IDENTIFICATION AND CLONING OF GENES INDUCED AND / OR REPRESSED UPON TREATMENTS OF WHEAT PLANTS (AVOCET S) WITH BTH, BABA AND *Trichoderma harzianum* Raifi KRL-AG2

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ABSTRACT

IDENTIFICATION AND CLONING OF GENES INDUCED AND / OR REPRESSED UPON TREATMENTS OF WHEAT PLANTS (AVOCET S) WITH BTH, BABA AND *Trichoderma harzianum* Raifi KRL-AG2

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One of the major problems concerning the production of food crops is the controlling of plant diseases to maintain the high quality and yield. Wheat diseases are caused by parasitic bacteria, fungi and viruses that are a major hazard in wheat production. Therefore, understanding of any resistance mechanism is prerequisite for the successful utilization of wheat crop species in modern agriculture. The phenomenon of induced resistance by fungi, bacteria, microbial elicitors and chemicals has been investigated widely and resulted in many discoveries that conclude a general realization that the disease resistance signaling pathway in plants shares a number of common elements with those leading to innate immunity but a few of them have been characterized at the molecular level yet. Therefore our goal in this study is to identify genes activated or repressed after treatment of wheat plants with biological elicitor fungus, *Trichoderma harzianum*, and chemical inducers, benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) and ß-aminobutyric acid (BABA).

mRNA differential display technique, which is a powerful tool to identify those genes that are differentially expressed between the two cell types has been extensively used in this study. The variety 'Avocet S' is used to identify putative genes activated or repressed after treatment of wheat plants with biological elicitor fungus, *Trichoderma harzianum,* and chemical inducers, BTH and BABA comparing to untreated 'Avocet S' wheat plants. The differentially expressed cDNA bands were cloned and sequenced. Nucleotide sequences of differentially expressed cDNA bands were searched in the Genbank. Sequence alignments between the fragments that represent a certain gene were also searched in ClustalX-1.81 computer programs. The sequences of the differentially expressed fragments were also confirmed by real time PCR that verify the gene expression differences observed between the biologically or chemically treated and untreated plants as a result of defense induction. The confirmed genes were found to be involved directly or indirectly in the induced disease resistance. These genes are important in terms of understanding the mechanism of systemic acquired resistance (SAR) signalling defense and helpful in producing transgenic wheat.

Key Words: Differential display (DD) technique, wheat, induced disease resistance genes.

ÖZ

BTH, BABA VEYA *Trichoderma harzıanum* Raifi KRL-AG2
UYGULAMASIYLA
ARTAN VE/VEYA AZALAN BUĞDAY BİTKİ (AVOCET S) GENLERİNİN
TANIMLANMASI VE KLONLANMASI

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Tahılların üretimindeki en önemli sorun, bitki hastalıklarını kontrol ederken yüksek kalite ve verim düzeyinin sürdürülmesidir. Buğday üretime en büyük zararı veren etmenler hastalığa neden olan parasitik bakteri, fungus ve virüslerdir. Bu nedenle, modern tarımda buğdaydan başarılı bir şekilde yararlanmak için ön koşul, herhangi bir dirençlilik mekanizmasının anlaşılmış olmasıdır. Fungus, bakteri, mikrobiyal elisitör ve kimyasalların dirençliliği uyarması olgusu yaygın biçimde araştırılmaktadır. Bu keşifler bitkilerdeki doğal bağışıklığa neden olan hastalığa dirençlik yollarının bazı ortak öğelerinin olduğunu, ancak bunların çok azının moleküler düzeyde tanımlandığını göstermiştir. Bu sebeple, çalışmamızda buğday bitkilerinin biyolojik bir elisitör olan *Trichoderma harzianum* küfüne ve benzo (1,2,3) tiyadiazol-7-karbotiyoik asit S-metil ester (BTH), ß-aminobütiric asit (BABA) kimyasal indükleyicilerine maruz bırakılması ile aktive olan veya baskılanan genlerin ortaya çıkarılması hedeflenmektedir.

İki hücre tipinde farklı ifade olmuş genlerin tanımlanmasında kuvvetli bir araç olan mRNA "farklılık gösterim" teknolojisi, bu tezde kapsamlı bir şekilde kullanılmıştır. Biyolojik elisitör *Trichoderma harzianum* küfü, kimyasal uyarıcılar BTH ve BABA ile muamele edilen 'Avocet' çeşidine ait buğday bitkileri, muamele edilmemiş 'Avocet' bitkileriyle karşılaştırıldığında aktive olan veya baskılanan genlerin bulunmasında kullanılmıştır. Farklı olarak ifade olduğu görülen cDNA bantları klonlanmış ve sekanslanmıştır. Farklı olarak ifade olduğu tanımlanan cDNA bantlarının nükleotit dizileri gen bankalarında taranmıştır. Belirli bir geni temsil eden parçalar, dizi sıralaması ve hizalama yapılarak ClustalX-1.81 bilgisayar programında da aranmıştır.

Biyolojik veya kimyasal muamele görenler ile muamele yapılmamış bitkiler arasındaki gen ifadesi farklılıklarının savunma reaksiyonu sonucunda oluştuğunu ispat etmek amacıyla, farklı ifade edilmiş olan parçaların dizileri gerçek zamanlı PCR ile doğrulanmıştır. Doğrulanan genlerin doğrudan veya dolaylı olarak uyarılmış hastalık dirençliliğinde rol aldığı bulunmuştur. Bu genler, sistemik olarak kazanılmış dirençlilik (SAR) sinyalleyen savunma mekanizmalarının anlaşılması ve transgenik buğday üretiminde yararlı olacaktır.

Anahtar kelimeler: farklılık gösterim tekniği, buğday, uyarılmış hastalık dirençliliği genleri.

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LIST OF ABBREVIATIONS

μg	: Microgram
μL	: Microliter
μM	: Micromolar
[α^{32}P]-dATP	: [α^{32}P]-deoxyadenosinetriphosphate
A	: Absorbance
AM protein	: Arbuscular mycorrhizal protein
AMP	: Antimicrobial protein
APS	: Ammoniumpersulfate
ATG	: Autophagy mRNA,
BDB	: Potato dextrose broth
BSA	: Bovine Serum Albumin
cRNA	: Complementary RNA
DNA	: Deoxyribonucleic acid
dNTP	: Deoxy-nucleotidetriphosphate
ds	: Double stranded
DTT	: Dithiothretiol solution
EDTA	: Ethylenediaminetetraacetic acid
ET	: Ethylene
GSPs	: Genes specific primers
GST	: Gutathione –transferase
h.	: Hour
HPRGs	: Hydroxyproline-rich cell wall glycoproteins
HR	: Hypersensitive response
ISR	: Induced systemic resistance
JA	: Jasmonic acid
kb	: Kilobase
LRR	: Leucine rich repeat
M	: Molar
MAPK	: Mitogen activated protein kinase
mCi	: Milicurie

mg	: Miligram
min.	: Minute
MJ	: Methyl jasmonate
mL	: Mililiter
mM	: Milimolar
NBS	: Nucleotide binding site
NEB	: New England Biolabs
ng	: Nanogram
nm	: Nanometer
PAL	: Inducible phenylalanine ammonia-lyase
PAL	: Phenylalanine ammonia-lyase
PCR	: Polymerase Chain Reaction
pmol	: Picomole
PR	: Pathogenesis related
QTL	: Quantitative trait linkage
RACE	: Rapid amplification of cDNA ends
RFLP	: Restriction fragment length polymorphism
RNA	: Ribonucleic acid
ROS	: Reactive oxygen species
rpm	: Rotation per minute
RT-PCR	: Reverse transcriptase polymerase chain reaction
SA	: Slicylic acid
SAR	: Systemic acquired resistance
SOD	: Superoxide dismutase
SSR	: Simple sequence repeat
Taq	: *Thermus aquaticus*
TEMED	: N, N, N’,N’-Tetramethyl ethylene diamine
U	: Unit
UBC	: Ubiquitin conjugating enzyme,
UPM	:Universal primer mix
UV	: Ultraviolet
v/v	: Volume per volume

CHAPTER I

INTRODUCTION

1.1. Wheat

Wheat is one of the most important agricultural crop in the world. The importance of wheat internationally is evident from production estimates of 17% of all cultivated land worldwide. It is also the main food for about 35% of the world's population providing 20% of calories consumed (Huang et al., 2003). In addition, wheat with its high protein content, is the single most important source of plant protein in the human diet (CIMMYT., 1996). Wheat has exceptions in several ways. Wheat is grown on more than 240 million ha, larger than for any other crop, and world trade is greater than for all other crops combined.

Wheat is a member of the *Triticeae* group of cereals. It belongs to the genus *Triticum*. Although, there is no wild hexaploid wheat, Hexaploid wheat evolved under domestication. Genetic analysis has shown that the original hexaploid wheat was the result of a cross between tetraploid domesticated wheat, such as *T. dicoccum* or *T. durum*, and a wild goat grass, *Aegilops. tauschii*. The polyploid wheats are tetraploid (4 sets of chromosomes), or hexaploid (6 sets of chromosomes). The tetraploid wild wheats are wild emmer, *T. dicoccoides*, and *T.araraticum*. Wild emmer is the ancestor of all the domesticated tetraploid wheats, with one exception: *T. araraticum* is the wild ancestor of *T. timopheevi*. (http://www.answers.com/topic/wheat-taxonomy).

Within bread wheat (*Triticum aestivum* L. 2*n* = 6x =42, genome formula is AABBDD) species there are hundreds of thousands of wild species, landraces, and local cultivars that constitute the wheats of the world. Anatolia is the main center of diversity of the species where *Triticum* species coexist in mixed populations and exhibit tremendous morphological and ecological diversity (Huang et al., 2003).

Today, the world's population is increasing very rapidly. Two hundred people are born every one minute. Therefore by the year 2050, the world's population will double to nearly 12 billion people. To feed this population, these people will require a great amount of food production (Tandon., 1993). Wheat breeding programs have made steady improvements in productivity through improvements of yield quantity and quality as well as raising the resistance to many diseases and insect pests. This progress, which started nearly a century ago, has been the result of many researches, that made available genetic variation, and which introduced breeding strategies and screening technologies that changed the scope and efficiency of wheat breeding. Now, the newer molecular genetic technologies offer the promise to sustain gains in plant breeding programs of the 21st century (Dalrymple., 1986).

1.2. Wheat improvement

The developments in molecular genetics in wheat have been relatively slow, especially when compared to other crops, such as maize, rice or tomato, due to wheat's ploidy level, the size and complexity of its genome, the very high percentage of repetitive sequences and the low level of polymorphism (Table **1.1.** adapted from Plaschke et al.,1996).

Table 1.1 Characteristics of the bread wheat genome that explain the slow progress in development in molecular genetics and mapping as compared to a diploid, highly polymorphic species such as maize**.**

Ploidy level	6x
Number of chromosomes	21
Genome size *(number of base pairs x 10^6)*	16 000
Polymorphism level	Low
RFLPs: probe x enzyme combinations (%)	20-30
SSRs: primer pairs (%)	40-50
Repetitive sequences (%)	>80
To construct linkage maps of same density (15 arkers/chromosome):	
Number of loci needed	315
Number of RFLP probes needed	1 000-1 500
Number of SSR primer pairs needed	700-800

Much fewer maps exist in wheat and far fewer quantitative trait linkage (QTL) studies have been reported when compared to other grass species. However, due to the large number of disease and pest resistances controlled by major genes, the mapping of such genes has dominated the research activities in wheat molecular genetics. On the other hand, the hexaploid nature of wheat and its suitability to cytogenetic manipulation have offered unique tools for molecular geneticists of wheat. These include the use of various aneuploid stocks, such as nullitetrasomic and ditelosomic lines, to assign molecular markers to specific chromosome arms (Plaschke *et al.,* 1996).

1.3 Wheat Diseases

One of the major problems concerning the production of food crops is the controlling of plant diseases to maintain the high quality and yield which producers and consumers expect. Disease, a major hazard in wheat production, causes losses through reduced yield and quality of grain. Wheat diseases are caused by parasitic bacteria, fungi and viruses. Wheat is subject to attacks from about 50 different pathogens, but not all of the diseases will occur in a particular area or in a certain year. Estimated annual wheat disease losses vary from 10 to 25 percent, depending upon many factors. When, the environmental conditions are suitable, it can result in yield losses up to 100%. Some disease losses cannot be prevented because no successful means have been devised for controlling some of the most destructive diseases. However, a considerable part of these losses can be prevented by using proven methods for disease prevention and control. (http://muextension.missouri.edu/xplor/agguides/crops/g04319.htm**)**

Table 1.2 Wheat plants major diseases and pathogens.

Pathogen	Disease
Fungal Diseases	Leaf Rust (Brown Rust), Stem Rust (Black Rust), Stripe Rust (Yellow Rust),Common and Dwarf Bunt (Stinking Smut), Karnal Bunt (Partial Bunt), Loose Smut, Flag Smut, Powdery Mildew, Septoria Tritici Blotch, Septoria Nodorum Blotch, Septoria Avenae Blotch , Spot Blotch (Helminthosporium Leaf Blotch), Tan Spot (Yellow Leaf Spot or Blotch), Alternaria Leaf Blight, Fusarium Leaf Blotch (Snow Mold), Scab (Head Blight), Ergot, Black Point, Downy Mildew, Eyespot (Strawbreaker), Sharp Eyespot and Rhizoctonia Root Rot, Common Root Rot, Foot Rot, and Crown Rot, Sclerotium Wilt (Southern Blight), Black Molds (Sooty Molds)
Bacterial Diseases	Bacterial Black Chaff and Bacterial Stripe, Basal Glume Rot and Bacterial Leaf Blight, Bacterial Spike Blight (Yellow Ear Rot)
Viral Diseases	Barley Yellow Dwarf
Insect Pests	Cereal Leaf Armyworms, Cutworms, and Stalk Borers, Stink Bugs, Aphids, Wireworms, White Grubs, Sawfly, Wheat Stem Maggot, Hessian Fly, , Thrips Beetle, Mites. Slugs, Snails, Grasshoppers, and Crickets,
Nematodes	Cereal Cyst Nematode, Seed Gall Nematode (Wheat Nematode or Ear Cockle) Root Knot Nematode

1.4 Disease control

Chemical (fungicide) control has been successfully used in some areas, however, there are many disadvantages of using fungicides, such as, known or unknown environmental hazards, occurrence of pathogens developing resistance to applied chemical, high cost of chemicals, limited shelf life and occurrence of pathogens developing resistance to applied chemical. Therefore, One application of biotechnology in addition to engineering disease resistant cultivars is to reduce the incidence of disease in agricultural crops, through the induction and enhancement of the plant's own defense mechanisms which would not involve the application of toxic compounds to plants such as pesticides.

Plants can acquire enhanced resistance to pathogens by "biotic" and "abiotic" inductions. Chemicals and biological inducers that can be used at very low rates are able to activate plant disease resistance in many plants (low cost). Also, there is no expected health risk to human and environment from using those elicitors.

1.4.1 Chemical inducers

The following list of chemicals represents the most important and the most commercially widely used chemicals for induction and enhancement of the plant's own defense mechanisms against wide variety of pathogens.

- benzo-(1,2,2)thiadiazole-7-carbothioic acid -S-methyl ester (BTH)
- Acibenzolar-S-methyl (ASM)
- Probenazole (ORYZEMATE)
- Beta-aminobutyric acid (BABA)
- 2,6-dichloroisonicotinic acid (INA)
- Salicylic acid (SA)
- 2-deoxy-D-glucose (DDG)

The most thoroughly investigated chemical inducer is BTH (benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester) which interferes with the salicylic acid (SA) pathway (Jakab et al., 2001).

BTH is highly effective at inducing of enhanced disease resistance. However, its mode of action and cellular targets are unknown (Liu et al., 2005). Another interesting group of elicitors of pathogenesis response in plant are chemical analogs of amino acids, such as BABA (DL-ß-aminobutyric acid) (Cohen et al., 1994). BABA is a non-protein amino acid which occurs rarely in nature and when applied as droplets on leaves, BABA penetrated with preferred accumulation in the youngest leaves (Jakab et al., 2001).

Little is known concerning the mode of action of BABA; some studies report an induction of PR after BABA treatment, whereas others state the contrary. Thus, the mode of action of BABA remains a matter of controversy (Zimmerli et al., 2000).

Figure 1.1 Chemical structure of benzo-(1,2,2)thiadiazole-7-carbothioic acid-S-methyl ester (BTH) and Beta-aminobutyric acid (BABA)

1.4.2 Biological inducers

In general, biological control, the use of specific microorganisms that interfere with the plant pathogens and pests, is a nature-friendly, ecological approach to overcome the problems caused by standard chemical methods of plant protection. There are three main microorganisms usually used as biological agents for induction of local and induced systemic resistance towards plant pathogens.

- Necrotizing pathogens
- Non-pathogen
- Root colonizing Bacteria

Both biologically as well as chemically induced resistance against pathogen attack effectively *in vivo* but they do not show any activity *in vitro*.

An alternative to chemical inducers that offer acceptable levels of disease control is a biological control by means of some micro-organisms such as *Trichoderma* spp.

(Elad., 2000). *Trichoderma spp.* are fungi that are present in nearly all agricultural soils, root ecosystem, avirulent plant symbionts, as well as being a producers of variety of compounds that induce localized or systemic resistance responses, and this explains their lack of pathogenicity. Root colonization by *Trichoderma* spp. also frequently enhances root growth and development and the uptake and use of nutrients. (Gary et al., 2004).

Although, the molecular basis of biological control is not clearly understood (Janice and Carlos, 2002), recent studies proved that, cell wall degrading enzyme (CWDE) genes from biocontrol fungi belonging to the genus *Trichoderma* have been demonstrated to encode proteins with high antifungal activity against a wide range of plant pathogenic fungi (Mei et al., 2004). *Trichoderma harzianum* has the ability to secrete a wide range of depolymerases and has been used as a biocontrol against several major plant pathogens (Williams et al., 2003).

The phenomenon of induced resistance by fungi, bacteria, microbial elicitors and chemicals has been investigated by a large number of studies with *Arabidobsis thaliana* (Hammerschmidt et al., 2001). These discoveries lead to the fact that the disease resistance signaling pathway in plants shares a number of common elements with those leading to innate immunity (Frank et al., 2004).

1.5 The application of biotechnology to wheat improvement

1.5.1 Critical Molecular Resources

The set of molecular resources and tools identified below are critical to the implementation of biotechnological approaches to wheat improvement. These methods permit discovery, identification, isolation, and transfer of genes from practically any source to important wheat varieties. In particular, these methods facilitate the rapid transfer of genes from one wheat or barley variety to another, and are especially useful for exploiting genes, such as disease and pest resistance, from wild species to cultivated wheat and barley.

1.5.1.1 Advanced DNA Marker Technology

1.5.1.1.1 Microsatellite DNA Markers:

Simple Sequence Repeats (SSR), also known as microsatellite, consists of tandem-repeated nucleotides with a core sequence ranging from 1–5. SSRs have been shown repeatedly as being one of the most powerful markers for the genetic studies in some crop species (Akkaya and Buyukunal-Bal., 2004). The major advantages of

microsatellites are their ease of use, low cost of analysis, and ability to detect genetic differences even among closely related individuals. Thus, microsatellite DNA markers offer the promise of DNA "Marker-Assisted Selection" that can function in wheat breeding programs for the development high yielding and resisting varieties. The disadvantage is the inability of the current research system to focus support for such high unit cost developments (Rajora et. al., 2001).

1.5.1.1. 2 Alternative Marker Strategies:

Amplified Fragment Length Polymorphism (AFLP) marker is a combination of restriction enzyme digestion and the PCR. DNA is digested with restriction enzymes producing sticky ends. Specific adaptors with a fixed sequence are ligated to these sticky ends. Subsequently these fragments are amplified with PCR technique using the adaptor sequences as primer sites. By extending the primer three ‘selective’ base pairs at the 3'-end, a selection of all restriction fragments in a reaction is amplified (Vrieling et al., 1997). Such newer methodologies may offer additional advantages and options but need further development (Zabeau and Vos. 1993).

1.5.1.2 Large Insert DNA Libraries

A fundamental requirement for isolating specific *Triticeae* genes and to understand genetic mechanisms of this gene is the availability of DNA clone libraries containing large fragments of the *Triticeae* genomes. One technology to accomplish this is to introduce genes of *Triticeae* into bacterial chromosomes to create 'bacterial artificial chromosomes' (BACs). This technology provides the opportunity to isolate a targeted gene from a cereal genome. Gene isolation then provides a potential for a gene transfer and to design new variants of the gene that wheat breeders can incorporate into breeding programs to improve disease resistance, grain quality, drought tolerance, and other important characteristics.

1.5.1.3 Transformation Technology

Transformation Technology is a process of introducing genes into plants by methods other than the sexual production process. New and valuable genes can be introduced into cereals *via* transformation that are not available through traditional breeding practices. However, the procedure is still too inefficient with monocots plants, and is limited to only a few varieties or types of wheat. In addition, of transgenic gene expression is insufficient. (Hoisington *et al.,* 1998)

1.6 The future of wheat molecular genetics

Genetic engineering opportunities enable plant breeders to incorporate genes isolated from organisms outside the gene pools to which they usually have access. This broadens the possibilities they have for overcoming a number of biotic and abiotic stresses. While the PCR-based marker systems have allowed more effective and efficient genotyping, DNA-array technology offers to increase the number of genes that can be analyzed Efforts are also underway to develop complete expressed sequence tag (EST) databases for wheat and related species. If this data can remain in the public sector (such databases for wheat are currently available in the private sector), chips containing a significant number of wheat genes will be produced and used in the not too distant future (Shalon *et al.,* 1996).

