CHANGES IN GENE EXPRESSION BY WHEAT IN RESPONSE TO INFECTION BY *Puccinia triticina*, CAUSAL FUNGUS OF WHEAT LEAF RUST

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PREFACE

The traditional approach to molecular biology research involves studying one gene at a time. This is based on the reductionist view that to understand the whole biochemical or metabolic pathway, one must study its parts. Recently, the field of functional genomics provides new strategies for analyzing expression of hundreds or thousands of genes simultaneously. Global analysis of gene expression through the functional genomics approach is fundamentally affecting research on plant-pathogen interactions.

Considerable progress has been made in the elucidation of the molecular mechanisms of plant disease resistance. However, substantial information on plant defense pathways is derived from studies on model plant systems. Due to the uniqueness and complexity of specific plant-microbe interactions, studies must be conducted on taxonomically diverse plant species. Hence, this research was done to analyze the wheat multi-component defense response to leaf rust infection at the molecular level. I hope that the results of this study will stimulate more fundamental and applied investigations that will facilitate the production of wheat varieties with improved resistance.

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CHAPTER I

INTRODUCTION

During the course of their co-evolution, plants and their natural enemies have developed complex relationships. Successful pathogen invasion ensues when the microorganism has evolved the ability to circumvent plant defense barriers. Once these defenses are circumvented, the plant then faces selection pressure to resist pathogen colonization. After new defense mechanisms have developed in the host plant, the pathogen again responds with an alternative mechanism that restores virulence. These continuing interactions between hosts and their pathogens have resulted in the development of highly specific and complicated attack and defense strategies (Jackson and Taylor 1996).

Fungi and Their Leaf Penetration Mechanisms

Fungi are small, generally microscopic, eukaryotic, usually filamentous, branched, spore-bearing organisms that lack chlorophyll (Agrios 1997). Very few fungal species cause disease in plants. Some fungi are obligate parasites that depend on the host for completion of their life cycle. Others are non-obligate parasites that depend on the host for parts of their life cycle. Almost all plants are attacked by some kinds of fungi, and specific fungi can attack one or several kinds of plants. Success of fungi in causing disease in plants is based on their ability to locate appropriate host surfaces and to elaborate specialized infection structures (Tucker and Talbot 2001).

Plant leaf diseases caused by fungal pathogens are initiated when fungal spores land on the host leaf surfaces. Adhesion to the leaf surface is critical in the initiation of infection. Spores are often coated with complex compounds that promote adhesion. Fungal adhesives contain glycoproteins, lipids, and polysaccharides (Xiao et al. 1994; Nicholson 1996). Spore attachment is also accompanied by production of fungal cutinases and esterases, which are enzymes that hydrolyze plant cuticular compounds (Deising et al 1992). The physical stimuli and hydration induces spore germination that leads to the formation of germ tubes (Read et al. 1992). A germ tube is a specialized fungal hypha that grows along the leaf surface towards a site of cuticular penetration. Germ tube growth and differentiation into a penetration structure known as an appressorium result from responses to plant signals including surface hardness (Dean et al 1994), hydrophobicity (Jelitto et al 1994), surface topography (Allen et al. 1991), wax components like 1,16-hexadecanediol and ethylene emissions (Kolattukudy et al. 1995). Efficient appressorial formation requires production of small-secreted hydrophobic proteins called fungal hydrophobins (Wessels 1994) and several transmembrane proteins of unknown function (DeZwaan et al. 1999). The appressoria develop into specialized infective structures that penetrate through the cuticle using cell expansion under high turgor pressure. In many cases, melanin biosynthesis is necessary for turgor generation (Takano et al. 1997). Cuticular penetration by other fungi also involves production of specific hydrolytic enzymes like endopolygalacturonase (Dumas et al. 1999), pectate lyase (Guo et al 1996) and cellobiohydrolases (Muller 1997). Alternatively, some

fungal species like *Puccinia triticina* use natural openings such as stomata and wounds for entry. After penetration, the fungus colonizes the cell by forming haustoria (Knogge 1996). Haustoria produced by biotrophic fungi are not intracellular as they are surrounded by a continuation of the plant plasma membrane (Heath 1997). Haustoria appear to have two functions: the regulation of the host-parasite interaction and uptake of nutrients (Mendgen et al. 2000).

