaction. Addition of Na_2CO_3 shifts the reaction pH to 11, which stops the reaction.

The method used here is adapted from Matchmaker^{TM} library construction and screening kit manual. Two millilitre of yeast culture was grown on selective medium overnight. In the next day, 8 ml of YPD medium was added to the overnight culture and the culture was further incubated for 3-5 hrs at 30 °C. After 3-5 hrs 1 ml of the culture was used to record the OD_{600}. When the culture reached mid-log phase (OD_{600} of 1 ml = 0.5-0.8), 1.5 ml of the culture was placed into three 1.5 ml microcentrifuge tubes and the tubes were centrifuged at 13,000 rpm for 30 seconds. The supernatant was removed and the cell pellet was resuspended in 1.5 ml of Z buffer. The cells suspension was centrifuged and the supernatant was removed. The cell pellet was resuspended in 300 µl Z buffer (the concentration factor is 1.5/0.3 = 5-fold) and 100 µl of the cell suspension were transferred into a fresh microcentrifuge tube. The tubes were placed into liquid nitrogen until the cells were frozen and then they were allowed to thaw at 37°C. This step was repeated twice to ensure that the cells have broken. For the ONPG assay, a blank tube with 100 µl of Z buffer was set, then 700 µl of Z buffer (+β-ME) were added to the samples and to the blank tubes. One hundred sixty microlitres of ONPG (4 mg/ml in Z buffer) was added immediately to the reaction tubes and the reactions were placed at 30°C until a yellow color develops. Four hundred microlitres of 1 M Na_2CO_3 was added to stop the reaction and the elapsed time was recorded in minutes. The reaction tubes were centrifuged for 10 min at 13,000 rpm to pellet cell debris and the supernatants was carefully transferred into clean tubes. The spectrophotometer was adjusted against the blank at OD_{420} and OD_{550}. The OD_{420} and OD_{550} of the reactions were measured relative to the blank.

$$\text{β-galactosidase units} = \frac{OD_{420} - (1.75 - OD_{550}) \times 1000}{OD_{600} * \text{time} * \text{volume}}$$

Time- time elapsed (in min) of incubation, Volume- 0.1 ml X Conc. Factor.

3.14.5 X-gal overlay assay

This assay was used to measure the qualitative activity of LacZ reporter genes in yeast. One percent of low-melting point agarose was added to Z-buffer and boiled. When the agarose became homogenous, it was cooled upto 40°C and the following reagents were added to a final concentration of; 6% of dimethylformamide, 0.1% of SDS, 0.25 mg/ml of X-gal, and 0.36% of β -mercaptoethanol. This agarose was poured upon the already spotted yeast cells on respective plates. The plate was wrapped in an aluminum foil and incubated at 30°C to develop the blue color.

Chapter 4

Isolation of CarWRKY1 gene & Expression analysis

4.1 Introduction

Activation of defense responses towards pathogen infection is associated with fine-tuned transcriptional regulation. Global gene expression profiling revealed that the major differences between PTI, ETI, basal defense, or SAR are quantitative and/or temporal rather than qualitative. The overlapping and graded transcriptional responses associated with various defense pathways indicate towards the existence of a complex regulatory circuit that is fine-tuned by transcriptional activators and repressors (Maleck *et al.*, 2001; Chen *et al.*, 2002; Scheideler *et al.*, 2002; Hahlbrock *et al.*, 2003; Tao *et al.*, 2003; Navarro *et al.*, 2004; Eulgem, 2005).

With the availability of genome sequence of model plants, *Arabidopsis* and rice, there is a shift from a 'gene-centric' to a 'genome-centric' perspective in plant biology. Transcription factors are the main players in genomic level expression studies. Members of various transcription factor families are implicated in plant defense. Few genes that appear in almost all the defense related transcriptional studies belong to ERF, TGA-bZIP, Myb, and WRKY family (Eulgem, 2005). Individual study of some members in these families revealed that a particular transcription factor could behave as positive and negative regulator depending upon host and pathogen that are under question. WRKY family members have been implicated in the regulation of defense transcriptome in response to elicitors and pathogens.

4.2 The WRKY transcription factors

The WRKY transcription factor superfamily consists of 74 and 102 members in *Arabidopsis thaliana* and *Oryza sativa*, respectively (Euglem and Somssich, 2007; Ross *et al.*, 2007). Although 74 genes are present in *Arabidopsis* genome but expressed cDNAs are available for 72 members till date (http://arabidopsis.org/browse/genefamily/WRKY.jsp). In other plants varying numbers of WRKY genes have been isolated; viz. *Nicotiana tabacum* (93), *Hordeum vulgare* (45), and *Glycine max* (64) (Mangelsen *et al.*, 2008; Rushton *et al.*, 2008; Zhou *et al.*, 2008).

The name of WRKY family is derived from the most prominent feature of its proteins i.e., the presence of conserved DNA-binding WRKY domain. This domain comprises of WRKYGQK peptide sequence and a zinc finger motif. These proteins generally binds to *cis*-element termed as W-box (C/TTGACT/C), although alternative binding sites are also known (Sun *et al.*, 2003; Cai *et al.*, 2008; Ciolkowski *et al.*, 2008;

van Verk *et al.*, 2008). Variants of the WRKYGQK signature peptide like WRKYGKK, WRKYGEK, and several other atypical motifs also exist (Wu *et al.*, 2005; Xie *et al.*, 2005). In terms of DNA-binding preferences, WRKYGQK type WRKY TFs binds to W-box while WRKYGKK type binds to WK-box (TTTTCCAC) (van Verk *et al.*, 2008). Maleck *et al.* (2000) found that W-box were overrepresented at 2.5-fold than statistically expected level in the promoters of a set of 25 *PR-1* coregulated genes, whereas Van Verk *et al.* (2008) found that WK-box were overrepresented 3.3-fold in this set. Moreover, in the 1kb upstream promoter regions of a set of 372 benzothiadiazole (BTH)-induced genes (Bülow *et al.*, 2007), the WK-box was found at twice the expected level, whereas the W-box was present at 1.4-fold.

WRKY family members are divided into three groups based on the number of WRKY domains and the features of their zinc-finger-like motif. Proteins with two WRKY domains belong to group I, whereas most proteins with one domain belong to group II. Group III with one WRKY domain has a zinc-finger motif pattern of C_2-HC (C-X_7-C-X_{23}-H-X_1-C) whereas group I and II has C_2-H_2 type motif (C-X_{4-5}-C-X_{22-23}-H-X_1-H). The single WRKY domains of the group-II and -III family members are more similar in sequence to the C-terminal WRKY domain of group-I. Although many WRKY genes were isolated from different plants before 2000, however Euglem *et al.*, 2000, presented the proper assembly and classification. The genes cloned earlier were from *Ipomoea batatas* (*SPF1*), *Avena fatua* (*ABF1, 2*), *Petroselinum crispum* (*PcWRKY1, 2, 3*), and *A. thaliana* (*ZAP1*). The NMR solution structure of AtWRKY4 and crystal structure of extended WRKY domain of AtWRKY-C is known but no crystal structure for full length WRKY is available (Yamasaki *et al.*, 2005; Duan *et al.*, 2007).

4.3 Origin and evolution of WRKY superfamily

Almost a decade ago, WRKY family was regarded as plant specific but recent ESTs data suggest that they are present in lower eukaryotes (Euglem *et al.*, 2000; Ülker and Somssich, 2004). The WRKY gene ESTs have been isolated from ferns (*Ceratopteris richardii*), mosses (*Physcomitrella patens*), green alga (*Chlamydomonas reinhardtii*), slime mold (*Dictyostelium discoideum*), and unicellular protist (*Giardia lambia*) (Eulgem and Somssich, 2007; Pan *et al.*, 2009). These WRKY ESTs resemble with that of the group I suggesting ancestral origin of this group, which dates back to 1.5-2 billion years ago. Although it is still in debate, Babu *et al.*, 2006 and Yamasaki *et al.*, 2008, proposed that *WRKY* genes have evolutionary links with transposons such as Mutator-

like elements and could have originated from a BED finger intermediate.

The comparative analysis of WRKY transcription factors in lower and higher plants indicates that the WRKY family expanded as plants have evolved from simpler (unicellular) to more complex (multicellular) forms. Rice group III genes have dramatically amplified due to tandem and segmental gene duplication as compared to *Arabidopsis*. This suggests that rice group III *WRKY* genes are evolutionarily more active and may have specific roles in monocots that are supposed to be most advanced and most successful in adaptability (Wu *et al.*, 2005; Zhang and Wang, 2005). Phylogenetic sequence analysis and comparative expression analysis in barley and *Arabidopsis* have revealed that *WRKY* genes have retained their functions (Mangelsen *et al.*, 2008). Zhang and Wang (2005) hypothesized that WRKY group II members originated from the loss of N-terminal WRKY domain of group I members. In any case, multiple domain acquisition and loss events appear to have shaped the WRKY family.

4.4 WRKY transcription factors and plant defense

WRKY genes are strongly and rapidly induced by pathogen infection, wounding, purified elicitors of various kinds, plant hormones, H_2O_2, and variety of abiotic stresses. From last ten years many publications have shown the importance of WRKY genes especially in biotic stress. In *Arabidopsis*, 49 out of 72 tested WRKY genes respond to bacterial infection and salicylic acid treatment (Dong *et al.*, 2003) and it is likely that more number may be activated during plant defense responses. They also control transcription of important regulators like *NPR1* of SA pathway (Yu *et al.*, 2001). Though the induction by salicylic acid of some genes is NPR1-independent, but NPR1 is required for full-scale induction of few genes like *AtWRKY53*, *AtWRKY54*, *AtWRKY58*, and *AtWRKY70* and for the expression of some genes its presence is must (Dong *et al.*, 2003; Wang *et al.*, 2006). Several groups have reported the importance of *AtWRKY70* in plant defense; it affects the balance between two signaling branches by promoting SA-dependent and suppressing JA-dependent responses (Li *et al.*, 2004). The *atwrky70* mutants are susceptible to bacteria *Erwinia carotovora* subsp *carotovora* and *Pst* DC3000 as well as to fungal biotroph *Erysiphe cichoracearum* (Li *et al.*, 2004; 2006), while the over-expression has opposite effect. Moreover, two T-DNA insertion mutants of *WRKY70* were susceptible to fungal necrotroph *Botrytis* (AbuQamar *et al.*, 2006), and it is required for full function of the SA-dependent *R*-gene *RPP4* against *Hyaloperonospora parasitica* (Knoth *et al.*, 2007).

In some plants, overexpression WRKY genes resulted in enhanced resistance to virulent pathogens. *Arabidopsis* plants overexpressing *AtWRKY29* showed enhanced resistance to *P. syringae* and *B. cinerea* (Asai *et al.*, 2002). *Nicotiana attenuata WRKY3* and *WRKY6* mediate plant's herbivore-specific defenses by differentiating mechanical wounding from herbivore attack at the level of jasmonic acid signaling (Skibbe *et al.*, 2008). Transgenic plants overexpressing *AtWRKY18 Arabidopsis* plants showed developmentally regulated enhanced PR genes expression and resistance to *P. syringae* (Chen and Chen, 2002). Ectopic overexpression of *AtWRKY33* in *Arabidopsis* increases resistance to necrotrophic fungal pathogens *Botrytis cinerea* and *Alternaria brassicicola,* while it increases susceptibility to *P. syringae* concomitant with the change in JA and SA mediated signaling pathways. The *wrky33* mutants have opposite effect to overexpression in response to fungal pathogens (Zheng *et al.*, 2006). Functional analysis based on T-DNA insertion mutants and transgenic overexpression lines indicates that *AtWRKY3* and *AtWRKY4* have positive role in *Arabidopsis* resistance to necrotrophic pathogen *B. cinerea* and *AtWRKY4* has a negative effect on plant resistance to biotrophic pathogens (Lai *et al.*, 2008). *OsWRKY13* act as an activator of rice resistance against bacterial blight and fungal blast by influencing physiological and metabolic pathway required for defense (Qiu *et al.*, 2007; 2008a). Overexpression of the *OsWRKY31* gene in rice was found to enhance resistance against infection with *Magnaporthe grisea* and negatively influences lateral root growth (Zhang *et al.*, 2008). Overexpression of the *OsWRKY89* gene in rice also enhanced resistance against blast fungus *M. grisea* and white-backed planthopper *Sogatella furcifera* as well as tolerance to UV-B irradiation (Wang *et al.*, 2007). Many other reports in various systems prove that WRKY genes improve tolerance against varied range of pathogens.

Several pathogen and elicitor inducible WRKY transcription factors act as negative regulators to fine-tune of plant defense responses. For instance, basal plant resistance triggered by a virulent *P. syringae* strain was enhanced in *Atwrky7,* and *Atwrky11/17* mutants (Journot-Catalino *et al.*, 2006; Kim *et al.*, 2006). Similarly, *AtWRKY48* also contributes negatively to basal resistance towards this bacterial pathogen (Xing *et al.*, 2008). The *OsWRKY62* gene, encodes for two splice variants (*OsWRKY62.1* and *62.2*) and interacts with Xa21, act as negative regulator of both basal and race-specific defense responses (Peng *at al.*, 2008). VIGS mediated gene silencing of *Capsicum annum CaWRKY1* resulted in decreased growth of *Xanthomonas axonopodis* pv. *vesicatoria* race1 while overexpression in tobacco accelerated the HR-related cell

death in response to TMV and *P. syringae* pv. *tabaci* (Oh *et al.*, 2008). Knockdown of *AtWRKY23* expression was shown to decrease susceptibility toward the parasitic cyst nematode *Heterodera schachtii* (Grunewald *et al.*, 2008). Mutations in *AtWRKY27* resulted in delayed symptom development in response to the bacterial wilt pathogen *Ralstonia solanacearum*, possibly by affecting nitric oxide signaling or trafficking between the phloem and the xylem (Mukhtar *et al.*, 2008).

4.5 Regulation of WRKY transcription factors

The majority of WRKY genes isolated from different plants respond to pathogenic stimuli. They are part of a complex and highly interconnected network that regulates various cellular processes in normal and stress conditions. The *WRKY* genes are regulated at all levels i.e., transcription, translation and at functional level by degradation, phosphorylation, protein-protein interaction, etc. Several *WRKY* genes are under direct positive or negative control of their own family members via specific feedback mechanisms (auto/ cross-regulation). Studies have shown that PAMP and pathogen responsive WRKY genes have unique arrangements of W-boxes in their promoters like *PcWRKY1* (Euglem *et al.*, 1999), *AtCMPG1* (Heise *et al.*, 2002), *AtTRXh5* (Laloi *et al.*, 2004), and *AtWRKY33* (Lippok *et al.*, 2007). A positive regulator of senescence and plant defense, AtWRKY6 was found to suppress its own promoter activity as well as that of closely related WRKY members (Robatzek and Somssich, 2002). *AtWRKY53* seems to be regulated by epigenetic mechanisms as chromatin reorganization takes place at its locus during senescence (Ay *et al.*, 2009). This gene is regulated by a MAP kinase kinase kinase (AtMEKK1) during defense and senescence as MEKK1 binds to its promoter and also phosphorylate it (Miao *et al.*, 2007). Six different proteins, including OsWRKY13, were identified in a yeast one-hybrid screen that binds to functionally important cis-regulatory DNA elements within the rice *OsWRKY13* promoter. However, their function needs to be tested.

Physical interactions within themselves and with other proteins are also necessary for their efficient function. *Arabidopsis* AtWRKY18, -40, and -60 forms homo- and hetero-dimers that imparts overlapping, antagonistic, and distinct roles to them during plant response to different type of pathogens (Xu *et al.*, 2006). Both AtWRKY38 and AtWRKY62 function additively as negative regulators of plant basal defense, but Histone Deacetylase19 (HD19) a positive regulator of basal defense interacts with them to balance defense response against bacterial pathogen (Kim *et al.*, 2008). Confusion still

exist on the role of NPR1 in regulation of certain WRKY genes as different groups uses different techniques. However, the expression of at least eleven *WRKY* genes, *AtWRKY18*, *-30*, *-38*, *-53*, *-54*, *-58*, *-59*, *-62*, *-66*, *-70*, and *-71* is dependent on NPR1 in SA pathway, suggesting that they may be under the control of TGA factors (Dong *et al.*, 2003; Wang *et al.*, 2006). Likewise, *AtWRKY51* also seems to be under the control of TGA2 as concluded from ChIP and whole-genome array analyses (Thibaud-Nissen *et al.*, 2006). In barley, negative regulation of defense by HvWRKY1/2 is disrupted when MLA10 (a CC-NBS-LRR type R protein) interacts with these transcription factors in an effector (Avr_{A10}) dependent manner (Shen *et al.*, 2007). Group-IId WRKYs seem to be regulated by pathogen triggered fluctuations in intracellular Ca^{2+} level as AtWRKY7 interact with calmodulin through its conserved structural motif (C-motif) (Park *et al.*, 2005). *Arabidopsis* MAP kinase 4 (MPK4) represses SA-dependent resistance and acts as regulator of plant defense through MKS1 (MAP kinase 4 substrate1) that interacts with AtWRKY25 and WRKY33 (Andreasson *et al.*, 2005). Upon infection by virulent *P. syringae*, MPK4 is phosphorylated, thereby releasing MKS1 and WRKY33 and thus allowing recruitment of WRKY33 to the promoters of target genes like *PAD3* and *CYP71A13* (Qiu *et al.*, 2008b). Atypical WRKY protein, RRS1 (AtWRKY52), interacts with *R. solanacearum* effector PopP2 (*Pseudomonas* outer protein P2) and gets targeted to nucleus. A cysteine protease RD19 (RESPONSIVE TO DEHYDRATION19) also interacts with PopP2 to form an active immune complex in nucleus (Deslandes *et al.*, 2003; Bernoux *et al.*, 2008). Several WRKY genes are the predicted targets of miRNAs suggesting smRNA-mediated regulation of WRKY transcription factors (Pandey and Somssich, 2009).

4.6 Role of WRKY genes in development and metabolism

Besides playing important role in plant defense, *WRKY* genes are also implicated in plant developmental processes, such as trichome and seed coat development, seed size regulation, senescence, somatic embryogenesis, phosphate acquisition, root development, lethality in interploidy crosses, regulation of growth hormone signaling, primary and secondary metabolism, etc. The *Arabidopsis* gene *TRANSPARENT TESTA GLABRA2* (*TTG2*/ *AtWRKY44*) plays a key role in trichome development, and mucilage and tannin synthesis in the seed coat (Johnson *et al.*, 2002). This gene is also involved in creating interploidy hybridization barriers (Dilkes *et al.*, 2008). The barley abscisic acid induced *HvWRKY38* gene is involved in salicylic-mediated suppression of seed

germination that is regulated by gibberellins (Xie *et al.*, 2007). Rice WRKY protein OsWRKY71 acts as a transcriptional repressor of GA signaling, while *OsWRKY24* encodes a protein that functions as negative regulator of abscisic acid and gibberellins signaling, in aleurone cells (Zhang *et al.*, 2004; 2009). The *Arabidopsis MINISEED3 (MINI3)*/AtWRKY10 regulates seed growth and size by regulating cellularization of the endosperm (Luo *et al.*, 2005). In *Arabidopsis, AtWRKY75* gene was found to be the regulator of phosphate starvation responses as well as root development and recently, as a positive regulator of plant defense (Devaiah *et al.*, 2007; Encinas-Villarejo *et al.*, 2009).

Down regulation and ectopic expression of a group-II WRKY gene from *Coptis japonica*, *CjWRKY1*, resulted in altered expression of all known genes involved in berberine biosynthesis, suggesting its importance as a positive regulator in production of this anti-microbial agent (Kato *et al.*, 2007). In barley, a group-I WRKY protein SUSIBA2 (sugar signaling in barley) acts as a regulatory factor in starch synthesis and carbohydrate anabolism (Sun *et al.*, 2003).

4.7. Results and discussion

To identify the genes involved in early molecular responses of a susceptible chickpea plant upon *Ascochyta rabiei* infection, at least three forward Suppression Subtractive Hybridization (SSH) cDNA libraries from early stages of infected tissues were generated and sequenced earlier in the laboratory. These three libraries were generated with following samples:

1. CarSSH1 library- Pusa 362 (susceptible) chickpea plants collected after 24h of *Ascochyta rabiei* spore inoculations.
2. CarSSH2 library- Pusa 362 (susceptible) chickpea plants treated with cyclohexamide (CHX) 30 min prior to the spore inoculation and samples were collected after 3h of *Ascochyta rabiei* spore inoculations.
3. CarSSH3 library- FLIP84-92C(2) (resistant) and PI359075 (susceptible) chickpea plants inoculated after 3h of *Ascochyta rabiei* spore inoculations.

Nearly 350 unique genes of chickpea were isolated from these libraries. Various genes related with basal defense, signaling, and transcription regulators were present in these libraries. A list of various transcription factors isolated is given in table 4.1. Among these transcription factors, based on expression analysis and literature available, an EST

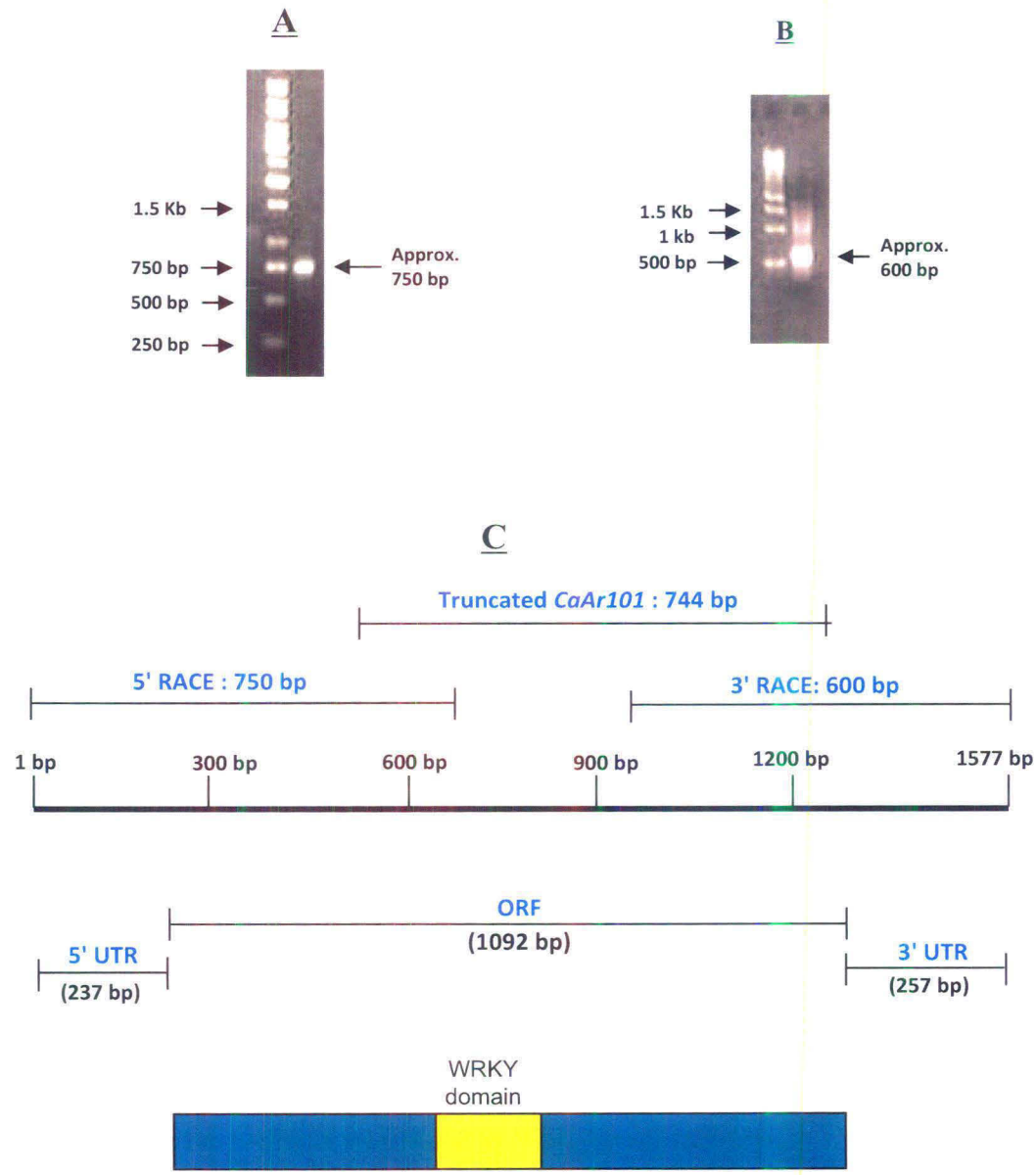


Fig. 4.1 Full length cDNA isolation of *CarWRKY1* using 5′ and 3′ RACE.