In this study, gene expression analysis approach was used as a limited alternative technique to DNA analysis.

1.7. Resistance mechanism in plant

1.7.1 Specific defense process

Specific defense response based on so-called *R*-genes specific resistance of a plant to pathogenic microorganisms (Kombrink and Schmelzer, 2001). *R* genes comprise a large and diverse group of related sequences in plant genome. Many *R* genes have been cloned from a wide variety of plant species. *R* genes have been isolated from

Arabidopsis as well as *solanaceous* species (tomato, potato, pepper, tobacco) and from barley, wheat, maize and rice, although only a few *R* genes have been cloned from monocots. So far *R* genes have been isolated that can provide resistance against viral, bacterial and fungal pathogens, as well as to nematodes and insect pests. (Martin et al., 2003).

During the last decade *R* genes have been described, in that they encode only five main classes of proteins. The majority of *R* proteins are classified as nucleotide-binding site leucine rich repeat (NBS-LRR) proteins with the *Arabidopsis* genome containing over 150 genes that encode this class of protein (Jones., 2001).

It is now clear that most *R* genes encode proteins that have a putative amino-terminal signaling domain, a nucleotide-binding site (NBS) and a series of carboxy-terminal leucine-rich repeats (LRRs) (Dilbirligi et al.,2004). The amino-terminal signaling domain recognizes the specific signals directly or indirectly produced by pathogen *Avr* genes. NBS domain is composed of several amino acid motifs that are highly conserved among family members (Cohn et al., 2001). Carboxy-terminal is thought to be involved in protein-protein interaction and pathogen recognition. LRRs consist of repeated imperfect amino-acid segments that fold into solvent exposed β-strand β-turn structures. It is considered that LRR region present in many *R* genes is a major factor responsible for the specificity of the pathogen recognition. Additionally, the TIR domain can also affect specificity (Ellis et al., 2000).

The NBS-LRR class which is cytoplasmic protein can be further divided into those that have *N* terminal homology to the Toll and Interleukin-1 receptor (TIR) genes (TIR-NBS-LRR), a leucine-zipper (LZ-NBS-LRR) or a coiled-coil motif (CC) (CC-NBS-LRR) (Jones 2001).

In addition to NBS-LRRs, there are three other classes of *R* genes*:* intracellular or extracellular LRRs, extracellular LRRs with a transmembrane an intracellular kinase domain, and serine/threonine kinases. All of the *R* gene families include a LRR domain except the serine/threonine kinases. (Feys and Parker., 2000).

Location and structure of the five main classes of plant disease resistance protein are shown in figure 1.1; Xa21 and Cf-X proteins carry transmembrane domains and extracellular LRRs. The recently cloned RPW8 gene product carries a putative signal anchor at the N terminus. The *Pto* gene encodes a cytoplasmic Ser/Thr kinase, but may be membrane associated through its N-terminal myristoylation site. The largest class of *R* proteins, the NB-LRR class, is presumably cytoplasmic (although it could be membrane associated domain) and carry distinct N-terminal domains (Dangl and Jones, 2001).

Figure 1.2 Re-presentation of the location and structure of the five main classes of plant disease resistance proteins (taken from Dangl and Jones., 2001).

Host recognition of a potential pathogen determined by a 'gene-for-gene' interaction initiates a very rapid defense response in the plant. The gene-for-gene theory is based on the recognition of an avirulence (*avr*) gene product in the pathogen by a corresponding resistance *R* gene product in the host. The pathogen possesses the *avr* (avirulence) gene and the host (plant) possesses the corresponding *R* (resistance) *gene* resulting in disease resistance). In most cases, this interaction occurs under high specificity. If the plant or pathogen lacks the appropriate *R* gene or *avr* gene, respectively, then activation of plant defense responses may be delayed or ineffective.

As a result, disease will occur on the host results in what is classified as a compatible reaction as it is illustrated in Figure 1.2 (Nimchuk et al., 2003).

Defense mechanisms are however commonly induced in plants lacking *R*-gene-mediated recognition, although activation of these mechanisms may occur later such that defenses are consequently less effective (Tao et al., 2003).

Figure1.3 Re-presentation of 'gene-for-gene' interaction model between a pathogen and host plant cell (www.plbio.kvl.dk\~dacoj3\resourse\receptor_model-bw.htm)

Plant defense responses can also be triggered by various pathogen-derived elicitors such as complex carbohydrates from fungal cell walls, microbial enzymes, polypeptides, proteins and lipids. Recognition of these elicitors by plant receptors triggers mitogen activated protein kinase (MAPK) cascades that cause the rapid and transient phosphorylation of specific nuclear, cytosolic and membrane bound proteins (Jonak *et al.,* 2002; Peck 2003). Recently, the first complete MAPK cascade in plants was shown in *Arabidopsis*. The cascade functions are downstream of the flagellin receptor "FLS2" to activate two plant specific transcription factors. Transient overexpression of components of this MAPK cascade provided resistance to bacterial and fungal pathogen ns (Asai et al., 2002).

The simplest model of plant–pathogen recognition is based on the interaction of pathogen elicitors with plant receptors and the subsequent transduction of this interaction into a defense response as illustrated in Figure 1.3. This can be subdivided into host-specific recognition through the interaction of a plant R protein with a corresponding pathogen avirulence protein (known as 'gene-for-gene' resistance, (Flor., 1971), or non-host resistance to provide resistance against all members of a specific pathogen species (Mysore and Ryu, 2004).

The cascade of resistance factors is induced when a plant recognizes that a potential pathogen is present, and compounds which are capable of triggering such responses are termed elicitors. The receptor participates in an initial step in a signal transduction cascade leading to the activation of a variety of defense responses.

1.7.2 Broad-spectrum defense responses

The mechanism includes the combined effects of Performed defense barrier and inducible resistance mechanisms. Performed barriers, such as a thick cuticle or inhibitory secondary metabolites in the outer cell layers of plant organs**,** in addition to that can be induced locally or systemically by biotic or abiotic agents (Oostendorp et al., 2001). Plants have evolved many natural defense mechanisms to survive in nature, only a few can be triggered by biological or chemical agents without deleterious side effects and in a sufficiently controlled way for practical use.

1.7.2.1. The hypersensitive response

One of the earliest plant defense responses following pathogen attack is a rapid increase in reactive oxygen species (ROS) in a process known as the oxidative burst. A first burst occurs in both susceptible and resistant plants within minutes of pathogen infection. In resistant plants a second, sustained burst develops a few hours later (Low and Merida., 1996).

Further defense mechanisms are employed from several hours to several days following this oxidative burst, and often include the development of the hypersensitive response (HR), characterized by localized cell death at the site of pathogen attack, and the induction of structural and biochemical barriers (Heath., 2000).

The HR is immediate, early defense responses of the directly invaded plant cells, starting with signal recognition and transduction and frequently leading to rapid cell death and is thought to confine the growth of biotrophic pathogens through the production of antimicrobial compounds and by limiting nutrient uptake (Kombrink and Schmelzer., 2001).

The rapid death of the cells at the infection site that is associated with pathogen limitation as well as with defense gene activation that lead to oxidative burst during which reactive oxygen species (ROS) are generated, is now universally accepted as a form of programmed cell death (PCD) (Michele., 2000). Hence, HR leads to activation of local defense response associated with cell wall strengthen and intracellular signal transduction mechanism. (Thulke and Conrath., 1998)

As a result, an extensive reprogramming of both primary and secondary metabolism at the gene expression level is initiated, and many of encoded proteins are either directly or indirectly inhibitory towards invading pathogens (Kombrink and Schmelzer., 2001). For example, the influx of Ca^{2+} and H^{+} ions and the efflux of K^{+} and Cl^{-} ions occurs through controlled protein phosphorylation and dephosphorylation events, and these are thought to be signals for the generation of superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), and reactive nitrogen species such as nitric oxide (NO) (Hancock et al., 2002).

In plant defense, ROS appear to have multiple roles. During the HR, H_2O_2 is thought to have not only a direct microcidal effect, but also to stimulate cell wall lignification and cross-linking, thereby strengthening the cell wall against the invading pathogen. H_2O_2 and NO can induce the expression of defense-associated genes such as phenylalanine ammonia-lyase (*PAL*), *PR* (pathogenesis related), and glutathione *S*-transferase (*GST*) genes (Desikan et al., 1998; Durner et al., 1998). The importance of NO in plant defense was highlighted in soybean cell studies where Delledonne *et al.,*

(1998) showed that NO potentiated ROS induced HR cell death and that NO synthesis inhibitors were able to block the HR in *Arabidopsis* induced by avirulent *P. syringae*.

Other signaling molecules implicated in the HR include salicylic acid (SA) and jasmonic acid (JA). Transgenic *Arabidopsis* plants unable to accumulate SA, if they express the *Pseudomonas putida NahG* gene which encodes salicylate hydroxylase to convert SA to catechol) do not produce a HR following some *R*-gene-mediated resistance responses (Heath, 2000).

1.7.2.2 Structural and biochemical barriers

Induced structural barriers include cell wall lignification, papillae formation, and the production of glycoproteins and vascular occlusions. Lignin is a tough, water-repellent plant polymer whose production is stimulated by H_2O_2 to add structural strength and protect the plant cell through its resistance to biodegradation (Yang et al., 1997). Lignin, in combination with cellulose, a polymer of β-1, 3-glucan, form papillae underneath the site of pathogen infection, and this was thought to help prevent pathogen spread following wounding or pathogen attack. Levels of hydroxyproline-rich cell wall glycoproteins (HPRGs) or extensins increase, and the production of vascular occlusions (gels, gums or tyloses) takes place. These occlusions block vessels to help limit the spread of a pathogen or its toxins (Turner., 1994).

Several *Arabidopsis* mutants with defects in cell wall composition leading to altered levels of disease resistance have been identified, including *cev1* (*constitutive expression of vegetative storage protein*) and *pmr* (*powdery mildew resistant*) mutants (Vorwerk et al., 2004). The *cev1* mutant has constitutive activation of the JA pathway and enhanced resistance to powdery mildew. *CEV1* encodes a cellulose synthase and is implicated in primary wall synthesis. It is proposed that a decrease of cellulose in *cev1* results in a defect in the cell wall that triggers increased JA production leading to enhanced disease resistance (Ellis et al., 2002).

Induced biochemical defenses include the production of phytoalexins and antimicrobial proteins (AMPs), which include PR proteins such as chitinases and glucanases. Phytoalexins include phenolics, terpenoids, polyacetylenes, and fatty acid derivatives and are produced by plants in response to diverse stresses, but predominantly against fungal infection (Pedras et al., 2000).

Following incompatible pathogen interactions, various AMPs are induced locally and then systemically in association with systemic acquired resistance (SAR) (Van Loon and Van Strein., 1999).

Originally five classes of *PRs* (*PR*1–5) were characterized in tobacco but this has since been increased to 14 (of which PRs1–5 and *PR*12 and 13 are the most studied in *Arabidopsis*) to include *PRs* from other plant species (Fritig et al., 1998).

1.7.2.3 Systemic defense responses

Systemic activation of genes encoding pathogenesis-related (PR) proteins which are directly or indirectly inhibitory towards pathogens and have been associated with the phenomenon of systemic acquired resistance (SAR). The first step in the development of SAR is the recognition of pathogen infection by plant. Once the plant reacts to the pathogen, signals are released that trigger resistance in adjacent as well as distant tissues.

Currently the markers that correlate with SAR initiation are cDNAs that are expressed in distant tissues in SAR induced plants. Conceivably, each taxonomic group of plants may have evolved its own set of SAR genes in response to evolutionary pressure from a specific spectrum of pathogens (Ryals et al., 1994).

This response can be induced locally or systemically by biotic or abiotic means and leads to generation of secondary signaling molecules, such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) which may activate defenses both locally at the site of infection and systemically in non-infected tissues (Oostendorp et al., 2001).

SAR results in long-lasting, systemic resistance to subsequent infection by a broad range of pathogens including bacteria, fungi, and viruses (Ryals *et al.,* 1996). Classically the increased resistance associated with SAR has been thought to be linked to the action of PR proteins. The function of some *PR* proteins is unknown, whilst others have been found to be B-glucanases and chitinases. Thus, given that B-glucans and chitin are major constituents of the fungal cell wall, such enzymes have an obvious anti-microbial role. However, it is less clear how such *PR* proteins could be effective against bacterial or viral pathogens. Therefore, there is a need for exploring an additional role for SA where this acts to enhance or "potentiate" a series of defence reponses (Mur et al*.,* 1996).

SAR can also be induced by functional SA analogues such as the synthetic chemicals benzo-(1,2,3)-thiodiazole-7-carothioc acid (BTH) and isonicotinic acid (INA) (Friedrich *et al.,* 1996). The phenomenon of induced resistance by fungi, bacteria, microbial elicitors and chemicals has been investigated by a large number of studies with *Arabidobsis.* The discoveries of these studies have come the realization that the disease resistance signaling pathway in plants shares a number of common elements with those leading to innate immunity (Hammerschmidt et al., 2001).

Using various genetic screens, the *A. thaliana* gene *NPR1* (for *n*onexpresser of *PR* genes; also called *NIM1* for *n*on*im*munity) was identified as a key regulator in transducing the SA signal leading to general acquired resistance responses, including SAR as well as local acquired resistance, the ability of plants to restrict the spread of virulent pathogen infections (Frank et al., 2004).

Avirulent pathogen resistance and *NPR1*-independent resistance both require SA- and JA/ET–mediated signaling pathways. JA/ET or sensitivity to JA/ET may be required for perception of the signal (elicitor) necessary to bypass the *npr1* mutation or for expression of downstream antimicrobial proteins. Therefore, HR-mediated local resistance is shown as a function of overlapping components from both SA- and JA/ET–mediated pathways (Figure1.4). Mutations in the *NPR1* gene result in a loss of resistance to virulent bacterial and fungal pathogens even when the plants are pretreated with SAR inducers. Expression studies demonstrated that although *NPR1* is

constitutively expressed in plants, its level can be further elevated by '2-folds after SA or INA treatment (or by pathogen infection. Upon SAR induction, activation of the NPR1 protein must also occur because constitutive expression of NPR1 in the absence of an inducer does not lead to constitutive expression of *PR* genes or resistance. These characteristics indicate that the SAR response may be enhanced through manipulation of *NPR1* either at the level of expression or the level of protein activity or both (Cao Hui, et al., 1998).

In summary, SAR, dependent on and characterized by an increase in SA, results in the expression of a subset of *PR* genes including *PR1, PR2* and *PR5*, while induction of SA-independent defense responses occurs *via* activation of JA and ET signal transduction pathways to induce a different subset of *PR* genes. Systemic acquired resistance acts non-specifically against a broad spectrum of pathogens.

Figure 1.4 Model of interacting defense response pathways; *NPR1*-dependent and *NPR1*-independent resistance with systemic resistance. (Modified from Clarke et al., 2000)**.**

1.8 Dissection of defense signaling pathways

Mutational analysis has been a very powerful approach for identifying genes involved in plant defense responses. *Arabidopsis* lends itself to this approach owing to its relatively small diploid genome, fast generation time, prolific seed generation, and the relative ease to transform or mutate it. Mutational analysis has been a very active research area. Research in *Arabidopsis* is starting to place and order genes within signal transduction networks that are proving to be tightly regulated and accommodate considerable cross talk between SA-dependent and JA / ET-dependent responses in synergistic or antagonistic fashions (Kunkel and Brooks., 2002).

1.8.1 SA-dependent signaling

SA has been shown to play a critical signaling role in activating local and systemic defense gene expression (Figure 1.5) (Vera et al., 1998). SA also was identified as an important signal in the systemic acquired resistance (SAR) transduction pathway (Bovie et al., 2004).

Figure 1.5 Classical model for systemic acquired resistance based on the dispersal of salicylic acid: (figure was adapted from Malamy et al., 1990).

Plants transformed with the *nahG* gene do not accumulate SA or PR and do not develop SAR in response to biological or chemical inducer. The *nahG* gene encodes salicylate hydroxylase, which converts SA into catechol, a product that does not induce resistance, (van Loom et al., 1998).

Transduction of the SA signal requires the function of NPR1 a regulatory protein that identified in *Arabidopsis* (Figure1.6). Therefore mutant *npr1* plants accumulate normal levels of SA after exogenous induction but are impaired in their ability to express *PR* genes and mount a SAR response (figure 1.6) (Spoel et al., 2003).

Figure 1.6 Signal transduction pathways leading to pathogen-induced systemic acquired resistance (SAR) and rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis thaliana*. (figure was taken from Van Loon et al., 1998).

Although the mobile signaling molecule(s) transported throughout the plant for SAR remain unknown, molecules implicated include a lipid derived signal (Kumar and Klessig 2003).

Recently, a salicylic acid-binding protein (SABP2) belonging to a hydrolase super family was identified in tobacco and found to exhibit lipase activity stimulated by SA binding (Kumar and Klessig 2003). SA has also been reported to bind to and inhibit the activity of a catalase enzyme, although the significance of this remains unclear (Chen et al., 1993).

Salicylic acid has been shown to play central roles in local plant defense responses and is required for the establishment of SAR. Adding to our knowledge of the roles of this signaling molecule, several mutants have been isolated with altered levels of SA or defects in SA sensitivity and have been positioned within the SA signaling pathway. For example, the *eds* (*enhanced disease susceptibility*) mutant *eds5–1* accumulates *PR-1* mRNA to only around 10% of wild type levels following virulent *P. syringae* infection, but is capable of mounting at least a partial SAR response with WT levels of *PR-2* and *PR-5* (Rogers and Ausubel., 1997).

1.8.2. JA-dependent signaling

Jasmonates are produced from the major plant plasma membrane lipid, linolenic acid, via the octadecanoid biosynthetic pathway. Jasmonic acid (JA) is an oxylipin-like hormone derived from oxygenated linolenic acid. The biological roles of jasmonic acid (JA) and some of its biosynthesis intermediates include fruit ripening, fertility, root growth, and responses to wounding, insects, microbial pathogens and abiotic stresses (Turner *et al.,* 2002).

Jasmonic acid (JA) serves as a signal for expression of a number compounds such as proteinase inhibitor (PINII), polyphenol oxidase and steriod glycoalkaloids. These appear to contribute to plant resistance against many insect attackers and some pathogens (Ryals etal., 1994).

JA or methyl jasmonate (MJ), induce expression of defence-related genes, such as the defensin *PDF1.2* (*PR-12*) and the thionin *Thi2.1* (*PR-13*) (Penninckx *et al.,* 1998).

We have recently shown that, levels JA increased strongly upon treatment of *Arabidopsis* with fungus *Alternaria brassicicola,* as well as exogenous application of methyl jasmonate (MeJA). JA-dependent defense response pathway leads to activation of PDF1.2 gene that encodes an antifungal peptide belonging to the family of plant defensin (Thomma et al., 1998). Ethylene (ET) often acts in concert with JA in

activating the expression of defense-related genes. However, there are some reports of negative interaction between ET and JA pathways (Maria et al., 2005).

1.8.3. ET-dependent signaling

The plant hormone ethylene (ET) is involved in plant growth and development and is produced in response to both biotic and abiotic stresses (Wang et al., 2002). Although most information regarding ethylene processes in plants is based on epistasis studies, ethylene can also induce the expression of plant defence genes such as *PDF1.2* (Penninckx et al., 1996). ET production is regulated by developmental signals and in response to biotic and abiotic stimuli (Wang et al., 2002).

Several mutants have been isolated with defects in ET responses based on seedling morphological characteristics known as the triple response and these include the ET insensitive mutants (*ein)* and ET resistant mutants (*etr*) (Guzman and Ecker 1990).

Components of the ET signaling pathway include the nuclear-localised transcription factor EIN3, which activates *ERF1* (*ethylene response factor1*), a member of the plant specific ethylene-responsive element binding protein (EREBP) family, which in turn binds to GCC box promoter elements to activate defense genes such as *PDF1.2* (Solano et al., 1998). The GCC box motif is associated with ET and pathogen-induced gene expression and is found in the promoters of many pathogen-responsive genes (Chen et al., 2002).

1.8.4 Induced systemic resistance (ISR-signaling)

Systemic resistance can also be achieved through a pathway not reliant on SA, in a process termed induced systemic resistance (ISR) (Pieterse et al., 1998). ISR develops in response to the colonization of plant roots by certain strains of non-pathogenic rhizobacteria. Rhizobacteria-mediated induced systemic resistance (ISR) has been demonstrated against fungi, bacteria, and viruses in *Arabidopsis*, bean, carnation,

cucumber, radish, tobacco, and tomato under conditions in which the inducing bacteria and the challenging pathogen remained spatially separated. Bacterial strains differ in their ability to induce resistance in different plant species, and plants show variation in the expression of ISR upon induction by specific bacterial strains. Bacterial determinants of ISR include lipopolysaccharides, siderophores, and salicylic acid (SA). Whereas some of the rhizobacteria induce resistance through the SA-dependent SAR pathway, others do not and require jasmonic acid and ethylene perception by the plant for ISR to develop as it was illustrated in figure 1.6. No consistent host plant alterations are associated with the induced state, but upon challenge inoculation, resistance responses are accelerated and enhanced (van Loon et al., 1998)

ISR induced by the non-pathogenic rhizobacterial strain *P. fluorescens* WCS417r against the pathogen *P. syringae* occurs through a novel signaling pathway, requiring components of the JA and ET response pathways, but regulated by NPR1 (Pieterse et al., 1998).