Plant Defense Mechanisms

Disease resistance involves a combination of structural and chemical barriers (Keen 1992). These barriers are components of two types of resistance mechanisms: the non-specific and race-specific (Scholthof 2001). Non-specific resistance is not mediated by dominant, single resistance genes and places severe restrictions on the host range of the pathogen (Heath 2000). On the other hand, race-specific resistance is mediated by dominant, single resistance genes. The most obvious manifestation of race-specific resistance is called the hypersensitive response (HR). HR is a localized defense reaction at the site of pathogen infection. HR-mediated resistance, as proposed by Flor (1971), is a consequence of the interaction of complementary genes between host and pathogen. This constituted the theoretical basis of the gene-for-gene model of host-pathogen interactions. The model postulates that the interaction between specific elicitor molecules encoded by the pathogen avirulence genes and receptor molecules encoded by the host resistance genes activates a cascade of host genes that leads to the HR (Staskawicz 1995).

Early events during HR in many plant-pathogen systems include changes in the level of active oxygen species, induction of ion fluxes across the cellular membrane and depolarization of the plant cell potential within hours after infection (Greenberg 1994). These cellular changes are perceived to activate diverse arrays of plant defense responses such as phytoalexin production, lytic enzyme generation, proteinase elicitation, pathogenesis-related protein accumulation, cross-linking of cell walls and localized host cell death (Dixon et al. 1994). It is not clear whether this localized cell death is a direct consequence of biochemical or physiological changes induced by the resistance-avirulence gene interaction (Baker et al. 1997). However, there is recent evidence of morphological markers of apoptosis, a programmed cell death in animals, associated with cell death in plant disease-related circumstances. The apoptotic-like morphologies include the digestion of DNA into fragments to produce a phenomenon known as DNA ladders in electrophoretic gels, and the appearance of apoptotic bodies in some plant disease-associated death (Mittler et al. 1995; Wang et al. 1996).

Another category of plant defense is called systemic acquired resistance (SAR), which acts nonspecifically throughout the plant. Expression of a number of defense related genes, in particular the pathogenesis-related genes, correlates with the establishment of SAR (Ward et al., 1991). Salicylic acid (SA) and its analogs such as 2,6-dichloroisonicotinic acid (INA) and benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) are found to induce SAR (Gaffney et al. 1993; Gorlach et al 1996; Metraux et al. 1991). However, a grafting experiment

showed that SA is not the systemic signal for SAR (Vernooij et al. 1994). Recently, jasmonates- and ethylene-involving signal transduction pathways have been identified as regulators of defense-related genes (Penninckx et al 1998; Thomma et al 1998; Clarke et al. 1998).

Plant Disease Resistance Genes

Expression of race-specific resistance is governed by plant resistance genes (R-genes). More than 30 R-genes have been isolated from different plant species (Hulbert et al. 2001). Several classes have been identified based on the molecular structures of the R-genes (Jones 2001; Hulbert et al. 2001). The largest R-gene group contains a nucleotide-binding site (NBS) and leucine-rich repeats (LRRs) domains. NBS is characterized by several sequence motifs found in animal ATP- or GTP- binding proteins whose functions are still unknown (Li et al. 1997). On the other hand, LRR structures are known to mediate protein-protein interaction and are major determinants of recognition specificity (Fluhr 2001). The NBS-LRR group is further subdivided into R-genes containing a domain with homology to the intercellular signaling domains of the Drosophila Toll and mammalian interleukin (IL)-1 receptors (TIR) or containing leucine zipper (LZ) structures (Dangl and Jones 2001). LZ structures are well known for their roles in homo- and hetero-dimerization of eukaryotic transcription factors as well as facilitating interactions between proteins with other functions (Hammond-Kosack and Jones 1996). Some R-genes consist of LRR with or without a serine/threonine kinase domain. The presence of a serine/threonine protein

kinase domain suggests the possible involvement in signal transduction mechanisms (Martin et al. 1993). Other R-genes are not receptors or signaling component of pathogen recognition like *Hm1* gene of maize, which has enzymatic properties.