(A) 5' RACE and **(B)** 3' RACE PCR products resolved on 1% agarose gel along with 1kb DNA ladder. **(C)** Schematic representation of the 5' and 3' RACE results with ORF and UTRs.

that the obtained sequence in 3'-RACE was part of *CarWRKY1* cDNA. Therefore, the full-length cDNA of 1586 bp was obtained and the gene was named as *CarWRKY1*, as this was the first WRKY gene isolated from chickpea. We have submitted its sequence in public database under GenBank accession number EU049488.

4.7.3 *In-silico* analyses of *CarWRKY1* sequence

The *CarWRKY1* cDNA contains an open reading frame (ORF) of 1092 bp that encodes for a protein of 364 amino acids. This ORF is flanked with 5'- and 3'-untranslated regions (UTRs) of 237 bp and 257 bp respectively. The deduced protein sequence of CarWRKY1 has a single WRKY domain with a C_2-H-C type zinc-finger-like motif (i.e., C-X_7-C-X_{23}-H-X_1-C) suggesting that this gene is a group-III member of WRKY superfamily (Euglem *et al.*, 2000) (fig. 4.2). The tblastx analysis of the ORF showed very high homology with some of the known WRKY proteins of various plants (Table 4.2).

Table 4.2 Proteins showing high homology with CarWRKY1 in tblastx analysis.

Results of tblastx	Size (amino acid)	Identity (%)	Similarity (%)
GmWRKY20 *Glycine max* (ABS18424)	268 (truncated)	69	81
PtWRKY *Populus trichocarpa* (XP_002304549)	342	53	66
RcWRKY *Ricinus communis* (XP_002509941)	338	52	65
BgWRKY *Bruguiera gymnorhiza* (BAG15875)	341	51	62
VvWRKY*Vitis vinifera* (XP_002272720)	342	47	60
CaWRKY30 *Capsicum annuum* (ACJ04728)	364	45	61
VaWRKY30 *Vitis aestivalis* (AAR92477)	349	44	57
AtWRKY53 *Arabidopsis thaliana* (NP_194112)	324	44	57
AtWRKY41 *Arabidopsis thaliana* (NP_192845)	313	38	52

High homology within WRKY domain region confirms the importance of this domain (Fig. 4.3). Interestingly, two closest homologs from *Arabidopsis* AtWRKY53 and -41 showed only 52% identitity (65% similarity) within themselves and both showed 38-44% homology with CarWRKY1. The divergence of ~50% (at amino acid level) between two group-III homologs of plant specific proteins from two different plants i.e., Chickpea and *Arabidopsis*, suggest that the WRKY group-III proteins have diversified at a faster rate. This conclusion was also derived from extensive studies on rice group-III genes (Zhang and Wang, 2005). The phylogenetic analysis of *Arabidopsis* group-III proteins and CarWRKY1 suggests closeness of AtWRKY53 and -41 (99% bootstrap value) (Fig. 4.4). The estimated molecular mass of CaWRKY1 protein is approximately

Fig. 4.2 The complete cDNA of CarWRKY1 with deduced protein sequence.

The translational start site and stop codon are marked with green and red respectively. The WRKYGQK amino acids are bold in blue color. Three cysteines and one histidine in the putative zinc-finger motif are bold and underlined. The probable nuclear localization signal (KKRK) is highlighted with orange color.

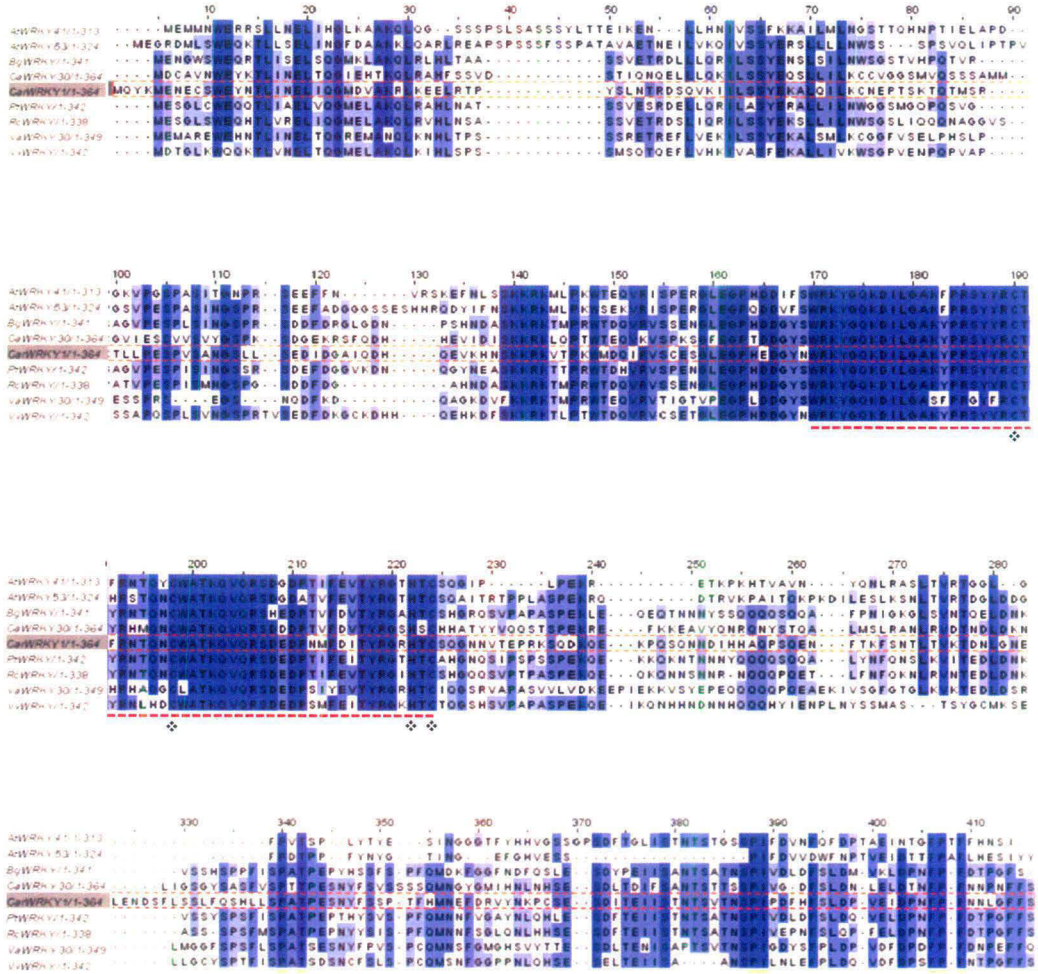


Fig. 4.3 Multiple alignment of CarWRKY1 sequence with other WRKY proteins.

Homologs from various plants were selected after tblastx. The amino acid sequences aligned are: *A. thaliana* WRKY41 and 53 (AAL35289 and AAK28442), *Bruguiera gymnorhiza* (BAG15875), *Capsicum annum* (ACJ04728), *Cicer arietinum* (EU049488), *Populus trichocarpa* (XP_002304549), *Ricinus communis* (XP_002509941), *Vitis aestivalis* (AAR92477), and *Vitis vinifera* (XP_002272720) Regions with high sequence similarity are shown in dark blue shade followed by light blue which are less similar. The WRKY domain is underlined and the zinc-finger ligands (C_2-H-C) are marked with star.

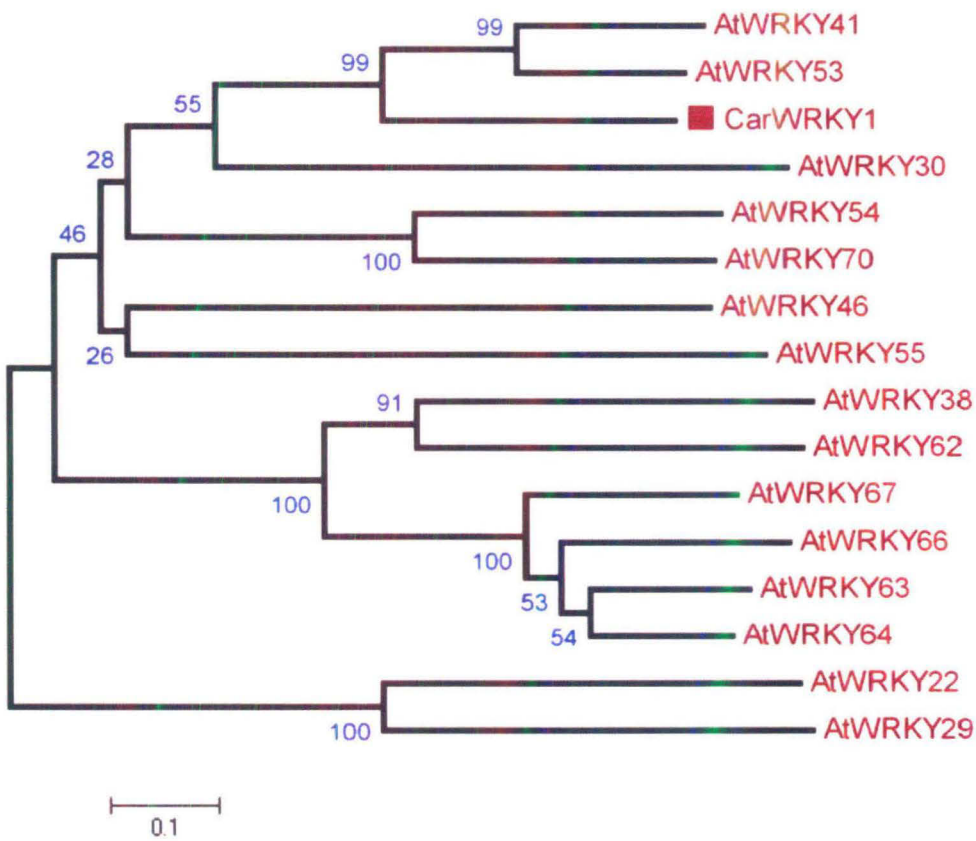


Fig. 4.4 Phylogenetic relationships between *Cicer arietinum* CarWRKY1 and *Arabidopsis thaliana* WRKY group III members.

The deduced amino acid sequence of CarWRKY1 was aligned with WRKY group III proteins of *Arabidopsis* using ClustalW and then tree was created using MEGA 4 software. Number along branches are bootstrap percentage values. AtWRKY22 and AtWRKY29 were taken as outgroup.

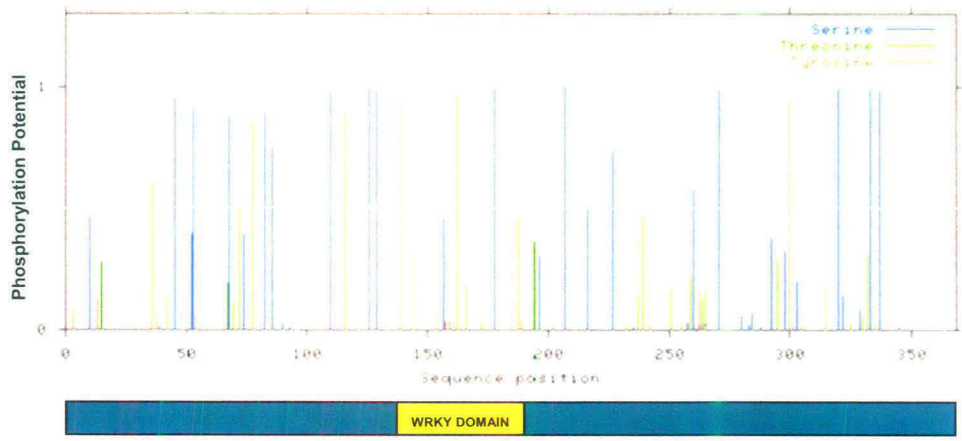
41.8 KDa and with a pI of 5.64. The gene structure of the CarWRKY1 agrees with most of the group-III members where an intron is placed in WRKY domain. The DNA binding activity and localization of transcription factor is modulated by phosphorylation in many cases, therefore potential MAPKs and protein kinase C phosphorylation sites were examined by online programs (Fig. 4.5). No clear additional structural features described in other WRKY proteins were found in CarWRKY1 protein sequence using the online programs used for analysis.

4.7.4 *Ascochyta rabiei* induces *CarWRKY1*

Many of the *WRKY* genes are known to get rapidly and strongly upregulated in response to pathogen infection. This may be due to PAMP or secreted elicitor responsiveness of these genes. As an initial step to evaluate the involvement of *CarWRKY1* in regulation of chickpea defense responses against *Ascochyta rabiei* infection, the expression pattern was analyzed by northern-blot analysis after challenging resistant [FLIP84-92C(2)] and susceptible [Pusa 362] chickpea cultivars with *Ascochyta* spores. FLIP84-92C(2) is known to have resistance against both pathotypes I and II of *Ascochyta rabiei* (Cho *et al.*, 2004). The northern blot showed biphasic induction kinetics. A low-level expression at 3 hpi (hours post inoculation) was observed and the induction at 12 hpi reaches to maximum at 24 hpi. No difference was observed in expression kinetics between resistant [FLIP84-92C(2)] and susceptible variety [Pusa 362] (Fig. 4.6). This expression kinetics was confirmed by biological replicates that again showed similar kinetics. The similar expression kinetics observed in both susceptible and resistant varieties correlates with the earlier observations of similar growth pattern of *Ascochyta* spores on resistant and susceptible varieties. Ilarsian and Dolar (2000) reported that *Ascochyta rabiei* spore germination, hyphae development, and slight swelling of appressoria on surfaces of both susceptible and resistant chickpea cultivars are identical. The biphasic nature of transcript induction in fungal infection is also observed for other defense-related genes like phenylalanine ammonia lyase (*PAL*) (Schmidt *et al.*, 2004).

4.7.5 Defense related hormones and wounding induces *CarWRKY1*

In order to examine whether salicylic acid activates *CarWRKY1,* the transcript expression pattern was analyzed after spraying Pusa-362 chickpea cultivar with 5 mM salicylic acid (SA). Three weeks old plants were given SA treatment both in aseptic environment and under green house conditions. Similar expression pattern was observed,



Position of amino acid	Context	score	Phosphorylated residues
45	NTRDSQVKI	0.950	S
53	ILLSSYEKA	0.916	S
110	VKHNSKKRK	0.982	S
126	QIRVSCESG	0.995	S
129	VSCESGLEG	0.985	S
178	QVQRSDEDP	0.997	S
207	EPRKSQDKQ	0.997	S
270	QENHSWVPP	0.989	S
320	NKPCSESDI	0.992	S
333	STNTSVTNS	0.987	S
337	SVTNSPIPD	0.985	S
162	YYRCTFRNT	0.996	T
139	HEDGYNWRK	0.929	Y
300	PESNYFSSP	0.950	Y

Fig. 4.5 NetPhos 2.0 prediction of putative phosphorylation sites of CarWRKY1.

Graph and CarWRKY1 cartoon shows the relative score and position of putative phosphorylation sites on CarWRKY1 protein sequence. Eleven serine, one threonine, and two tyrosine phosphorylation sites with score are depicted.

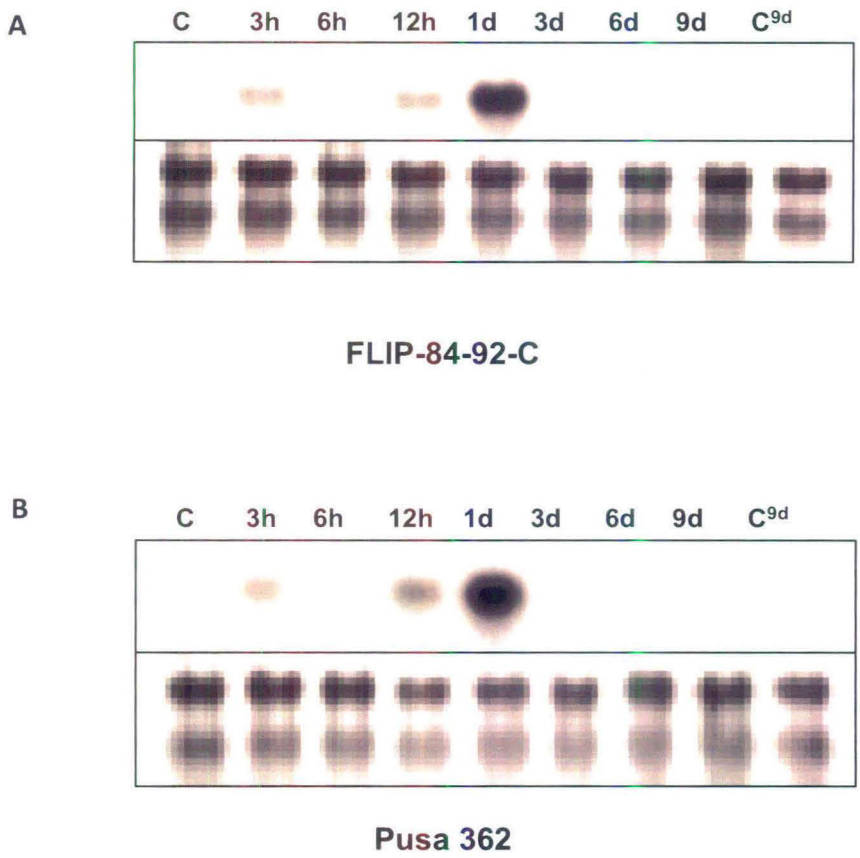


Fig. 4.6 Expression of *CarWRKY1* gene during *Ascochyta rabiei* infection on *Cicer arietinum*.

Total RNA was isolated from indicated time point after *A. rabiei* spore inoculation on chickpea resistant variety FLIP-84-92-C **(A)** and susceptible Pusa 362 **(B)**. RNA was fractionated on 1.2% denaturing agarose gel before transferring it to nylon membrane and hybridization with *CarWRKY1* full length cDNA as probe.

which validates the expression kinetics under both the kinetics. The transcript level reached maximum within 1 hr of SA spray confirming early SA mediated induction of *CarWRKY1* (Fig. 4.7A). Salicylic acid mediated induction of genes in *Arabidopsis* is NPR1 dependent and independent also. The SA mediated expression of most members of *Arabidopsis* WRKY group-III is NPR1-dependent but few show NPR1 independent expression in response to SA. The *Arabidopsis AtWRKY53* seem to be interesting in this aspect as its early induction by salicylic acid is NPR1-independent but for maximum level induction NPR1 is required (Yu *et al.*, 2001; Kalde *et al.*, 2003). The induction of *CarWRKY1* in *Ascochyta* infection and externally applied SA suggest that in chickpea defense against *Ascochyta* endogenous SA levels may increase. The salicylic acid is formed in plants through two different pathways one using phenylalanine ammonia lyase and the other using iso-chorismate synthase enzyme. It will be interesting to check whether some correlation exists between *CaWRKY1* induction and the increase in activity of these two enzymes leading to SA biosynthesis. As such, SA-pathway in also required for phytoalexin biosynthesis that plays important role in defense against necrotrophic fungi.

The *CarWRKY1* transcript level was also modulated by 100 µM of JA treatment. The maximum level of transcript was observed after 6 hr of JA treatment that seemed slower and lower than SA mediated induction (Fig. 4.7B). In a study, Cho *et al.* (2004) has proposed that *Ascochyta* blight resistance in chickpea is SA and JA-independent, and the susceptible varieties are insensitive to SA and JA. Although there is no difference between the expression pattern of *CaWRKY1* in resistant and susceptible varieties, but Pusa-362 doesn't seem to be insensitive to SA- and JA- treatments as it responds to these treatments rapidly. Jasmonic acid and ethylene mediated pathways helps in building resistance against necrotrophic fungi. Therefore, the induction of *CarWRKY1* by SA and JA hormones also showed its significance during defense.

The *CarWRKY1* gene also responds to wounding within 30 min of mechanical injury and this transcript level is maintained up to 1hr and then decreases (Fig. 4.7C). During the *Ascochyta rabiei* infection wounding takes place at sites of fungal invasion. In an earlier report, in wounding and *Ascochyta* infection of chickpea, induction of copper amine oxidase (CuAO) gene has been shown.

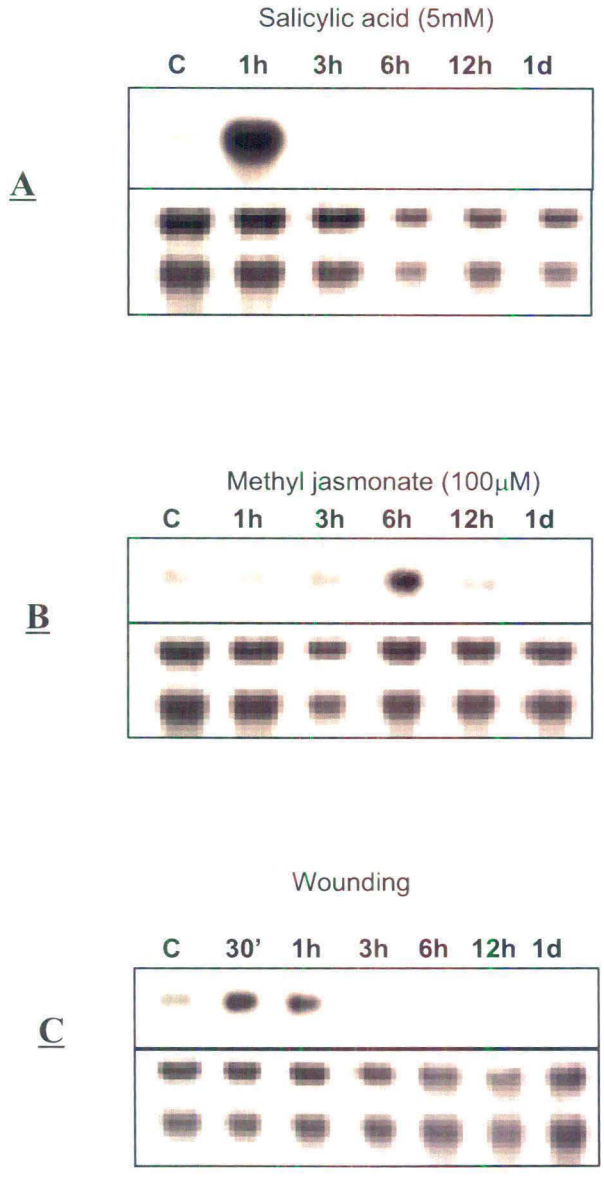


Fig. 4.7 Expression of *CarWRKY1* transcripts in response to chemical treatments and wounding.

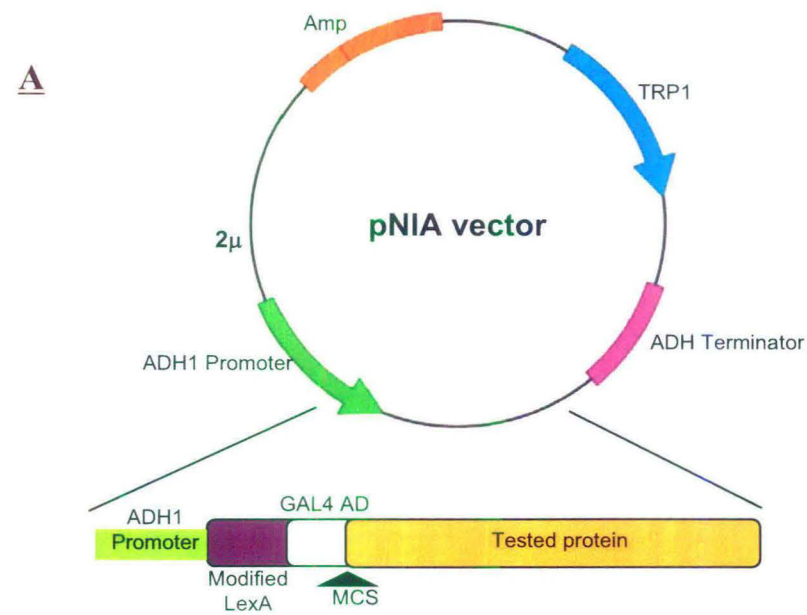
Total RNA was isolated from chickpea variety Pusa362 at the indicated time point samples after exposure to (A) Salicylic acid (B) Jasmonic acid (C) and Wounding.