1.8.5 Interactions between signaling pathways

Although SA-dependent and JA / ET-dependent pathways induce the expression of different subsets of *PR* genes and provide resistance against specific pathogens, there is both synergism and antagonism between the pathways (Kunkel and Brooks., 2002). The SA and JA pathways often seem to act antagonistically. For example, SA has been shown to have an inhibitory effect on JA biosynthesis and JA-responsive gene expression (Gupta et al., 2000) and *NahG* plants exhibit increases in JA and JA-responsive gene expression in response to biotrophic pathogens indicating that pathogen-induced SA accumulation suppresses JA formation and JA-responsive gene expression (Spoel et al., 2003). In addition to the negative effect of SA on JA signaling, JA is also reported to negatively regulate SA signaling (Kachroo et al., 2003).

However, SA and JA can also act synergistically to induce defence-associated genes (Schenk et al., 2000). The ET and JA pathways often work synergistically. For example, most genes induced by ET in a microarray study were also induced by MeJA

(Schenk *et al.,* 2000). Furthermore, both JA and ET are required for *PDF1.2* expression (Ellis and Turner., 2001), and the SA-independent systemic resistance ISR requires JA and ET signaling (Pieterse et al., 1998).

Additionally, they found some genes whose expression were JA dependent but ET independent, and another group-regulated by JA / ET signaling and an unknown signaling process that did not include SA. The results confirm that in most cases in response to virulent bacterial pathogen attack, SA and JA signaling oppose each other, JA and ET signaling generally act together, and that SA and ET signaling tend to oppose each other.

1.9. Engineering plant resistance to pathogen

The cloning of R genes has opened doors for producing disease-resistant crop plants. Transgenic plants that resist pathogen attacks can be engineered either by insertional mutagenesis or by map-based cloning methodology (Gachomo et al., 2003).

1.9.1. Insertional mutagenesis method of transformation

A fragment of DNA is inserted into the coding region of a gene, which results in disruption of gene expression. After this, one proceeds to the cloning of the plant DNA that flanks the integrated insertional mutagen by plasmid rescue. The cloned plant DNA can then be used as a hybridization probe to isolate the gene by screening a lambda or a cosmid library constructed from the wild-type plant. The final test is to introduce the cloned gene by transforming sensitive plants and examine them in order to determine whether they have become disease-resistant. The most commonly used DNA molecules for insertional mutagenesis is transposon and T-DNA from Ti plasmid.

1.9.2. Map based cloning (positional cloning)

In the map-based cloning which is also known as positional cloning, one need to determine the chromosomal location of the gene of insert. The restriction fragment length polymorphism (RFLP) is the most frequently used method for linking the chromosomal position to the picture trait. In this case, co-inheritance of the disease

resistance trait with one specific RFLP DNA probe defines the approximate location of the R gene on the chromosome.

1.10. Genome wide Expression Analysis

Global analysis of gene expression which is referred to as genome-wide expression profiling, is considered to be one of the most promising tools in functional genomics. In the past few years several technologies for expression profiling have been developed. These methods depend on three different principles: hybridization of probes to microarrays, counting of sequence tags from cDNA fragments and gel-based analysis of cDNA fragments (Zabeau 2001).

1.10.1. Description of SAGE

Serial analysis of gene expression (SAGE) is a method for comprehensive analysis of gene expression patterns. Three principles underlie the SAGE methodology (Figure 1.7) (Velculescu, et al., 1995)

1. A short sequence tag (10-14bp) contains sufficient information to uniquely identify a transcript provided that the tag is obtained from a unique position within each transcript.
2. Sequence tags can be linked together to from long serial molecules that can be cloned and sequenced.
3. Quantitative of the number of times a particular tag is observed provides the expression level of the corresponding transcript.

By this way experimenter can determine the absolute measure of gene expression levels. Another advantage is that; independent data sets can be studied in a single database allowing comparison of different data sets. The main disadvantage of the technique is the requirement of large number of ESTs (expressed sequence tags) in order to monitor rarely expressed genes. Another limitation of the method is that the obtained tags are very short and therefore unambiguous.

Figure 1.7 Schematic representing the three principles that underlie the Serial analysis of gene expression (SAGE) methodology.

1.10.2. Microarray Technology

Microarray technology represents a powerful functional genomics technology, which permits the expression profiling of thousands of genes in parallel (Schena et al., 1995). Microarray Technology is based on hybridisation of complementary nucleotide strands (DNA or RNA). Microarray chips consist of thousands of DNA molecules (corresponding to different genes) that are immobilized and girded onto a support such as glass, silicon or nylon membrane. Each spot on the chip is representative for a certain gene or transcript (Figure 1.8). The expression levels of all genes in cells taken from a tissue sample or a cell line culture can be determined by isolating the total amount of mRNA, which is defined to be the trascriptome of the cell at the given time. Fluorescently or radioactively labeled nucleotides (targets) that are complementary to the isolated mRNA are prepared and hybridised to the immobilized molecules. Target molecules that did not bind to the immobilized molecules (probes) during the hybridisation process are washed away.

The relative abundance of hybridised molecules on a defined array spot can be determined by measuring the fluorescent or radioactive signal. This method provides the advantage that it can interrogate the level of transcription of several thousands of different genes from one sample in one experiment. The drawback of this technology is the requirement of sequence knowledge or an available cDNA clone. In addition to the difficulty of differentiation between different transcripts which share a high degree of homology. Especially in plants substantial fraction of the genes are members of gene families. Sensitivity of the hybridization and the requirement of large amounts of RNA are also problems for the technology. Finally the costs of the chips are high for our purposes and it can only be used.

Figure 1.8 The basic construction of affymetrix chips that consists of thousands of DNA molecules (corresponding to different genes) that are immobilized and girded onto a support such as glass, silicon or nylon membrane.

The production of target molecules includes some further steps. After the isolation of mRNA and the reverse transcription to cDNA, the cDNA strands are converted to double stranded DNA molecules. After this step, the DNA is converted to cRNA molecules using a special polymerase (T7 polymerase) and fluorescently labelled. This cRNA is hybridised to the microarray. Unbound cRNA are washed away and signal intensities are scanned.

The additional steps that lead to the production of cRNA have two advantages. First, the initially isolated mRNA will be multiplied during the conversion from DNA to cRNA which enables the detection of signals that are based on very low numbers of expressed mRNA. Secondly, hybridisation of RNA to DNA has a higher sensitivity compared to hybridisation from DNA to DNA.

1.10.3. Subtractive cloning

Subtractive cloning is a relatively new method that modernizes subtractive hybridization. Many of the limitations of subtractive hybridization have been eliminated, including quantity of RNA, speed to finished product, and amplification of rare transcripts. The major disadvantage, however, is that like differential and subtractive hybridization, only two cell populations can be compared.

cDNA is synthesized from 0.5 to 2.0 µg of poly$(A)^+$ RNA isolated from the two populations of interest (this is still more RNA than is required for DDRT-PCR). The reference cDNA is called the driver, and the cDNA where the differentially expressed transcripts are to be found is called the tester. The tester cDNA population is split, and different adapters are ligated to each of the two pools of tester cDNA; adapters are not added to the driver cDNA. Common cDNAs between driver and tester populations anneal in a hybridization reaction (figure1.6) in the presence of excess driver cDNA. This result in a single-stranded DNA population enriched for differentially expressed products. In a second hybridization reaction, all cDNAs are mixed and annealing occurs only between single-stranded DNAs, since the pools are not first denatured. PCR amplification is performed with primers complementary to each of the adapter

sequences, so only hybrids with two adapter sequences will be amplified (Figure 1.9) (Sturtevant., 2000).

Figure 1.9 General outline of subtractive hybridization that is used for development a new method called Subtractive cloning. (Sagerstrom et al., 1997)

1.10.4. Differential Display

The method of Differential Display developed by Liang and Pardee 1992 has gained large audience and is now the method of choice to quickly identify and isolate differentially expressed genes in several experimental systems. The method is powerful due to its inherent simplicity and allows the simultaneous comparison of up- and down-regulated genes with small amounts of raw material.

The principal advantage of DD is that it allows genome-wide analysis in any species without prior sequence knowledge and that both known and unknown genes can

be studied. DD offers reliable results while reducing both the time needed and the necessary amount of tissue.

Most criticisms concerned false positives generated by the PCR step. In addition, the identification of differentially expressed genes remains tedious and limited by the fact that the amplified cDNA fragment generally corresponds to the trailer region of mRNA. Additional difficulties are (a) the contamination of bands recovered from display gel by heterogeneous sequences and (b) the strong bias in favour of the abundant mRNAs.

For these reasons many researches attempted to improve the method, either through different primer designs (by using longer primers instead of the original 10mers) suggested by Liang and Pardee 1992. Longer primers were expected to favour more specific hybridisation in the PCR. According to this design PCR is performed in two steps: (i) few initial low-stringency cycles allowing frequent mispairing between the arbitrary primer and the cDNA template, followed by (ii) several high stringency PCR cycles favouring perfect matches with the anchored primers.

As an initial step in DD molecular technique is that, cDNA is constructed by using oligo dT with reverse transcription taking the advantage of poly A tail present on most eukaryotic mRNAs. In the next step PCR is performed by using two primer combinations: a 5′ arbitrary P primer and a 3′ T primer which contains two additional 3′ bases to allow the $T_9N_{-1}N$ primer to bind the 5′ end of the poly A tail. Initial 3 steps of PCR are performed at low stringent conditions to allow the arbitrary primers to bind the template. Subsequent PCR reactions are carried out at high stringent conditions. Products are labeled radioactively during the PCR when α-32 P dATP is used. When α -32 P ATP is used as one of the primers is labeled before PCR. The PCR products are displayed on a sequencing gel. A schematic diagram of differential display and expected results are illustrated in Figure 1.10 &1.11 respectively.

Figure 1.10 Schematic diagram of the Differential Display; cDNA were synthesized from poly(A)$^+$ RNA isolated from the populations of interest. cDNAs were differentially expressed by DDRT-PCR

Figure 1.11 Schematic of expected results in a DD analysis. The signs (*) indicates the selected differentially expressed fragments either induced or repressed for further studied including re amplification, cloning and sequencing.

Our purpose in this study was to identify putative genes with induced or repressed expression after treatment of wheat plants with biological elicitor fungus, *Trichoderma harzianum,* and chemical inducers, BTH and BABA, in addition to knowing the potential involvement of these genes in the signal transduction pathway leading to SAR or to protein responsible for the state of resistance.

In the first set of experiments, we tested different biological and chemical activating agents that have been reported to be effective in inducing acquired resistance in wheat.

The mRNA differential display (DD) method (Lang and Pardee., 1992) was then used to identify the putative genes. Many differentially expressed cDNAs were identified and some of them were cloned and sequenced.

The expression level of the identified genes was analyzed by real time PCR. Some of the genes in this context are transiently activated after infection or elicitor treatment.

CHAPTER II

MATERIALS AND METHODS

2.1 Plant materials

Bread wheat cultivar is (Avocet S) is susceptible to all the known races of the yellow rust spores. We have obtained the seeds of (Avocet S) from International Center of Agricultural Research in Dry Area (ICARDA), Syria. The seeds were maintained and distributed to other centers.

2.2 Germination and growth

Single seeds were wrapped by filter papers. They were soaked in water and incubated at 4°C for 2 days. After that period, they were incubated at room temperature in the dark for one week. Seedlings were transferred to hydroponic medium and incubated at growth chamber for 15 days to be ready for treatments with chemicals and biological agents. Growth conditions were 18 °C at light period for 12 h, and 12 °C at dark period for 12 h. Humidity was kept covalent as 40 % during light and dark periods. After treatments with chemicals and biological agents, plant samples were collected from leaf tissues of plants and were frozen in liquid nitrogen and then they were transferred to – 80 °C until use.

2.3 Nutrient solution preparation for seedling growth

Plant seedlings were grown in a 7 liter container that contains all necessary nutrients for optimal growth. Growth nutrients were dissolved in double distilled water. Macronutrient, micronutrient, $ZnSO_4$ and Fe-EDTA stock solutions were prepared separately. Macronutrient solution contains 0.88mM K2S04, 0.25mM KH_2PO_4, 1.0 mM $MgSO_4$, 2.0 mM Ca $(NO3)_2$. Micronutrient solution contains 1µM H_3BO_3, 0.5 µM $MnSO_4$, 0.2 µM $CuSO_4$. Fe-EDTA concentration was 10 µM. Zinc

was supplied as $ZnSO_4$ at concentration of 4µM for zinc sufficient growth conditions.

The final concentrations of these compounds in the hydroponic medium are as follow; 2mM calcium (Ca), 4mM Nitrate (NO_3), 1mM magnesium (Mg), 2mM potassium (K), 0.2mM phosphorus (P), 1µM boron (b), 1µM manganese (Mn), 0.2µM copper (Cu), 100 µM iron (Fe), 10 µM ammonium (NH_4). Nutrient solutions were changed every 2 days.

2.4 Growing of fungal cells (*Trichoderma harzianum* Raifi KRL-AG2)

A small amount of dry *Trichoderma harzianum* Raifi KRL-AG2 spores (piece of toothpick 0.8-01 cm) was used to inoculate the surface of 100 ml potato dextrose broth PDB in 500 ml conical flask. Fungal cells were allowed to grow for 10 days at 25 degrees temperature with shaking in dark conditions. The culture was then filtered through filter paper to separate the mycelia mat from the culture that was diluted with water (1:1) and was used to treat t.

2.5 Treatments of the plant samples:

Plant materials were grown in the hydroponic medium for 15 days, then they were treated with two chemical inducers for disease resistance which are benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) and ß-aminobutyric acid (BABA), in addition to Biological inducer (*Trichoderma harzianum* Raifi strain (KRL-AG2).

Table 2.1 Avocet plant samples treatments with different concentrations of chemical and biological elicitors and the time period of harvesting the treated samples.

Sample (#) (3-5 seedlings)	**Types of treatment**	**Concentrations used in treatment**	**Harvesting time after treatment**
P1	Water control		2 days
P2	*Trichoderma*	(1water:1culture)v\v	2 days
P3	Water control		4 days
P4	*Trichoderma*	(1water:1culture)v\v	4 days
P5	Water control		4 days
P6	BTH	0.3 mM	4 days
P7	BTH	1 mM	4 days
P8	Water control		10 days
P9	BTH	0.3 mM	10 days
P10	BTH	1 mM	10 days
P11	BABA	1mM	4 days
P12	BABA	3.5 mM	4 days
P13	BABA	3.5 mM	10 days
P14	BABA	3.5 mM	10 days

2.6 RNA Isolation from plant leaf tissue

RNA isolations were pereformed using the TRIzol reagent according to the manufacturer's protocol. For each sample, 100 mg of plant leaf tissue from each treatment and time points of combined individual seedlings were used for RNA isolation according to manufacturer’s protocol.

2.6.1 Homogenization

Tissue samples were powdered using a mortar and liquid nitrogen. Powdered tissue was homogenized in 1 ml of TRIzol reagent per 100 mg of tissue in a 2 ml sterile tube.

2.6.2 Phase Separation:

Homogenized samples were incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Then 0.2 ml of chloroform per 1 ml of TRIzol reagent was added. Sample tubes were capped securely. Tubes were shaken vigorously by hand for 15 seconds and incubated at room temperature for 2 to 3 min. Then, the samples were centrifuged at 15,000 rpm for 15 min at 4°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an inter-phase, and a colorless upper aqueous phase. RNA remains exclusively in the upper aqueous phase.

2.6.3 RNA Precipitation

The upper phase was transferred to a fresh tube. RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. 0.5 ml of isopropyl alcohol was used per 1 ml of TRIzol reagent used for the initial homogenization. Samples were incubated at room temperature for 10 min and centrifuged at 15,000 rpm for 10 min at 4°C. The RNA precipitate observed was a gel like pellet on the side and bottom of the tubes.

2.6.4 RNA Wash

Supernatant occurred from the last centrifugation was removed and the RNA pellet was washed once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIzol Reagent used for the initial homogenization. Samples were mixed by vortex and centrifuged at 10,000 rpm for 5 min at 4°C.

2.6.5 Re-dissolving the RNA:

At the end of the procedure, the RNA pellet was briefly dried (10 – 15 min) at room teperature. RNA was dissolved in DEPC treated sterile water and incubated for 10 min at 55°C-60°C.

2.7 Determination the Concentrations of RNA samples

RNA samples were diluted 1/1000 to 0.5 ml in double distilled water and their absorbance values were measured at 230 nm, 260 nm, and 280 nm in

Schimadzu UV-1601 spectrophotometer. RNA absorbs UV light at 260 nm, but it is also required to know the absorbance values of proteins at 280 nm and phenolic compounds at 230 nm in order to evaluate the contamination level of them. Concentration determination of DNA samples was made according to the equation given below:

$1A_{260}$ = 40µg/ml RNA

Concentration of RNA (mg/µL) = A_{260} value(recorded) x dilution factor x 40 mg/ml RNA/A_{260}

Table 2.2 List of determined concentrations and A values of total RNAs of the parents and the bulks at different wavelengths.

RNA samples	A230	A260	A280	A260/ A280 Ratio	Concentrations µg/µL
P1 RNA	0.026	0.047	0.029	1.62	1.88
P2 RNA	0.029	0.046	0.028	1.64	1.84
P3 RNA	0.022	0.048	0.024	2.00	1.92
P4 RNA	0.009	0.048	0.025	1.92	1.92
P5 RNA	0.023	0.049	0.024	2.04	1.96
P6 RNA	0.017	0.046	0.022	2.09	1.84
P7 RNA	0.021	0.039	0.023	1.69	1.56
P8 RNA	0.021	0.041	0.028	1.64	1.64
P9 RNA	0.023	0.047	0.024	1.95	1.88
P10 RNA	0.023	0.043	0.021	2.04	1.72
P11 RNA	0.028	0.046	0.019	2.42	1.84
P12 RNA	0.029	0.046	0.033	1.39	1.72
P13 RNA	0.024	0.048	0.029	1.66	1.92
P14 RNA	0.025	0.047	0.024	1.96	1.88

2.8 Bulk RNA samples

The previous RNA samples listed in figure 2.1 were bulked into only 7 samples listed in the following table based on their concentrations (Concentrations are almost equals).

Table 2.3 List of bulked total RNA samples isolated from Avocet S leaf tissues

Sample Number	Combinations of bulk RNAs	Descriptions of treatments and time points.
B1	1	*Trichoderma* harvested after 2days(control)
B2	2	*Trichoderma* treated harvested after 2 days
B3	3	*Trichoderma* control harvested after 4 days
B4	4	*Trichoderma* treated harvested after 4 days
B5	5&8 (equal amounts)	control for BTH & BABA
B6	6&7&9&10 (equal amounts)	BTH treated
B7	11&12&13&14 (equal amounts)	BABA treated

2.9 Purity of RNA samples

In order to determine the quality of RNA (lack of DNA contamination), PCR reaction was performed on RNA bulked samples together with a positive control DNA isolated from Avocet S plants in parallel for which constitutively expressed actin1 gene primers were used. PCR amplification reaction was performed using *Taq* DNA polymerase enzyme as follow: In 50 µL of final volume the following components were mixed; 5 µl new isolated RNA and\or double strand DNA, 1x PCR Buffer (75 mM Tris-HCl with pH 8.8 at 25°C, 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20), 0.25 mM dNTP mix (DNA Amp), 1,5 mM $MgCl_2$ (DNA Amp), 0.05 mM dNTP mix (DNA Amp), 1,5 mM $MgCl_2$ (DNA Amp), 1 ul of *Taq* DNA polymerase, 10 pmol forward primer and 10 pmol reverse primer sterile distilled water up to 50 µl volume. PCR cycling conditions were 94°C for 2 min as initial denaturation, 30 cycles of three steps as denaturation at 94°C for 1 min, annealing at

53°C for 1 min and extension at 72 °C for 1 min and final extension at 72°C for 5 min.

2.10 Synthesis of first strand cDNA

Synthesis of first strand is achieved by using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's procedure.

Following components were combined in a 200 µL sterile PCR tube: 10 pmol cDNA synthesis primer (Gibco BRL), 10 pmol Smartoligo (Gibco BRL), total RNA (1 ng-5), 0.625 mM dNTP (DNA Amp) mix, Sterile distilled water up to 12 µL. Mixture was incubated at 65 ^{0}C for 5min and quickly chilled on ice. Mixture was spun briefly and the following components were added: 1x first strand buffer (Invitrogen), 0.005 M DTT (Invitrogen), 30 U RNase inhibitor (Ambion). The content of the tube was spun briefly and incubated at 42 ^{0}C for 2 min. Finally 200 U of the enzyme SuperScript III reverse transcriptase was added (Invitrogen). Reaction was carried out at 50 ^{0}C for 2 h and stopped by incubating at 70 ^{0}C for 15 min**.**

2.11 Visualization of Constructed cDNA by using actin1 amplification

In order to check the succeeding in constructing ds-cDNA, PCR reaction was performed on the constructed ds-cDNA by using actin 1 gene primers. Constructed ds-cDNA was visualized by running 10µL of amplified actin1 gene on 1% agarose gel and ethidium bromide staining. Successfully constructed ds-cDNAs were used in Differential Display.