R-genes have been isolated using map-based cloning or transposon tagging. The *Pto* gene of tomato was isolated using positional cloning (Martin et al. 1993). This gene confers resistance to races of *Pseudomonas syringae pv. tomato* that carry the avirulence gene *avrPto*. The amino acid sequence of *Pto* revealed relatedness to serine-threonine protein kinases, suggesting that it may play a role in signal transduction. Arabidopsis *RPS2*, which confers resistance to the bacterial pathogen *P. syringae pvs. tomato* and *maculicola* expressing the avirulence gene *avrRpt2*, was also isolated using map-based strategies (Mindrinos et al. 1994). *RPS2* encodes a novel 105 kDa protein containing a leucine zipper, a nucleotide-binding site and 14 leucine-rich repeats. Likewise, the *Arabidopsis thaliana RPM1* gene conferring resistance to *P. syringae* isolates expressing the *avrRpm1* gene was isolated based on RFLP markers and yeast artificial chromosomes. This gene contains features found in the predicted polypeptide sequences of the other R genes: a potential leucine zipper, two nucleotide binding site motifs and 14 leucine-rich repeats. The tomato *Cf9* gene, which confers resistance to the fungal pathogen *Cladisporium fulvum* expressing the avirulence gene *avr9*, was isolated by transposon tagging using the maize Ds element (Jones et al. 1994). The isolated *Cf9* gene encodes a putative membrane-anchored extracytoplasmic glycoprotein, which shows homology to

the receptor domain of several receptor-like protein kinases in *Arabidopsis*. The tobacco *N* gene which confers resistance to TMV (Whitmam et al. 1994) and the flax L6 gene which confers resistance to the fungal pathogen *Melamspora lini* (Lawrence et al. 1995) were identified by transposon tagging using the maize Ac transposable element. Sequence analysis of the *N* gene found that it encodes a protein of 131.4 kDa with an amino-terminal domain similar to the cytoplasmic domain of the *Drosophila* Toll protein and the interleukin-1 receptor in mammals, a nucleotide binding site and four leucine-rich repeats. Similarly, the L6 gene has a nucleotide binding site and a leucine-rich region. Another unique class of R-genes is the rice *Xa21* gene that was isolated based on positional cloning, but has no known interaction with a corresponding avirulence gene. The gene contains both a leucine-rich repeat motif and a serine-threonine kinase-like domain, suggesting a role in cell surface recognition of a pathogen ligand and subsequent activation of an intracellular defense response (Song et al. 1995).

Gene-for-gene resistance is initiated when the product of a plant resistance gene recognizes the product of a pathogen avirulence gene. Limited evidence of direct interactions between products of avirulence and resistance genes has led to the guard hypothesis (van der Biezen and Jones 1998). The guard hypothesis was put forward to explain the requirement of Pto-AvrPto interaction for another protein, the NB-LRR Prf, to activate plant defense (Salmeron et al. 1996). The Pto gene of tomato is an R-gene that confers resistance to *Pseudomonas syringae* strains carrying *avrPto*. According to the guard hypothesis, Pto is the target of AvrPto and their interaction suppresses the

defense pathway. Prf is thus an NB-LRR protein that "guards" Pto, detects and inhibits the suppression by AvrPto and then activates defense. The guard hypothesis is by no means proven and the elicitor/receptor model may still be true for some systems (Dangl and Jones 2001).

Other Genes Involved in Localized and Systemic Acquired Resistance

Several plant genes required for localized and systemic acquired resistance have been identified through genetic approaches. Mutants have been isolated with impaired salicylic acid (SA) biosynthesis and signaling. Zhou et al. (1998) identified an *Arabidopsis* mutant, *pad4*, that shows no accumulation of SA upon infection with an incompatible pathogen. The gene PAD4 encodes for lipase-like proteins that act upstream of SA accumulation. Similarly, *sid (SA-induction deficient)* mutants compromise SA biosynthesis in response to both virulent and avirulent forms of *Pseudomonas syringae* and *Peronospora parasitica* (Nawrath and Metraux 1999).