4.7.6 Nuclear localization of CarWRKY1

In order to check the nuclear localization, a method developed in yeast system was used (Rhee *et al.*, 2000). In this system, the bacterial LexA protein was modified (mLexA) to abolish its intrinsic nuclear localization signal (NLS) and fused to the activation domain of the yeast Gal4p (Gal4AD). This fusion protein cannot localize to nucleus by itself. In case, a tested protein fused to mLexA-Gal4AD contains a functional NLS, it will enter the yeast cell nucleus due to its intrinsic NLS. Once the fusion protein is inside the nucleus, its LexA DNA binding domain will bind to LexA operator sequences and activation domain will activate the reporter gene expression. We made a translation fusion construct of CarWRKY1, in the pNIA vector using *Bam*HI and *Pst*I restriction enzyme sites (Fig. 4.8A). The pNIA-CarWRKY1 construct was transformed in the yeast L40 strain competent cells and the transformants were selected on SD/-Trp plates. Along with the experimental construct, we also transformed positive control having VirD2 gene and negative control having VirE3 gene.

The nuclear import assay was performed by spotting of yeast cells. The *CarWRKY1*, positive control and negative control construct transformed yeast cells were spotted on SD/-Trp and SD/-Trp/-His plates. The growth of positive control and CarWRKY1 containing yeast cells on SD/-Trp/-His showed nuclear localization of both the proteins. The appearance of blue color in X-gal overlay assay also confirms the nuclear localization of CarWRKY1 (Fig. 4.8B). This result proves that CaWRKY1 possess a functional nuclear localization signal. After proving the nuclear localization of CarWRKY1 in yeast, we made a translational fusion construct of CarWRKY1 with the N-terminal of green fluorescence protein (GFP). The construct was made by cloning ORF of CarWRKY1 in a modified pBI121 vector. This modified pBI121 vector has *mGFP5* gene cloned downstream to multiple cloning site. We have transformed this construct in *Nicotiana tabacum* cv. SR1. The analysis of this CarWRKY1 fusion protein will be interesting as some genes shuttle between nucleus and cytoplasm under certain conditions.

The region that acts as a functional NLS is yet to be proved in WRKY transcription factors. Many groups have predicted a peptide stretch of KKRK, towards N-terminal of WRKY domain, as a probable NLS as it is very conserved in many WRKY proteins (Fig. 4.3) (Xu *et al.*, 2004). Available online softwares also failed to predict the NLS region though they have predicted the nuclear localization of CarWRKY1.



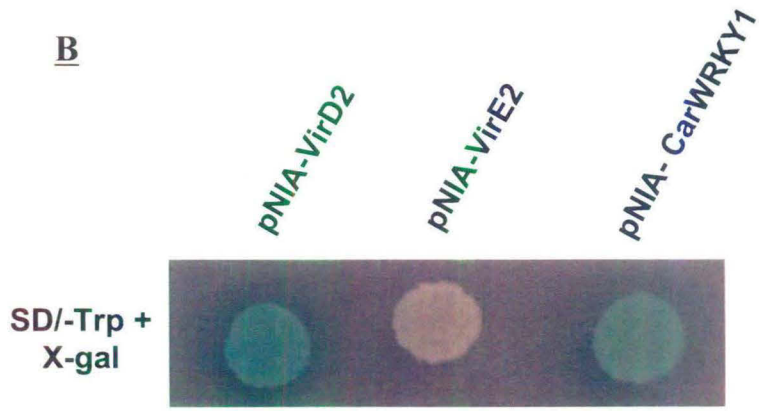


Fig. 4.8 Yeast based nuclear import assay (yeast NIA).

(A). Plasmid map of pNIA (a modified vector suitable for NIA assay). **(B)** X-gal agarose overlay assay to check the activation of *LacZ* reporter gene in yeast strain L40; VirD2, VirE2, and CaWRKY1 are positive control, negative control and experimental constructs, respectively. Blue color indicates the localization of protein in the nucleus.

4.7.7 Identification of transcriptional activation domain in CarWRKY1

Several classes of activation domains have been identified (Triezenberg, 1995) but a clear patch of amino acids that may behave as transcriptional activation domain was missing in CarWRKY1. To confirm that CarWRKY1 act as a transcriptional activator, the full-length protein needs to be used as an effector construct in yeast one-hybrid system. For the same, we have cloned CarWRKY1 gene in pGBKT7 at restriction sites *Nco*I and *Bam*HI. This resulted in a fusion protein of GAL4-DNA-binding domain and CarWRKY1. The pGBKT7-CarWRKY1 construct and a negative control (pGBKT7 vector alone) were transformed separately in AH109 strain. The transformed colonies were selected on SD/-Trp plates. These transformed yeast cells were assayed for transcriptional activation by spotting on Sd/-Trp and SD/-Trp/-His/-Ade plates. The growth on SD/-Trp/-His/-Ade confirmed the transcriptional activation property of CarWRKY1.

To delineate the transcriptional activation domain, we have developed deletion constructs from C-terminal of CarWRKY1. The AtWRKY53 protein also have C-terminal end region that behaves as a transcriptional activation domain (Miao *et al.*, 2004) (Fig. 4.9B). These deletion constructs were cloned in pGBKT7 vector at *Nco*I and *Bam*HI restriction sites using primers shown in table 4.3. After transformation of these constructs in AH109 and selection on SD/-Trp plates, the expression of the *LacZ* reporter gene was visualized by an X-gal agarose overlay assay and was quantified by a β-galactosidase assay. The β-galactosidase activity is considered equivalent to the trans-activation potential. The table 4.3 shows the summary of the constructs and their β-galactosidase activity obtained from them. The N-terminal deletion construct showed higher transcriptional activation potential (almost double of the full-length protein), which suggests that N-terminal may behave as a negative regulator of transactivation domain or the structure of this truncated CarWRKY1 construct favors transactivation. The result showed that C-terminal region (~30 amino acid) is responsible for major transcriptional activity in yeast.

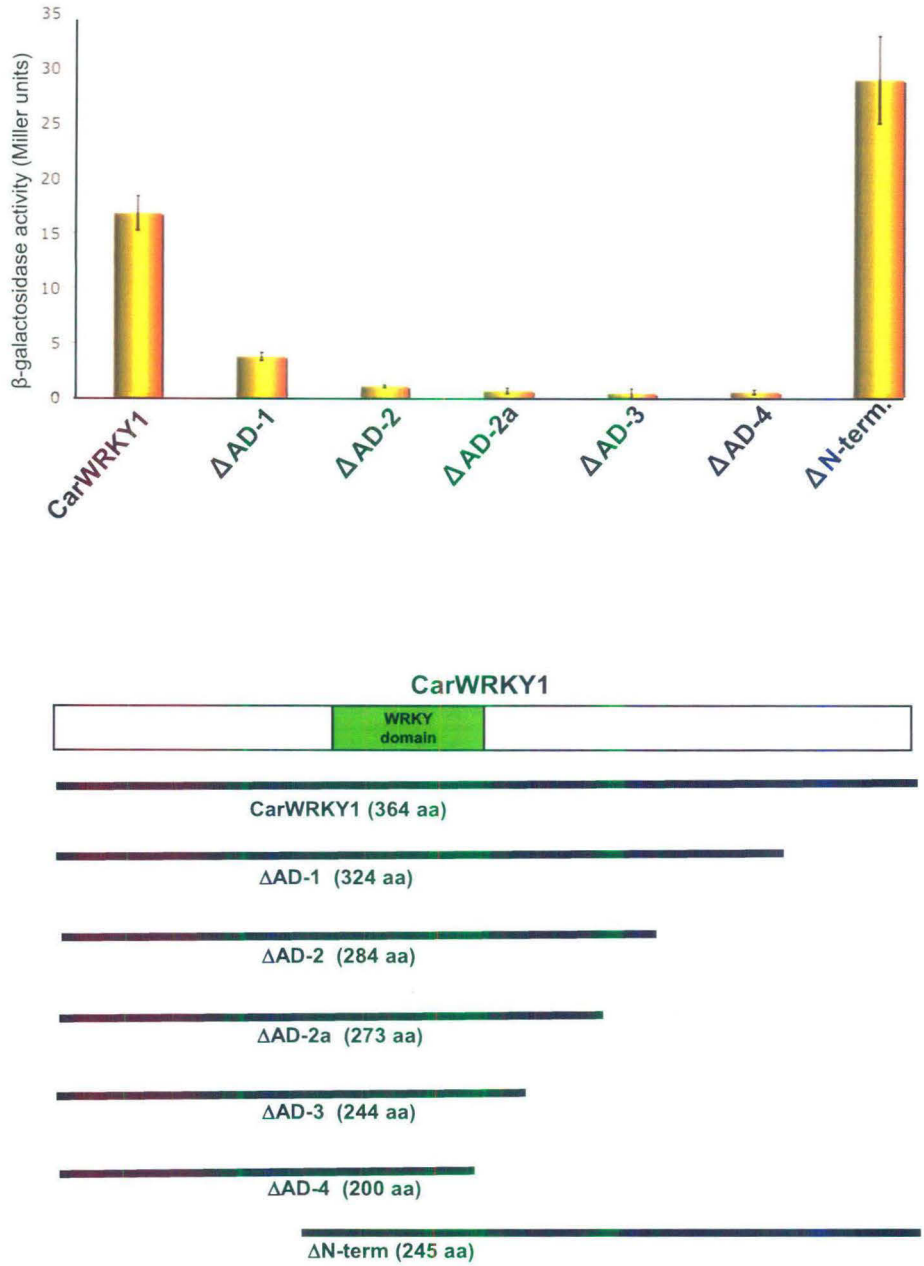


Fig. 4.9 Transcription activation analysis of CarWRKY1 in yeast.

(A). Transcriptional activation potential of CarWRKY1 constructs in yeast cells analyzed with β-gal assay using ONPG as substrate. **(B)**. Schematic representation of various deletion constructs of CarWRKY1.

Table 4.3 Summary of yeast one-hybrid constructs and transcriptional activation assay results. Primers sequences are shown in section 3.14.4.

S. No.	Constructs	Forward primer	Reverse primer	Size of protein (amino acids)	beta-gal. activity (SD±)
1.	CarWRKY1	KT7EW1F	KT7EW1R	364	16.81 (1.57)
2.	ΔAD1	KT7EW1F	W1ADR1	324	3.74 (0.35)
3.	ΔAD2	KT7EW1F	W1ADR2	284	1.03 (0.12)
4.	ΔAD2a	KT7EW1F	W1ADR5	273	0.61 (0.26)
5.	ΔAD3	KT7EW1F	W1ADR3	244	0.37 (0.45)
6.	ΔAD4	KT7EW1F	W1ADR4	200	0.50 (0.21)
7.	ΔN-term	W1ADF2	KT7EW1R	245	29.01 (3.99)

4.7.9 Isolation of other *WRKY* genes from Chickpea

To isolate other members of WRKY family from chickpea those are induced by *Ascochyta rabiei* infection, primers were designed from conserved WRKY domain. The cDNA template used for this work was made from the pooled RNA, isolated from different time points of *A. rabiei* infection on chickpea. The total RNA from 6h, 12h and 24h *Ascochyta rabiei* infected Pusa 362 chickpea samples was pooled and cDNA was constructed using oligo(dT) primer. Using this cDNA as template, a PCR was performed with WRKY domain specific primer and a primer against the oligo (dT) adaptor region. The amplified products were cloned in PCR cloning vector (pDRIVE, Qiagen). Size variants of colony PCR were further selected for sequencing. The analysis of eighty-seven sequenced clones with tBLASTx showed that many of them are WRKY genes and the *in silico* CAP3 analysis showed that many of them were redundant. This work resulted in isolation of five WRKY transcription factors, out of which four were new and also the earlier isolated *CarWRKY1*. The results of this exercise are summarized in table 4.3.

Table 4.3 WRKY family members isolated from chickpea.

S. No.	Name	Redundancy	Homology with	WRKY subfamily
1.	CarWRKY1	12	Glycine max GmWRKY20	Group III
2.	CarWRKY2	16	WRKY4 of *Vitis*	Group II
3.	CarWRKY3	4	SPF1 of Sweet potato	Group I
4.	CarWRKY4	1	WRKY4 of *Vitis*	Group II
5.	CarWRKY5	1	SPF1 like of *Cucumis*	Group I

Isolation of full-length sequences of these new genes and their expression analyses will give insight into the importance of these WRKY family members in

chickpea defense response. Recent reports have showed the importance of WRKY members in defense mechanism against necrotrophic fungi (AbuQamar *et al.*, 2006; Zheng *et al.*, 2006).

4.7.9 Conclusion

The WRKY superfamily of transcription factors are induced in many stresses and play a major role plant defense by maintaining the complex regulatory circuits. Their amplification in higher plants is correlated with the evolutionary success of highly adapted land plants. Here, we have shown the isolation and characterization of a WRKY group-III transcription factor from *Cicer arietinum*, *CarWRKY1*. Our data showed that it is highly induced in response to *Ascochyta rabiei* infection, salicylic acid, jasmonic acid and wounding at transcriptional level. This seems to be interesting as most of genes are either induced by SA-mediated pathway or by JA- induced pathway. The CarWRKY1 behaves as transcriptional activator in yeast that is similar to its homologs from other plants. The *in-silico* analyses suggest that it may be regulated at protein level by phosphorylation. It behaves a transcriptional activator in yeast but these results needs further confirmation in plants. We have also isolated four additional WRKY genes from chickpea and their characterization is presently in progress. Further, studies on targets of *CarWRKY1* and its role in plant defense especially against necrotrophs is required. As this gene is highly and rapidly induced, therefore, finding its targets would be interesting. Its promoter analyses will be useful in finding *Ascochyta* responsive elements that can be used in genetic engineering of plant defense.

Chapter 5

Functional analysis of CarWRKY1 in Transgenic plants

5.1 Introduction

Reprogramming of cellular functions in response to external stimuli involves complex changes in gene expression. Regulation of gene expression at the level of transcription is a major control point in many biological processes, and plant genome devotes approximately 7% of their coding sequence to transcription factors (Udvardi *et al.*, 2007). Transcription factors acts in concert with other components of the transcriptional machinery to modulate the expression of target genes in a temporal and spatial manner. Several members of various transcription factor families are linked with plant defense responses and specific gene regulation.

Although discovered relatively recently, WRKY transcription factors are becoming one of the best-characterized classes of plant transcription factors and are at the forefront of research on plant defense responses. Various features and the role played by WRKY proteins in plant defense were reviewed in chapter 4. In this chapter, we describe the overexpression and 5'-upstream regulatory region analyses of a chickpea WRKY family of transcription factor.

5.2 Overexpression studies of WRKY transcription factors

The overexpression studies with individual members of large family of transcription factors are important because many genes may have redundant function (Zhang, 2003). The down-regulation or T-DNA mutants cannot clearly define the role played by that particular gene in such large families. However, ectopic expression leading to nonphysiological concentrations of the protein can also affect a plethora of regulatory networks and yield multiple phenotypes. Most of the results of overexpression of WRKY transcription factor are from the model plant *Arabidopsis thaliana,* however, few from other plants were also reported recently.

In transgenic tobacco, overexpression of *Vitis vinifera VvWRKY1* and *VvWRKY2* provides tolerance against various fungal pathogens (Marchive *et al.*, 2007; Mzid *et al.*, 2007). Overexpression of rice *OsWRKY53* lead to enhanced resistance against fungal pathogen *M. grisea* race 007. There are some common set of genes, like PR genes, peroxidases, and genes involved in oxidative stress that get induced in *WRKY* gene overexpressiong lines probably because of their wide range of actions downstream to various pathways. In rice, overexpression of *OsWRKY45* led to enhanced resistance against blast fungus while, the transformants overexpressing *OsWRKY62*, *-76*, and *-19* became susceptible to this fungal pathogen (Shimono *et al.*, 2007). The overexpression

studies with *Arabidopsis AtWRKY6* has shown that this gene is involved in plant defense as well as regulating senescence. Its expression is induced in response to pathogen and during the senescence stage (Robatzek and Somssich, 2002). The growth condition of overexpressing plants also influences resistance and susceptibility suggesting that many other factors also contribute to the signaling pathways. The overexpression of *OsWRKY13* enhances the rice resistance against bacterial blight and blast fungus at both seedling and adult stages, making in a good target for transgenic plant generation in rice (Qui *et al.*, 2007).

In some cases the overexpression of WRKY genes has lead to lethal phenotype as overexpression of *OsWRKY03* gene in rice produced severe dwarfism, reduced root and shoot elongation, and ultimately plant died (Liu *et al.*, 2005). Negative effect on plant defense is also seen in some cases of overexpression, probably when these factors acts as suppressors of defense related pathway. The *Arabidopsis* WRKY group-II genes that contain a leucine zipper at N-terminal, *AtWRKY18*, *-40*, and *-60* are implicated in repressing basal defense to virulent hemibiotrophic *P. syringae*. The homologs of these proteins from barley HvWRKY1/2 also acts as repressors. The repression activity of HvWRY1/2 proteins in barley is relieved by the effector-induced MLA10 resistance gene (Shen *et al.*, 2007).

Many WRKY transcription factors impart tolerance against abiotic stresses. Recently, overexpression of *AtWRKY25* and *AtWRKY23* provided tolerance to *Arabidopsis* against salinity and increased sensitivity to ABA (Jiang and Deyholos, 2009). In rice, enhanced heat and drought tolerance was provided by overexpression of *OsWRKY11* under the control of *HSP101* promoter (Wu *et al.*, 2009). Overexpression of the *Glycine max* GmWRKY57B in tobacco conferred the transgenics tolerance to drought stress (Lan *et al.*, 2008). In *Arabidopsis*, ectopic expression of soybean WRKY transcription factors *GmWRKY13, -21, and -54* provided tolerance to various abiotic stresses (Zhou *et al.*, 2008). Unlike earlier mentioned reports, a *TcWRKY53* gene from *Thlaspi caerulescens* negatively regulates osmotic stress in tobacco (Wei *et al.*, 2008). Varying sets of downstream candidate target genes were identified in the lines overexpressing the different WRKY transgenes, suggesting a certain degree of specificity for the individual factors. Further studies are required to understand the complexity of WRKY transcription factors.

5.3 Transcriptional regulation by WRKY genes.

The WRKY superfamily of genes are induced almost by all the stresses and elicitors which makes them good target for the study of gene regulation under stress conditions. These studies can also elucidate new signaling cascades and *cis*-acting elements. Nearly all studied WRKY factors appear to have a stereotypic binding preference to one DNA element termed as W-box. Recent studies have shown that the sequences adjacent to the W-boxes contribute to the specificity of WRKY protein binding on their targets (Ciolkowski *et al.*, 2008). The presence of adjacent W-box elements has synergistic effect on transcription as seen in parsley *PcWRKY1* (Euglem *et al.*, 1999). The barley Hv-WRKY38 factor also requires two adjacent W-boxes for efficient DNA binding (Maré *et al.*, 2004). Few WRKY factors can influence expression of specific genes without directly binding to the DNA. The rice WRKY factor OsWRKY51, although failing to bind itself, was shown to enhance specific binding of OsWRKY71 to the *Amy32b* gene promoter (Xie *et al.*, 2006). Further studies are required to understand the discrete binding site selectivity of the large set of WRKY factors to their *in vivo* target sites.

5.4 Results and discussion

5.4.1 Construction of *CarWRKY1* overexpression construct

To elucidate role of *CarWRKY1* in plant defense and development, it was desired to overexpress in *Nicotiana tabacum* cv. xanthi under the constitutive promoter *CaMV35S*. The *CarWRKY1* was amplified with a proofreading enzyme using OE2W1F1 and OE2W1R1 primers (Table 3.7). These primers were designed to amplify a fragment of ~1400 bp that included 46 bp 5′-UTR, 1095 bp ORF, and 223 bp of 3′-UTR. The amplified product was cloned in pDRIVE U/A cloning vector after A-tailing with native Taq polymerase. From the pDRIVE-*CarWRKY1* clone, *CarWRKY1* fragment was excised out by digesting this clone with *Xba*I and *Sac*I. The gel-eluted fragment of desired size was cloned in the pBI121 vector at *Xba*I and *Sac*I restriction sites by replacing *uidA* gene. The cloning was confirmed by digestion and sequencing. Thus, *CarWRKY1* was cloned in pBI121 binary vector under the constitutivly active *CaMV35S* promoter.

5.4.2 Transformation of *Nicotiana tabacum* cv. xanthi with pBI121-CarWRKY1 construct

The pBI121-CarWRKY1 was transformed to LBA4404 strain of *Agrobacterium tumefaciens* using freeze-thaw method (section 3.13.1). The transformed *Agrobacterium* cells were selected on LB plates containing kanamycin (50µg/ml) and Rifampicin (25µg/ml). The transformed colonies were confirmed by colony PCR. The *Agrobacterium*-mediated transformation of *Nicotiana tabacum* cv. xanthi leaf discs was performed, as described in section 3.13.3, for pBI121 blank vector and pBI121-CarWRKY1 constructs. The regenerating plantlets were selected on kanamycin (100 µg/ml). The regenerated plants with proper shoot were transferred to the rooting medium and allowed to grow in presence of kanamycin. Plants originated from different leaf-discs were regarded independent transgenic lines. We have generated about 49 plants of 21 independent lines that survived on kanamycin. The genomic PCR was performed to check the integrity of *CarWRKY1* expression cassette on tobacco genome. The genomic PCR and Southern analysis was used to confirm the transgenic lines and results showed that 16 lines were positive. The RNA was also isolated from these lines to check the ectopic expression of CarWRKY1. All these lines showed increased level of expression using RT-PCR of gene specific primers except the OE-P1 line that showed low-level expression. The positive T_0 lines with varying expression level were grown under natural conditions in clay pots.