2.12 Differential display using single stranded cDNA

Differential display conditions were optimized in our laboratory. Initially, Differential display reactions were performed using ss-cDNA constructed by SuperScript III M-MLV reverse transcriptase and *Taq* polymerase which has potential ability to amplify bands that have sizes of 1.5-2 kb. All incubation reactions for differential display were performed in MJ Research PTC 100 type thermal cycler. Primers used in reactions are listed in Table 2.4.

Table 2.4 List of sequences of the primers used in differential display analysis

Primer name	Primer sequence (5'-3')	T_m C^o)
T1	CAT TAT GCT GAG TGA TAT CT$_{(9)}$ AA	65,1
T2	CAT TAT GCT GAG TGA CT$_{(9)}$ AC	64,9
T3	CAT TAT GCT GAG TGA TAT CT$_{(9)}$ AG	65,0
T4	CAT TAT GCT GAG TGA TAT CT$_{(9)}$ CA	67,0
T5	CAT TAT GCT GAG TGA TAT CT$_{(9)}$ CC	67,8
T6	CAT TAT GCT GAG TGA TAT CT$_{(9)}$ CG	68,5
T7	CAT TAT GCT GAG TGA TAT CT$_{(9)}$ GA	67,0
T8	CAT TAT GCT GAG TGA TAT CT$_{(9)}$ GC	68,6
T9	CAT TAT GCT GAG TGA TAT CT$_{(9)}$ GG	68,5
P1	ATTAACCCTCACTAAATGCTGGGGA	66.1
P2	ATTAACCCTCACTAAATCGGTCATAG	63.7
P3	ATTAACCCTCACTAAATGCTGGTGG	61.3
P4	ATTAACCCTCACTAAATGCTGGTAG	59.7
P5	ATTAACCCTCACTAAAGATCTGACTG	60.1
P6	ATTAACCCTCACTAAATGCTGGGTG	61.3
P7	ATTAACCCTCACTAAATGCTGTATG	58.1
P8	ATTAACCCTCACTAAATGGAGCTGG	61.3
P9	ATTAACCCTCACTAAATGTGGCAGG	61.3
P10	ATTAACCCTCACTAAAGCACCGTCC	63.0

2.12.1 PCR amplification

In a 200 µL sterile PCR tube, following components were mixed ; 1x PCR Buffer (75 mM Tris-HCl with pH 8.8 at 25°C, 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20), 0.25 mM dNTP mix (DNA Amp), 1,5 mM $MgCl_2$ (DNA Amp), 2 u of *Taq* DNA polymerase, 15 pmol forward primer, 15 pmol reverse primer and sterile distilled water up to 20 µl volume were mixed in a 200 µl PCR tube. PCR cycling conditions were 94 °C for 2 min as initial denaturation, 35 cycles of three steps as denaturation at 94 °C for 1 min, annealing at 42°C for 1 min and extension at 72 °C for 1 min and 1 cycle of 5 min extension at 72°C. Reaction was terminated by

adding 4 μL of stop solution (95% formamide, 20 mM EDTA, 0.25% bromophenol blue and 0.025% xylene cyanol).

2.12.2 Radioactive labelelling of PCR product

PCR products are labeled radioactively during the PCR reaction using [α-^{32}P]-dATP or [α-^{33}P]-dATP. The PCR products are displayed on a sequencing gel.

2.12.3 Detection and analysis of Differential Display bands

Differential Display products were denatured at 94 °C before loading on the denaturizing gel. Samples were loaded on 6 % denaturizing polyacrylamide gel [5.7% acrylamide, 0.3% N, N'methylene-bis-acrylamide, 8M urea, 1X TBE (90mM Tris base, 90mM boric acid, 2mM EDTA)]. To 60 mL of gel solution, 650μL of 10% APS (ammoniumpersulfate) and 25μL of TEMED (N, N, N', N'-tetraethyl ethylene diamine) was added and gel was poured between two sigma coat treated sequencing gel plates immediately. Radioactively labeled differential display products were electrophoresed at a constant power of 60 Watt (Biometra High Voltage Power Supply, Pack P30, Germany). Electrophoresis continued until the xylene cyanol dye migrates 10 cm after the bromophenol blue dye goes out of the gel. After completion of electrophoresis, gel was transferred on Whatman paper (3MM), and covered with stretch film. The gel was left on the gel dryer (Savant SGD 2000) and dried at 76°C for 30-40 minutes. After drying procedure, an X-ray film was exposed to the gel. Films were developed at METU Health Center for a period depending on the half-life of the radioactive material (7-15 days).

Different combinations of forward P primers and reverse T primers, which were designed to attach to 5′ and 3′ ends of the wheat cDNA samples that were used in these analysis.

2.12.4 Differential display autoradiograph:

More than 70 mRNA Differential Display autoradiographs were performed using ss-cDNA of wheat total RNA using (P1,P2,P3,P4,P5,P6,P7,P8,P9,P10) forward primers and (T1,T2,T3,T4,T5,T6,T7,T8,T9,T10) reverse primers. The differentially expressed bands that are present in the treated samples and missing in the control in the case of induction occurrence or vice versa in the case of repression are cut to be the subject of further studies.

2.13 Re-amplification of differentially expressed fragments

Reactions were carried out in 40 µL of final volume and following components were mixed; 5 µl re-dissolved product, 1X PCR buffer (75 mM Tris-HCl with pH 8.8 at 25°C, 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20), 0.25 mM dNTP mix (DNA Amp), 1,5 mM $MgCl_2$ (DNA Amp), 0.05 mM dNTP mix (DNA Amp), 1,5 mM $MgCl_2$ (DNA Amp), 1 u of *Taq* DNA polymerase, 10 pmol forward primer and 10 pmol reverse primer sterile distilled water up to 50 µl volume. PCR cycling conditions were 94°C for 2 min as initial denaturation, 25 cycles of three steps as denaturation at 94°C for 1 min, annealing at 42°C for 1 min and extension at 72 °C for 2 min and final extension at 72°C for 5 min. Re-amplified bands were loaded on 1% agarose gel and screened. Re-amplified bands were cut and redissolved in 20 µl distilled water to be cloned.

2.14 Cloning of differentially expressed bands

Differentially Expressed Bands detected in DD gel were cloned in order to be sequenced. As an initial step, differentially expressed bands were cut form sequencing gel and dissolved in 30 µL distilled water. Dissolved products were re-amplified by PCR.

2.15 Ligation of the re-amplified fragments to pTZ57R-T-Easy vector:

Re-dissolved DNA that was cut from agarose gel was ligated to PTZ7R-T-Easy vector (Fermentas#K1214) (appendix A) with a final volume of 15 µL in a PCR tube. Following components were combined; 6 µL re-dissolved DNA, 5 ng pTZ57R-T Easy vector (Fermentas # K1214), 1 x Ligase Buffer (Fermentas#K1214) and 2 units T_4 DNA Ligase enzyme (Fermentas # K1214). Mixture was incubated at 22°C overnight (Approximately 12 hours). The vector uses the TA cloning strategy.

2.16 Preparation of *E.coli* competent cells

A single colony of *E.coli* Dh5-α cells was inoculated into 2 ml LB medium. Cells were grown at 37°C with moderate shaking (220rpm) overnight. 1 mL of overnight grown culture was inoculated into 500-ml flask containing 100 ml of LB medium and grown at 37°C, shaking (220rpm), until OD_{600} reach 0.5; this usually

takes 2-2.5 hr. Culture was a liquated into two 50-mL pre-chilled tubes and left on ice for 20 min. and then cells were collected by centrifugation for 5 min at 15000 rpm at 4°C. Supernatant was poured off and each pellet was re-suspended in 10 mL ice-cold TSS solution (Transformation and Storage Solution for Chemical transformation) which consists of; 85% LB medium; 10% PEG (wt/vol, MW8000), 5%DMSO (vol/vol) and 50mM $MgCl_2$. Now the competent cells are ready to be transformed or they can be stored at 4°C for maximum of 6 hr. without significant loss of competency. The same competent cells can be stored at -80 °C for long-term storage (pre-treated with liquid nitrogen or dry ice).

2.17 Transformation of *E.coli* competent cells with ligation products:

E.coli Dh5-α competent cells were transformed with ligation products according to the following procedure; In a sterile 2 mL tube following components were combined; 15 μL ligation product, and 150 μL *E.coli* Dh5-α competent cells. Mixture was placed on ice for 30 min with occasional mix. Then heat shocked at 42 °C for 2 min. After heat shock mixture was put on ice for 2 min. Cells were allowed to recover in 0.5 ml SOC Medium (2% Bactotryptone,0.5%Bactio yeast extract ,10 mM $NaCl_2$, 2.5 mM KCl ,10 mM $MgCl_2$,10mM $MgSO_4$,20 mM glucose).

Cells were incubated 37 °C for 60 minutes and quickly chilled on ice for 2 minutes, and were spread on plates as 100 μL/plate (Agar LB plates contain IPTG, X-Gal and Ampeciline). Plates were incubated at 37 °C overnight. After the incubation, white colonies were selected among grown colonies and these colonies transferred to 100 μL LB medium containing sterile 2 ml tubes. Selected colonies were PCR amplified.

2.18 PCR amplification of DNA from recombinants

1X PCR buffer (DNAmp), 0.2 mM dNTP mix (DNAmp), 0.8 pmol M13 forward and reverse primers (TIB Molecular Biology), 1.2 mM $MgCl_2$ (DNAmp), 1 unit *Taq* Polymerase enzyme, 1 μL DNA from colonies and sterile PCR water up to 25 μL final volume combined in a sterile PCR tube. PCR cycling conditions were initial denaturation at 94°C for 2 minutes followed by 30 cycles 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min.

PCR products were run on 1.5% agarose gel to detect positive and false positive clones (colonies that do not carry the expected sized DNA fragment). Positive clones were selected and used in plasmid isolation step.

2.19 Plasmid isolation from colonies

Plasmids were isolated using RapidPURE Plasmid Mini Kit (BIOGEN) acording to the manufacturer's protocol.

Bacterial culture of 1.5 mL was harvested by centrifugation for 5 min at 15,000 rpm. Supernatant was poured off, tube was inverted and blotted to on a paper towel to remove excess media. 50 μL of Pre-Lysis buffer was added to tube and cell pellet was completely resuspended by vortexing. 100 μL 0f Alkaline Lysis solution was added, then the tube mixed by gently inverting four times, incubated at RT for approximately 3 minutes. 100 μL of Neutralizing solution was added and mixed by inverting 4 times. Sample tubes were centrifuged at 12,000 rpm for 2 minutes at RT. Supernatant was transfered into aclean, user-supplied microcentrifuge tube, 250μL of RapidPURE Mini Salt was added, then the tube mixed by gently inverting four times,then the mixture was poured into a spin column, provided by manufacturer, inserted into collection tube, centrifuged at 12,000 rpm for 1 minute at room temperature.

After the centrifugation, lower phase in the collection tube was discarded and collection tube reinserted. 350 μL of Column Washing solution Buffer was added to spin column, centrifuged at 12,000 rpm for 1 minute at RT, flowthrough discarded and the collection tube reinserted again and to remove residual wash buffer completely additional 2 min centrifugation was involved. The contents of spin column was transferred to a new sterile 1.5 ml tube, the plasmid DNA was eluted by adding 100 μL of PCR Water, by letting sit for 5 min at RT and centrifugation at 12,000 rpm for 1 min. Spin column assembly was removed and harvest stored at –20 °C.

2.20 Visualization of isolated plasmid

In order to re-confirm the ligation, plasmid DNA was digested with EcoRI restriction enzyme, allowing the release of insert DNA; 5 µL purified plasmid, 1X NE Buffer (New England Biolabs), 1 U of EcoRI enzyme (New England Biolabs) and PCR ddH_2O were combined in a PCR tube with a final volume of 10 µL. Mixture was incubated at 37°C for 3 hours. After the incubation samples were electrophorased on 1.5% agarose gel.

2.21 Sequencing and BLAST analysis

Plasmids were isolated from the randomly selected clones using Qiagen QIAperp Spin Miniprep kit. Plasmids have made sequenced at The Ohio State University Plant-Microbe Genomics Facility pmgf@osu.edu using T7 primers. NCBI databanks were searched for finding homolog sequences. Sequence analysis was performed using Editseq 4.0 (DNA Star Inc.), sequence analysis software. Bioinformatics analyses were performed using utilities and databases at the National Centre for Biotechnology Information (http:// www.ncbi.nlm.nih.gov). DNA sequences were searched by using tblastn, tblastx, and tblastp were used to assign putative functions to the sequences (Altschul *et al.*, 1997).

2.22 Sequence and homology analysis

NCBI databanks were searched for finding homolog sequences that are available. Sequence analysis was performed using Editseq 4.0 (DNA Star Inc.) expert sequence analysis software. The sequence alignments were obtained using ClustalX 1.81 and BoxShade (http://www.ch.embnet.org/software/ BOX_form.html) computer program.

2.23. Real time PCR analysis

2.23.1 Clean up of single strand RNA for qRT-PCR

Single strand RNA was cleaned up by treatment of RNA with DNAse and lithium chloride precipitation. DNAse treatment was performed using Turbo DNAse (AMBION INC. Lott 095K81). Following components were combined in a 200 µL sterile PCR tube: 15 ul ssRNA which is equal to 1µg, 1 µL DNAse enzyme.

Samples were incubated at 37 °C for 30 minutes and then reaction was in activated by heating for 15 minutes at 65 °C.

2.23.2 Lithium chloride precipitation

In order to separate pure RNA from the mixture, following components were added into a 2 mL sterile tube containing 15 µL ssRNA: one tenth volume of lithium chloride (Ambion salt, 7.5M, #9480), 2.5 volume of 100% ethanol. Then tubes were frozen at -20 °C for 30 minutes for dilute small sizes. Samples were mixed by vortex and centrifuged at 10,000 rpm for 5 min at 4°C. Following centrifugation, supernatants were removed and pellets were washed with 70% ethanol made with nuclease free water. Finally, samples were centrifuged at 10,000 rpm for 5 min at 4°C and pellets were re-suspended in 15 µL of nuclease free water.

2.23.3 qRT-PCR analysis

Currently, some housekeeping genes are well described for the normalization of expression signals. The most common is actin1.

Expression level differences of the genes identified were confirmed and quantified by comparative qRT-PCR. The measurements were performed in a Stratagene MX3005p QPCR System using the Brilliant SYBR Green qPCR Master mix (Stratagene, Cat no: 600548). Total RNAs leaf samples of *Trichoderma* treated and untreated plants have been used for qRT-PCR experiments to confirm the genes expressed upon treatment of wheat plants with *Trichoderma harzianum*. Also, total RNAs from leaf samples of BTH as well as BABA treated and untreated samples have been used for qRT-PCR experiments to confirm the genes expressed upon treatment of wheat plants with BTH and BABA. Q-RT-PCR was performed in triple replicates for each RNA sample/primer combination. The amounts of RNA in each Q-RT-PCR reaction were normalized using primers specific for Actin-1 gene ($^{5'}$AATGGTCAAGGCTGGTTTCGC$^{3'}$ and $^{5'}$CTGCGCCTCATCACCAACATA$^{3'}$ forward and reverse, respectively). Primer pairs for real-time PCR analyses were designed using Primer3 software (Rozen and Skaletsky, 2000). Threshold (Ct) values and gene transcript numbers were used to determine the gene expression levels from different cDNA samples. Real-time PCR data were analyzed with the REST software (Bustin et al., 2005) in comparison to constitutively expressed wheat

actin1, gene for normalizations. Among those 57 putative differentially expressed clones, 15 were selected to be confirmed with Q-RT PCR analysis. Primers were designed using the Primer3 software. For Q-RT PCR confirmation analysis

Table 2.5 Clones' names of and primers sequences of the differentially expressed clones used in qRT PCR analysis.

Clone name	**Forward Primer sequences 5'-3'** **Reverse Primer sequences 5'-3'**	**Tm ^{o}C** **F-R**
Zea mays ubiquitin conjugating enzyme (UBC) mRNA, complete cds	ATTACCCCTTCAAGCCACCA GTGGGCAATCTCAGGAACAA	65.3-65
Oryza sativa (japonica cultivar-group) cDNA clone:006-306-E01, full insert sequence.	CGAGTCATGCTTCCACGATT AAGCGGTTTCCGTGAAGAAT	65.1-64.6
Pyrus communis partial gene for putative nucleotide binding site leucine-rich repeat disease resistance protein, clone RGA06.	AAACTGGTTACACCCCAATCG CATGTGAGAAAGTCGTTATTGCAG	64.6-64.5
Gossypium hirsutum mRNA for transcription factor myb109	CCGCGGGAATTCGATTATTAAC GTCATTATGCTGAGTGATATCTTT	66.8-57.8
Lycopersicon esculentum ethylene overproducer-like 1 (EOL1) mRNA.	CGCGGGAATTCGATTATTAACC TCATTATGCTGAGTGATATCTTT	66.8-57
Oryza sativa blast-resistant mRNA, partial sequence	TTTTTAGACAAAGTACCAAAAG CCCTCACTAAATGCTGGTAGCA	54-.-65
Fragaria x ananassa gene encoding methionine sulfoxide reductase	CACGCACATACACTCATCCA CTTTCGCTTTCTTCCCTTCC	63.3-63.9
Triticum aestivum inducible phenylalanine ammonia-lyase mRNA ,partial cds.	GAGTGCCCCAAGGAGTGGAAC CACAGTCTTGAACCACTTCTATAT	69.1-60
Triticum aestivum autophagy mRNA, complete cds	GACGTCGCAACC TGAACA TC GCTTCCTGTACATGACTTACAGT	60-61
Dendrobium grex Madame Thong-IN ovg15 mRNA,partial sequence	AGGGGACGCAAGAGAGAGTG AACCCTCACTAAATGCTGTATGG	65.8-62.8
Pisum sativum L. cultivar Frisson mRNA for arbuscular mycorrhiza protein	CAGGCTGGAGTGCAGAGGTAC ACAGACATGCACCACCACATC	66.2-61
Nicotiana tabacum DNA, TJ sequence	TTGGTGTACACGGACAGAGCT GGCCTCTTCGCTATTACGCCA	64.6-69.1
Triticum aestivum cytosolic acetyl-CoA carboxylase psi-Acc-2,1 pseudogene, partial sequence	CCAATTTGGTCATTTCCCTTC GGGGTTGCTCAAATCAATCTTC	64.1-65.5
Lycopersicon hirsutum clone PK12_340 RGA marker sequence	GGGCAAGCCGGACTATATT TTGCATTGGAACAAGGTGAAG	62.7-64.8
putative pentatricopeptide (PPR) repeat-containing protein	AAGACGACGACGACGATGATGA TCGCTTATCTTTGAAAGCAACA	69-63.5

CHAPTER III

RESULTS AND DISCUSSION

3.1 RNA isolation from plant leaf tissue for differential display analysis (DD)

Intactness of RNA is one of the most important criteria in DD. Therefore, isolation of pure and intact full length RNA is so crucial for the rest of the experiments. "TRIzol" total RNA isolation reagent is used for this purpose. As DD is based on comparing the mRNA levels, not only the integrity of RNA but also the correct and equalized concentration determination is important. Concentrations of samples must be determined very carefully, since concentrations will result in as false positives in DD analysis when comparing the RNA levels of the treated and untreated samples. Thus, concentrations of the samples were measured very carefully using the spectrophotometer. Another important factor, is to maintain the intactness of RNA by taking extreme caution to avoid RNase activity. All the glass equipments were treated with hypo-chromic acid solution and washed with DEPC treated ddH_20 before use. Other non-glass or plastic equipments were treated with NaOH washed with DEPC treated ddH_20 and autoclaved. Non-plastic equipments were stored at 180°C two days before isolation. All the solutions used in isolations were prepared by using DEPC treated ddH_20. Isolated total RNA was also redissolved in DEPC treated ddH_20 in order to avoid degradation by RNase after isolation. Aliquots were prepared from samples and held in -80°C until use.

Integrity of RNA was verified by running the samples on 2% formaldehyde-agarose RNA gel in (Figure 3.1). It is a common practice that, if the rRNA appear non-degraded on the agarose gels, then it is expected that, mRNAs is intact as well. In this study, the integrity of RNA samples was verified on 2% formaldehyde-agarose RNA gel.

Figure 3.1 Appearance of total RNA samples isolated from leaf tissues on 2% formaldehyde-agarose RNA gel. Lane numbers are indicating the treated plant samples with different chemicals and biological agents, and the time of harvesting as it is summarized in the Table 2.1 of chapter II.

3.2 Bulk RNA samples

The replicates of the treated RNA samples were bulked into only 7 samples to facilitate the comparison of complicated DD lanes. Then 2 µL of each bulked sample were separated on 2 % formaldehyde-agarose RNA gel (Figure 3.2) to confirm the intactness and the purity of RNA. Bulking of samples depended on the concentration of each isolated RNA individuals. Although the concentrations were almost equal, the small variations were taken in considerations when they are pooled.

Figure 3.2 Appearance of bulked RNA samples isolated from leaf tissues on 2% formaldehyde-agarose RNA gel. Lanes are labeled as 1&3: Avocet water treated (harvested after 2&4 days respectively), 3&4: Avocet *Trichoderma* treated (harvested after 2&4 days respectively), 5: Avocet water treated control for BTH&BABA, 6: Avocet BTH treated bulked RNA, 7: Avocet BABA treated bulked RNA.