5.4.3 Analyses of *CarWRKY1* transgenic (T_0) plants

All the transgenic plants were transferred in pots at the same time along with the control plants transformed with blank vector. These plants were checked for morphological changes associated with the overexpression of *CarWRKY1* in T_0 plants. The first clear difference that appeared on *CarWRKY1* overexpressing lines as compared to control blank vector transgenic lines was the appearance of necrotic spots on leaves especially on the lower leaves (Fig. 5.1A). It was supposed that the necrosis was due to accumulation of H_2O_2 in adult stage. To visualize the accumulation of H_2O_2, leaves of transgenic and blank vector tobacco plants were stained with 3,3-diaminobenzidine (DAB). The areas that had more necrosis showed the presence of H_2O_2 as evident by reddish-brown color accumulation (Fig. 5.1B) (Thordal-Christensen *et al.*, 1997). In most of the CarWRKY1 overexpressing plants, flower buds appeared about a month earlier than control plants (Fig. 5.2). This preliminary data indicates that overexpression

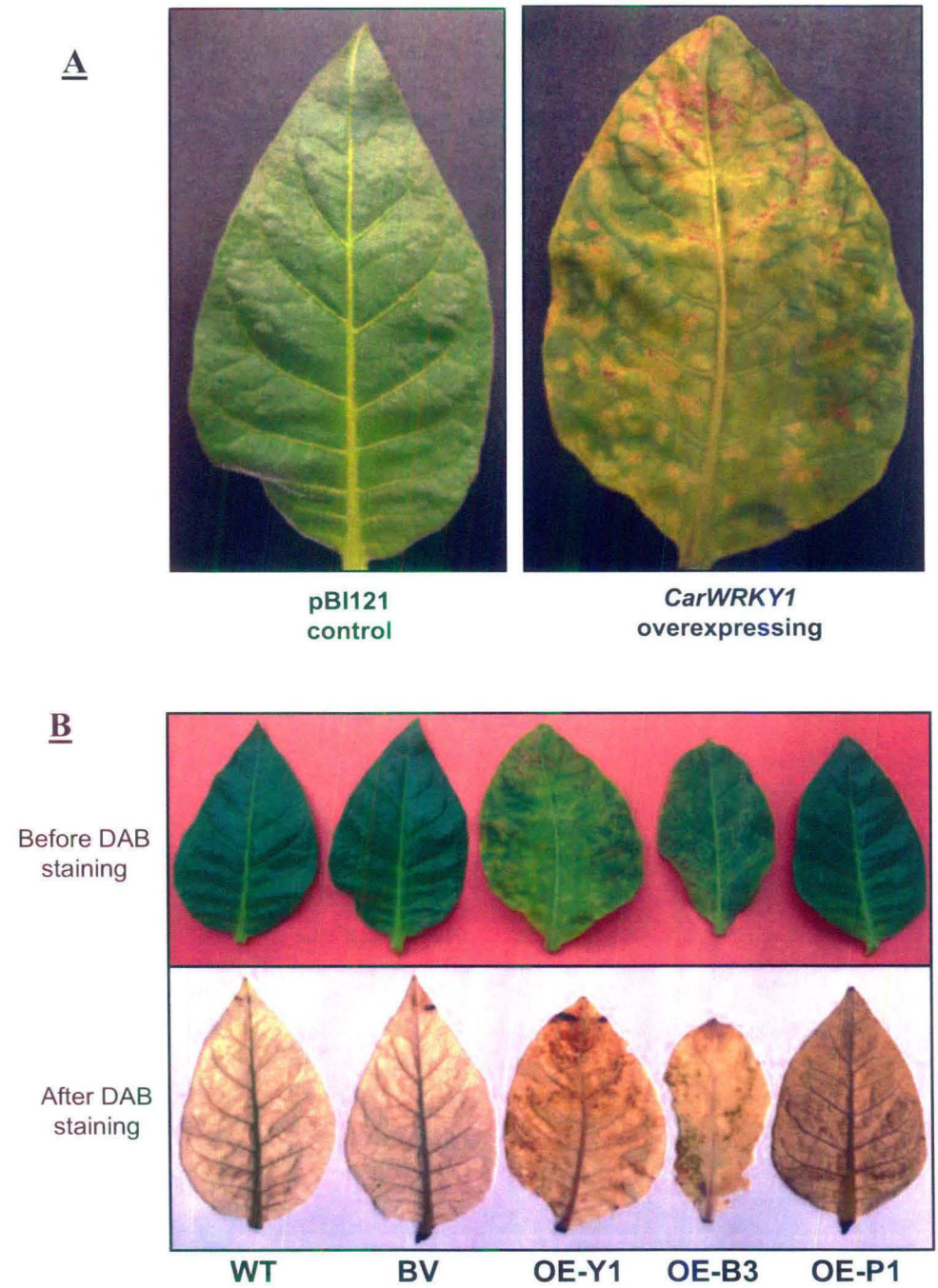


Fig. 5.1 Overexpression of *CarWRKY1* in tobacco results in necrosis on adult plant leaves in T_0 generation

(A). Necrosis symptoms on leaves of *CarWRKY1* overexpressing tobacco plants. Blank vector pBI121 (control) transgenic plants leaves are normal. **(B)**. DAB staining showing accumulation of H_2O_2 in leaves of WT- wild type, BV- blank vector pBI121 control and OE-Y1, B3, P1 transgenic tobacco lines overexpressing *CarWRKY1*.

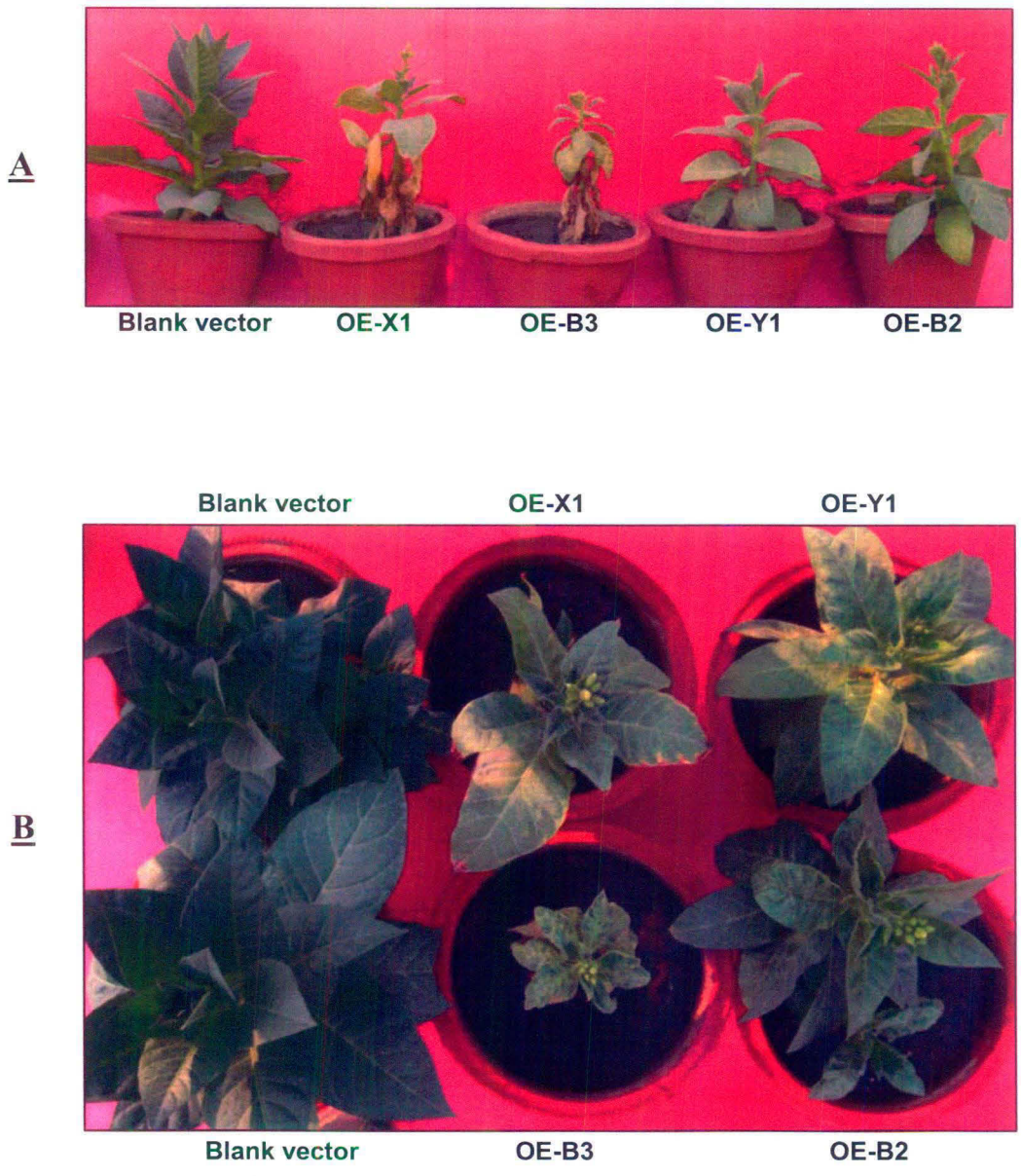


Fig. 5.2 The *CarWRKY1* overexpressing tobacco (T_0) lines showed early senescence and early flowering

(A). Lateral view of control (blank vector pBI121) and *CarWRKY1* overexpressing (OE) transgenic tobacco plants showing early senescence in lower leaves. **(B)**. Aerial view of control (blank vector) and *CarWRKY1* overexpressing (OE) transgenic tobacco plants to show early appearance of flower buds.

of *CarWRKY1* induce early flowering, a common phenotype associated with the overexpression of defense related genes. Some of the overexpression lines also showed stunted growth (Fig 5.3A). When compared in context to the number and size of seed bearing pods these lines had few small pods. Second common observation was the early appearance of senescence in overexpression lines as compared to control blank vector lines. The senescence was very clear in lower leaves of overexpressing lines. At the same time, wild type plants were showing vegetative growth while on overexpression lines flowers had already appeared (Fig. 5.2A).

The stunted growth with partly necrotic leaves and early flowering was also observed in the T_0 and T_1 generation of plants overexpressing *AtWRKY6*, *AtWRKY18*, *AtWRKY70* (Chen and Chen, 2002, Robatzek and Somssich, 2002; Ülker and Somssich, 2004; Li *et al.*, 2004). However, when *AtWRKY53*, the *Arabidopsis* homolog of *CarWRKY1,* was overexpressed this symptom appeared until T_2 generation (Miao *et al.*, 2004). The reason behind these developmental defects may be due negative regulation of developmental pathways or inappropriate activation of defense responses that generally put negative influence on development of plant. This gene was induced by salicylic acid and salicylic induced pathway also generates these type of defects in plants (Du *et al.*, 2009). The SA analog BTH downregulates the expression of a significant number of genes involved in auxin transport, reception and response. *Arabidopsis* mutants with high levels of endogenous SA had relatively low IAA levels (Wang *et al.*, 2007; Kazan and Manners, 2009). In another study, the wound induced jasmonates were shown as inhibitors of plant growth by inhibiting mitosis (Zhang and Turner, 2008). Though extensive studies will give more insight into its diverse roles, the observations presented here shows that *CarWRKY1* is a functional homolog of defense related WRKY genes like AtWRKY53 and can be an important plant defense regulator in chickpea.

5.4.3 Analyses of *CarWRKY1* transgenic (T_1) plants

Among the segregating population of T_0 seeds, the kanamycin positive plantlets were allowed to grow up to adult stage to get T_1 seeds. These plants were again checked for presence of *CarWRKY1* by genomic PCR. For each transgenic line about ten plants were grown. In T_1 generation also the early senescence and early flowering phenotype appeared but the stunted growth phenotype and appearance of necrotic lesions was not so pronounced as compared to T_0 generation plants (Fig. 5.3B). The analysis of tolerance for bacterial and fungal pathogens will now give insight to the role of *CarWRKY1*

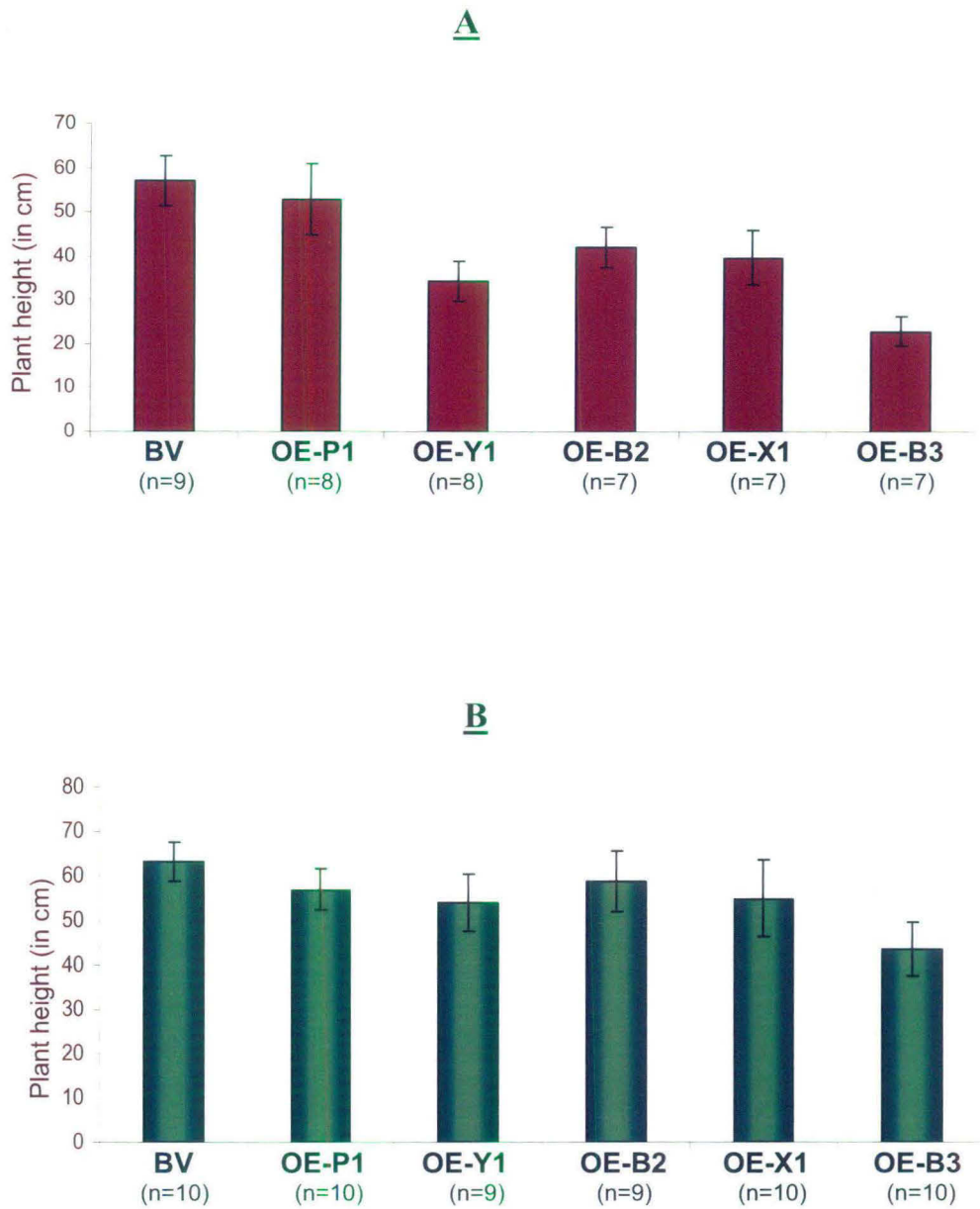


Fig.5.3 Transgenic plants overexpressing *CarWRKY1*

Height attained by the T_0 (A) and T_1 (B) generation tobacco transgenic lines at the time of flower bud appearance. The number of plants measured are indicated by 'n' below respective lines.

in plant defense.

5.4.4 Isolation of *CarWRKY1* 5'-upstream sequences

The *CarWRKY1* gene showed rapid and high-level expression, therefore, it was desired to isolate the 5'-upstream sequences of *CaWRKY1* to better understand the mechanism by which the expression of the gene is regulated. The Universal GenomeWalker kit (Clontech) was used to isolate 5'-upstream sequence as described in section 3.12. Chickpea variety Pusa 362 genomic DNA was digested and adapter ligated to get genomic library. This library was used as a template to amplify 5'-upstream sequence of *CarWRKY1*. The primary and nested PCRs were performed using WRKYP1 and WRKYP2 primers with GenomeWalker adapter primers (Table 3.7). A single band of 1.3 Kb was obtained in the primary PCR of library which was constructed using *Hinc*II restriction enzyme. The nested amplification product was cloned and sequenced.

After sequencing, the obtained 5'-upstream sequence was analyzed using online programs like PlantCARE (http://intra.psb.ugent.be:8080/PlantCARE/) and PLACE (http://www.dna.affrc.go.jp/PLACE/) in order to identify the putative *cis*-elements present. Several putative regulatory motifs, which are homologous to the *cis*-acting elements involved in activating the defense genes in plants, were identified in the 5'-upstream region of the *CarWRKY1* gene. The obtained results are depicted in fig 5.4 and the known functions of these elements are summarized in table 5.1. The possible with functions and positions with respect to transcription start site (+1) are also mentioned.

Table 5.1 Summary of CarWRKY1 5'-upstream sequence analysis using PlantCARE and PLACE programs.

***cis*-element**	**Core sequence**	**Functions**
A-box	TA(A/T)C	Sequence conserved in α-amylase promoters
ABRE	ACGT	ABA responsiveness
AuxRE	CTCA	Part of an auxin responsive element
ERE	ATTT	Ethylene responsive regions
GCN4 motif	GTCA	Involved in endosperm expression
Skn-1 motif	GTCA	Required for endosperm expression
TGA element	AACG	Auxin responsiveness
WUN motif	AATT	Wound-responsive element
W - box	TGAC	WRKY binding site
TAAAG element	TAAAG	Dof binding site at KST1 gene
SP8b element	TACTATT	SPF1 binding site
Dof binding site	AAAG	for binding of Dof transcription factor
GT1 Gm S-CAM4	GAAAAA	GT-1 motif plays a role in pathogen- and salt-induced Soybean calmodulin CaM-4 gene expression
OsBIHD1	TGTCA	Binding site of OsBIHD1, a rice transcription factor having role in disease resistance

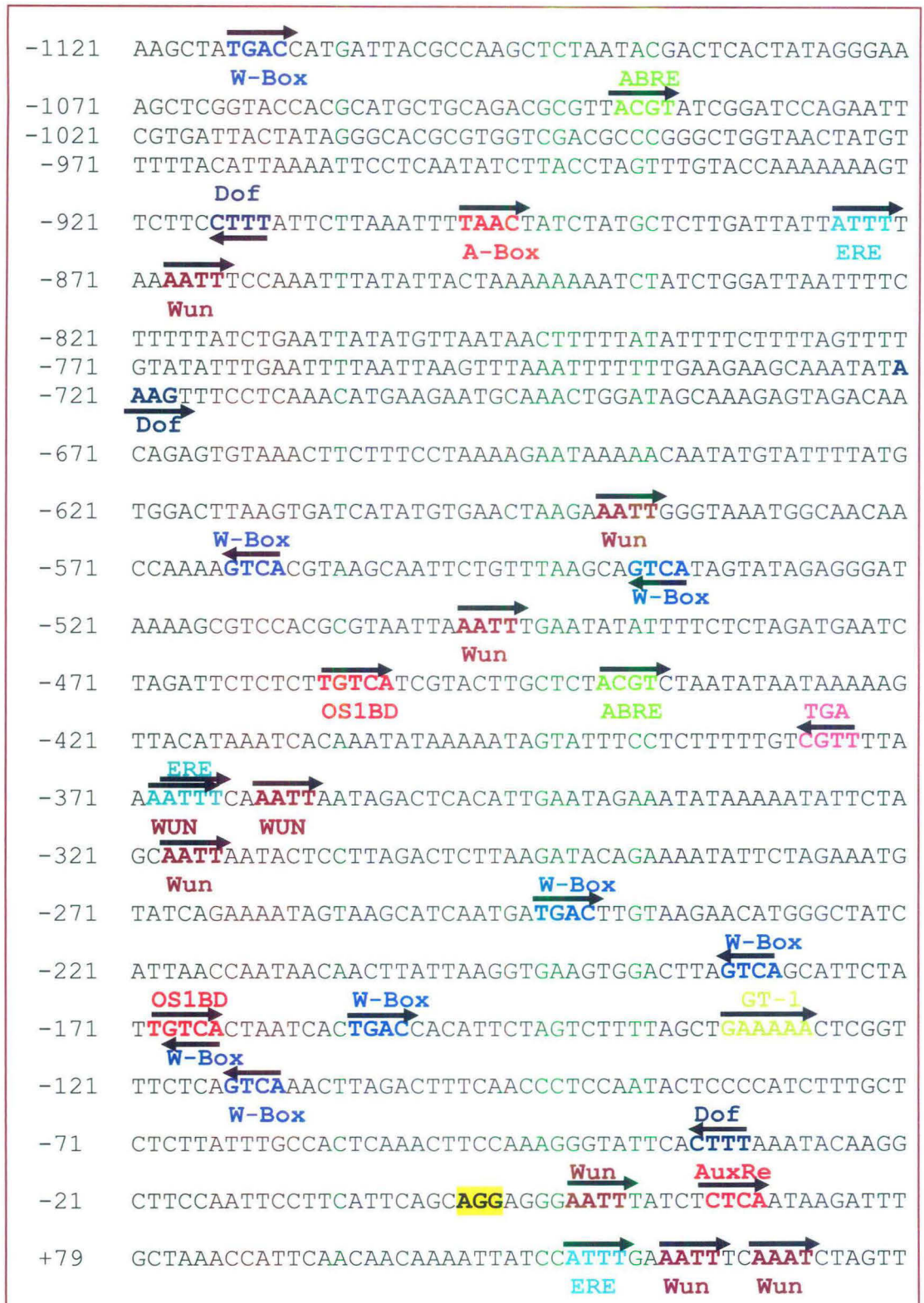


Fig. 5.4 5′Upstream sequences of CarWRKY1 depicting important *cis*-acting element using PlantCare and PLACE analysis tools. The direction of arrows indicates the location of *cis*-elements on sense (⟶) and antisense (⟵) strands.

The elements in the 5'-upstream sequence of *CarWRKY1* supports the expression analysis data of chapter 4. In addition to the typical TATA and CAAT boxes located close to the putative transcription start site (CAG), several *cis*-acting elements including ERE, ABRE, and WUN motif, that were previously shown to be involved in the activation of gene expression by ethylene, abscisic acid, and wounding, respectively (Montgomery *et al.*, 1993; Song and Goodman, 2002). The Dof-binding site, present in *CarWRKY1,* is prerequisite for SA mediated induction of *Sar8.2b* gene in tobacco (Song and Goodman, 2002). The presence of many W-boxes core motif suggests auto-regulation as well as binding of other WRKY family transcription factor for regulation of its expression as observed in *AtWRKY6* and *AtWRKY53* genes (Robatzek and Somssich, 2002; Miao *et al.*, 2004). The W-box core motif also overlaps with the *as-1* element motif, requited for TGA factors binding on SA responsive genes. The WRKY proteins also bind to other sites, like SPF1 to TACTATT (Ishiguro and Nakamura, 1994). Therefore, it will be interesting to study the regulation of *CarWRKY1 in vivo* by various elements. Whether the presence of these *cis*-acting elements function *in vivo* during early defense signaling is a matter of conjecture.

5.4.5 Generation of deletion constructs of 5'-upstream regulatory region of *CarWRKY1* and their transformation in *Nicotiana tabacum* cv. xanthi

In order to check the effects of various *cis*-acting elements, dispersed randomly throughout 5'-upstream regulatory region of CarWRKY1, 5'- deletion constructs of regulatory region were prepared. The amplified PCR products were first cloned in pDRIVE U/A cloning vector and then in pBI101.2 binary vector upstream to the reporter *uidA* gene using *Hind*III and *Bam*HI enzyme restriction sites using the primer combinations mentioned in table 5.2. We have obtained seven constructs of which six have 5'- deletions and seventh has an extended 5'-UTR of *CarWRKY1* gene (Fig. 5.4a).

Table 5.2 Summary of *CarWRKY1* regulatory region deletion constructs. Primers sequences are mentioned in table 3.7

S. No.	Construct	Forward Primer	Reverse primer	Size of deletion (bp)
1.	CarW1PΔ1	W1PF1	W1PR1	1053
2.	CarW1PΔ2	W1PF2	W1PR1	854
3.	CarW1PΔ3	W1PF3	W1PR1	643
4.	CarW1PΔ4	W1PF4	W1PR1	477
5.	CarW1PΔ5	W1PF5	W1PR1	279
6.	CarW1PΔ6	W1PF6	W1PR1	149
7.	CarW1PΔ7	W1PF1	W1PR2	1254

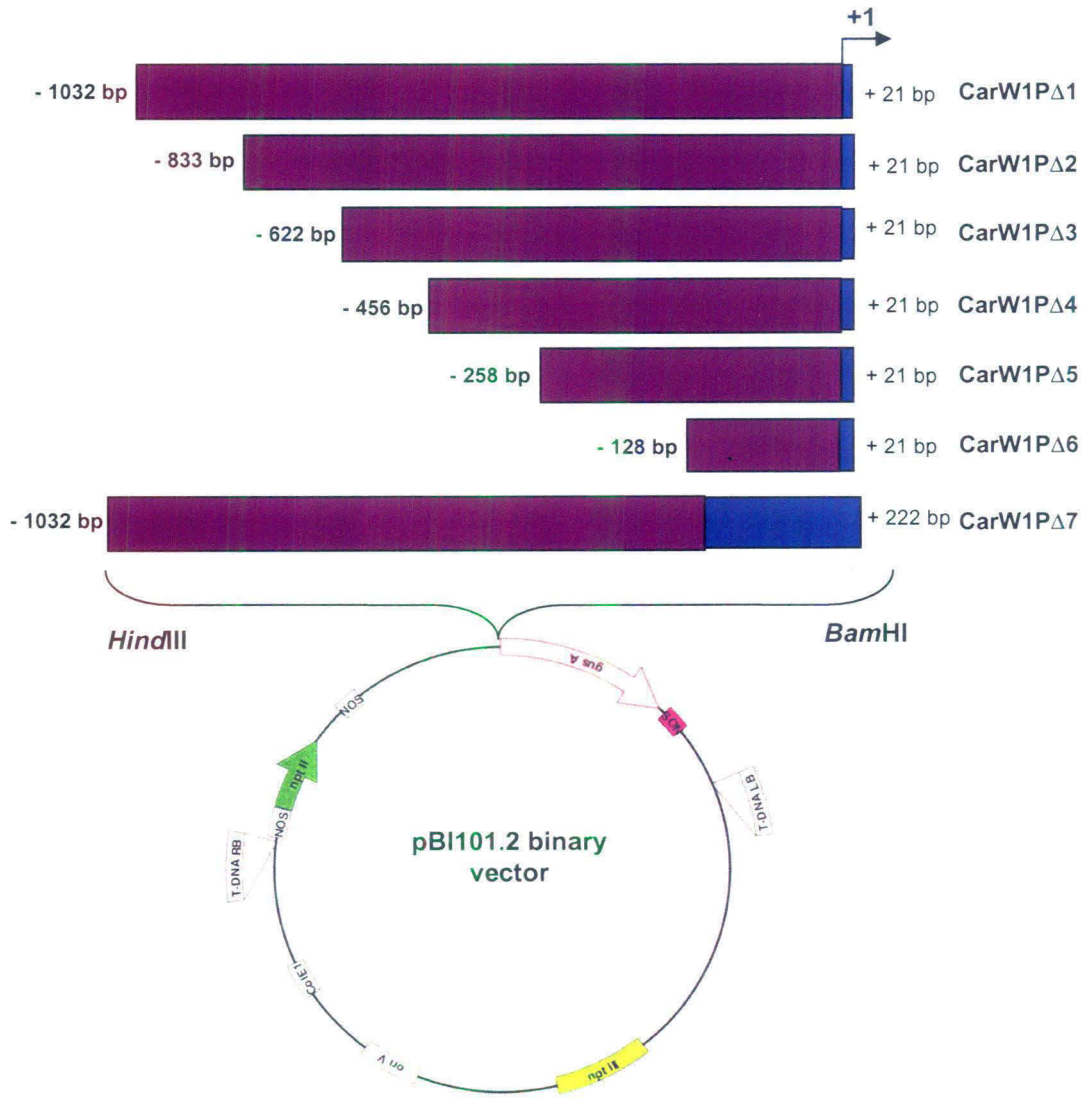


Fig.5.4a Deletion constructs of the *CarWRKY1* promoter

Fragments of the *CarWRKY1* promoter representing a 5′ deletion series were fused to the *uidA* reporter gene. The length of fragments from transcription start site is mentioned. The fragments were cloned in pBI101.2 binary vector at *Hind*III and *Bam*HI restriction sites.

The transcription start site of *CarWRKY1* was used in all the deletion constructs for uniformity and avoiding misinterpretation of results. The CarW1PΔ7 construct has few more bases of the 5'- untranslated region, as many wound responsive elements were present in this region. These constructs were mobilized in LBA4404 strain of *Agrobacterium tumefaciens*. All the seven constructs were transformed in *N. tabacum* cv. xanthi to generate transgenic plants. After confirming with genomic PCR, transgenic plants were transferred in clay pots for the generation of seeds and further analysis.

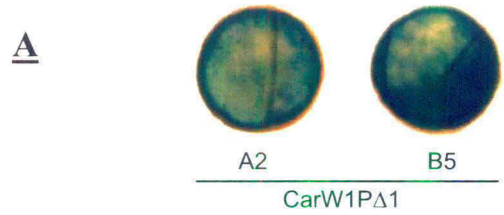
5.4.6 Analyses of *CarWRKY1's* 5'-upstream regulatory region in tobacco

Two transgenic lines having CarW1PΔ1 construct were analyzed for wound-induced expression of *uidA* reporter gene in tobacco leaves. The cork borer excised leaf-discs showed high GUS activity at margins suggesting that this construct has *cis*-elements for wound-induced expression (Fig. 5.5A). Mechanical wounding induced *CarWRKY1* in chickpea plants. There are many dispersed wound responsive *cis*-elements in the analyzed regulatory region. Further analysis of remaining 5′-regulatory region deletions will certainly give insight into the role played by these dispersed wound-responsive elements.

The GUS staining was carried out for flowers and all the floral parts of various developmental stages. High expression level of *uidA* gene controlled by *CarWRKY1* regulatory region was found in petals of tobacco floral stage twelve and thirteen. These are the stages when pollination occurs and after it the petal goes for senescence (Fig. 5.5B&C). The GUS staining was also seen in pollens and at the upper region of gynoecia (Fig. 5.6). These studies on 5′-regulatory region of *CarWRKY1* suggest that it may be playing diverse roles both in defense and development. In two *Arabidopsis* genes, *AtWRKY6 and -53* are similarly involved in senescence and plant defense. The targets of these genes and the phenotype of their overexpression suggest this role of these WRKY proteins (Robatzek and Somssich, 2002; Miao *et al.*, 2004). The extensive study of these deletions will certainly prove useful for delineating the regulatory regions of *CarWRKY1* promoter and will help in isolation of novel regulators of defense and development.

5.4.7 Conclusion

Various studies have proven that WRKY genes are important regulators of plant defense and development. The group-III WRKY gene isolated in this study from chickpea is the first report of any WRKY gene isolation from this crop legume. Therefore, to study its



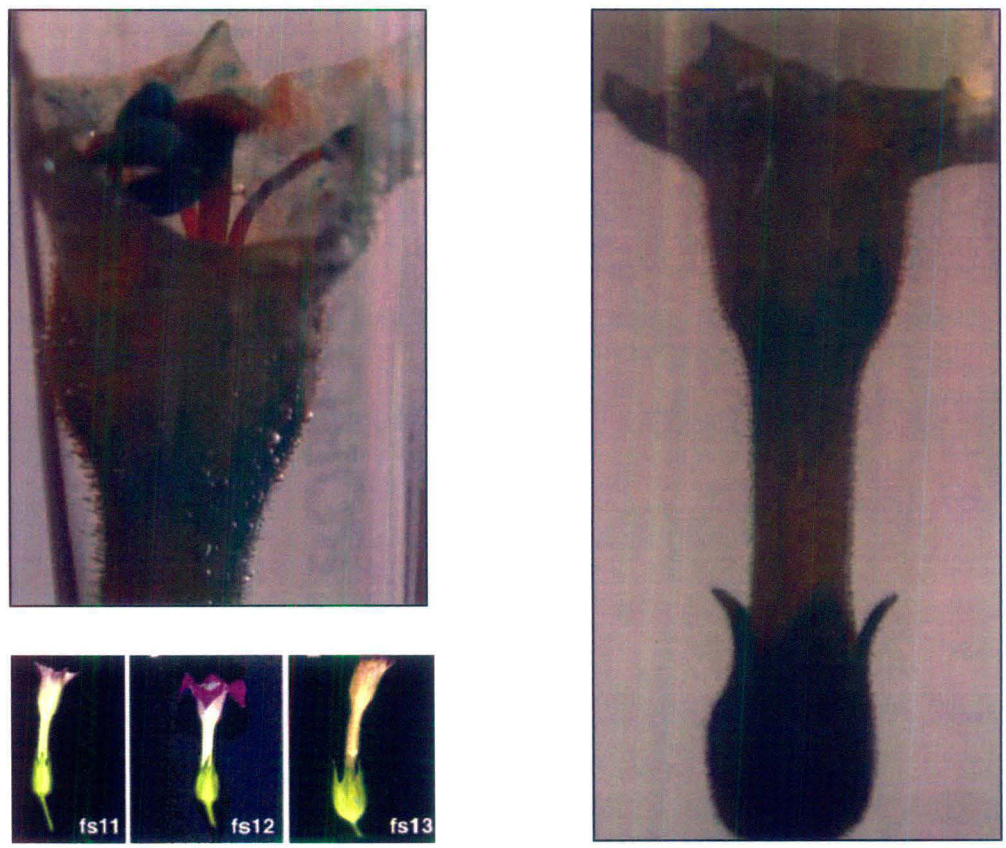


Fig 5.5 The *CarWRKY1* 5′ regulatory region expression in T_0 transgenic tobacco lines

Histochemical localization of GUS activity in transgenic tobacco lines expressing the *uidA* reporter gene under the control of *CarWRKY1* 5′-regulatory region (CarW1PΔ1). **(A)** GUS activity in cork-borer excised leaf discs of two different transgenic lines. **(B)** and **(C)** Flowers of two independent lines in floral stage 12. GUS activity can be visualized in petal, gynoecia, and anthers **(D)** Representative photographs of tobacco floral stage-fs11, fs12, and fs13.

A

B

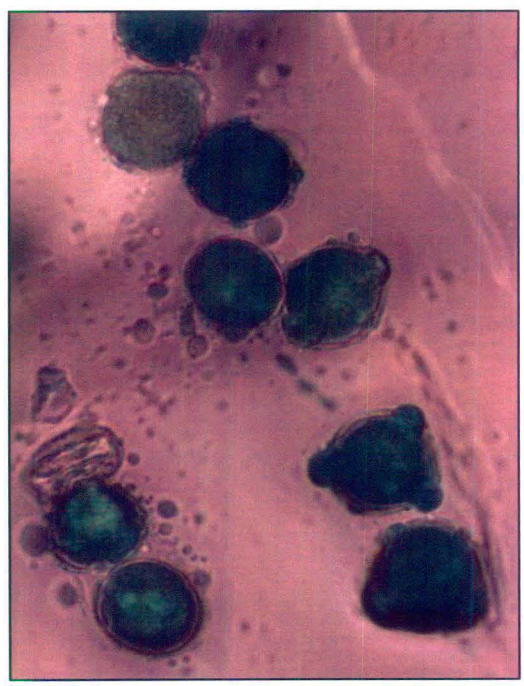


Fig. 5.6 The *CarWRKY1* promoter expression in tobacco floral parts

(A). Histochemical localization of GUS activity in anthers and stigma of transgenic tobacco lines expressing the *uidA* reporter gene under the control of *CarWRKY1* promoter (CarW1PΔ1). **(B)**. GUS activity in pollen grains

function and regulation of its transcript extensively, we have developed transgenic plants overexpressing *CarWRKY1* and 5′ upstream regulatory region deletions. The plants overexpressing *CarWRKY1* showed developmental defects in T_0 and T_1 generation. Similar phenotypes were observed in other defense regulators and WRKY family members of *Arabidopsis* (Robatzek and Somssich, 2002). This suggests functional similarity with WRKY family members from other plants and *CarWRKY1*. The plant senescence and defense showed overlap between pathways associated with hypersensitive response, suggesting that some transcriptional regulators may be common (Gepstein *et al.*, 2003; Ülker *et al.*, 2007). One such regulator is *AtWRKY53*, which is involved in basal defense and is an important regulator of senescence. The expression of *uidA* reporter gene by *CarWRKY1* 5′ regulatory sequences in flower parts including pollens suggests its role in regulating development and senescence. Analysis of T_2 generation transgenic lines will elucidate the role of *CarWRKY1* with respect to regulation of early defense signaling and defense pathways induced against bacterial and fungal pathogens.

Chapter 6

Isolation of Interacting proteins by Y2H

6.1 Introduction

Physical association between different proteins, so-called protein-protein interactions (PPIs), are intrinsic to virtually every cellular process ranging from the formation of cellular structures and enzymatic complexes to the regulation of signaling pathways. In fact, the performance of proteins is highly dependent on interactions with other proteins or protein complexes (Cusick *et al.*, 2005). Therefore, the identification of protein interactions and protein networks provides one important clue to their molecular function. The diversity of proteins indicates the need of different experimental strategies to uncover the entity of protein interaction networks and the detailed characterization of individual protein interactions. In this context, an immense variety of methodologies have been established in recent years, including high-throughput assays, to identify protein-protein interactions and to cope with the huge amount of data generated by sequencing multiple genomes. Many laboratories around the globe use these techniques regularly according to their requirements. But to date, most of the new interactions that have been detected experimentally relied on one of the two technologies, the yeast two-hybrid system (Fields and Song, 1989) and mass spectrometry of co-affinity purified protein complexes (co-AO/MS) (Pandey and Mann, 2000). The available methods can be divided into *in vivo* and *in vitro* approaches (Table 6.1; adapted from Lalonde *et al.*, 2008).

Table 6.1 Methods for analyzing protein-protein interactions

	In vitro	***In vivo***
Affinity purification	Affinity purification-mass spectrometry	Affinity purification-mass spectrometry
Genetic test systems		Yeast two hybrid systems
Fluorescence	Fluorescence resonance energy transfer (FRET), Bioluminescence resonance energy transfer (BRET)	FRET/BRET, Split-GFP
Plasmon resonance	Quantitative surface Plasmon resonance	
Calorimetry	Quantitative analysis of protein interactions	
Atomic force microscopy	Detection and quantitative analysis of protein interaction	
NMR	Quantitative analysis of large complexes	STINT-NMR
Protein arrays	Identification and selectivity of protein interactions	

The aim of this introduction is to survey the important techniques for the identification and confirmation of novel PPIs as this chapter deals with the isolation of interacting partner(s) of CarWRKY1 using yeast two-hybrid screening. Thus, it will focus on yeast two-hybrid systems, which can be used to study trans-activating proteins.

6.1.1 Identification of protein-protein interactions *in vitro*

The classical biochemical techniques for detecting protein interactions *in vitro* are immuno-precipitation and pull-down assays, both of which are based on affinity purification of a bait protein associated with its binding partners. Of the various high-throughput experimental methods used so far to identify PPIs, tandem affinity purification (TAP) of affinity-tagged proteins followed by mass spectrometry has provided the best coverage and accuracy (Rigaut *et al.*, 1999; Puig *et al.*, 2001; von Mering *et al.*, 2002). TAP-tagged baits expressed in a target cell are allowed to form complexes with untagged physiologically expressed prey proteins. Protein complexes are subsequentaly purified using the TAP-tag in two consecutive steps of purification under mild conditions. Proteins from complexes are electrophoresed before subjecting them to mass spectroscopy. Two groups used the TAP procedure in comprehensive surveys of the entire yeast proteome, in which all ORFs were tagged (Gavin *et al.*, 2006; Krogan *et al.*, 2006). In various other reports this method has been used to isolate complex members of an individual protein.

The Cross-and-Capture system, a simple-one step pulldown approach in *Saccharomyces cerevisiae* that can be used to confirm known or predicted protein associations, as well as to screen for novel protein complexes is developed recently (Suter *et al.*, 2007). Various other methods like LUMIER (Luminescence-based Mammalian Interactome mapping), QUICK (Quantitative Immunoprecipitation Combined with Knockdown), protein microarrays, phage display, etc. have been developed and some are in the process of development.

6.1.2 Direct visualization of protein interactions *in vivo*

Fluorescence resonance energy transfer (FRET) using variants of the green fluorescent protein (GFP) has become a very important application for the characterization of PPIs in living cells. FRET is a direct, radiationless energy transfer between two spectrum-overlapped fluorophores: the donor (CFP, cyan; BFP, blue) and the acceptor (variant GFP, green; YFP, yellow) molecules. The bait protein of interest is linked to the donor whereas the prey protein is linked to the acceptor. An interaction between bait and prey brings the donor and acceptor fluorophores into close proximity with one another, resulting in FRET and emission of fluorescence from the acceptor. The technique is ideally suited to assess the real-time dynamics of complex formation and dissociation *in vivo*. Furthermore, the sub-cellular compartment where an interaction takes place can be

readily determined. A drawback of standard FRET methods is that they often suffer from fluorescent background or photobleaching. Bioluminescence resonance energy transfer (BRET) was developed to avoid these problems by replacing the donor fluorophore with luciferase from *Renilla reniformis* (Jares-Erijman and Jovin, 2003, 2006).

In bimolecular fluorescence complementation (BiFC), a PPI induces the complementation of a fluorescent protein from two dissected halves. BiFC is therefore an adaptation of protein fragment complementation (PCA). Fragments of fluorescent proteins with different spectral characteristics allow the simultaneous visualization of interactions between different proteins in the same cell (multicolor BiFC) (Hu *et al.*, 2002; Hu and Kerppola, 2003).

6.1.3 Yeast Two-Hybrid (Y2H), a genetic method of protein-protein interaction

A breakthrough in the screening of protein interactions *in vivo* was made 20 years ago by inventing the yeast two-hybrid (Y2H) method (Fields and Song, 1989). This technique was inspired by the modular nature of transcription factors GAL4 containing a DNA-binding domain (DBD) and a transcription activation domain (AD). Splitting the DBD and AD inactivates this yeast transcription factor. But when a DBD-fused protein X (bait) and an AD-fused protein Y (prey) are expressed in yeast, a quasi-native functional GAL4 transcription factor is generated, if proteins 'X' and 'Y' interact. Consequently, this quasi-native functional GAL4 activates common auxotrophic reporter genes such as *HIS3*, *LEU2*, *URA3*, *ADE2* and *LacZ* and *MEL1* markers, such that yeast cells grow on selective medium or turn blue in a β-galactosidase assay (Fig. 6.1).

Since the Y2H method is rapid and easy to use, it has become the most popular *in vivo* method for the identification of proteins and was shown to be very suitable for large-scale applications in several organisms. Several groups and companies have modified this method to screen cDNA library.

6.1.4 Modified yeast two-hybrid methods

Some protein classes, including transcription factors and membrane proteins cannot be studies in the classical yeast two hybrid system. To outwit these constraints and to broaden the scope of interaction studies with less false positives, the two-hybrid systems underwent several modifications and evolved into many directions. Some of the methods are mentioned below:

A. The split-ubiquitin system

The split-ubiquitin system takes advantage of highly specific cleavage by ubiquitin binding proteases (UBPs). The expression of a fusion protein consisting of ubiquitin and a C-terminal attached reporter in yeast results in fast and complete cleavage by UBPs. Separation of ubiquitin in N-terminal (Nub) and C-terminal half (Cub) leads to the spontaneous reassociation of the so called "split-ubiquitin", which is functional since it is recognized by UBPs. Mutations were engineered into Nub by replacing the isoleucine residues at position 3 and 13 into glycine, alanine or valine to reduce its affinity for Cub and thereby suppress the spontaneous reassembly of Cub and mutated NubG (mutated Nub). The reconstitution of split-ubiquitin is only possible if bait and prey proteins forces NubG and Cub into close proximity. Subsequently, a Cub-attached reporter is cleaved off by UBPs from split-ubiquitin (Johnsson and Varshavsky, 1994).

Two modifications of the split-ubiquitin system have been described. First, the rUra3 based split-ubiquitin system based system takes advantage of the N-end rule pathway of protein degradation, according to which proteins that carry a destabilizing amino acid at the N-terminus are rapidly degraded by ubiquitination (Bachmair *et al.*, 1986; Varshavsky, 1997). In the rUra3-based system, a destabilizing Ura3 protein (rUra3) is fused as reporter to Cub that is attached to bait protein. Since Ura3 converts the compound 5-fluoro-orotic acid (5-FOA) into a toxic metabolite, cells that express the X-Cub-rUra3 fusion protein are unable to grow on plates supplemented with 5-FOA. If an interacting protein of 'X' is fused to NubG then rUra3 will be cleaved and degraded, thus yeast cells will now grow in presence of 5-FAO (Varshavsky, 1996).

In the second modification, rUra3 was replaced with a fusion protein LexA-VP16 that acts as a transcription factor. When bait and prey interact, LexA-VP16 is released and diffuses into the nucleus where it activates reporter genes. Based on this transactivator split-ubiquitin system, two modified systems developed and marketed by DualsystemsBiotech. They are membrane two-hybrid system (MY2H) (Stagljar *et al.*, 1998; Thaminy *et al.*, 2003) and cytosolic yeast two-hybrid system (cytoY2H) (Fig. 6.2) (Möckli *et al.*, 2007).

B. The SOS recruitment system

The SOS recruitment system is based on the essential Ras signal transduction pathway (Aronheim *et al.*, 1994) and was developed to study transcriptionally active proteins or proteins which are associated with the membrane. Ras guanyl exchange factor Cdc25p

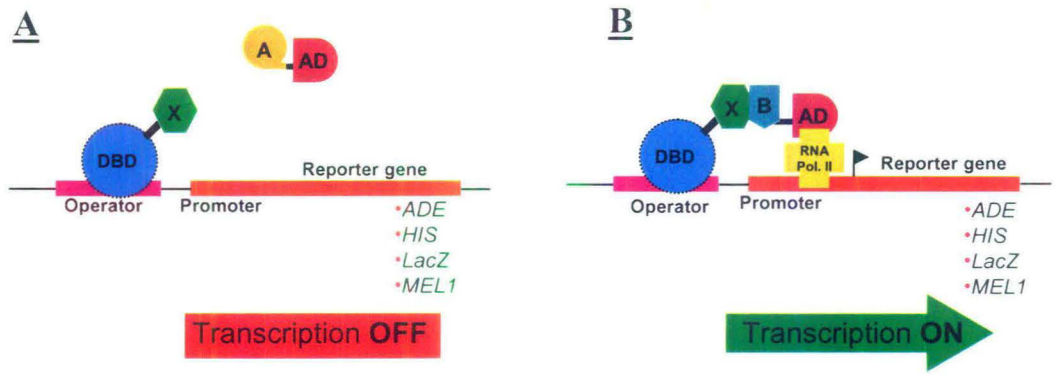


Fig. 6.1 The yeast two-hybrid system

(A). Co-expression of a DBD-fused to bait 'X' and a non-interacting AD-fused prey 'A' does not lead to a functional hybrid transcription factor. Therefore, transcription of reporter gene(s) is not activated in the Y2H-assay and yeast fails to grow on selective media. **(B)**. When the DBD-fused bait interacts with an AD-fused prey 'B' and thereby activates the reporter gene(s) leading to positive growth selection or a colorimetric readout.

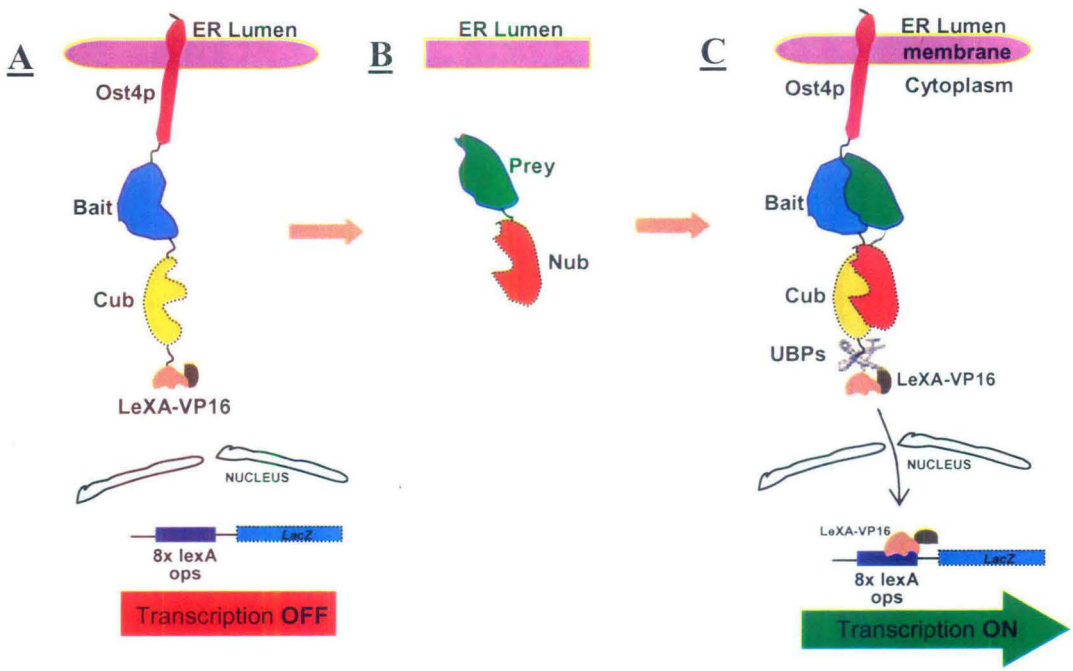


Fig. 6.2 The DUALhunter system

(A). A protein of interest (bait) is inserted between the membrane protein Ost4p, which anchors the protein in the endoplasmic reticulum (ER) membrane, and the C-terminal half of ubiquitin (Cub) followed by the artificial transcription factor LexA-VP16. **(B)**. The prey is fused to the mutated N-terminal half of ubiquitin (NubG). **(C)**. If bait and prey interact, Cub and NubG complement to form split ubiquitin, which attracts cleavage by ubiquitin-specific proteases (UBPs). As a result, the transcription factor is released and translocates to the nucleus, where it can activate transcription of endogenous reporter genes.

stimulates guanyl nucleotide exchange on Ras and promotes downstream signaling events that ultimately lead to cell growth. A Cdc25-2 temperature sensitive yeast strain can be complemented by the human homolog SOS, which is active but unable to localize at the plasma membrane. A soluble protein X (bait) is fused to SOS, whereas a protein y (prey) is associated by myristoylation with the membrane. Since the Ras pathway is absolutely dependent on the membrane recruited human SOS, cells expressing an interaction between bait and prey grow on the non-permissive temperature of 36°C (Fig. 6.3) (Young *et al.*, 1998).