3.3 Checking the purity of RNA samples

Single strand RNAs of the bulked samples as well as double stranded DNA of Avocet S plant sample were used as templates for PCR amplification of a constitutively expressed *actin1* gene. Checking the purity of RNA is an important step before cDNA synthesis to get rid of false positives in DD analysis that may arise due to DNA contamination, when used as template. In the case of contamination there will be a PCR product in the reactions which uses sole RNA as template. DNA template is included as a positive control that to show two bands of *actin1* gene (Figure 3.3). Because, hexaploid wheat genome is a cross between a tetraploid domesticated wheat, *T. dicoccum* or *T. durum*, and a wild goat grass, *Aegilops tauschii*. The expected size of the *actin1* gene PCR product was 745bp using (5'AGGGCTGTTTTCCCTAGCAT3' and 5'AAACGAAGAATGGCATGAGG3', forward and reverse primers, respectively). *Actin1* gene, Accession number: AF326781, is in Appendix D).

Figure 3.3 Absence of PCR product of constitutively expressed *actin1* gene using total RNA in addition to DNA control of Avocet S samples on 1% agarose DNA gel and presence of PCR product in DNA control. Lanes are labeled as 1&3: Avocet water treated (harvested after 2&4 days respectively), 3&4: Avocet *Trichoderma* treated (harvested after 2&4 days respectively), 5: Avocet water treated control for BTH & BABA, 6: Avocet BTH treated bulked RNA, 7: Avocet BABA treated bulked RNA**.**

In the lanes (1-7), there appear no PCR products, indicating the absence of DNA contamination. Therefore there is no need for DNase treatment of RNA samples.

3.4. Optimization of cDNA synthesis reaction

It is also important to represent all the mRNA population as cDNA to acquire reliable results. Therefore, SuperScript II (Gibco BRL) MMLV reverse transcriptase which can generate cDNA up to 12.3 kb was used for synthesis of first strand of cDNA. First strand cDNA was synthesized by Clontech's smart cDNA synthesis method as described in Chapter I. The single strand cDNA synthesis saves considerable time, since reverse transcriptase is an expensive reagent, it reduces costs as well. We also find the subsequent PCR reaction more reproducible, therefore we used single strand cDNA instead of double strand cDNA.

3.5 Verifications of constructed cDNA by using *actin1* amplification

Constructed single strand cDNA was tested for reproducible *actin 1* gene PCR amplification using actin 1 gene primers $^{5'}$AATGGTCAAGGCTGGTTTCGC$^{3'}$ and $^{5'}$CTGCGCCTCATCACCAACATA$^{3'}$ forward and reverse, respectively, that amplify band with about 180bp as presented in appendix D. PCR products were separated on 1% agarose gel and ethidium bromide staining. Successfully constructed ss-cDNAs were used in Differential Display analysis. The expected PCR products were obtained at the equal concentrations using RNA samples (Figure 3.4).

Figure 3.4 PCR product of constitutively expressed Actin1 gene using ss-cDNA of wheat total RNA in addition to negative control on 1% agarose DNA gel. Lanes are labeled as 1&3: Avocet water treated (harvested after 2&4 days respectively), 3&4: Avocet *Trichoderma* treated (harvested after 2&4 days respectively), 5: Avocet water treated control for BTH & BABA, 6: Avocet BTH treated bulked RNA, 7: Avocet BABA treated bulked RNA**.**

3.6 Optimization of differential display

Differential display analysis technique was optimized in our laboratory. Initially, because of the sensitivity of DD detecting variation in cDNA levels, PCR temperature profiles of all reaction steps were performed very carefully. Because the principle basis of DD depends on comparing the mRNA levels between the two cell populations and finding the induced, repressed and/or appeared and lost bands in one of the samples according to their length, it is important to have a cDNA population representing all the messages.

Finally we have applied the DD RT-PCR method according to Clontech's Delta DD method in which the ss-cDNA is used as template in DD analysis. PCR cycles of this approach were optimized by performing different PCR conditions such as varying annealing temperature. Reactions were performed using the following primer combinations in Table 3.1.

Table 3.1 Primers combinations and the resulting differentially expressed bands (+; induced, - ; repressed).

Band Number.	Primers combination	Trichoderma 2 days treatment results	Trichoderma 4 days treatment results	BTH Treatment results	BABA Treatment results
1	P8-T8	+	+	+	+
2	P8-T8			+	+
3	P6-T6			+	+
4	P6-T2				+
5	P8-T7	+	+	+	+
6	P6-T3	+	+		
7	P8-T9	+	+		
8	P6-T6	+	+		
9	P8-T9	+	+		
10	P9-T8	+	+	+	+
11	P9-T7	-	-		
12	P7-T1	+	+	+	+
13	P7-T1	+	+	+	+
14	P7-T3	+	+	+	+
15	P2-T3	+	+		
16	P2-T2	+	+	+	+
17	P2-T3	-	-		
18	P2-T2	+	+		

"**Table 3.1** continued"

19	P2-T4			+	+
20	P3-T8	+	+		
21	P3-T7	+	+		
22	P4-T4	+	+		
23	P3-T8	+	+	+	+
24	P3-T8	+	+		
25	P4-T3	-	-		
26	P4-T4	+	+		
27	P10-T2	+	+	+	+
28	P10-T3	+	+	+	+
29	P4-T4			+	+
30	P4-T3	+	+		
31	P4-T3			+	+
32	P3-T7	+	+	+	+
33	P3-T8	+	+		
34	P3-T7	+	+	+	+
35	P4-T3	+	+	+	+
36	P4-T4	+	+		
37	P3-T7	+	+		
38	P4-T2	+	+		
39	P3-T7	+	+	+	+
40	P3-T8	+	+		
41	P3-T9	+	+		
42	P3-T7	+	+		
43	P3-T9	+	+		
44	P3-T7	+	+		
45	P3-T7	+	+		
46	P3-T7			+	+
47	P5-T4				+
48	P5-T4	+	+		
49	P5-T4			+	
50	P2-T9	+	+		
51	P2-T9	+	+		
52	P2-T8	+	+		
53	P9-T9	+	+		
54	P2-T8	-	-		
55	P2-T9	+	+	+	+
56	P6-T5	+	+	+	+
57	P2-T8	+	+		

Sequences of the primers used are listed in Table 2.4 in chapter II. Results of these experiments are presented in Figures 3.5 to 3.16**.**

3.7 Evaluation of Differential Display autoradiographs

We have tried more than 70 primer combinations for DD/RT PCR analysis method involving the ss-cDNAs of chemically, biologically treated, and untreated plant samples. Some primers combinations (P and T primer) produced fewer bands than the others. Number of differentially expressed bands were also fewer. This is probably due to the less homology of P primers to the 5′ region of the mRNA.

Many differentially expressed bands between the treated and the untreated bulks have been detected. The arrows attached to DD autoradiographs indicate the differentially expressed bands that are either induced (present in the treated samples and missing in the control) or repressed (present in the control samples and missing in the treated one).

Table 3.2 Descriptions of the type of treatments and time points and the corresponding lanes appear in DD autoradiographs.

Lane number	**Descriptions of the type of treatments and time points.**
1	Water treated sample harvested after 2days (control for #2)
2	*Trichoderma* treated harvested after 2 days
3	Water treated sample harvested after 4days (control for #4)
4	*Trichoderma* treated harvested after 4 days
5	Water treated sample (control for BTH & BABA treatments)
6	BTH treated
7	BABA treated

Figure 3.5 Part of mRNA Differential Display autoradiography. Lanes are labeled as 1&3: Avocet water treated (harvested after 2&4 days respectively), 3&4: Avocet *Trichoderma* treated (harvested after 2&4 days respectively), 5: Avocet water treated control for BTH & BABA, 6: Avocet BTH treated bulked RNA, 7: Avocet BABA treated bulked RNA**.**

Figure 3.6 Part of mRNA Differential Display autoradiography. Lanes are labeled as 1&3: Avocet water treated (harvested after 2&4 days respectively), 3&4: Avocet *Trichoderma* treated (harvested after 2&4 days respectively), 5: Avocet water treated control for BTH & BABA, 6: Avocet BTH treated bulked RNA, 7: Avocet BABA treated bulked RNA**.**

Figure 3.7 Part of mRNA Differential Display autoradiography. Lanes are labeled as 1&3: Avocet water treated (harvested after 2&4 days respectively), 3&4: Avocet *Trichoderma* treated (harvested after 2&4 days respectively), 5: Avocet water treated control for BTH & BABA, 6: Avocet BTH treated bulked RNA, 7: Avocet BABA treated bulked RNA**.**

Figure 3.8 Part of mRNA Differential Display autoradiography. Lanes are labeled as 1&3: Avocet water treated (harvested after 2&4 days respectively), 3&4: Avocet *Trichoderma* treated (harvested after 2&4 days respectively), 5: Avocet water treated control for BTH & BABA, 6: Avocet BTH treated bulked RNA, 7: Avocet BABA treated bulked RNA**.**

Figure 3.9 Part of mRNA Differential Display autoradiography. Lanes are labeled as 1&3: Avocet water treated (harvested after 2&4 days respectively), 3&4: Avocet *Trichoderma* treated (harvested after 2&4 days respectively), 5: Avocet water treated control for BTH & BABA, 6: Avocet BTH treated bulked RNA, 7: Avocet BABA treated bulked RNA**.**

Figure 3.10 Part of mRNA Differential Display autoradiography. Lanes are labeled as 1&3: Avocet water treated (harvested after 2&4 days respectively), 3&4: Avocet *Trichoderma* treated (harvested after 2&4 days respectively), 5: Avocet water treated control for BTH & BABA, 6: Avocet BTH treated bulked RNA, 7: Avocet BABA treated bulked RNA**.**

Figure 3.11 Part of mRNA Differential Display autoradiography. Lanes are labeled as 1&3: Avocet water treated (harvested after 2&4 days respectively), 3&4: Avocet *Trichoderma* treated (harvested after 2&4 days respectively), 5: Avocet water treated control for BTH & BABA, 6: Avocet BTH treated bulked RNA, 7: Avocet BABA treated bulked RNA**.**

Figure 3.12 Part of mRNA Differential Display autoradiography. Lanes are labeled as 1&3: Avocet water treated (harvested after 2&4 days respectively), 3&4: Avocet *Trichoderma* treated (harvested after 2&4 days respectively), 5: Avocet water treated control for BTH & BABA, 6: Avocet BTH treated bulked RNA, 7: Avocet BABA treated bulked RNA**.**

Figure 3.13 Part of mRNA Differential Display autoradiography. Lanes are labeled as 1&3: Avocet water treated (harvested after 2&4 days respectively), 3&4: Avocet *Trichoderma* treated (harvested after 2&4 days respectively), 5: Avocet water treated control for BTH & BABA, 6: Avocet BTH treated bulked RNA, 7: Avocet BABA treated bulked RNA

Figure 3.14 Part of mRNA Differential Display autoradiography. Lanes are labeled as 1&3: Avocet water treated (harvested after 2&4 days respectively), 3&4: Avocet *Trichoderma* treated (harvested after 2&4 days respectively), 5: Avocet water treated control for BTH & BABA, 6: Avocet BTH treated bulked RNA, 7: Avocet BABA treated bulked RNA

Figure 3.15 Part of mRNA Differential Display autoradiography. Lanes are labeled as 1&3: Avocet water treated (harvested after 2&4 days respectively), 3&4: Avocet *Trichoderma* treated (harvested after 2&4 days respectively), 5: Avocet water treated control for BTH & BABA, 6: Avocet BTH treated bulked RNA, 7: Avocet BABA treated bulked RNA

Figure 3.16 Part of mRNA Differential Display autoradiography. Lanes are labeled as 1&3: Avocet water treated (harvested after 2&4 days respectively), 3&4: Avocet *Trichoderma* treated (harvested after 2&4 days respectively), 5: Avocet water treated control for BTH & BABA, 6: Avocet BTH treated bulked RNA, 7: Avocet BABA treated bulked RNA.

3.8 Evaluation of results obtained from DD analysis

57 bands from the P forward and T reverse primer combinations were chosen to be cloned. These 57 selected fragments were precisely cut from DNA denaturing gels and re-amplified with PCR using the same P&T combinations that in DD profiles obtained. They are successfully re-amplified. There are also many short bands appeared in the DD autoradiographs which were not chosen for reamplification because of their limited available sequence information.

3.9 Cloning of differentially expressed bands

Differentially expressed bands shown in DD autoradiographs were cloned in order to be sequenced. Cloning of the fragments was achieved using pTZ57R-T-Easy vector (Appendex A) and *E.Coli* (*Dh5-*α cells) as indicated in chapter II.

PCR amplification from the selected colonies was performed to confirm the plasmids carrying the expected sized inserts, using primers annealing to M13 sites of the vector as shown in Figure 3.17. Plasmids were isolated from these colonies and prepared for sequencing.

Figure 3.17 M13 PCR products of the reamplified clones number 1-57 amplified by using M13 forward and reverse primers on 1 % agarose gel. The size marker used in this figure was Lambda DNA *Eco*RI+*Hin*dIII and the arrows are indicating the length of 564 bp DNA fragment.

3.10 Verification of the presence of the insert in the selected clones

Additionally, plasmid DNA samples were digested with *Eco*RI+*Hin*dIII restriction enzymes to release the inserts and then separated on 1% agarose gel Figure 3.18.

Figure 3.18 Appearance of *Eco*RI+*Hin*dIII digested plasmids from the selected colonies carrying the cloned fragments of putative gene fragments number1-57 on 1.5% agarose gel. The size marker used in this figure was Lambda DNA *Eco*RI+*Hin*dIII and the arrows are indicating the length of 564 bp DNA fragment.

3.11 Results of the obtained sequences

The obtained sequences were searched in the NCBI (National Center for Biotechnology Information) database for comparison with available genes and DNA sequences. Out of 57 sequenced bands we got 35 bands that have homologous sequences in the Genbank. Majority of these fragments were not

containing stop codons. The others did not result any significant similarity with the sequences in the gene bank. The result of Blast search for sequence similarities of these 35 bands are shown in Table 3.3.

The sequence readings unfortunately were very poor. In most of the readings, very short sequences information was obtained. It is suggested that the plasmid isolation and /or the sequencing reactions were not optimized to obtain full sequence information of all the results in the clones. Therefore, sequence alignment analysis had to be performed in most cases using these short sequence information. As a result, out of 57 clones sequenced 22 did not align to any sequence presented in databank. Even in the samples with detected alignments the sequences were very short. Nevertheless, due to high % homology values it was possible to predict the putative identity of these differentially expressed genes.

Table 3.3 Homologous sequences found in Genbank, using Blast algorithm v2.2.3.

Bands No.	Primer Combination & Figure	Blast Hits	Accession Number	Score (Bits)	E Value	Identities %
1	P8-T8 3.5	Pyrus communis partial gene for putative nucleotide binding site leucine-rich repeat disease resistance protein, clone RGA06	AJ581773	46.1	5e-04	23/23 (100%)
3	P6-T6 3.5	Pyrus communis partial gene for putative nucleotide binding site leucine-rich repeat disease resistance protein, clone RGA06	AJ581773	50.1	2e-04	25/25 (100%)
4	P6-T2 3.6	Pyrus communis partial gene for putative nucleotide binding site leucine-rich repeat disease resistance protein, clone RGA06.	AJ581773	38.2	0.57	19/19 (100%)
5	P8-T7 3.5	Zea mays ubiquitin conjugating enzyme (UBC) mRNA, complete cds	AF034946	492	5e-137	373/341 (91%),
6	P6-T3 3.6	Cocos nucifera microsatellite DNA, clone CnCir L3	AJ888988 AJ937270	61.9	5e-08	39/38 (97%)
7	P8-T9 3.5	Pyrus communis partial gene for putative nucleotide binding site leucine-rich repeat disease resistance protein, clone RGA06	AJ581773	50.1	2e-04	25/25 (100%)
11	P9-T7 3.7	Oryza sativa (japonica cultivar-group) cDNA clone:006-306-E01, full insert sequence.	AK104220	432	2e-119	227/230 (98%)
13	P7-T1 3.8	Glycine max unknown protein mRNA, partial cds	AY352267	52.0	3e-05	26/26 (100%)
14	P7-T3 3.8	Pyrus communis partial gene for putative nucleotide binding site leucine-rich repeat disease resistance protein, clone RGA06.	AJ581773	38.2	1.1	19/19 (100%)
15	P2-T3 3.9	Gossypium hirsutum mRNA for transcription factor myb109	AJ549758	61.9	7e-08	38/39 (97%)

'' **Table 3.3** continued"

16	P2-T2 3.9	Lycopersicon esculentum ethylene overproducer-like 1 (EOL1) mRNA,complete cd	DQ099681	50.1	2e-04	27/28 (96%)
21	P3-T7 3.12	Pyrus communis partial gene for putative nucleotide binding site leucine-rich repeat disease resistance protein, clone RGA06.	AJ581773	42.1	0.033	21/21 (100%)
23	P3-T8 3.12	Pyrus communis partial gene for putative nucleotide binding site leucine-rich repeat disease resistance protein, clone RGA06	AJ581773	40.1	0.28	20/20 (100%)
27	P10-T2 3.11	Dendrobium grex Madame Thong-IN putative phenylalanine ammonia-lyase (ovg43) mRNA, partial cds.	AF100336	80.3	0.076	24/25 (96%)
30	P4-T3 3.12	Oryza sativa blast-resistant mRNA, partial sequence	AF450251	46.1	0.005	23/23 (100%)
31	P4-T3 3.12	Fragaria x ananassa gene encoding methionine sulfoxide reductase	AJ297967	50.1	6e-04	25/25 (100%)
32	P3-T7 3.12	Oryza sativa blast-resistant mRNA, partial sequence	AF450251	46.1	0.006	23/23 (100%)
33	P3-T8 3.12	Medicago truncatula clone mth2-10e13, complete sequence	AC125476	38.2	1.1	19/19 (11%)
36	P4-T4 3.12	Oryza sativa blast-resistant mRNA, partial sequence	AF450251	50.1	2e-04	25/25 (100%)
37	P3-T7 3.12	Triticum aestivum inducible phenylalanine ammonia-lyase mRNA , partial cds.	AY005474	347	1e-93	187/191 (97%),
38	P4-T2 3.12	Oryza sativa (japonica cultivar-group) chromosome 11 clone OSJNBa0010E20	AC152996	36.2	0.60	18/18 (100%)
39	P3-T7 3.12	Triticum aestivum inducible phenylalanine ammonia-lyase mRNA, partial cds.	AY005474	194	8e-48	110/114 (96%)
40	P3-T8 3.12	Oryza sativa blast-resistant mRNA, partial sequence	AF450251	50.1	1e-04	25/25 (100%)
41	P3-T9 3.12	Triticum aestivum autophagy mRNA, complete cds	AF542187	190	1e-46	103/104 (99%),
43	P3-T9 3.12	Triticum aestivum inducible phenylalanine ammonia-lyase mRNA, partial cds.	AY005474	157	1e-36	89/93 (95%)
44	P3-T7 3.12	Oryza sativa blast-resistant mRNA, partial sequence	AF450251	52.0	7e-05	26/26 (100%)
45	P3-T7 3.12	Unknon protein [Arabidopsis thalian	NP_177513	36.2	3.0	21/22 (95%)
46	P3-T7 3.12	Arabidopsis thaliana T-DNA flanking sequence, right border, clone559 E06.	AJ840172	48.1	0.003	56/68 (82%)
47	P5-T4 3.13	Dendrobium grex Madame Thong-IN ovg15 mRNA,partial sequence	AF100329	52.0	7e-05	26/26 (100%)
48	P5-T4 3.13	Pyrus communis partial gene for putative nucleotide binding site leucine-rich repeat disease resistance protein, clone RGA06	AJ581773	42.1	0.080	21/21 (100%)
51	P2-T9 3.16	Pisum sativum L. cultivar Frisson mRNA for arbuscular mycorrhiza protein.	AJ006305	244	1e-62	170/186 (91%),
54	P2-T8 3.16	Nicotiana tabacum DNA, TJ sequence.	D84238	287	2e-67	157/163 (96%)
55	P2-T9 3.16	Triticum aestivum cytosolic acetyl-CoA carboxylase psi-Acc-2,1 pseudogene, partial sequence.	AF305209	63.9	6e-08	133/161 (82%)
56	P6-T5 3.15	Lycopersicon hirsutum clone PK12_340 RGA marker sequence	AF534347	40.1	0.56	20/20 (100%)
57	P2-T8 3.16	putative pentatricopeptide (PPR) repeat-containing protein	XM_480482	97.6	2e-18	58/61 (95%)

3.12 Sequence alignment analysis of the differentially expressed gene fragments

Among the obtained 35 sequence bands, we found some bands are carrying sequences belonging to the same gene as we can see in Table 3.3. Therefore, we searched ClustalX-1.81 computer program in order to obtain sequence alignments between the fragments that represent a certain gene. Only 15 unique gene fragments were identified. They were re-assessed by comparative real time RT-PCR (sequencing results are in Appendix B).

3-13 Real time qRT-PCR

Expression level differences of the genes identified were confirmed and quantified by comparative qRT-PCR technology which is widely used to aid in quantifying cDNA because the amplification of the target sequence allows for greater sensitivity of detection than could otherwise be achieved. In an optimized reaction, the target quantity will approximately double during each amplification cycle.

In quantitative PCR (qRT-PCR), the amount of amplified product is linked to fluorescence intensity using a fluorescent reporter molecule. The measurements were performed in a Stratagene MX3005p QPCR System using the Brilliant SYBR Green qPCR Master mix (Stratagene, Cat no: 600548) as explained in details in chapter II.

The Quantitative PCR experiment uses a standard curve to accurately quantify the amount of target present in Unknown samples. In Quantitative PCR experiments, the Mx3005P instrument detects the fluorescence during each cycle of the thermal cycling process and at the end of the reaction (endpoint qPCR). In endpoint qPCR, fluorescence intensity values are detected after the amplification reaction has been completed, usually after 30–40 cycles, and this final fluorescence is used to back-calculate the amount of template present prior to PCR.

Results are typically displayed in amplification plot which reflect the change in fluorescence during cycling.

The initial copy numbers of RNA or DNA targets can be quantified using real-time PCR analysis based on threshold cycle (Ct) determinations. The Ct is defined as the cycle at which a statistically-significant increase in fluorescence is detected. The threshold cycle is inversely proportional to the log of the initial copy

number. In other words, the more template that is present initially, the fewer the number of cycles required for the fluorescence signal to be detectable.

The SYBR Green experiment has two major phases: Amplification and Dissociation. The Amplification phase corresponds to the PCR portion of the experiment, and results in the generation of dsDNA. In the Dissociation phase, the dsDNA product is melted into ssDNA by a stepwise increase in temperature. Dissociation curve, used to verify that the majority of fluorescence detected can be attributed to the labeling of specific PCR products, this to verify the absence of primer-dimers and sample contamination.

The accumulation of dsDNA can be assessed by the incorporation of SYBR Green I dye into a PCR reaction. A major advantage of this approach is that no fluorescence-labeled probes are required. A limitation is that dye binding to both specific and non-specific PCR products generates the same fluorescence signal. Including a dissociation curve in the experiment facilitates the process of optimizing the assay for conditions that minimize the formation of nonspecific products, and provides a means for verifying the predominance of specific products in each run.

Fluorescence results are typically displayed in an Amplification plot**,** which reflects the change in fluorescence during cycling.

Normalization of expression signals were achieved by using some constitutively expressed genes. The most common are *actin1*, glyceraldehyde-3-phosphate dehydrogenase and ribosomal genes (Stürzenbaum and Kille., 2001). qRT-PCR was performed in triple replicates for each RNA sample per primer combination. The amounts of RNA in each qRT-PCR reaction were normalized using primers specific for constituvely expressed wheat *actin-1* $^{5'}$AATGGTCAAGGCTGGTTTCGC$^{3'}$ and $^{5'}$CTGCGCCTCATCACCAACATA$^{3'}$ forward and reverse, respectively).

The selected bands to be confirmed with qRT PCR analysis were used to design primers to each one for qRT-PCR confirmation analysis as it was shown in table 2.6 in chapter III. To confirm that the gene expression differences observed between the treated and / or untreated plants as a result of defense induction, we have used RNA that is purified very well and treated with DNase and precipitated with lithium chloride to make samples absolutely free of DNA contamination.

3.14. Evaluation of the results of Real Time qRT-PCR

Although all of the 15 genes were tested by qRT-PCR, among them only 5 were confirmed as differentially expressed. The differentially expressed genes are; *UBC*: Ubiquitin conjugating enzyme**,** PAL: Inducible phenylalanine ammonia-lyase, *ATG*: Autophagy mRNA, AM: arbuscular mycorrhiza protein. In addition to repressed one gene; Oryza sativa (japonica cultivar-group) cDNA clone: 006-306-E01, full insert sequence (Accession number: AK104220) as it is illustrated in Table 3.4 and Figure 3.19.

Table 3.4 Summary of qRT-PCR results: Including expression level fold changes of the genes induced or repressed upon the chemical and biological treatment of Avocet S wheat plants; Td: Trichoderma, BTH: benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester, BABA: ß-aminobutyric acid.

No	elicitor	Clone name	Accession Number	Expression changes
5	Td BTH BABA	Zea mays ubiquitin conjugating enzyme (UBC) mRNA, complete cds	AF034946	4 folds up 3 folds up 3 folds up
11	Silent in Td	Oryza sativa (japonica cultivar-group) cDNA clone: 006-306-E01, full insert sequence.	AK104220	2 folds down
14	Td BTH BABA	Pyrus communis partial gene for putative nucleotide binding site leucine-rich repeat disease resistance protein, clone RGA06.	AJ581773	No Change No Change No Change
15	Td BTH BABA	Gossypium hirsutum mRNA for transcription factor myb109	Aj549758	No Change No Change No Change
16	Td BTH BABA	Lycopersicon esculentum ethylene overproducer-like 1 (EOL1) mRNA.	Dq099681	No Change No Change No Change
30	Td	Oryza sativa blast-resistant mRNA, partial sequence	AF450251	No Change
31	BTH BABA	Fragaria x ananassa gene encoding methionine sulfoxide reductase	AJ297967	No Change No Change
37	Td BTH BABA	Triticum aestivum inducible phenylalanine ammonia-lyase mRNA ,partial cds.	AY005474	5 folds up 3 folds up 3 folds up
41	Td	Triticum aestivum autophagy mRNA, complete cds	AF542187	4 folds up
47	BABA	Dendrobium grex Madame Thong-IN ovg15 mRNA,partial sequence	AF100329	No Change
51	Td	Pisum sativum L. cultivar Frisson mRNA for arbuscular mycorrhiza protein.	AJ006305	2.5 folds up regulated
54	Td BTH BABA	Nicotiana tabacum DNA, TJ sequence	D84238	No Change
55	Td	Triticum aestivum cytosolic acetyl-CoA carboxylase psi-Acc-2,1 pseudogene, partial sequence.	AF305209	No Change
56	Td	Lycopersicon hirsutum clone PK12_340 RGA marker sequence	AF534347	No Change
57	Td	putative pentatricopeptide (PPR) repeat-containing protein	XM_480482	No Change

Figure 3.19 Summary of expression level fold changes of the genes induced and / or repressed upon the chemical and biological treatments of Avocet S wheat plants; UBC: Ubiquitin conjugating enzyme**,** PAL: Inducible phenylalanine ammonia-lyase, ATG: Autophagy mRNA, AM: arbuscular mycorrhiza protein and the repressed gene; Oryza sativa (japonica cultivar-group) cDNA clone: (Accession number: AK104220)**.**

The amplification plots show plots of cycles versus fluorescence for plateau on which data are gathered. Available fluorescence data type is: dR (baseline-corrected raw fluorescence. The fluorescence data designated as dRn refers to data normalized according to the passive reference dye used.

When basing plots on normalized fluorescence (dR), the threshold fluorescence value for each dye is listed on the command panel and is marked on the graph by a horizontal line that if a dye is hidden, the line corresponding to threshold fluorescence for that dye will not be shown on the graph.

For our experiments that use SYBR Green detection and that include a Dissociation segment, the Dissociation Curve screen, view the dissociation profile. This screen shows a plot of the fluorescence (or its first derivative) as a function of

temperature for the five genes that confirmed to be induced or repressed as it is shown in the Figures 3.20-3.26.

Figure 3.20 Real Ttime PCR profiles illustrate the comparison between treated and untreated Avocet S seedlings with *Trichoderma harzianum* non-pathogenic fungal cells suspension. **A**: Normalization of mRNA levels using Actin1 gene expression with triplicates for treated and untreated samples. **B**: Expression level differences observed for the ubiquitin conjugating enzyme (UBC) mRNA between the treated and untreated samples. C & D are the Real time PCR dissociation phases of A & B, respectively

Figure 3.21 Real Time PCR profiles illustrate the comparison between treated and untreated Avocet S seedlings with BTH and BABA. **A**: Normalization of mRNA levels using Actin1 gene expression with triplicates for treated and untreated samples. **B**: Expression level differences observed for the ubiquitin conjugating enzyme (UBC) mRNA between the treated and untreated samples. C & D are the Real time PCR dissociation phases of A & B, respectively.

Figure 3.22 Real Time PCR profiles illustrate the comparison between treated and untreated Avocet S seedlings with *Trichoderma harzianum* non-pathogenic fungal cells suspension. **A**: Normalization of mRNA levels using Actin1 gene expression with triplicates for treated and untreated samples. **B**: Expression level differences observed for the *Oryza sativa* (japonica cultivar-group) mRNA: 006-306-E01, full insert sequence mRNA between the treated and untreated samples. C & D are the Real time PCR dissociation phases of A & B, respectively.

Figure 3.23 Real Time PCR profiles illustrate the comparison between treated and untreated Avocet S seedlings with *Trichoderma harzianum* non-pathogenic fungal cells suspension. **A**: Normalization of mRNA levels using Actin1 gene expression with triplicates for treated and untreated samples. **B**: Expression level differences observed for the *Triticum aestivum* inducible phenylalanine ammonia-lyase mRNA between the treated and untreated samples. C & D: are the Real time PCR dissociation phases of A & B, respectively.

Figure 3.24 Real Time PCR profiles illustrate the comparison between treated and untreated Avocet S seedlings with BTH and BABA. **A**: Normalization of mRNA levels using Actin1 gene expression with triplicates for treated and untreated samples. **B**: Expression level differences observed for the *Triticum aestivum* inducible phenylalanine ammonia-lyase mRNA between the treated and untreated samples. C & D are the Real time PCR dissociation phases of A & B, respectively.

Figure 3.25 Real Time PCR profiles illustrate the comparison between treated and untreated Avocet S seedlings with *Trichoderma harzianum* non-pathogenic fungal cells suspension. **A**: Normalization of mRNA levels using Actin1 gene expression with triplicates for treated and untreated samples. **B**: Expression level differences observed for *Triticum aestivum* autophagy mRNA between the treated and untreated samples. C & D are the Real time PCR dissociation phases of A & B, respectively.

Figure 3.26 Real Time PCR profiles illustrate the comparison between treated and untreated Avocet S seedlings with *Trichoderma harzianum* non-pathogenic fungal cells suspension. **A**: Normalization of mRNA levels using Actin1 gene expression with triplicates for treated and untreated samples. **B**: Expression level differences observed for *Pisum sativum* L. cultivar Frisson mRNA for arbuscular mycorrhiza protein between the treated and untreated samples. C & D are the Real time PCR dissociation phases of A & B, respectively.

The functions of the genes and relevance of their functions to our experiments are discussed bellow in details.

The first gene is **ubiquitin conjugating enzyme (*UBC*);** induced by *T. harzianum*, BTH and BABA (band # 5). It has been shown recently that the ubiquitin-proteasome pathway plays a key role in many cellular processes including

cell cycle control and flower dependent hormonal signal transduction (Kuroda et al., 2002). Also, ubiquitin-proteasome pathway plays role in shifting the elicitation system to the active state (Becker et al., 2000). The ubiquitin-proteasome dependent proteolytic pathway is found in all eukaryotes. It degrades a wide range of proteins in the nucleus and cytoplasm as well as membrane proteins and protein exported from the cytoplasmic reticulum (ER) (Ingvardsen and Veierskov, 2001). Ubiquitin becomes attached to substrate in three enzymatic steps: (i) Ubiquitin is activated by an ATP dependent thiol coupling of its carboxy terminus to an activating enzyme (E1); (ii) A subsequent transthiolation leads to the transfer of ubiquitin from E1 onto a ubiquitin-conjugating enzyme E2; (iii) Finally, ubiquitin is transferred to ε-amino group of an internal lysine residue of an appropriate target protein. In some cases, this transfer requires a third type of enzyme, called ubiquitin-protein ligase (E3) (Feussner et al., 1997). The ubiquitinated substrates are either degraded by the large enzyme complex, the 26S proteasome or are de-ubiquitinated by a special set of enzymes, the de-ubiquitinating enzymes (Kawasaki et al., 2005).

Ubiquitin is a 76 amino-acid polypeptide that has a compact globular structure with a flexible protruding C-terminal tail, three hydrophobic side chains that are exposed on the surface for the recognition of the polyubiquitin chains by the 26S proteasome and seven lysine residues for degradation by 26S proteasome. In eukaryotes, the 26S proteasome is a multi-catalytic proteinase complex comprising the 26S core particle that performs proteolysis and the 19S regulatory particle that recognizes the protein targeted for degradation, especially poly-ubiquitin targeted protein (Suty et al., 2003).

E1s are highly conserved: Plants have several E1s, as two have been reported from *Arabidopsis* and three from wheat. All E2s have a relatively conserved catalytic core domain of approximately 150 amino acids, the UBC domain, showing a sequence identity of at least 35%. The ubiquitin conjugating enzymes have been divided into several families, most of which have plant representative. The largest so far is the the UBC4 / 5 family, which has members from large range of organisms. The enzymes in this family are involved in stress response and regulation of transcription factors. Ubiquitin conjugating enzymes from the UBC1 family (UBC4-like) also seem to be involved in stress. Most Ubiquitin conjugating enzymes are found in the cytosol (Ingvardsen and Veierskov, 2001).

The multiplicity of UBC/E2 identified in a number of organisms suggests that each UBC may be important in controlling the ubiquitination of a specific subset of proteins (Clark et al., 1997). UBC then donate ubiquitin directly to specific target protein by cooperating with compatible ubiquitin-protein ligases (E3s). And then is recognized by one or more receptors within the 26S proteasome that breaks down the target protein into short peptides (Vierstra and Callis 1999).

In the case of our study on chemically and biologically induced resistance, since UBC involved in ubiquitin-proteasome pathway is found to be induced 3-4 folds, we suggest that this pathway plays a responsive role in these treatments. It is also known that ubiquitin-proteasome pathway is involved in regulation of the elicitation profile of SA and JA as it refered by several studies bellow; SA and JA accumulate in response to chemically and biologically induced resistance. Induced resistance is regulated by network of interacting signal transduction pathways in which SA and JA function as key signaling molecules. SA and JA-dependent defense pathways have been to cross-communicate, providing the plant with regulatory potential to fine-tune the defense reaction depending on the type of attacker encountered (Spoel et al., 2003). The transduction of SA signal requires the function of NPR1, a regulatory protein that was identified in *Arabidopsis*. Upon induction of SAR, NPR1 is translocated to the nucleus; where it interacts with membranes of the TGA / OBF subclass of basic domain –Leu Zipper (bZIP) transcription factors that are involved in SA-dependent activation of PR genes. So, NPR1 acts as positive regulator of SA pathway in nucleus. The activation of SAR has been shown to suppress JA signaling in plants, whereby prioritizing SA-dependent resistance to certain pathogens over JA-dependent defense against different pathogens. Moreover, pharmacological and genetic experiments have shown that SA is a potent suppressor of JA-inducible gene expression. Recently it has been reported that NPR1 has a novel function in cytosol that plays a crucial role in cross-communication between SA and JA-dependent plant defense response (Spoel et al., 2003). The mode of action of NPR1 in the cytosol is unknown, but it is tempting to interfere with the previously identified SCF ^{COII} ubiquitin-ligase complex that regulates JA-responsive gene expression through targeted ubiquitination and UBC then donate ubiquitin directly to specific target protein by cooperating with compatible ubiquitin-protein ligases (E3s), subsequent proteasome-

mediated degradation of negative regulator of JA signaling (Maria et al., 2005). Additional key elements that are involved in pathway cross-talk have been identified. For instance, the *Arabidopsis* transcription factor WRKY70 acts as both activator of SA-responsive genes and repressor of JA-inducible genes, thereby integrating signals from these antagonistic pathways. In addition, the transcription factor ethylene response factor 1 (ERF1) integrates signals from JA and ET pathways in activating defense related genes that are responsive to both JA and ET (Pieterse and Van Loon, 2004; Maria et al., 2005).

Protein degradation by ubiquitin-dependet pathway is highly regulated. It is the specificity of recognition that causes difference in protein turnover sometimes accompanied by phosphorylation. The E2-E3 complexes are believed to recognize specific degradation signals on substrates. But only a few of these signals are known. A degradation signal consists of a binding site for E2 and / or E3 complex (the recognition site) and a lysine residue (the attachment site). Unfolded proteins might be recognized by chaperons as that *Hsp70*, which if not able to refold the protein, deliver the protein to the degradation machinery. The recognition signals involved in chaperon-mediated degradation machinery by ubiquitin-dependent pathway are not known (Ingvardsen and Veierskov, 2001).

The cross-talk and overlap between SA and JA pathways revealed the complexity of the interaction between the pathways, and support the hypothesis stated by Maria et al., 2005 said that plant has a flexible defense response to fine-tune the appropriate mechanism by tightly regulating the concentrations of the different signals.

The recent advances in genetic studies have extended the function of the ubiquitin and ubiquitin-like systems for simple providing a degradation signal to having more dynamic roles such as the activation of kinases, provision of localization signal, and protein lipidation (Devoto et al., 2003). For example;

(1) Ubiquitin is the best studied protein modifier in plants and plays an important regulatory role in auxin signal transduction pathway (Lois et al., 2003). Several auxin proteins have been shown to directly interact with E2- SCF. This interaction is very rapidly promoted by Indol acetic acid (IAA); the most important auxin in higher plants in concentration dependent manner (Berleth et al., 2004). Another player in defence signalling interactions, the plant hormone abscisic acid (ABA), has

recently been shown to have antagonist interactions with JA/ET mediated defence gene expression (Anderson *et al.,* 2004). ABA signalling had a negative effect on JA/ET regulated gene expression. Further to this, mutants impaired in ABA signalling had, in addition to increased JA/ET regulated defence gene expression, increased resistance to a necrotrophic fungal pathogen. These results highlight another level of complexity in the regulation of defence signalling pathways.

(2) A small ubiquitin-like modifier (SUMO) has been known to be structurally related but functionally different from ubiquitin. Also, SUMO resembles ubiquitin in its mechanism of ligation to target protein that involves the sequential action of activating (E1), conjugating (E2), and ligase (E3) enzymes. The modification of substrates by SUMO is reversible, and desumolation is mediated by a family of SUMO proteases. Meanwhile the general consequence for SUMO modification remains unclear and may vary depending on the target. The role of SUMO in pathogen defense response has been studied in tomato and tobacco plants. The tomato SUMO ortholog (LeSUMO) interacts with ethylene-inducing xylanase (EIX) from fungus *Trichoderma viridae* in yeast two hybrid assays. The expression of LeSUMO in tobacco transgenic plants suppressed the induction of the defense response by EIX, suggesting that LeSUMO may act as repressor of plant defense ET-dependent pathway. It is also found that; LeSUMO modulates the signal of hormone Abscisic acid (ABA) which mediates plant response to environmental stresses, such as cold, drought and high salinity. Plants with increased sumoylation levels showed an attenuation of ABA-mediated growth inhibition (Lois et al., 2003).

(3) The third protein modifier is called related-to-ubiquitin (RUB) protein (called Nedd8 in some species). RUB is conjugated to the cullin subunit of SCF (SCF is composed of SKP1, Cullin, F-Box) which is class of ubiquitin protein ligase E3 through a series steps similar to ubiquitin conjugation. A single RUB molecule is linked to specific lysine residue near the C terminal of protein. Although the precise role of RUB modification is unclear, studies indicate that the modification is important for SCF function. In *Arabidopsis*, RUB modification of CUL1 is required for normal function of SCFs that have broad role in plant. For instance, SCF^{TIR1} is implicated in response to the plant hormones and SCF^{COII} is implicated in response to jasmonate. In *Arabidopsis* the RUB conjugating enzyme is named RCE1 protein (Dharmasiri et al., 2003).

The second gene is **autophagy protein (*ATG*);** induced by *T. harzianum* (band # 41). It is literally means self eating (bulk degradation of long-lived cytosolic protein and organells) whereas ubiquitin-proteasome system degrades specific short lived proteins (Yurimitsu and Klionsky, 2005 ; Allison and Richard, 2005).
Autophagic process involves three types (i) Chaperon-mediated autophagy which is a mechanism that allows the degradation of cytosolic proteins that contain a particular pentapeptide consensus motif (ii) Micro-autophagy involves the direct engulfment f cytoplasm at the surface the degradative organelle by protrusion, septation, and / or inragination of the limiting membrane (iii) Macro-autophagy involves the sequestration of cytoplasm into a double-membrane cytosolic vesicle, termed an autophagosome (Yurimitsu and Klionsky, 2005). In addition to chaperon-mediated autophagy, micro and macro-autophagy, a fourth type can be distinguished: autophagy by permeabilization or rupture of the lysosome or tonoplast (because the idea of rupture implies permeabilization to large molecules, It is used the general term permeabilization). This permeabilization appears to be common in plant programmed cell death (PCD). It results in the release of vacuolar hydrolases, which can degrade whatever left in the cell (Wouter et al., 2005).

Autophagy has been suggested to play a central role in several physiological and developmental responses in plants, including (i) the formation and degradation of protein storage vacuoles (PSVs) during seed development and germination (ii) *de novo* vacuolar biogenesis (iii) nutrient recycling during starvation, senescence, and apoptotic processes such as xylem and schlerid cell morphogenesis and (iv) the pathogen-induced hypersensitive response (Allison and Richard, 2005). Another study reported that even when nutrient conditions are non-limiting, plant cells dispose of abnormal plastids into vacuoles by autophagy, therefore they suggest that autophagy may have a quality control function within the cell (Niwa et al., 2004).

To date, there are various examples of PCD in plants that occurs as the endpoint of cell differentiation, for example, petal witting following pollination or as an adaptation to stress. This PCD that occurs during normal differentiation is called developmental PCD (Wouter et al., 2005). Another example of PCD in plant is the hypersensitive response (Wan et al., 2002), a rapid death that is associated with all forms of resistance, including, race specific, race non-specific and non-host. And it

is found in all tested plant species. The differential HR effectiveness has lead to the proposal that differential thresholds are responsible for activating HR and different resistance related genes (Kombrink and Schmelzer, 2001).