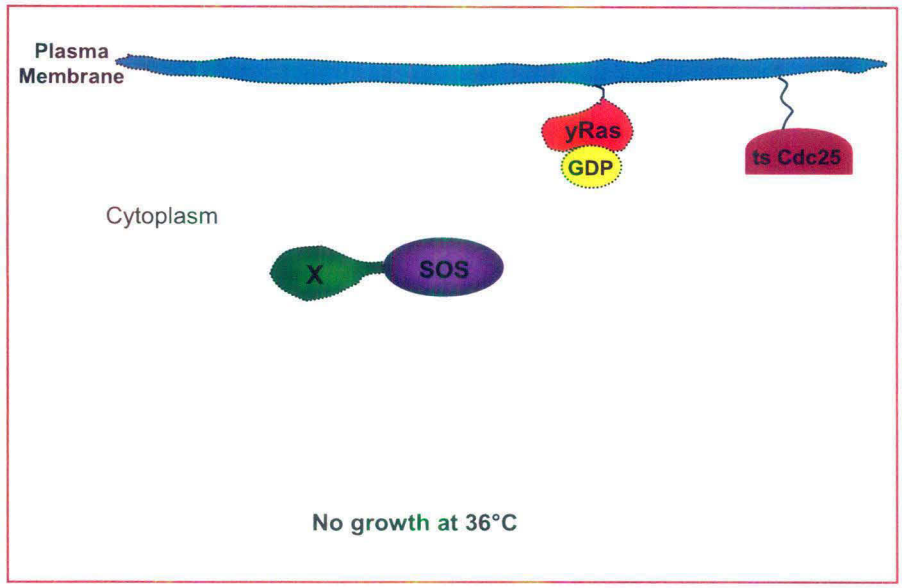
C. Ras recruitment system

The Ras pathway was also exploited for the Ras Recruitment System (RRS) (Broder *et al.*, 1998) and the reverse RRS (Hubsman *et al.*, 2001). Similar to the SOS system, a Cdc25-2 temperature sensitive yeast strain is used in both the RRS and the reverse RRS. In the RRS, a soluble protein under investigation X is expressed as a fusion with activated mammalian Ras (mRas), localized in the cytoplasm. Since the mRas is constitutively active, no Cdc25p is required for the guanyl nucleotide exchange. The only requirement for activating the Ras pathway is the recruitment of mRas to the plasma membrane. Co-expression of X-mRas with an interacting protein Y that was targeted to the plasma membrane by a myristoylation signal recruits X-mRas to the plasma membrane. As the endogenous Cdc25-2 mutant is active at 30°C, but inactive at 36°C, only those cells are selected for growth at 36°C which harbor an interaction between the membrane anchored prey Y and X-mRas (Fig. 6.4).

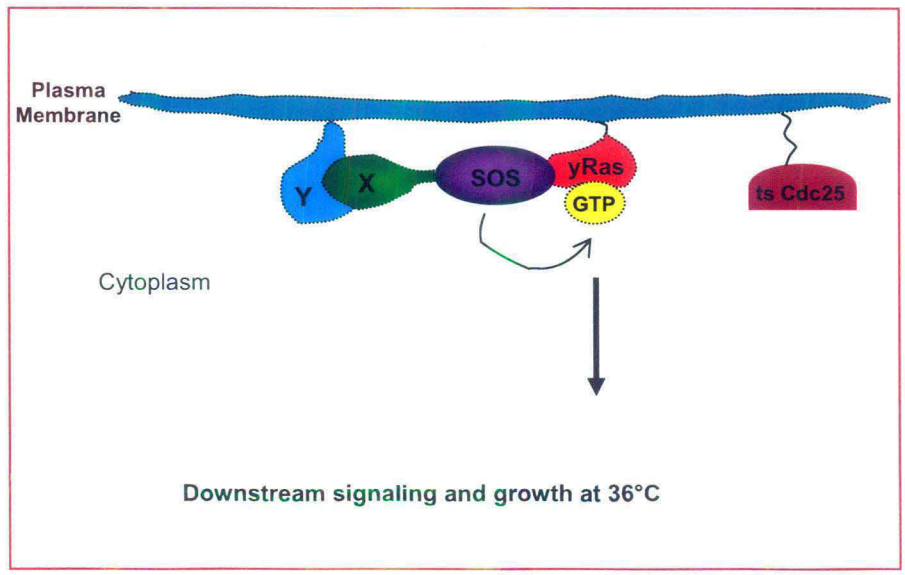
The RRS method can be applied on cytosolic and nuclear bait proteins, but not for membrane associated or integral membrane proteins. To circumvent this problem the reverse RRS system was created, for which an integral membrane protein serves as bait protein. The unmodified bait is coexpressed with a binding partner Y fused to activated mRas. This leads to the recruitment of mRas-Y to the membrane protein X upon interaction and positive growth selection at 36°C (Fig. 6.5). However, the reverse RAS is very limited for screening procedures since membrane proteins expressed as bait (RRS) or from a cDNA library (reverse RRS) will lead to unspecific results.

D. The RNA polymerase III based two-hybrid system

The RNA polymerase III system was designed to screen and study protein interactions of transcription factors as well. The method exploits the fact that polymerase II and III



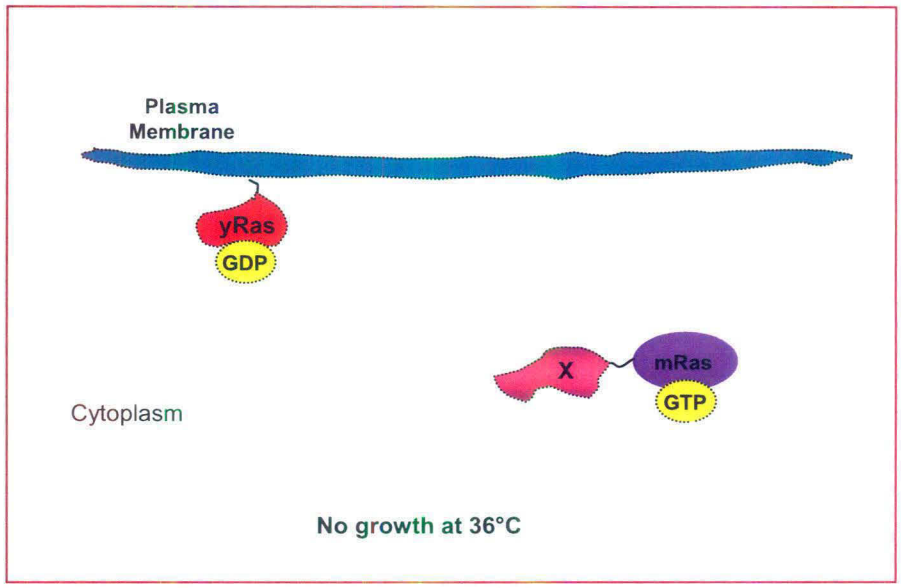
A



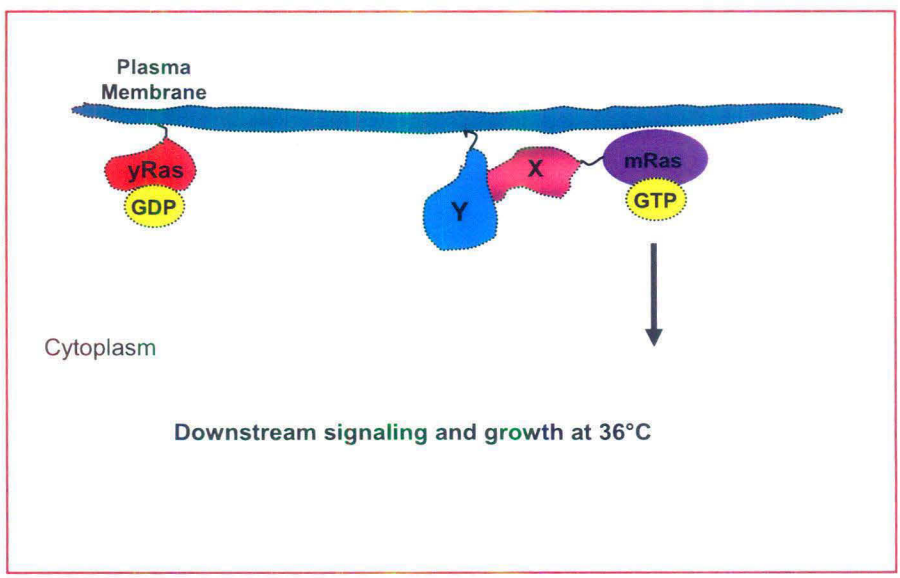
B

Fig. 6.3 SOS recruitment system

(A). No selective growth at 36°C, due to a lack of binding partner 'Y'. The guanidine exchange factor SOS remains in the cytoplasm. **(B)**. The membrane anchored prey 'Y', recruits 'X'-SOS to the membrane, since X and Y interacts the nucleotide exchange by SOS renders yeast Ras active and downstream signaling occurs. Yeast cell can grow at 36°C.



A



B

Fig. 6.4 Ras recruitment system

(A).The Ras recruitment system use the same temperature sensitive Cdc25-2 yeast strain as in the SOS system. **(B)**. Activated 'X'-mutantRas (mRas) is directed to the membrane, upon interaction between the bait 'X' and a membrane associated prey 'Y'. The downstream signaling leads to growth at 36°C.

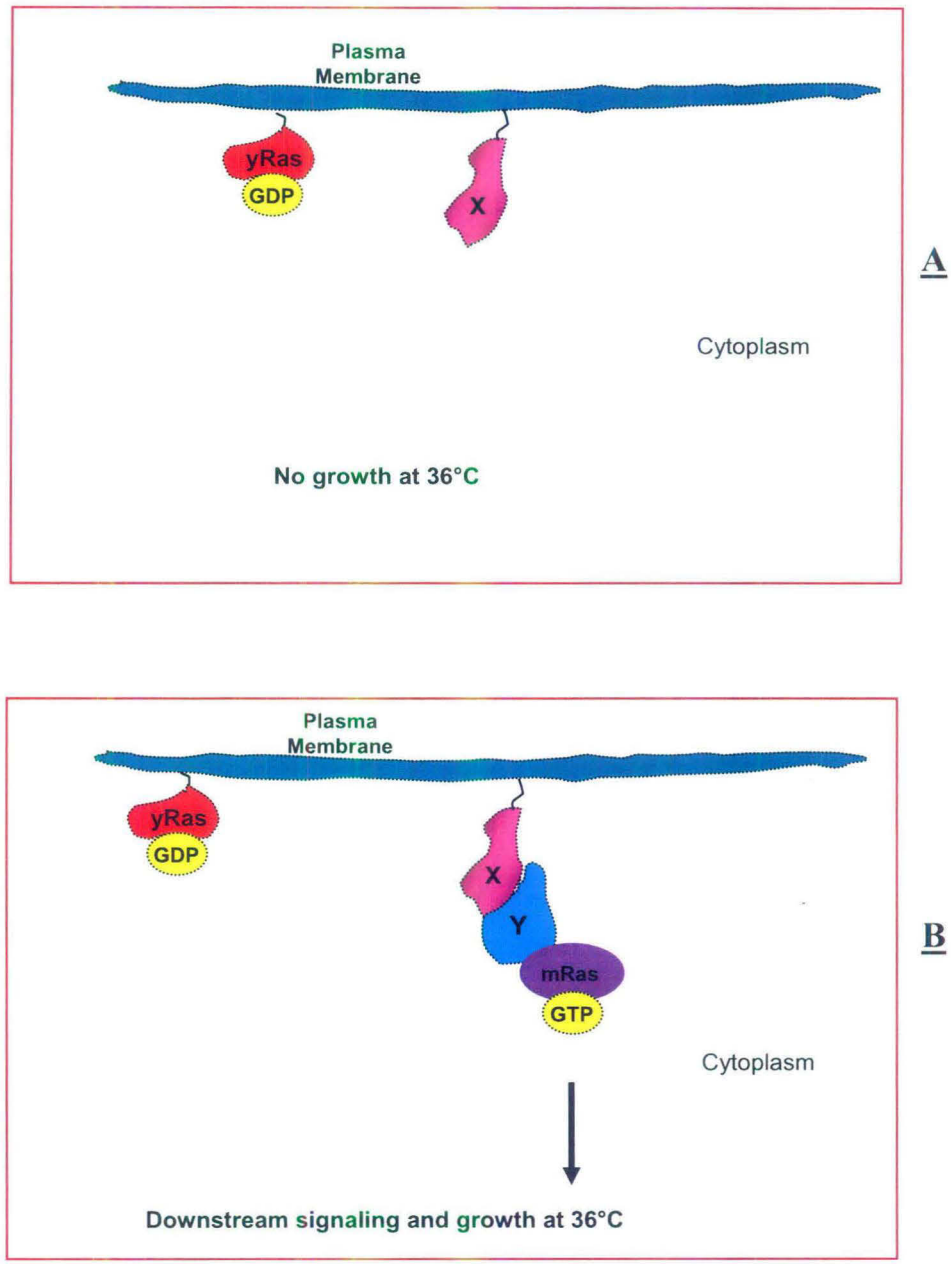


Fig. 6.5 Reverse Ras recruitment system

(A). The Reverse Ras recruitment system uses the same temperature sensitive Cdc25-2 yeast strain as in the SOS system. The Reverse Ras uses an integral membrane protein as bait 'X'. Contrary, to the Ras recruitment system, here the prey 'Y' is fused to activated mRas. **(B)**.The interaction of bait and prey leads to growth at non-permissive temperature.

based transcription is mediated by different transcription factors and is based on the activation of a modified *SNR6* reporter gene transcribed by the polymerase III (Marsolier *et al.*, 1997). U6, the transcript of the *SNR6* gene, is an essential small nuclear RNA involved in splicing. Transcription of *SNR6* is stimulated by binding of the transcription factor τ138p, a subunit of TF-IIIC, to a specific DNA sequence located downstream of the *SNR6* gene, the so-called B-block. Deletion of this B-block abolishes binding of τ138p and inactivates transcription of *SNR6*. The reporter construct of the RNA polymerase system bears five Gal4p binding sequences (UAS_G) in place of the original B-block (UAS_G-*SNR*). This otherwise inactive reporter gene can be activated by expression of a fusion protein between τ138p and the Gal4p-DBD. Activation of UAS_G-*SNR* can also be achieved by fusing τ138p to a protein that interacts with a known partner fused to the Gal4p-DBD, analogous to the conventional yeast two-hybrid system. For screening procedures a *SNR6* knock out strain and a temperature sensitive episomally expressed *SNR6* mutant is used (survival construct), which allows growth at 30°C, but not at 37°C (Petrascheck *et al.*, 2001). Plasmids encoding the survival construct, the τ138p- and Gal4p- fused potential interactors are inserted into the *SNR6* deleted reporter strain by triple transformation. Yeast harboring an interaction then is selected at the non-permissive temperature of 37°C (Fig. 6.6).

E. The repressed transactivator system

The yeast general transcription repressor TUP1 is recruited by MIG1 to upstream repressing sequences of glucose repressed genes. Gene repression by TUP1 is dependent on the corepressor SSN6 and might involve modulation of the RNA polymerase II holoenzyme function and organizing nucleosomes (Hirst *et al.*, 2001). To detect protein interactions with transactivating baits by the repressed transactivator method, the bait is fused to the DBD of the GAL4 yeast transcription factor. The repressing domain (RD) of TUP1 is attached to the prey. Without an interaction, the Gal4-DBD-bait is directed to the upstream activating sequences of GAL4 (UAS_{GAL4}) and induces transcription of the reporter gene *URA3*. If the DBD-bait is coexpressed with an interacting prey-TUP1-RD fusion, *URA3* is repressed and the readout of the interaction follows 5-FOA resistant yeast cells (Fig. 6.7).

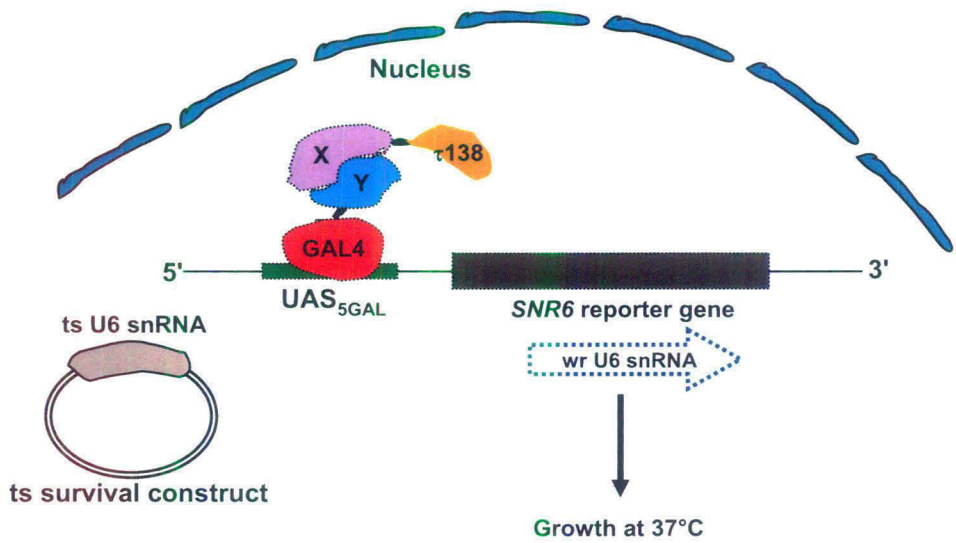


Fig. 6.6 RNA polymerase system- principle
Since the temperature sensitive (ts) survival construct is not functional at 37°C, only those cells are selected at 37°C which harbor an interacting τ138-X and Y-Gal4-AD The hybrid transcription factor binds to the operator sites, recruits the RNA pol. III, which leads to the expression of the essential U6 snRNA.

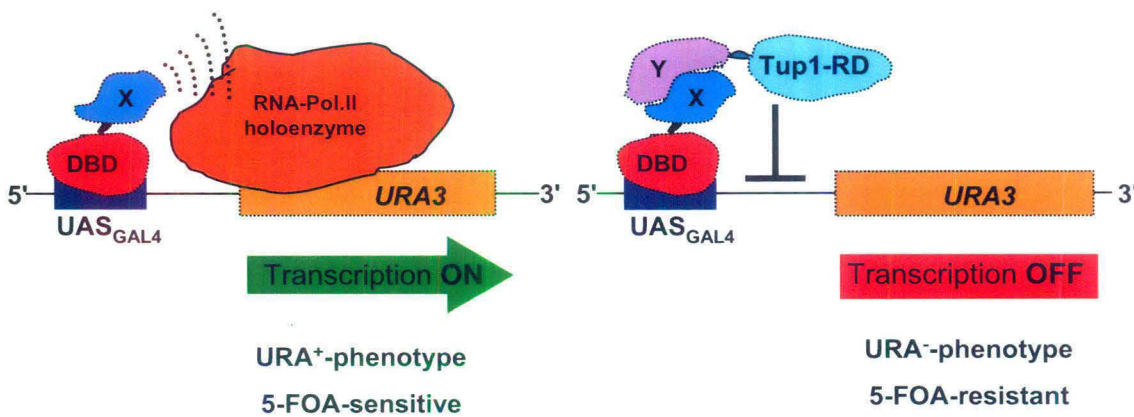


Fig. 6.7 The repressed transactivator system (RTA)- the principle

A transactivating bait is fused to the GAL4-DBD. The bait is recruited to the upstream activating sequences of GAL4 (UAS_{GAL4}) by the GAL4-DBD. Due to the intrinsic transcriptional activity of the bait, it is capable of recruiting the RNA polymerase II complex and activates transcription of *URA3*. The expression of *URA3* leads to a $URA3^+$ phenotype and 5-FOA sensitivity in the reporter strain. Upon interaction between the DBD-fused transactivator bait with a prey fused to the TUP1-repressor domain (Tup1-RD) the *URA3* reporter gene is repressed. Thus, interaction can be selected by 5-FOA resistance.

6.2 Results and discussion

Both defense-activating and -repressing WRKY proteins have been identified from different plants. This indicates that these proteins are important regulators of differential and graded plant defense responses to distinct types of microbial pathogens. Understanding the regulation of these WRKY proteins by other functional or signaling proteins will help in understanding the molecular basis of plant defense mechanisms. We have earlier discussed the isolation and expression of *Ascochyta rabiei* induced *CarWRKY1* gene from *Cicer arietinum* (chapter 4). The sequence and functional relationships of *CarWRKY1* with defense regulators like *AtWRKY53* and *-41* suggests its involvement during defense-signaling pathways. Therefore, it was desired to isolate the components of signaling cascade. These proteins may influence the binding or transcriptional activation activity that may ultimately influence its functions and chickpea defense responses.

6.2.1 Yeast two-hybrid library screening using CarWRKY1 as bait

The yeast two-hybrid screening system was selected to isolate CarWRKY1 interacting partners because it's a generic system. Among the conventional yeast two-hybrid screening kits, MATCHMAKER library construction and screening kit (Clontech) is widely used because of the ease to make cDNA library and the options of reporter genes in yeast strain AH109. Yeast two-hybrid library screening for transcriptional activator will activate the reporter gene without an interacting partner; therefore, it is always suggested to delete the transactivation domain. Therefore, a truncated CarWRKY1 (259 amino acids) protein was used. This truncated version, ΔCarWRKY1 was cloned in pGBKT7 at *Nco*I and *Bam*HI sites by using KT7EW1F and W1ADR6 primers (Table 3.7). Before library screening, its transcriptional activation potential was checked in comparison to other truncated versions. No yeast growth was observed by this truncated version of CarWRKY1 in auto-activation test and in X-gal agarose overlay assay. Therefore, we preferred it for further library screening.

To construct the library for Y2H screening, the RNA was isolated from *A. rabiei* infected chickpea. The cDNA library was prepared by pooling RNA from *A. rabiei* spore inoculated *C. arietinum* variety Pusa 362 (6 hpi and 24 hpi) samples. The protocols used for library construction, yeast strain AH109 transformation, and selection is described in section 3.14. Instead of co-transforming yeast with pGBKT-ΔCarWRKY1, cDNA library, and pGADT7-Rec simultaneously, we preferred to transform yeast with

pGBKT7-CarWRKY1 alone. The pGBKT7-ΔCarWRKY1 transformed cells were used to make competent cells to achieve high efficiency of cDNA and pGADT7-Rec co-transformation in yeast. From the ~3 x 10^5 yeast cells transformed, 110 colonies were selected on SD-Leu/-Trp/-His/-Ade (SD-QDO) plates after three days. They were patched again on SD-QDO + 20 mM 3-AT plates and then the cloned cDNA containing plasmids were rescued in *E. coli* for sequencing. Plasmid PCR performed using isolated plasmid mix from yeast clones as template revealed that many of the yeast clones possess more than one prey vector. Glycerol stock of each rescued clone was prepared after comparing the size of clones from yeast plasmid PCR and *E. coli* colony PCR. Nearly 20 clones that showed good growth after re-patching were selected for sequencing and further analysis. Surprisingly, most of the clones were found to be truncated and out of frame after sequencing (Table. 6.2). In the next batch of 20 clones, we observed similar problem along with redundancy. A few in-frame clones were selected for further confirmation by re-transformation with blank bait vector, pGBKT7-CarWRKY1and bait-dependency test with Δp53 protein, were showing non-specific interaction with most of the clones showed sticky proteins (Finley, 2008). Two clones that passed the initial verification tests showed homology with *Arabidopsis* AtRACK1A (for Receptor for Activated C-Kinase 1A) and Cysteine proteinase. The AtRACK1A and OsRACK1A from *Arabidopsis* and rice, respectively, have shown as important regulators of plant immune responses (Chen *et al.*, 2006; Nakashima *et al.*, 2008). Therefore, confirmation of the interaction of these two proteins with CarWRKY1 was further carried out.

Table 6.2 Summary of putative CarWRKY1 interacting clones isolated by Matchmaker based yeast two-hybrid screening.

S. No.	Clone	Homology	Frame
1.	Y2H-5	*Arabidopsis* Receptor for Activated C-Kinase 1a	+1
2.	Y2H-7	*Cicer arietinum* Cysteine proteinase	+1
3.	Y2H-10	Aspartate aminotransferase	+1
4.	Y2H-12	Cyclase family protein	+1
5.	Y2H-13	*Cicer* ribosomal protein S12	+3
6.	Y2H-22	*Vitis vinifera* Unknown protein	+2
7.	Y2H-27	Zinc finger protein	+2
8.	Y2H-28	*Arabidopsis* SAP18	+2
9.	Y2H-42	40S ribosomal protein S14	+1
10.	Y2H-46	Photosystem II polypeptide	+3
11.	Y2H-47	Na^+/H^+ antiporter	+3
12.	Y2H-49	*Cicer arietinum* Protease inhibitor/LTP family protein	+2
13.	Y2H-52	Unknown function	+1
14.	Y2H-54	Photosystem I P700 apoprotein A2	+1
15.	Y2H-69	Chlorophyll a/b binding protein	+1

6.2.2 Validation of yeast two-hybrid screened clones

The two clones, named as *CarRACK1A* and *CarCysProt*, were again transformed in AH109 strain along with pGBKT7-CarWRKY1 construct and the interaction of truncated clones was confirmed by their growth on SD-QDO plates. The truncated clones were also cloned with GAL4BD domain i.e., in pGBKT7 vector at *Nde*I and *Bam*HI restriction sites. The growth on SD-QDO and color development on X-α-gal plates confirmed the CarWRKY1 interaction with truncated CarRACK1a and CarCysProt in yeast. To further confirm this interaction with full length CarRACK1A and CarCysProt in yeast, it was desired to isolate their full-length cDNA. The 5′ truncated cDNA region of *CarRACK1a* was isolated by genome walking using CarRACK1R1 and CarRACK1R2 primers (Table 3.7). The CarRACK1A is a WD-domain containing protein and is highly conserved in various organisms. No intron is present between the primer binding site (used for genome walking) and the transcription start site. The full-length cDNA sequence of CarCysProt is available in the database. The coding sequences of both genes were cloned in-frame with GAL4AD- and GAL4BD-domains in pGADT7 and pGBKT7 vectors respectively. The interaction of CarRACK1 full-length turned out to be false as it showed auto-activation with GAL4AD. Full-length CarRACK1A in pGBKT7 vector did not show interaction with CarWRKY1. The full-length CarCysProt also showed negative results in both the constructs. Therefore, it was speculated that either these genes showed false positive results or the limitations of classical yeast two-hybrid technique hinders in the isolation of interacting partners for a transcription activator like CarWRKY1.

6.2.3 Testing of CarWRKY1 interaction with CarRACK1a and CarCysProt using DUALhunter system

The split-ubiquitin based DUALhunter system (Dualsystems Biotech) is a newly developed system to isolate interacting partners of transcriptionally active proteins, sticky proteins, and proteins that have clusters of highly acidic amino acids. This kit functions on the principle described in section 6.5.1. It has certain advantages like the correct expression and background growth of bait can be checked using prey control vectors pAI-Alg5 and pDL2-Alg5. The two constructs express a fusion of endogenous endoplasmic reticulum (ER) protein Alg5 to the wild-type Nub portion of yeast ubiquitin (pAI-Alg5) or to the Nub portion bearing the isoleucine to glycine mutation at position 13 (pDL2-Alg5). If bait is properly inserted into the ER membrane, co-expression with

pAI-Alg5 will result in activation of the reporter genes as wild-type NubI has a strong affinity for the Cub in bait fusion protein. On the other hand, co-expression with pDL2-Alg5 will not activate the reporter genes as NubG has virtually no affinity for Cub and Alg5 itself does not interact with protein of interest.

Many attempts to clone CarWRKY1 in pDHB1 (bait vector with Cub part and reporter LexA-VP16) at *Sfi*I site have failed. Such problem may arise due to the secondary structures formed by GC-rich regions at end of the fragment. Therefore, we cloned it at a single site *Nco*I of the vector pDHB1. The orientation of *CarWRKY1* insert was checked by PCR and later confirmed by sequencing. The correct expression of full-length CarWRKY1 in pDHB1-CarWRKY1 construct was confirmed by transforming it with pAI-Alg5 and pDL2-Alg5 plasmids in yeast strain NMY51. After checking the expression, pDHB1-CarWRKY1 was also transformed with plasmids of pPR3-N-CarRACK1 and pPR3-N-CarCysProt constructs (Fig. 6.8). This kit also did not confirm the interactions; therefore full length CarRACK1A and CarCysProt do not interact with CarWRKY1 in yeast.

6.2.4 Modifications of pPR3-N vector for recombination based cloning of cDNA in yeast

Since screening of CarWRKY1 interacting proteins by MATCHMAKER kit was inconclusive, DUALhunter kit was used for further experiments. The prey vector pPR3-N used in this system is not suitable for library screening by cDNA digestion based cloning which is labour intensive, less efficient, and requires large amount of cDNA. As the numbers of steps are more so the representation of clones with subsequent steps also decreases. Therefore, it was decided to incorporate the recombination based cloning method in this prey vector to generate a new modified vector.

Other modification in this vector was aimed to express cloned cDNAs in all three open reading frames. This was based on the observation that a cDNAs or genomic library made to express in all six frames by different vectors showed increased efficiency during Y2H screening (Maier *et al.*, 2008.). Another modification was the incorporation of nuclear export signal (NES) at *Bam*HI and *Nco*I restriction enzyme site. The purpose was to keep the prey proteins in the cytoplasm as many prey proteins can get localized to nucleus using their NLS.

We have amplified the MCS of pGADT7-Rec vector using primers KKF1 and KKR1 to clone in pPR3-N. This amplified product was cloned in pDRIVE U/A PCR

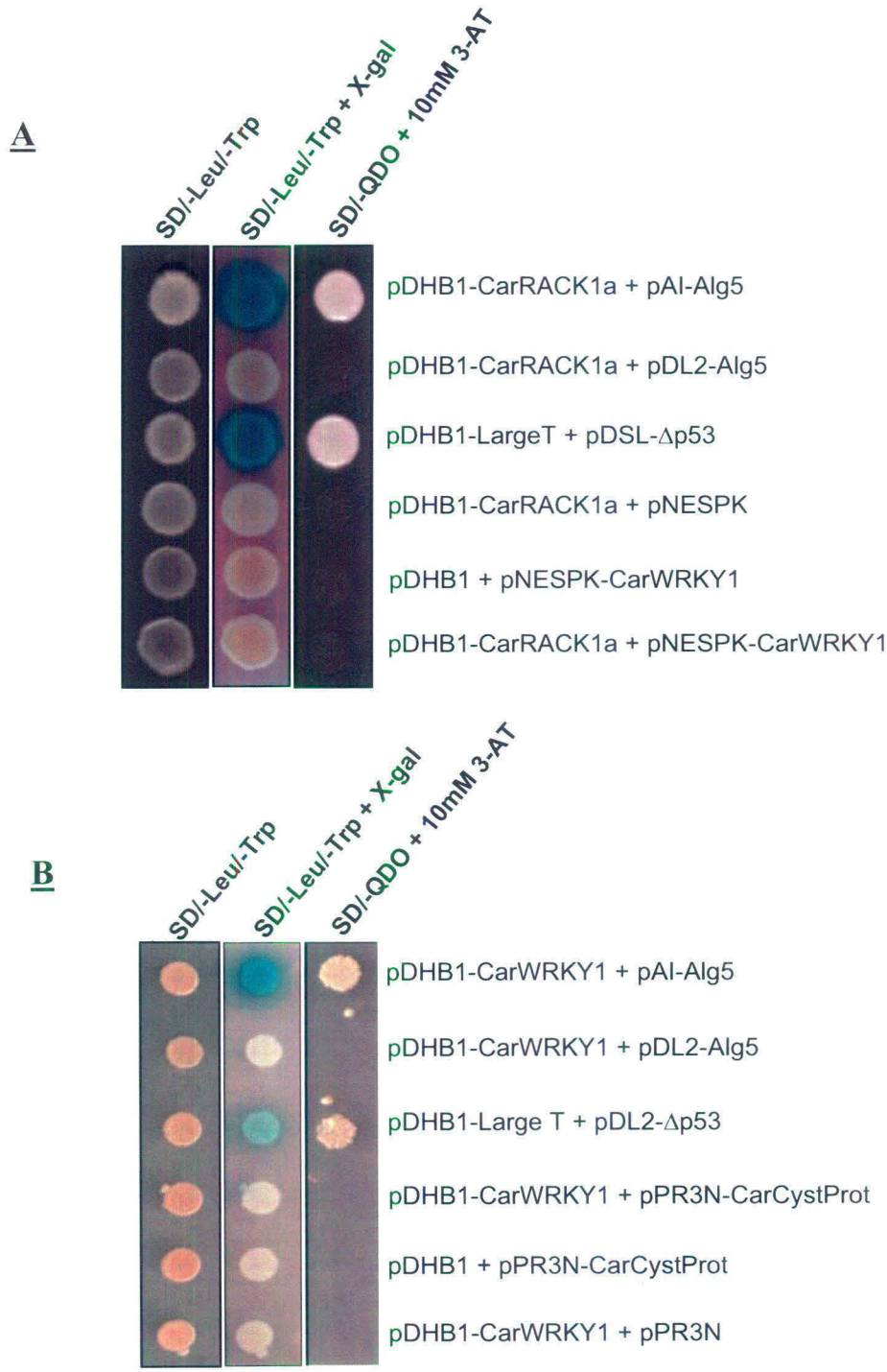


Fig. 6.8 CarRACK1a and CarCysProt interaction analysis with CarWRKY1 in yeast

The interaction of CarWRKY1 with full length proteins of CarRACK1a **(A)** and CarCysProt **(B)** was checked using DUALhunter kit. Both these clones failed to show any interaction with CarWRKY1 in yeast. Appropriate controls are used to rule out any false positive results.

cloning vector and from there it was cloned in pPR3-N vector at *Bam*HI and *Sal*I restriction sites. This new vector with MCS equivalent to pGADT7-Rec was named as pPR3MK.

Further modification in pPR3MK was related to the use of a nuclear export signal, which has been used for various studies in yeast. The earlier studied nuclear export signal of protein kinase inhibitor (PKI) was found appropriate for our vector modification (Klemm *et al.*, 1997; Kosugi *et al.*, 2008). The complementary oligonucleotides PKINESF and PKINESR (Table 3.7) were used to incorporate NES in pPR3MK at *Bam*HI and *Nco*I restriction sites of pPR3MK. This new vector was designated as pNESPK. In pNESPK vector, further modifications were carried out to delete a *Sfi*I site and addition of adenine nucleotides in such a way that the cloned cDNA can be expressed in all the three frames. This was done by using complementary oligonucleotides- Frame1/cFrame1, Frame2/cFrame2, and Frame3/ cFrame3 (Table 3.7) and cloning them at *Nco*I and *Sma*I restriction sites of pNESPK. This new series of yeast two-hybrid library screening vectors were named as pSUPF1, pSUPF2, and pSUPF3 (for Split-Ubiquitin Prey vector Frame1, 2, and 3) (Fig. 6.9). The validity of the nuclear export signal incorporation in pSUPF vector was checked by cloning of Δp53 in pDHB1 and pSUPF series vectors along with SV40 largeT in both the bait and prey vectors. The Δp53 contains both NES and NLS while LargeT protein only contains NLS. Therefore, the difference in reporter gene activation by the accumulation of prey protein (here LargeT) in cytoplasm through NES could be detected. The initial results validated the use of NES. However, more quantitative assays by mutating NLS of LargeT are required for final confirmation.

6.2.5 Y2H cDNA library screening using DUALhunter system

To identify the interacting partners of CarWRKY1, a new yeast two-hybrid screening was carried out by using the same protocol as mentioned in section 3.14. The difference was in the bait (now pDHB1-CarWRKY1) and prey vectors, which are *Sma*I linearized and dephosphorylated pSUPF series vectors. The cDNA library was transformed with each pSUPF series of vectors, thus in total three libraries were screened. After selection on SD-QDO plates for 5 days, eleven colonies appeared. These colonies were patched on SD-QDO+10mM 3-AT plates. After selection for 3 days the colonies were inoculated in the liquid SD-QDO+10mM 3-AT for plasmid isolation. Using the plasmid isolated from yeast clones as template, the size and number of inserts in a yeast colony was checked by

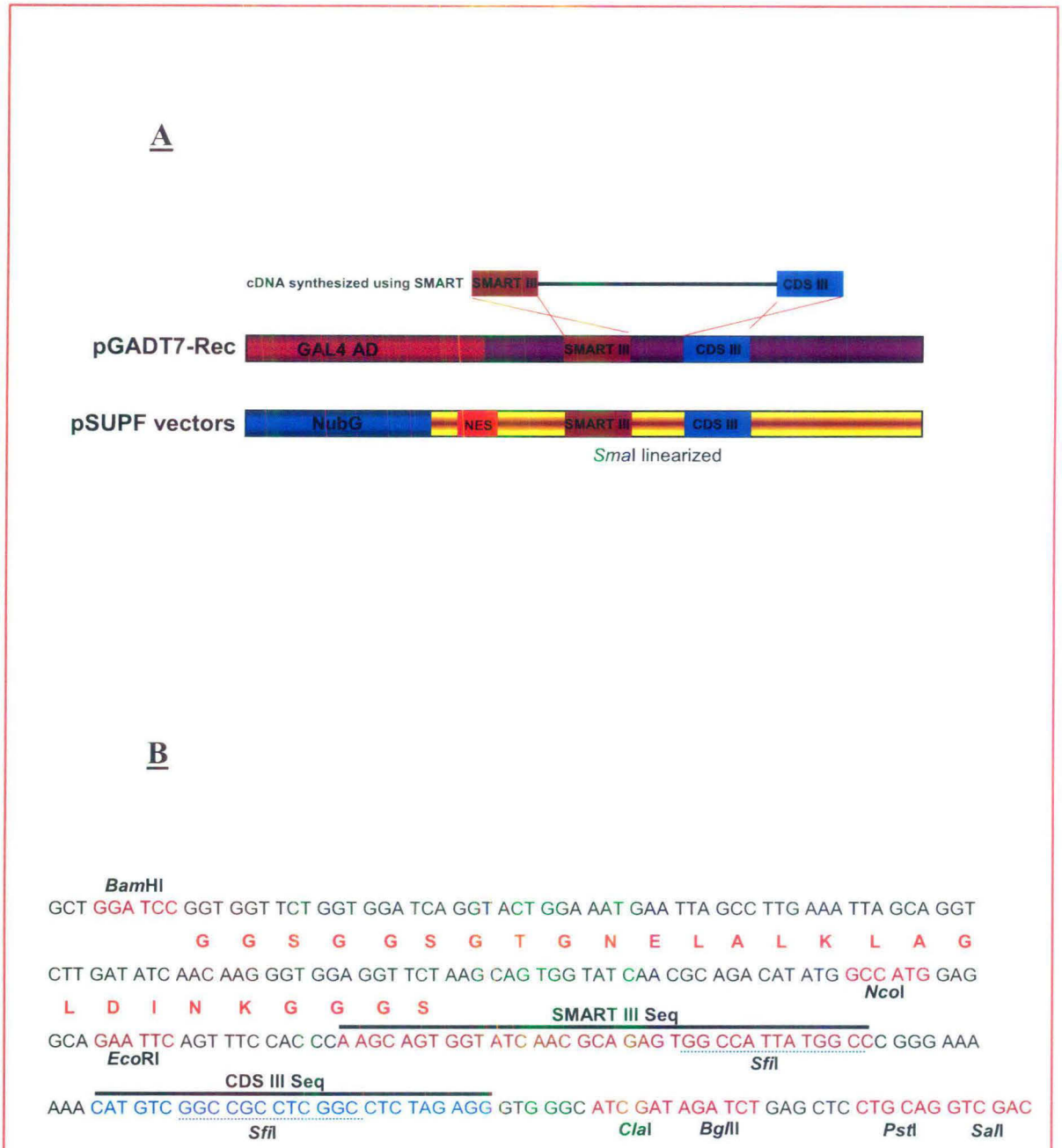


Fig. 6.9 Modified pSUPF vectors for split-ubiquitin based yeast two-hybrid

(A). The MCS which recombines with the cDNA made using SMART technology was incorporated in pPR3-N with some modifications. pSUPF (Split-Ubiquitin Prey vector Frame). **(B)**. The sequence of pSUPF1 near MCS region that are modified in pPR3N to make it suitable for recombination. Amino acids incorporated for NES are shown in orange color. Enzyme restriction sites are duly marked.

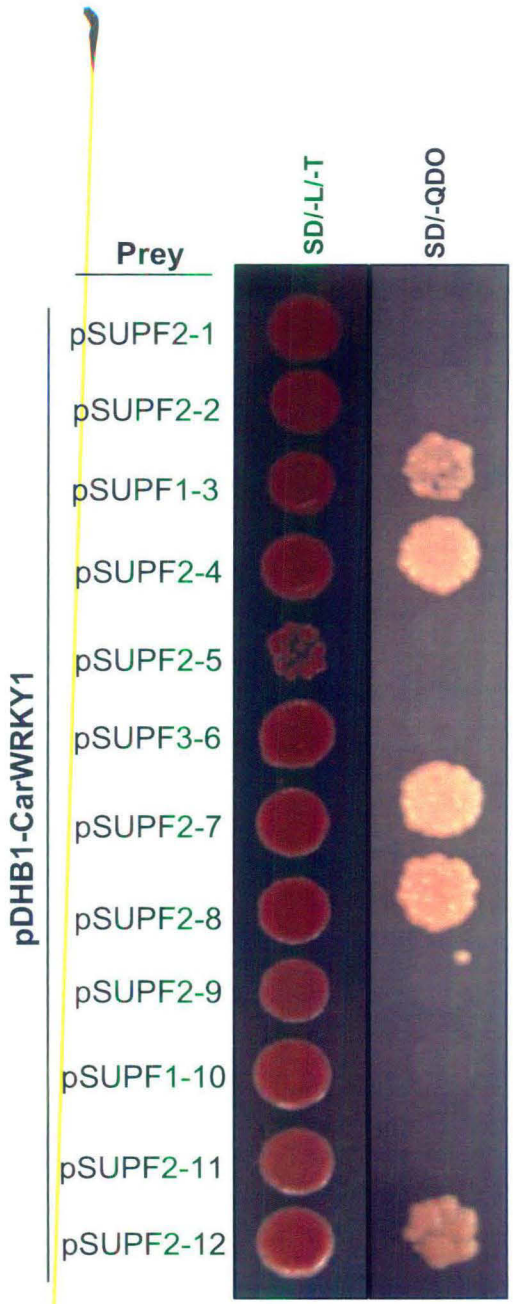


Fig. 6.10 Confirmation of CarWRKY1 interaction with primary screened clones of modified split-ubiquitin based two-hybrid system.

The pDHB1-CarWRKY1 construct was retransformed with rescued plasmids from primary screened yeast clones and were selected on SD/-Leu/-Trp/-His/-Ade (SD-QDO) for growth to confirm interaction. Clones 3, 4, 7, 8, and 12 showed growth at high stringency pointing towards them being the putative interacting partners.

PCR and digestion. These plasmids were rescued in *E. coli* and sequenced (Table 6.3). The result of retransformation and selection on SD-Leu/-Trp and SD-QDO is shown in Fig. 6.10. The retransformation eliminated the earlier observed false positive clones, mainly by small fragments.

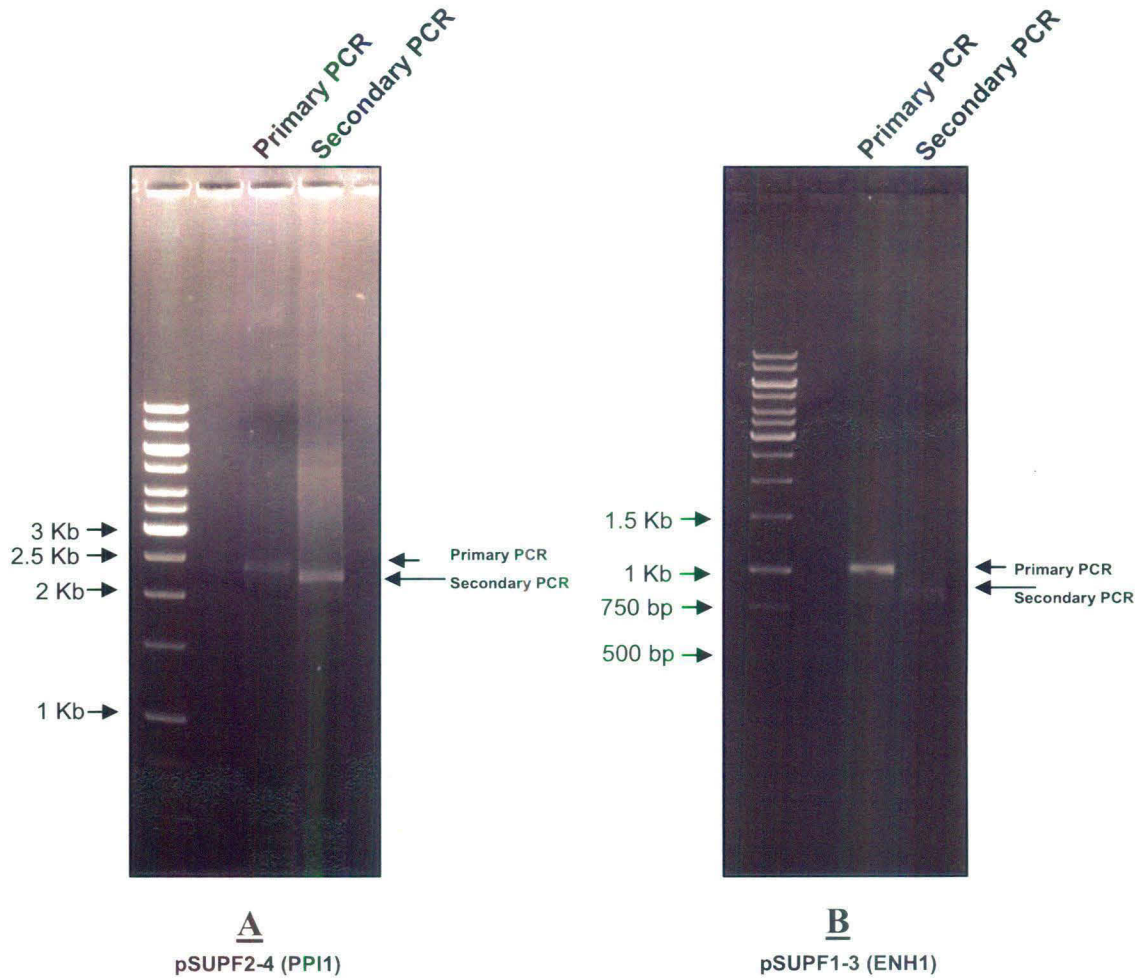
Table 6.3 Isolated clones of split-ubiquitin based Y2H screening

S. No.	Clone	Homology
1	pSUPF2-1	Unknown
2	pSUPF2-2	Unknown mRNA *Medicago*
3	pSUPF1-3	(Enhancer of SOS3-1) ENH1
4	pSUPF2-4	Proton Pump Interactor (PPI1) At4g27500
5	pSUPF2-5	Unknown
6	pSUPF3-6	Unknown
7	pSUPF2-7	Unknown
8	pSUPF2-8	Unknown
9	pSUPF2-9	Unknown
10	pSUPF1-10	Unknown
11	pSUPF2-11	Unknown
12	pSUPF2-12	Unknown

The isolated clones were truncated from 5′-cDNA ends. Therefore, 5′-RACE of two-selected clone was performed. First was the pSUPF1-3 clone that showed homology with *ENH1* (*enhancer of sos3-1*) and second was pSUPF2-4 clone that showed homology with *PROTON PUMP INTERACTOR1* (*PPI1*) (Fig. 6.11)(Hirabayashi *et al.*, 2004; Zhu *et al.*, 2007). These clones need to be examined further by bait dependency analyses and downstream confirmatory tests.