Here, we are attempting to categorized wheat plant PCD process as autophagy process (vacuolar degradation of cytoplasmic components).

Here in the present study we suggest that treatment of wheat plants with the culture of non-pathogenic fungus, *Trichoderma harzianum* induces HR that is associated with the activation of autophagic mechanism. However, little is known about the molecular mechanism underlying autophagy in higher plants (Yushimoto et al., 2004). Also, very little is known about the mechanism of programmed cell death (PCD) in plants and primary signal triggering this PCD is as yet unknown (Zhivotovsky, 2002).

Plant cells commonly respond to elicitors or microbial pathogens with an " oxidative burst" during which reactive oxygen species (ROS) are penetrated, usually extra-cellulary, and this ROS have been demonstrated to play a role in the expression of a non-host hypersensitive response (Michele, 2000).

A general type of resistance against a broad spectrum of pathogen is known as non-host resistance. This inherent plant resistance response is a complicated interaction that involves the recognition of multiple elicitors. Such elicitors may be components of the pathogen such as chitosan, a derivative of a deacetylated derivative of fungal cell wall chitin, or pathogen exudate. These elicitors may also be derived from the host and include various plant polysaccharides from the digested cell wall matrix. Disease resistance, in general, is initiated by recognition events that trigger a rapid HR (Jane et al., 2001).

Our study supports the last demonstration and explains the function of autophagy in the plant immune response that concert with the hypothesis stated by (Greenberg, 2005) that said; localized programmed cell death PCD is part of widespread defense mechanism in plant and autophagy is essential for preventing uncontrolled local and systemic PCD during infection. And with the hypothesis stated by (Liu et al., 2005) that said; autophagy regulates PCD during the plant innate immune response in that PCD must be restricted to infection sites to prevent HR from playing a pathogenic rather than protective role.

In pioneering genetic screens by various laboratories, a large collection of autophagy-related genes (ATG) genes (also known as APG, AUT, CVT and GSA) were discovered that effect micro or macro-autophagy. The sequencing of the *Arabidopsis* genome has allowed the identifications homologues of the yeast autophagy genes and animal autophagy genes, suggestin that the autophagic systems directed by these proteins are remarkably conserved among eukaryotes (Allison and Richard, 2005).

We have found ATG gene in susceptible wheat plant, Avocet S indicating that comparable micro and macro-autophagic systems exist in wheat plants.

The third gene is **inducible phenylalanine ammonia lyase (*PAL*);** induced by *T. harzianum*, BTH and BABA (band # 37). Phenylalanine ammonia lyase (PAL) is a key enzyme in phenylpropanoid pathway by which several secondary metabolites are produced such as SA, lignin-like polymers, phytoalexins, lignins and flavonoids (Kohler et al., 2002). These compounds help protect plants from pathogen attack. PAL function in phenylpropanoid pathway is catalyzing the first commited step in phenylpropanoid biosynthesis, namely, the deamination of phenylalanine to yield *trans* cinnamic acid and $NH4^+$, figure 3.27 (Wang et al., 2004).

The transcription of PAL is induced by several stimuli, such as UV light, wounding and attack by pathogens. However some pathogens have evolved mechanisms for reducing the activation of this gene, so they increase their chance of successfully infecting plants. To reduce the PAL activity, pathogens secrete so-called suppressors, which reduce the transcription of PAL and, consequently, produce a reduction in enzyme activity and, finally result in a reduce amount of secondary metabolites required for defense against pathogens (Schmidt et al., 2004).

The defense responses associated with PAL expression may include structural alterations and the production of a wide range of plant defense molecules such as antimicrobial proteins (Schenk et al., 2000).

Figure 3.27 Route of SA biosynthesis pathway.

Expression of defense genes encoding anti-microbial pathogenesis-related proteins (PR) or enzymes involved in secondary metabolism, like PAL, as well as the production of anti-microbial low molecular weight compounds constitute further key processes in the plant defense response (Zeier et al., 2004).

PAL genes are induced by pathogen infection and specific elicitor molecules. The promoter sequences responsible for this induction have been identified in some genes including Parsley PAL1, Pea PS PAL1 and Carrot gDcPAL1. Many of these *cis*-elements share a conserved AC rich element, the mutation or deletion of which can result in a reduction or loss of induced activation following pathogen infection or elicitor treatment (Wang et al., 2004).

Few studies have dealt with monocots PAL genes. To date, only PALs in rice, wheat and barley have been cloned, and only PALs of rice and barley have been shown to be induced following pathogen infection (Wang et al., 2004).

We reported here the induction of wheat PAL gene upon treatment of wheat plants with BTH, BABA, and *Trichodermal* infiltrate elicitors.

The fourth gene is **arbuscular mycorrhiza protein (*AM*)**; induced by *T. harzianum* (band # 51) Arbuscular mycorrhizal symbioses are the most common

underground symbiosis, which around 80% of terrestrial plant species able to associate with members of fungal phylum Glomeromycota (Glassop et al., 2005). AM fungi are all members of the class Zygomycota. AM fungi have not been cultured in the absence of the host plant and this has hampered their mass production and utilization within crop systems. However, it is possible to grow AM fungi in sterile culture with plant root explants, because AM fungi are obligatory biotrophic micro-organisms (Harrier, 2001).

Arbuscular mycorrhizal fungi (AMF) peneterate into cortical cells of the root plant forming branched hyphae called arbuscule this arbuscule formation increases the expression of JA (Liu et al., 2003). Colonization also enhances plant's resistance to biotic and abiotic stresses. In addition, arbuscular mycorrhizal symbiosis improved nutrient uptake (mainly phosphorus), enhance drought resistance, and protection from pathogens (Fehlberg et al., 2004).

The development of Arbuscular mycorrhizal symbiosis requires significant alteration in both symbionts that are assumed to be coordinated via reciprocal signal exchange (Liu et al., 2003). Penetration into the root and intercellular growth of AM fungi involves a complex sequence of biochemical and cytological events and intracellular modification that lead AM fungus to differentiate arbuscules. The mechanisms controlling AM development are largely unknown. However, evidence suggests that, as in plant-pathogen interactions, the induction/suppression of mechanisms associated with plant defense play a key role in AM fungal colonization and compatibility with its host (Garcia-Garrido and Ocampo, 2002).

Signaling components that are required for the development of AM symbiosis have also been uncovered. A plant respons to signals from AM fungi has not been identified so far, but there is an evidence that fungal signals that activate plant gene expression before physical contact with the host support the existence of the host plant response (Harrison and Baldwin., 2004).

Nowadays, it has been demonstrated that, some soil bacteria, which have been named mycorrhiza helper bacteria (MHB), could enhance the development of mycorrhizal symbiosis. This effect has been shown with different host plants, including herbaceous species (Duponnois and Plenchette, 2003).

Fungi such as *Trichoderma* seem to affect positively on the development of mycorrhizal symbiosis by means of volatile and soluble exudates produced by these fungi (Martinez et al., 2003).

In 2003 a study done by Srinath and others showed that inoculation of Ficus plants with AM fungus *Glomus mosseae* together with *Trichoderma harzianum* or the mycorrhiza helper bacteria (MHB), *Bacillus coagulance*, enhance mycorrhizal root colonization and sporulation by *Glomus mosseae* compared to the plants inoculated with *Glomus mosseae* alone. Since, *Bacillus coagulance* was described as mycorrhiza helper bacterium (MHB). It has been suggested that, *Trichoderma harzianum* also should be categorized as mycorrhizal helper organism (MHO).

From the previous studies we can deduce a hypothesis that, *Trichoderma harzianum* volatile and soluble exudates may induce the expression of some mycorrhizal related genes in host plant. And these genes may be involved in the signal interaction between the host plants and AM fungi even before any physical contact between the two symbionts.

Finally, the repressed gene **Oryza sativa (japonica cultivar-group cDNA** accession number: AK104220, induced by *T. harzianum* (band # 11).

In the case of our study, we found this gene is repressed in wheat plant Avocet S after inoculation with *Trichoderma harzianum*. Three other AK clones are also mapped on the same locus "Os03g0146500" according to the description in Rice annotation project database RAP-DB (Figure 3.28) but none of them has meaningful functional annotation currently.

Figure 3.28 Details Rice annotation project database: AK clones that are mapped on the same locus "Os03g0146500" http://rapdb.lab.nig.ac.jp/cgi-bin/gbrowse/IRGSP

This gene may deserve to be added to the list of the genes that are repressed upon chemical or biological treatments stated by (Schenk et al., 2000) on *A. thaliana* cv. Columbia plants that were either inoculated with non-pathogenic strain of *A. brassicicola* or treated with the signal molecules SA, MJ, or ethylene, approximately 10% of responsive genes found with known functions or previously were implicated in plant defense, whereas approximately 90 % had unknown functions were found. Of 270 genes with no known function, 106 genes showed significant induction or repression profiles after *Alternaria* inoculation, SA, MJ and ethylene treatments. The majority of repressed genes are involved in (i) high molecular weight signal regulation, (ii) cell maintenance and development and (iii) other unclassified genes (Table 3.5).

Table 3.5 Expression profiles of repressed genes after *Alternaria* inoculation, SA, MJ and ethylene treatments and the negative ratio of repression. The complete list can be accessed at http:yywww.tpp.uq.edu.au/ microarrayyresponsivegenes.htm or from supplemental data at www.pnas.org. (Schenk *et al.,* 2000).

Putative function	**GeneBank accession**	**A ratio**	**SA ratio**	**MJ ratio**	**ET ratio**
High moleculat weight signal regulator					
Transcription factor OBF4	H36941	2.24	6	2.97	1.37
Receptor like protein kinase	T44408	1.64	4.9	3.73	0
Response regulator ATRR2	T21859	1.53	4.3	3.26	0
CLV1 Receptor kinase like protein	H37524	1.46	1.3	2.5	3.2
Receptor protein kinase	T13648	1.35	2.1	1.53	5.5
Kinase associated protein phospatase	T75985	1.86	1.4	1.38	3.9
Cell maintenance and development					
glutathioneS-conjugate transp. ATPase	T03460	2.9	1.68	1.21	1.06
AtCesA3	N96510	2.9	1.33	1.26	1.02
Heat shock protein	H37687	2.43	4.18	2.81	1.93
extinsine-like protein	T42336	1.33	2.38	1.75	2.74
Ribulose bisphosphate carboxylase	H36115	1.4	1.56	5.4	1.23
Carbonic anhydrase, chloroplast	H37128	1.38	0	13	2.73
NADPH Photochlorophy deoxidase	T46041	1.09	1.68	2.72	6.3
Other unclassified					
No description	N97042	2.09	2.45	8.58	1.58
No description	N97219	2.24	3.74	2.75	2.21
AtG5	T46089	1.2	5	2.8	1.2
No description	T42977	0	0	8	2.58

The rest of the genes that we were unable to confirm by qRT-PCR are as in the following:

Acetyl-CoA carboxylase (*ACCase*); induced by *T. harzianum*, BTH and BABA (band # 55), exists as a multifunctional protein in some organisms but a multiprotein complex in others. In plants, ACCase activity controls carbon flow through the fatty acid pathway and therefore may serve as an important regulation point of plant metabolism (Podkowinskii,et al., 1996).

Pyrus communis partial gene for putative nucleotide binding site leucine-rich repeat disease resistance protein, clone RGA06; induced by *T. harzianum*, BTH and BABA (band # 14). A large number of resistance genes display a similar characteristic domain identified as a nucleotide binding site (NBS), often associated with a leucine rich repeat motif (LRR) (Sallaud, et al., 2003).

Blast resistance genes in rice (*R genes*); induced by *T. harzianum* (band # 30), have been studied extensively since the 1990s as a result of the increasing availability of DNA molecular markers. To date, at least 25 R genes have been identified to confer resistance of rice blast (Wu et al., 2005).

Transcription factor *myb*109; induced by *T. harzianum* (band # 15). The *MYB* class is responsive to osmotic stress and wounding. Dehydration, osmotic or salinity stress, and abscisic acid (ABA) application enhance *AtMYB102* transcript levels. Also, methyl jasmonate (MeJa) enhances xpression combination with ABA, whereas MeJa alone has a limited effect. It is proposed that *AtMYB102* is a regulatory component (Denekamp et al., 2003).

Putative pentatricopeptide (*PPR*) repeat-containing protein; induced by *T. harzianum* (band # 30). A large number of gene family encoding proteins with a pentatricopeptide repeat (PPR) motif exists in flowering plants but not in algae, fungi, or animals. Intracellular localization analysis demonstrated two PPR proteins in chloroplasts (cp), whereas the cellular localization of the other three PPR proteins is unclear. The genes of the cp-localized PPR513-10 and PPR566-6 were expressed differentially in protonemata grown under different light–dark conditions, suggesting they have distinctive functions in cp (Hattoria et al., 2004).

Methionine sulfoxide reductase (*PMSR*); induced by BTH and BABA (band # 31). It is an enzyme that repairs oxidatively damaged proteins. (PMSR) catalyzes the reduction of Met sulfoxides back to Methionine. Therefore, PMSR is

proposed to act as a last-chance antioxidant, repairing proteins damaged from oxidative stress. (Romero et al., 2004).

Glycine max protein; induced by *T. harzianum*, BTH and BABA (band # 13), in soybean has been reported to be involved in plant disease resistance. However, little is known about the pathway by which this specific gene induces the defense signal and then up-regulates pathogenesis-related (PR) genes (Park et al., 2004).

Ethylene overproducer (*Eto*); induced by *T. harzianum*, BTH and BABA (band # 16). It overproduces ethylene. Three Eto loci have been identified to play arole in post-transcriptional regulation of aminocyclopropane-1- carboxylic acid (ACC) synthase (ACS) that is responsible for ethylene biosynthesis (Woeste et al., 1999)

There were also other genes with unknown functions. These include Dendrobium grex Madame *Thong-IN* ovg15 mRNA, partial sequence, Nicotiana tabacum DNA, *TJ* sequence, and Lycopersicon hirsutum clone *PK*12_340 *RGA* marker sequence

CHAPTER IV

CONCLUSIONS

Wheat is universally regarded as the most important crop in the world as it represents the major diet component because of the wheat plant's agronomic adaptability, ease of grain storage and ease of converting grain into flour for making edible, interesting and satisfying foods (Orth and Shellenberger, 1988).

One of the major problems concerning the production of wheat crops is the difficulty of controlling plant diseases to maintain the high quality and yield. The range of organisms that attack plants is diverse and includes viruses, mycoplasma, bacteria, fungi, nematodes, protozoa, and parasites. Therefore, the elucidation of the genes involved in pathways and/or mechanisms of wheat disease resistance is a prerequisite for the successful utilization of wheat crop species in modern agriculture.

Resistant wheat crops have been produced successfully for many years by conventional breeding techniques. The advent of molecular biology has enabled the genes that confer disease resistance to be analyzed at a molecular level, an analysis that is generating novel insights into the complexity of plant defense. This knowledge has also enabled more sophisticated breeding strategies to be employed by using marker-assisted selection and, potentially, offers biotechnological solutions for the ancient and perennial problem of wheat crop protection.

One critical application of modern biotechnology is to reduce the incidence of disease in agricultural wheat crops, through the induction and enhancement of the plant's own defense mechanisms which would not involve the application of toxic compounds to plants such as pesticides. The induced resistance is associated with an enhanced capacity to cellular defense response, a process called 'priming'. Although, the phenomenon has been known for years, major progress in the understanding of priming was made only recently.

Of the many natural defense mechanisms plants have evolved to survive in nature, only a few can be triggered by biological or chemical agents without deleterious side effects and in a sufficiently controlled way for practical use. The best-known example is systemic acquired resistance (SAR), which in nature is activated by localized infections with necrogenic pathogens or non-pathogens including viruses, bacteria, or fungi in addition to treatments with some chemicals in a very low concentration. The activated state of SAR is characterized by broad-spectrum resistance against viruses, bacteria, and fungi, and by a set of biochemical responses; both the spectrum of protection and biochemical responses vary according to plant species.

Research on biological and chemical activation of disease and insect resistance has taught us that plants possess complex networks of inducible defense pathways that can interact with each other. Of these pathways, the SA dependent SAR pathway seems to be the most robust to be exploited for practical crop protection, as well as JA or ET dependent (induced sysyemic resistance) ISR pathway.

Unfortunately, much less is known about the signaling pathways involved in biological or chemical stimulation of disease resistance in monocots. However, the experience with the chemical plant activators available so far suggests that some basic inducible broad-spectrum defense responses are conserved across the plant kingdom.

The wheat genome is large and highly repetitive (80%). Because of this complexity isolation of genes by map based cloning and transposon tagging have not been achieved effectively. Therefore, to find the chemically or biologically induced genes that confers resistance against a wide range of pathogens and directly or indirectly can help us to understand the molecular mechanism of biological and chemical activation of induced disease resistance (immunity resistance) in wheat plants by other means strategies is important to be seeked.

Therefore, we have used an alternative approach; mRNA differential display to detect the genes involved in this resistance mechanism. The variety 'Avocet S', susceptible wheat plant has been utilized for differential display analysis for the detection of induced or repressed gene expression upon treatments of Avocet S plants with BTH, BABA as well as non-pathogenic fungus *T. harzianum*.

We have tried more than 70 combinations of primers for DD molecular analysis. Many differentially expressed fragments have been detected. As a

representative of these differentially expressed fragments, 57 of them have been cloned and sequenced. 35 of the sequences were read. The sequences of these fragments have been searched for homologies with the available sequences in the Genbanks. We used ClustalX-1.81 computer program in order to obtain sequence alignments within the fragments that represent a certain gene. We found that, there are homologous sequences the same fragments that represent one gene.

Therefore, the obtained 35 sequences bands were lowered to 15 bands that have homology to some distinct genes (sequencing results are in appendix B). The sequences of the last 15 differentially expressed fragments were also tested for confirming by real time PCR that verify the gene expression differences observed between the biologically or chemically treated and-or untreated plants as a result of defense induction. The confirmed genes have a strong relation to the enhanced disease resistance either directly or indirectly therefore they might be helpful in understanding the mechanism of SAR signaling defense and producing transgenic wheat.

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APPENDIX A

Cloning vector: pTZ57R-T Easy vector

APPENDIX B

DNA Sequences of the differentially expresses gene fragments

1-Zea mays ubiquitin conjugating enzyme (UBC) mRNA, complete cds

CATCCTCAAGGAACTGAAGGACCTGCAGAAGGACCCGCCCACATCATGC
AGCGCAGGTCCTGCTGGTGAGGACATGTTTCATTGGCAAGCAACAATTA
TGGGACCCCCTGACAGTCCCTATGCCGGCGGTGTTTTCTTAGTGAACATT
CATTTCCCTCCGGATTACCCCTTCAAGCCACCAAAGGTATCTTTTAAGAC
AAAGGTCTTCCATCCTAATATCAACAGCAATGGAAGCATATGCCTTGAT
ATTCTTAAGGAGCAGTGGAGCCCTGCTTTGACGATCTCCAAGGTCTTGCN
NNCTATCTGTTCCCTGCTGACCGATCCCAACCCGGATGATCCCCTTGTTC
CTGAGATTGCCCACATGTACAAGACG

2-Oryza sativa (japonica cultivar-group) cDNA clone:006-306-E01, full insert sequence (Silent gene)

TACCATTTCCATTTCGAGCATTACCTGTTGAAGCCATTGAATCAAGTGGC
AAGCAAGTGCTTGAGCAGTTACTCCGAGTCATGCTTCCACGATTTCTGAA
GCAGCTTGATAAAGACTACCGAGCTTGGGCCTCGGGCGATTCTTCACGG
AAACCGCTTGGCACTGGGGAAATATGAAGCTTCGCAGTGTCTCCATTAG
GCATTATAGTCTTTTCTTGTTGCATTAGTTAT

3-Pyrus communis partial gene for putative nucleotide binding site leucine-rich repeat disease resistance protein, clone RGA06.

GCNTCTGCGTCGAGGGCCCGGGANCGATTGAAACTGGTTACACCCCAAT
CGCGGTGAAAATAACTGAGTAACTTCGTGCTCCATGAACCCCCTGCAAT
AACGACTTTCTCACATGTGACAATACCTTCACTTGTAATAACTCCAGTCA
CTTTATTATTTTCAATACAGTACCAGCTACAAATCTAGATGCATTCGCGA
ANNTNAGTNGGNNGCACGNNTGNNNNCATCNNNTGGNNCTCA

4-Gossypium hirsutum mRNA for transcription factor myb109

GCNTGCTCCCGGCCGCCTGGCGGCCGCGGGAATTCGATTATTAACCCTCA
CTAAAGATCTGACTGCAACGGGCAATATGTCTCTGTGTGGATTAAAAAA
AAAGATATCACTCAGCATAATGACNGNNTTCNTGAGTGNNNNTACNNCG
NNTACNCTANANANNGTNTTTGNNTNNTGCGCTNTGCT

5-Lycopersicon esculentum ethylene overproducer-like 1 (EOL1) mRNA.EO1-

GNNTCNCAGNTCCCGGCCGCCTGGCNGCCGCGGGAATTCGATTATTAAC
CCTCACTAAANNTNTGACTGAAAAAAAAAGATATCACTCAGCATAATGA
TNCGNTACNCTANANACAGTNTTNGNNTCTGCGCTCTGNTNA

6-Oryza sativa blast-resistant mRNA, partial sequence blast resistant gene

CTCTGCGTCNANGGCCCGGGNTCCGATTCNNATGCTGAGTGATATCTTTT
TTTTTAGACAAAGTACCAAAAGTATTTATTNTCAAAATAAAAGATAAAG
ACTAGCCTATATGGTTACGCTAGCCCCTCCATCAATATTTAGTTGCTACC
AGCATTTAGTGAGGGTTAATAATCTAGATGCATTCGCGAANNTNAGTTN
GGGTGCNCG

7-Fragaria x ananassa gene encoding methionine sulfoxide reductase Methionine sulphoxide reductase

TGCGTCGAGGGCNNNNNANCGANTCTNNTGCTGAGTGATATCTTTTTNN
NACAATNAGTACNANNACAAGCGCTCATATACACGCACATACACTCATC
CATATGAACGCACATAGCACACCCTANTCCTACCTACCAGCATTTAGTGA
GGGTTAATAATCTAGATGCATTCGCGACGCCTAAACNGAACCTAAAGGA
GCCCCGATTTACAGCCTGACGGCAAAGCCCGCAACGNNCNNAAAGGAA
GGGAAGAAAGCGAAAGGACGGGCNCTGGNCGNTGCAACTGTAGCATCA
CNNTNCNNNAATCNACCCCCCCNCCCCNCTNANGCGCCGNTACNNNNCN
NAAGTGGACTTTTTGGGGAATGNNNNGNACCCCANTGATANTTNTTANN
NCTTACAAATGNNACCNTNNTAANNCACACCTATNAAAGCTNAANATAT
ANAAAGAAAAGATNATNTTAAAATTCNNNTNACCTTATTTCCTTTTG

8-Triticum aestivum inducible phenylalanine ammonia-lyase mRNA ,partial cds.phenylalanine

CGGGGAGGAGCTGAACAAGGTGCTGGTGGCCATGAACGAGCGCAAGCA
CATCGACCCGCTGCTCGAGTGCCCCAAGGAGTGGAACGGCGAGCCCCTG
CCGCTCTGCTGAGCAGTCGACGAGAGAAGGTAACACCAAAGGAAAAGA
AAACACGTACACACAAAGTGAATATAGAAGTGGTTCAAGACTGTGA

9-Triticum aestivum autophagy mRNA, complete cds Autophagy

AGACGTCGCAACCTGAACATCCAT CATTT ATT TAC AGT GGC AGC GCA
CAT GGA TTA GGC AGA GCC GAA AGT GTT CTC GCC ACT GTA AGT
CAT GTA CAG GAA GC

10-Dendrobium grex Madame Thong-IN ovg15 mRNA,partial sequence (Unknown protein)

ATCTNNTGCTGAGTGATATCTTTTTTTTTACGTCATTCTCCGGGGGCAGC
AGACAGACAGGCAACGCAACGCAACGCAGGGGACGCAAGAGAGAGTGC
TCCGATCCCATCCCCTGCCTGCCCGCGGTCTCCGCGAGCTCTTCTCCATA
CAGCATTTAGTGAGGGTTAATAATCTAGATGCATTCGCGA

11-Pisum sativum L. cultivar Frisson mRNA for arbuscular mycorrhiza protein Arbscular protein

AGACGAAGTCTCGCTCTGTCGCCCAGGCTGGGTGCAGAGGTACAATCTN
AGCTCACTGCAACCTCCACCTCCTGGATTCAAGAGATTCTCCTGCCTCAG
CCTCCCCAGTAGGTGGGATTACAGACATGCACCAC CAC ATC TGG CTA
ATT TTT GTA TTTTTAGTG GAG ACAGGG TTTCACCATGTT

12-Nicotiana tabacum DNA, TJ sequence

CTGCGTCGANGGCCCGGNANCGATTCTNNTGCTGAGTGATATCTTTTTTT
TTGGTGTACACGGACAGAGCTCGCCTTAATCTAGATGCATTCGCGAGGT
ACCGAGCTCGAATTNANTGGCCGNNGTTTNACAACGTCGTGACTGGGAA
AACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGC
CAGNTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAG
TTGCGCAGCCTGCNTGGCAAATGCN

13-Triticum aestivum cytosolic acetyl-CoA carboxylase psi-Acc-2,1 pseudogene, partial sequence AcetylCoA-Carboxylase

AAGGGGAAGCCANANNNGGGNATACGTANNCCCCATTTCGTTCTTCTTC
TTTTATTTATTTGCGTCATAGGACCTTCGTACTATTAAGAGGGATATAGG
TTCTCAAAGCTTAGATCCATTGTGATGCTTAGCCCCAAACCTATAAAAGA
TGCGACTGAAATCTCTTTATCCTCTGAATGATTACCTGGCAGCAGTAGAC
ATTGTTCTTTGTGCCACATGAGATATCTTCTGAACTGAGGAGTTAGTGCT
ACTTTCTCTGCAAGTAATGCCANNNTTNTGCTCCAATTTCCGACGAGTCA
TTGGCTACTCCTCGTGTCCAATTTGGTCATTTCCCTTCAGGGAATCTTTGG
CTATAAATAGCCACCCCGCTCCAACATTGTCCATTGGCTGCTCCGCTTGA
GATTTCTGAGAAGATTGATTTGAGCAACCCCTCTCCGATTGATTTGAGTT
TGAGATCCACCGTGAGAAAAATCAAGAGCCCAAACTTAGACAATAGTTT
AGCATCACAGGGGTTCTGAGTTAGAGTTCGTGTACCTGTTACTCTAGTGG
TTGCGAATCTAGATGCATTCGCGA

14-Lycopersicon hirsutum clone PK12_340 RGA marker sequence PK1-RGA MARKER

GCNNGNNTGNGGGCCCGGGTTCCGANCTNATGCTGNGTGATATCTTTTTT
TTTAATTTTGATTATCTTTGATCTTTGAATGTGAAATACACATGCATGAA
AATACATTATGATGATTCCAAATATTTTACACTGCTGTAACTACTATACA
CTATATATTGAGACAATGACGGGCAAGCCGGACTATATTTTTAAGTGCCC
TCAAATGGGGAATAGGAGCCGAAAAAGTATAACAAGAGACAAGAATCT
CCCCTCTGCCCCGAACACATGAAATGAACATAGCTATCACAACAAAACA
TAGGTTACACACAAATGGCACTATACTCCAGGTGGAAAATTGCTGACTT
CACCTTGTTCCAATGCAATCTAGATGCATTCGCGA

15-putative pentatricopeptide (PPR) repeat-containing protein PPR

CTCGCGAATGCATCTAGATTGGGGGGGTGGGGAAGACGACGACGACGAT
GATGACGATTACGAGGAAATGGATGATGAAGAGTTAGATTTTGTTGCTT
TCAAAGATAAGCGAGAACTAAATTTTGCGACTTGATATGTTGAAGCATT
GGTGTATATTTTCTTGCTGCAAAACTTATGATAGCTTTTCCTATGAAAAA
AAGGACTTTGTGGTATTCTCGAAATGTGTCAATACAGCTGGGCTGGATG
GTGTTTTTGCNGGNNNAAAAAAAGATATCACTCAGCATAAGAATCGNNC
CCGGGCCNTCGNCGc

APPENDIX C

Alignments of the confirmed differentially expresses gene fragments:

1-Zea mays ubiquitin conjugating enzyme (UBC) mRNA, complete cds

>gi|2668743|gb|AF034946.1|AF034946 Zea mays ubiquitin conjugating enzyme (UBC) mRNA, complete cds

```
Query  1    CATCCTCAAGGAACTGAAGGACCTGCAGAAGGACCCGCCCACATCATGCAGCGCAGGTCC  60
            ||||||||||| |||||||||||||||||||||||| ||||||||||||||| ||||||||
Sbjct  55   CATCCTCAAGGAGCTGAAGGACCTGCAGAAGGACCCCCCCACATCATGCAGTGCAGGTCC  114

Query  61   TGCTGGTGAGGACATGTTTCATTGGCAAGCAACAATTATGGGACCCCCTGACAGTCCCTA  120
            ||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||
Sbjct  115  TGCTGGTGAGGACATGTTTCATTGGCAAGCAACAATTATGGGACCACCTGACAGTCCCTA  174

Query  121  TGCCGGCGGTGTTTTCTTAGTGAACATTCATTTCCCTCCGGATTACCCCTTCAAGCCACC  180
            ||| || |||||||| |||||||||||||||||||| || ||||||||||||||| || ||
Sbjct  175  TGCTGGTGGTGTTTTTTTAGTGAACATTCATTTCCCGCCAGATTACCCCTTCAAACCTCC  234

Query  181  AAAGGTATCTTTTAAGACAAAGGTCTTCCATCCTAATATCAACAGCAATGGAAGCATATG  240
            |||||| ||||| |||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  235  AAAGGTTTCTTTCAAGACAAAGGTCTTCCATCCTAATATCAACAGCAATGGAAGTATATG  294

Query  241  CCTTGATATTCTTAAGGAGCAGTGGAGCCCTGCTTTGACGATCTCCAAGGTCTTGCNNNC  300
            |||||| ||||| || |||||||||||||||||||||||| || || |||||| |||   |
Sbjct  295  CCTTGACATTCTCAAAGAGCAGTGGAGCCCTGCTTTGACAATTTCTAAGGTCCTGCTCTC  354

Query  301  TATCTGTTCCCTGCTGACCGATCCCAACCCGGATGATCCCCTTGTTCCTGAGATTGCCCA  360
             ||||| |||||||||||||| ||||||||||| || || ||||| || ||||||||||||
Sbjct  355  CATCTGCTCCCTGCTGACCGACCCCAACCCGGACGACCCTCTTGTCCCGGAGATTGCCCA  414

Query  361  CATGTACAAGACG  373
            |||||||||||||
Sbjct  415  CATGTACAAGACG  427
```

2-Oryza sativa (japonica cultivar-group) cDNA clone:006-306-E01, full insert sequence (Silent gene)

>gi|32989429|dbj|AK104220.1| Oryza sativa (japonica cultivar-group) cDNA clone:006-306-E01,

```
Query  1    TACCATTTCCATTTCGAGCATTACCTGTTGAAGCCATTGAATCAAGTGGCAAGCAAGTGC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  581  TACCATTTCCATTTCGAGCATTACCTGTTGAAGCCATTGAATCAAGTGGCAAGCAAGTTC  640

Query  61   TTGAGCAGTTACTCCGAGTCATGCTTCCACGATTTCTGAAGCAGCTTGATAAAGACTACC  120
            |||||||  |||||||||||||||||||||||||| ||||||||||||| ||||||||||||
Sbjct  641  TTGAGCAACTACTCCGAGTCATGCTTCCACGATTTTTGAAGCAGCTTGTTAAAGACTACC  700

Query  121  GAGCTTGGGCCTCGGGCGATTCTTCACGGAAACCGCTTGGCACTGGGGAAATATGAAGCT  180
             ||| ||||| ||||| ||||||||| ||||||| ||||||||||| ||||||||||||||
Sbjct  701  AAGCATGGGCTTCGGGTGATTCTTCAAGGAAACCACTTGGCACTGGTGAAATATGAAGCT  760

Query  181  TCGCAGTGTCTCCATTAGGCATTATAGTCTTTTCTTGTTGCATTAGTTAT  230
            ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  761  TCGCAGTGTCTCCATTAGGCATTATAGTCTTTTCTTGTTGCATTAGTTAT  810
```

3-Triticum aestivum inducible phenylalanine ammonia-lyase mRNA ,partial cds.phenylalanine

>gi|9965439|gb|AY005474.1| Triticum aestivum inducible phenylalanine ammonia-lyase mRNA,
partial cds

```
Query  1     CGGGGAGGAGCTGAACAAGGTGCTGGTGGCCATGAACGAGCGCAAGCACATCGACCCGCT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527  CGGGGAGGAGCTGAACAAGGTGCTGGTGGCCATGAACGAGCGCAAGCACATCGACCCGCT  1586

Query  61    GCTCGAGTGCCCCAAGGAGTGGAACGGCGAGCCCCTGCCGCTCTGCTGAGCAGTCGACGA  120
             ||| ||||||| ||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  1587  GCTGGAGTGCCTCAAGGAGTGGAACGGCGAGCCCCTGCCGCTCTGCTGAGCAGCCGACGA  1646

Query  121   GAGAAGGTAACACCAAAGGAAAAGAAAACACGTACACACAAAGTGAATATAGAAGTGGTT  180
             || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1647  GACAAGGTAACACCAAAGGAAAAGAAAACACGTACACACAAAGTGAATATAGAAGTGGTT  1706

Query  181   CAAGACTGTGA  191
             |||||||||||
Sbjct  1707  CAAGACTGTGA  1717
```

4-Triticum aestivum autophagy mRNA, complete cds Autophagy

>gi|32401364|gb|AF542187.1| Triticum aestivum autophagy mRNA, complete cds Length=574

```
Query  1    AGACGTCGCAACCTGAACATCCAT-CATTTATTTACAGTGGCAGCGCACATGGATTAGGC  59
            |||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
Sbjct  481  AGACGTCGCAACCTGAACATCCATTCATTTATTTACAGTGGCAGCGCACATGGATTAGGC  422

Query  60   AGAGCCGAAAGTGTTCTCGCCACTGTAAGTCATGTACAGGAAGC  103
            |||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  AGAGCCGAAAGTGTTCTCGCCACTGTAAGTCATGTACAGGAAGC  378
```

5-Pisum sativum L. cultivar Frisson mRNA for arbuscular mycorrhiza protein Arbscular protein

>gi|3901090|emb|AJ006305.1|PSA6305 Pisum sativum L. cultivar Frisson mRNA for arbuscular mycorrhiza protein

```
Query  1    AGACGAAGTCTCGCTCTGTCGCCCAGGCTGG-GTGCAGAGGTACAATCTNAGCTCACTGC  59
            |||||||||||||||||||||||||||||||| |||||| |||   |||| ||||| ||||
Sbjct  237  AGACGAAGTCTCGCTCTGTCGCCCAGGCTGGAGTGCAGTGGTGTGATCTTAGCTCGCTGC  178

Query  60   AACCTCCACCTCCTGGATTCAAGAGATTCTCCTGCCTCAGCCTCCCCAGTAGGTGGGATT  119
            |||||||||||||  ||||| |||||||||||||||||||| |||  || || |||||||
Sbjct  177  AACCTCCACCTCCCAGATTCCAGAGATTCTCCTGCCTCAGCATCCTGAGGAGCTGGGATT  118

Query  120  ACAGACATGCACCACCACATCTGGCTAATTTTTGTATTTTTAGTGGAGACAGGGTTTCAC  179
            |||| || |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  117  ACAGGCACGCACCACCACATCTGGCTAATTTTTGTATTTTTAGTGGAGACAGGGTTTCAC  58

Query  180  CATGTT  185
            ||||||
Sbjct  57   CATGTT  52
```

APPENDIX D

Triticum monococc actin (*ACT-1*) gene

Sequence Size: 950 bp DNA
Accession: AF326781
Forward primer; 5'AGGGCTGTTTTCCCTAGCAT3'
Reverse primer; 5'AAACGAAGAATGGCATGAGG3'
Product size: 745

```
  1 ATGGCTGACGGTGAGGACATCCAGCCCCTTGTCTGTGACAATGGAACCGGAATGGTCAAG

 61 GTCAGGAACTCTCCCCTAAACTGTTACACATGTCATCTCCTGCATCTGTTAACACATGTC

121 ATTTTGTTGTGGTTTATTATCGTCTAGGCTGGTTTCGCTGGAGATGATGCACCAAGGGCT
                                                          >>>>>>

181 GTTTTCCCTAGCATAGTTGGTCGCCCACGGCACACTGGTGTCATGGTAGGGATGGGGCAG
    >>>>>>>>>>>>>>

241 AAGGATGCCTATGTTGGTGATGAGGCGCAGTCCAAGAGAGGTATCCTCACACTCAAGTAC

301 CCGATCGAGCACGGCATCGTGAGCAACTGGGATGACATGGAGAAAATCTGGCATCACACT

361 TTCTACAATGAGCTCCGTGTGGCACCCGAGGAGCACCCTGTGTTGCTCACTGAGGCTCCT

421 TTGAACCCAAAAGCCAACAGAGAGAAGATGACCCAGATTATGTTTGAGACTTTCAATGTT

481 CCTGCCATGTACGTCGCAATTCAGGCCGTGCTTTCCCTCTATGCAAGTGGTCGTACTACT

541 GGTATGGACTGTTTCCAACTTTTATAGTTCACTGATGCTTTGCTCAGTAACTTACCTTTA

601 TGATTCTGTTACATTATGCTGCACCCTTCTGATTAATTGAATTATTTTCAACCTTCAATT

661 GTTCAGCCATGATTATGCTTACTGTGGCTTGCATAATTTCAATATACTGCAGTATTGATT

721 GCTGCTTATTTCTTACTGCTGTATTGCATTAGGGAAAACAATTGCAACTAATATATCAAG

781 AAGTATAATGTAGAGGATTACTTTGTTGTGGCTGAAATTTTTCTCTGTTTGTAGGTATCG

841 TTCTCGACTCTGGTGATGGTGTCAGCCACACTGTGCCCATTTACGAAGGATATGCGCTTC
                                                                <

901 CTCATGCCATTCTTCGTTTGGATCTCGCTGGTCGGGATCTCACGGACTCC
    <<<<<<<<<<<<<<<<<<<
```

Triticum monococc actin (*ACT-1*) gene 580 bp mRNA

For RT-qPCR

Sequence Size: 580 bp mRNA
Accession: AF326781
Forward primer; 5'AATGGTCAAGGCTGGTTTCGC3'
Reverse primer; 5'CTGCGCCTCATCACCAACATA3'
Product size: ~180bp

```
  1 ATGGCTGACGGTGAGGACATCCAGCCCCTTGTCTGTGACAATGGAACCGGAATGGTCAAG
     >>>>>>>>>>>>>>
 61 GCTGGTTTCGCTGGAGATGATGCACCAAGGGCTGTTTTCCCTAGCATAGTTGGTCGCCCA

121 CGGCACACTGGTGTCATGGTAGGGATGGGGCAGAAGGATGCCTATGTTGGTGATGAGGCG

181 CAGTCCAAGAGAGGTATCCTCACACTCAAGTACCCGATCGAGCACGGCATCGTGAGCAAC

241 TGGGATGACATGGAGAAAATCTGGCATCACACTTTCTACAATGAGCTCCGTGTGGCACCC
                   <<<<<<<<<<<<<<<<<<<<<
301 GAGGAGCACCCTGTGTTGCTCACTGAGGCTCCTTTGAACCCAAAAGCCAACAGAGAGAAG

361 ATGACCCAGATTATGTTTGAGACTTTCAATGTTCCTGCCATGTACGTCGCAATTCAGGCC

421 GTGCTTTCCCTCTATGCAAGTGGTCGTACTACTGGTATCGTTCTCGACTCTGGTGATGGT

481 GTCAGCCACACTGTGCCCATTTACGAAGGATATGCGCTTCCTCATGCCATTCTTCGTTTG

541 GATCTCGCTGGTCGGGATCTCACGGACTCC
```

CURRICULUM VITAE

PERSONAL INFORMATION:

Surname, Name: Al-Asbahi, Adnan
Nationality: Yemeni (YE)
Date and Place of Birth: 6 December 1973, Yemen
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EDUCATION

Degree	**Institution**	**Year of graduation**
MS	AL al-Bayt University (Jordan)	2001
BS	Omar Al Mukhtar University (Libya)	1996
High School	AL-Kuwait Secondary School (Yemen)	1991

WORK EXPERIENCE

Year	**Place**	**Enrollment**
1997-1998	Hadhramout University of Science & Technology (Yemen)	Research assistant
2002-Present	METU Graduate School of Natural & Applied Sceinces	Graduate Student

FOREIGN LANGUAGES

Advanced English
Fluent Turkish

POSTER PRESENTATIONS

1. Günel, A. Al-Asbahi, A. Zeybek, A. Akkaya, M. "Purification and 2D-PAGE analysis of BTH induced wheat proteins" International conference on proteomics, 11-14 October, 2006, LUXEMBOURG

2. Aslıhan Günel, Adnan Al-Asbahi, Mahinur S. Akkaya "Purification and 2D-PAGE analysis of BTH induced wheat proteins" Uluslararası Katılımlı Proteomik: Protein yapı ve fonksiyonu çalıştayı. 20 - 23 EYLÜL 2006, Antalya, TURKEY.

3. Adnan AL-Asbahi., Mahinur S. Akkaya "Identification and cloning of genes induced and/or repressed upon treatments of wheat plants (AvocetS Yr10) with BTH,BABA, and *Trichoderma harzianum* Raifi KRL-AG2" XIV. Ulusal Biyoteknoloji Kongresi. 31 Ağustos-2 Eylül 2005. Eskişehir, TURKEY.

4. Mahinur S. Akkaya**,** Figen Yildrim, Osman Bozkurt, Mine Turktas, Yasemine Aktas, Mehmet Somel, Turgay Unver, Adnan Al-Asbahi "Strategies for the determination of genes involved in disease resistance and zinc uptake in plants" 13. National Biotechnology congress, 25-29 Augest, 2003, Canakkale, TURKEY.

PROGRAMMING SKILLS

Word, Excel, Power Point, Primer detective, Clustal, Popgen32 vs., Blast, Photoshop, Photo-express, Windows 98/XP, Internet usage, Network settings.