6.2.6 Interaction of *Arabidopsis* MPKs with CarWRKY1

Some of the WRKY members are shown as direct targets of mitogen activated protein kinases (MAPKs/MPKs) (Menke *et al.*, 2005). Recently, out of 55 used WRKY proteins of *Arabidopsis* 25, were shown to be the targets of activated MPKs (Popescu *et al.*, 2009). This protein microarray data was further validated for AtWRKY6, -53, -62, and -65 by checking the phosphorylation status through mobility shifts in SDS-PAGE. The AtWRKY25 and 53 are shown to be indirect target of *Arabidopsis* MAPK4 through MKS1 (MAP kinase 4 substrate 1) while AtWRKY22 and 29 are considered as targets of flg22 activated MAPK cascade (Asai *et al.*, 2002; Andreasson *et al.*, 2005). OsBWMK1 from rice mediates SA-dependent defense responses by activating the transcription factor OsWRKY33 (Koo *et al.*, 2009). Beside these, AtWRKY53 is phosphorylatd by AtMEKK1 and tobacco WRKY1 is phosphorylated by SIPK to mediate HR-like cell



C

>CarENH1 protein

MSAMVAGTSSSFSATFTTTRSSKNSHLTPSKTLTSLPCQKTSLLGLSLHEAKRGVSVSFLGD
NKNGTYSISRRRFEINARTAGASKTIEVEVDKPLGLTLGQKNGGGVVITAVEGGGNAARAGL
KSGDQVLYTSSFFGDELWPADKLGFTKTAIQAKPDSVYFVVSRGADVDVKRLPKRPAPPRFG
RKLTETQKARATHICLDCGYIYFLPKSFDDQPETYSCPQCQAPKKRFAPYDVNTGRAIGGGG
L**PPIGVIIGLVAGLGAVGALLVFGLQ**

Fig. 6.11 The 5′-RACE of Y2H Clone(pSUPF1-3) and Clone (pSUPF2-4).

Primary and secondary (using nested primers) PCR products of 5′-RACE of chickpea homologs of **(A)** *PPI1* and **(B)** *ENH1* resolved on 1% agarose along with 1 Kb DNA ladder (MBI Fermentas). **(C)**. The deduced protein sequence of CarENH1 after RACE. Putative trans-membrane domain is highlighted.

death (Menke *et al.*, 2005; Miao *et al.*, 2007).

The *C. arietinum* MPK1 having high homology with AtMAPK3 was earlier isolated in the laboratory and it showed *Ascochyta* induced expression pattern. Therefore, we first checked its interaction with CarWRKY1. The CarWRKY1 was not interacting with CarMPK1 in yeast. The conservation of MAPK cascade from lower to higher eukaryotes has prompted many groups to use MAPKs from heterologous organisms (Teige *et al.*, 2004). Therefore, we proceeded with the verification of CarWRKY1 interaction with seven *Arabidopsis* MAPKs belonging to three classified groups (Fig. 6.12A) The seven MPKs obtained as pGAD425 clones were digested with various combinations of restriction enzymes to clone in pSUPF series vectors (Table 6.4). The obtained clones were confirmed by digestion and sequencing (Fig. 6.12B) The interactions were verified by transforming seven pSUPF cloned MPKs with blank pDHB1 vector as negative control and with pDHB1-CarWRKY1 as experimental. The spotting of transformed clones on SD/-Leu/-Trp and SD-QDO plates and β-galactosidase activity confirmed that MAPK7 strongly interacts with CarWRKY1 in yeast. However, AtMPK4 also showed autoactivation with blank pDHB1 vector (Fig. 6.13).

Table 6.3 Summary of AtMPKs cloning in pSUPF2 vector.

Clone	MAPKs cloning site in pGAD425	MAPKs ORF size (bp)	MAPKs cloning sites in pSUPF2
#273, MPK1-pGAD425	*Bam*HI, *Sal*I	1113	*Nco*I, *Sal*I
#445, MPK2-pGAD425	*Nco*I, *Not*I	1131	*Nco*I, *Sal*I
#258, MPK3-pGAD425	*Bam*HI, *Sal*I	1113	*Sma*I, *Sal*I
#226, MPK4-pGAD425	*Bam*HI, *Sal*I	1131	*Sma*I, *Sal*I
#446, MPK5-pGAD425	*Nco*I, *Not*I	1131	*Nco*I, *Sal*I
#270, MPK6-pGAD425	*Bam*HI, *Sal*I	1188	*Sma*I, *Sal*I
#447, MPK7-pGAD425	*Nco*I, *Not*I	1107	*Nco*I, *Sal*I

The MAPK7 also interacts with AtWRKY53 and phosphorylates, which is dependent upon the activated MKK7. Transient overexpression of wild type or activated MKK7 results in extensive cell death in *Nicotiana benthamiana* (Popescu *et al.*, 2009). The *bushy and dwarf1* (*bud1*) mutant of *Arabidopsis* have increased expression of MKK7 leading to SA overproduction, may leads to defects in polar auxin transport and lateral root growth (Dai *et al.*, 2006). This mutant has increased basal defense and systemic acquired resistance. Therefore, MKK7 may be a link between auxin signaling and plant defense (Zhang *et al.*, 2007). Further studies on this cascade will give insight for AtWRKY53 like WRKY proteins in mediating such effects.

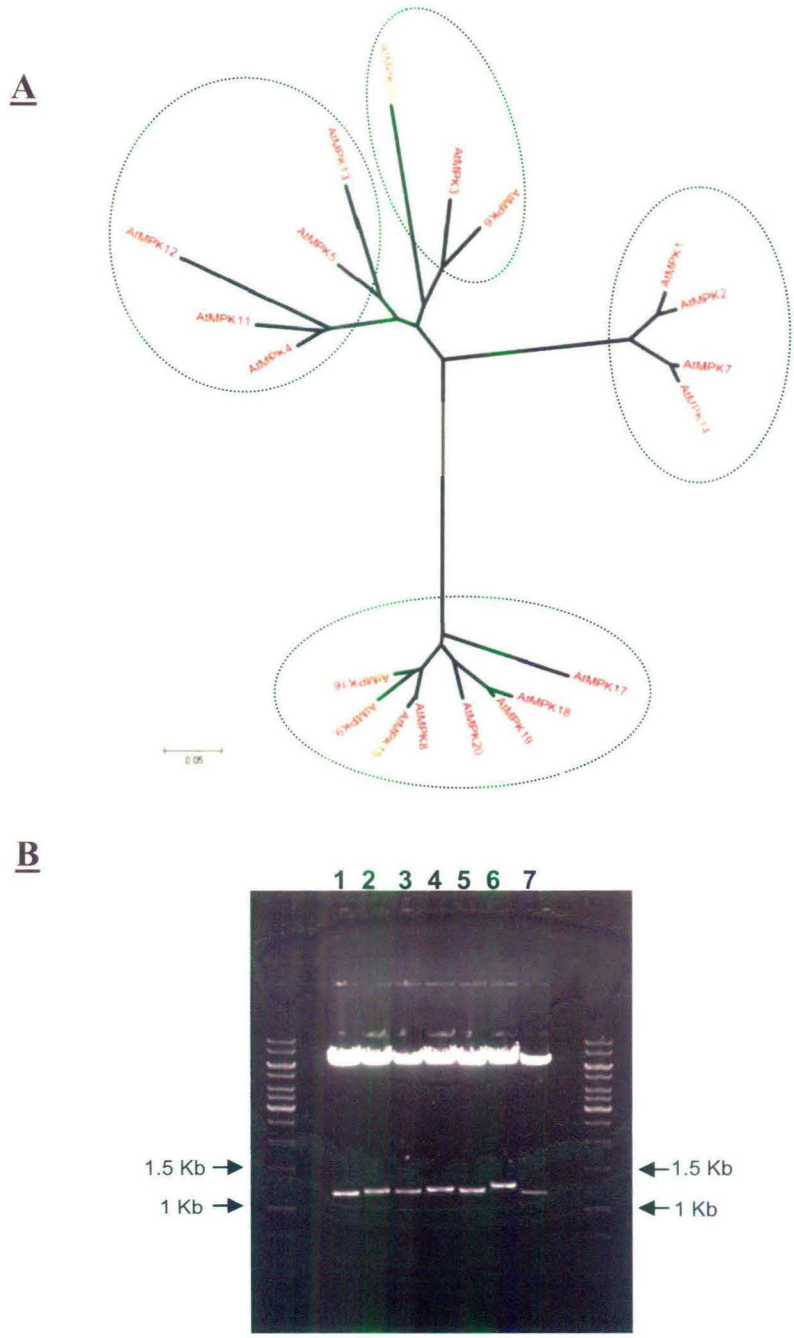


Fig 6.12 (A) Phylogenetic analysis of AtMPK from *Arabidopsis*.

Alignment of twenty *Arabidopsis* AtMPKs was performed using ClustalW and phylogenetic tree was made using MEGA 4 software. Four different subgroups of AtMAPKs are encircled.

(B). Confirmation of AtMPKs cloning in pSUPF2 by digestion.

To confirm cloning in pSUPF2, digestion was performed with appropriate restriction enzymes used for cloning of AtMPKs. The digested products were resolved on 1% agarose gel along with 1 Kb DNA ladder.

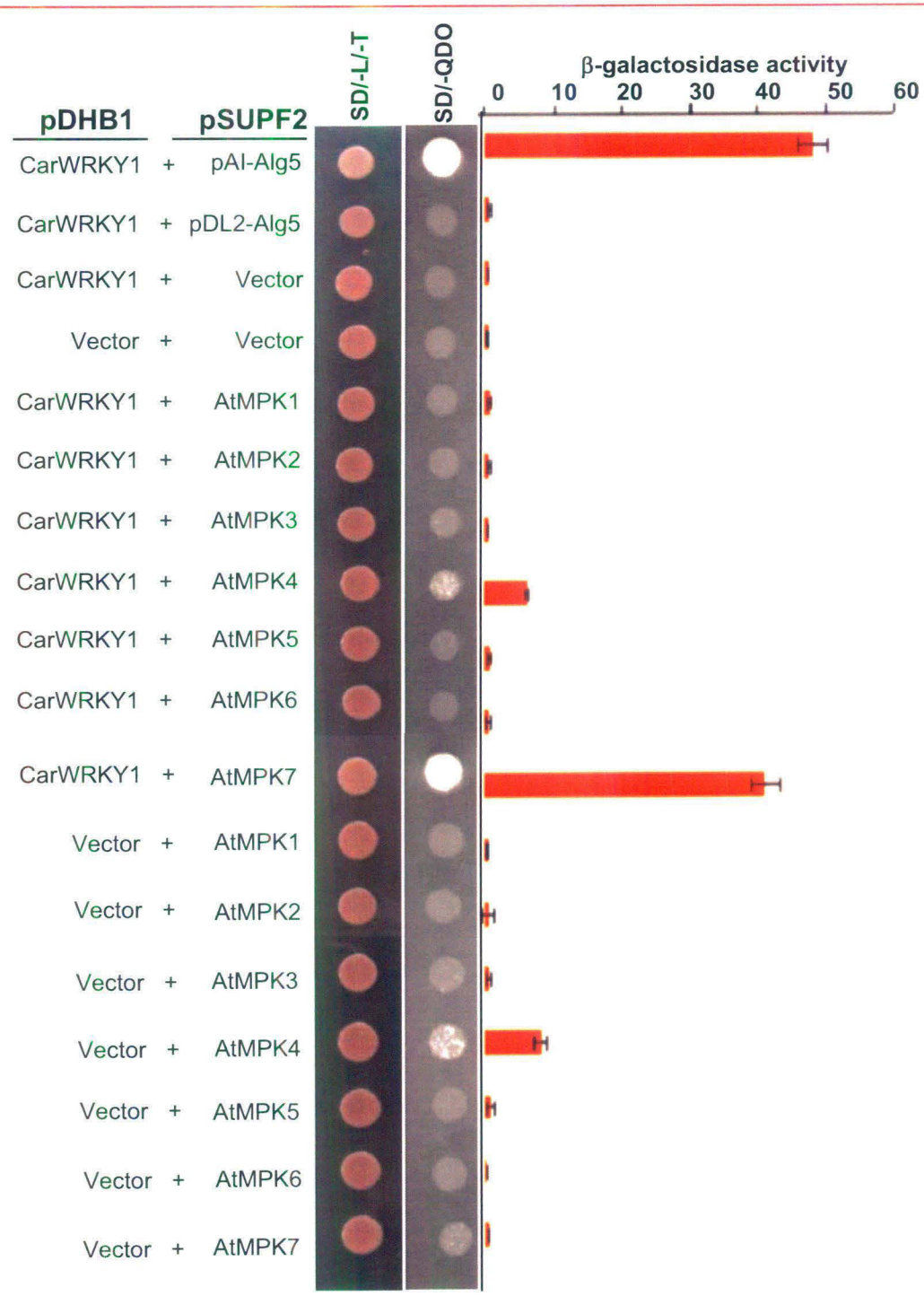


Fig. 6.13 CarWRKY1 interacts with *Arabidopsis* AtMAPK7 in yeast

Interaction of chickpea CarWRKY1 was checked with seven *Arabidopsis* AtMPKs by DUALhunter kit. The transformed yeast cells were spotted on SD/-Leu/-Trp and on SD/-Leu/-Trp/-His/-Ade and growth monitored after 3 days. The quantitative measurement of interaction was done by β-gal assay using ONPG as substrate. AtMPK4 construct shows non-specific interaction.

6.3 Conclusion

Because of the importance of protein interactions for biology, a wide spectrum of advanced methods has been developed. But most of the knowledge about protein-protein interaction networks in all organisms studied so far is largely derived from yeast two-hybrid. A number of variants have emerged for yeast two-hybrid according to need and convenience but no one is full proof. Every interaction needs to be checked by at least two other systems or confirmatory tests like bait-dependency with two unrelated proteins. We have screened library to identify signaling components of CarWRKY1 with a commercially available kit based on classical two-hybrid. The use of truncated CarWRKY1 protein also trans-activated the reporter gene in presence of some sticky proteins. Some cDNAs were found to be out of frame but still activated the reporter genes by encoding non-functional truncated proteins. Thus, by using this kit we failed to isolate true interacting proteins of CarWRKY1 despite isolation of many clones in primary screening. These false results may also be contributed by inappropriate folding of truncated CarWRKY1 used.

We have modified a split-ubiquitin based pPR3-N vector to pSUPFs for direct cloning of cDNAs, made by SMART technology, into this prey vector through recombination. Further, we improved this vector by incorporating NES and made three vectors, pSUPF1, pSUPF2 and pSUPF3, which express cDNAs in three different open reading frames. The initial result for the verification of NES incorporation in pSUPFs with LargeT and Δp53 were positive. Therefore, we screened for yeast two-hybrid, using modified split-ubiquitin based system with full-length CarWRKY1 protein as bait. The primary Y2H cDNA library screening resulted into isolation of few clones. The retransformation of these rescued clones with CarWRKY1 bait yielded only five clones as positive. Validation assays like bait dependency, vector shuffling, etc are in progress to confirm these interactions and to prove the validity of this modified Y2H system. We also checked for the interaction of seven *Arabidopsis* AtMPKs with CarWRKY1 in yeast. This experiment resulted in the interaction of AtMPK7 with CarWRKY1. This activated AtMPK7 is also known to phosphorylate AtWRKY53, an important plant defense and development regulating WRKY protein. The functional similarity of CarWRKY1 with AtWRKY53 and its interaction with AtMPK7 are encouraging for further studies in chickpea defense signaling.

Chapter 7

Summary

Plants have layered defense mechanisms to protect against invading pathogens. If one layer of defense is breached then second layer protects and so on. Thus, in nature we see disease is rare, though environment is full of potential pathogens. Detection of a pathogen by a plant triggers a complex set of signal transduction pathways and a battery of defense mechanism gets activated. Upon perception of pathogen/microbe-associated molecular patterns (PAMPs), plants can activate distinct mitogen-activated protein kinase cascades and associated signaling events, leading to PAMP-triggered immunity (PTI). Successful pathogens suppress PTI through secreted effector proteins and, as a result, cause diseases. Coevolution of plants with the virulent pathogens can give rise to specific plant disease resistance (R) proteins that recognize pathogen effectors and activate highly efficient effector-triggered immunity (ETI). Both PTI and ETI are associated with the accumulation of defense signal molecules such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). *Arabidopsis* mutants defective in SA biosynthesis or signaling are compromised in resistance to biotrophic pathogens. The JA- and ET-mediated signaling pathways, on the other hand, often mediate defense against necrotrophic pathogens. A complex web of interactions between different hormonal pathways contributes to plant development and defense.

Chickpea is world's third most important grain legume. It is a major source of dietary protein and a significant contributor to agricultural sustainability by fixing atmospheric nitrogen. The yield potential of chickpea is limited by a series of biotic and abiotic stresses, including Ascochyta blight, Fusarium wilt, *Helicoverpa armigera* infestation, drought, and salinity. Ascochyta blight (AB), caused by a necrotrophic pathogen *Ascochyta rabiei*, is a major factor in the low productivity of chickpea across the globe sometimes causing 100% yield losses. Despite many reports on QTLs for resistance to AB, the use of marker-assisted selection in breeding for resistance to AB in chickpea is limited. This could be attributed to variability in resistance and virulence mechanisms of chickpea and *A. rabiei* respectively, which changes with environment. To understand the resistance mechanisms of chickpea against *A. rabiei*, we have isolated many defense-related genes induced by *A. rabiei* from resistant and susceptible cultivars using suppression subtractive hybridization (SSH). The roles played by some of the isolated genes in chickpea defense are under various stages of investigation. One such gene of WRKY superfamily group III member, *CarWRKY1*, was selected for the present study.

Many evidences have shown that WRKY transcription factors play important roles in regulation of genes associated with plant defense responses. Pathogen infection or treatment with elicitors or signaling molecules induces rapid expression of *WRKY* genes from a number of plants. In *Arabidopsis*, expression of 49 out of 72 tested WRKY genes were differentially regulated after pathogen infection or SA treatment. A number of defense-related genes contain WRKY binding element, W- and WK-boxes, in their respective promoters.

The *CarWRKY1* gene gets rapidly induced after *A. rabiei* spore inoculation. In this analysis, *CarWRKY1* showed biphasic induction in both resistant [FLIP84-92C(2)] and susceptible [Pusa 362] varieties. The biphasic induction is mainly associated with the genes that are induced during hypersensitive response, as two peaks of reactive oxygen species burst occur in most plants upon pathogen invasion. The *CarWRKY1* gene also responds to jasmonic acid and mechanical wounding, which suggests diverse role of this gene in stress responses. Jasmonic acid is also an important hormone in regulating defense responses against necrotrophic pathogens. Therefore, *CarWRKY1* could be a component of chickpea defense mechanism against the necrotrophic fungus *Ascochyta rabiei*.

The cDNA of *CarWRKY1* encodes for a protein of 364 amino acids and has a single WRKY domain with WRKY superfamily group III like C_2-HC type zinc-finger motif. Many putative phosphorylation sites were detected in the CarWRKY1 protein sequence. This is consistent with the recent reports of WRKY proteins being a major target of various signaling cascades operated through MAPKs. Based on the sequence, *CarWRKY1* is homologous to *Arabidopsis AtWRKY53* gene. The *AtWRKY53* gene is an important regulator of basal defense, systemic acquired resistance, and development in *Arabidopsis*. It also showed homology with other WRKY family members from different plants. The yeast 'nuclear transport assay' revealed CarWRKY1 localization in nucleus. The presence of conserved KKRK signal in the CarWRKY1 protein may contribute for its nuclear localization. In the yeast transcriptional activation assay, various deletion constructs of CarWRKY1 were used to delineate the functional activation domain. The growth on auxotrophic SD-QDO plates, X-gal overlay assay, and quantification analysis by β-galactosidase activity were used to confirm transactivation potential in yeast. These assays revealed that a C-terminal region of ~30 amino acids was contributing to transactivation by interacting with RNA polymerase II holoenzyme complex proteins.

In order to elucidate the role of *CarWRKY1* gene in plant defense, it was constitutively overexpressed in tobacco under the *CaMV35S* promoter. The T_0 tobacco lines that showed high expression of *CarWRKY1* transcript and also showed visible developmental defects. Many lines showed necrosis on leaves before flowering. This correlated with the increased amount of H_2O_2 at those sites, demonstrated by appearance of brownish color after the DAB staining. The *CarWRKY1* overexpressing tobacco plants showed early senescence and flowering with respect to control plants that were transformed with blank pBI121 vector. In the overexpressing lines flowering occurred approximately one month earlier than the control plants. The overxpressing T_0 plants showed stunted growth compared control plants. Some high overexpressing lines showed premature death before reaching to the flowering stage. The flower buds were small in size and less in number in some of the overexpressing lines compared to control plants. Morphological defects like stunted growth and leaf necrosis were rescued to some extent in T_1 generation plants but early flowering and senescence was consistent with T_0 generation. There are reports of similar phenotypes when some WRKY family members were overexpressed in rice and *Arabidopsis*. The analyses of T_2 generation transgenic plants are underway at the time of the submission of thesis. These observations and results will surely give an insight to understand the role of *CarWRKY1* in plant defense.

We have observed rapid and high level of *CarWRKY1* transcript accumulation in response to *A. rabiei*, SA, JA, and wounding. These data points towards *CarWRKY1* being an early responsive gene. To delineate the *cis*-acting elements present in its 5′-regulatory region the promoter was isolated and various 5′-deletions were made which were further used to transform tobacco. The resulting transgenic plants showed activity of *uidA* reporter gene in floral parts. These transgenic plants also responded to wounding and the GUS activity was observed around the wounded regions of leaves. The detailed analysis of *CarWRKY1* 5′-regulatory regions deletion constructs in transgenic plants will be useful in order to elucidate their role in biotic stress response.

Many WRKY members are important regulators of defense signaling and this is the first study of a WRKY gene isolation from chickpea. Therefore, we screened for its interacting partners to isolate other important proteins involved in defense regulation and signaling. The screening through classical yeast two-hybrid system resulted in isolation of many false-positive interacting partners of this transcriptional activator. Therefore, we have modified a split-ubiquitin based yeast two-hybrid system. Using this modified

system, we screened for interacting partners of CarWRKY1 that resulted in the isolation of few putative clones. The validity of these interactions with CarWRKY1 in yeast is under progress. The MAPK cascade forms an important part of signaling in every eukaryotic organism and especially in plant defense. Therefore, we have cloned seven MAPKs from *Arabidopsis* in our modified yeast two-hybrid system and checked interaction with CarWRKY1. The AtMAPK7 showed interaction with CarWRKY1, which is parallel with the interaction of this MAP kinase with AtWRKY53, a close homologs of CarWRKY1.

Therefore, *CarWRKY1* is an important gene induced by *A. rabiei*, whose functional similarity with known defense regulators makes it a good candidate for further analysis in crop plants. Future work would be concentrated on the generation of *CarWRKY1* overexpressing and down-regulated chickpea plants to demonstrate its role during chickpea-*Ascochyta* interactions.

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