In contrast, Cao et al. (1994) isolated the *npr1* (nonexpresser of PR genes) mutant that lacks the expression of PR genes and systemic acquired resistance even upon exogenous application of SA. NPR1 encodes an ankyrin (see Appendix B) repeat protein and was found to interact with a basic leucine zipper transcription factor that binds to PR1 promoter elements (Cao et al. 1997; Zhang et al. 1999). NDR1 and EDS1 mutations suppress resistance mediated by R-genes of the LZ-NBS-LRR and TIR-NB-LRR types, respectively (Parker et al.

1996, Aarts et al. 1998). EDS1 encodes a lipase-like protein and functions upstream of SA-mediated plant defense responses (Falk et al. 1999).

Other mutations produced enhanced disease resistance. Bowling et al. (1994) identified the *cpr1* (constitutive expressor of PR genes) mutant that shows elevated expression of SA and PR genes. Because of the mutant's phenotype, the Cpr1 gene was proposed to act upstream of SA as a negative regulator of SAR. In addition, *dnd1* (defense no death 1) and *mpk4* (mitogen activated protein kinase 4) mutants also show elevated SA expression in the absence of necrotic lesions (Yu et al. 1998; Petersen et al. 2000). The focus of recent studies is on determining the relationships of the different genes involved in SA signaling, and double mutant analyses have begun to unravel the order in which they function (Glazebrook 2001).

"One Gene at a Time Analysis" of Gene Induction and Plant Defense Responses

Plant responses to pathogen attack are complex and involve the induction of various genes. However, traditional techniques of detecting differential gene induction in plant defense responses, such as northern blot analysis, are usually limited to small numbers of genes. Benito et al. (1996) identified three cDNA fragments, ddB-2, ddB-5 and ddB-47, which represented *Botrytis cinerea* genes that are up-regulated during its interaction with tomato. Northern blot analysis showed that the transcripts detected with the cDNA clones ddB-2 and ddB-5 accumulated at detectable levels only at late time points during the interaction. The cDNA clone ddB-47 detected two different sizes of transcripts displaying

distinct, transient expression patterns during the interaction. Sequence analysis and database searches revealed no significant homology to any known sequence.

Clive Lo et al. (1999) studied the accumulation of PR-10 and chalcone synthase (CHS) in *Cochliobolus heterostropus* and *Colletorichum sublineolum* infected sorghum. Coordinated expression of PR-10 and CHS genes was localized to the area of inoculation along with the accumulation of phytoalexins. Inoculation with *C. heterostropus* resulted in the rapid accumulation of PR-10 and CHS transcripts after appressoria became mature. Accumulation of these transcripts was delayed in plants inoculated with *C. sublineolum* until penetration of host tissue had been completed and infection vesicles had formed. Results suggest that different recognition events are involved in the expression of resistance to the two fungi, and *C. sublineolum* suppresses the non-specific induction of defense responses.

Uncinula necator, the causal agent of grapevine powdery mildew, was found to elevate the activity of the pathogenesis related proteins, chitinase and β-1, 3 glucanase in leaves and berries of a number of grapevine cultivars (Jacobs, 1999). PR genes encoding extracellular proteins were strongly induced in the infected leaves including an acidic class III chitinase, a basic class I glucanase and a thaumatin-like-protein. Van Der Vlutgt-Bergmans et al. (1997) reported that catalase gene *cat1* of tomato is induced from the time of fungal penetration onwards, suggesting that H_2O_2 is produced during the interaction.

High Throughput Gene Expression Analysis and Plant Defense Responses

High throughput gene expression analysis is a powerful tool that can be applied to elucidate the molecular mechanisms of plant defense responses. In recent years, several high throughput techniques of expression profiling have been developed like representational difference analysis (Hubank and Schatz 1994), subtractive suppressive hybridization (Diatchenko et al. 1996), differential display (Liang and Pardee 1992), serial analysis of gene expression (Velculescu et al. 1995), oligonucleotide arrays (Lockhart et al. 1996) and cDNA arrays (Schena et al. 1995). These techniques have the capability of analyzing hundreds or thousands of genes in a range of experimental conditions. The common use of these techniques is to determine differential expression of genes in two or more populations of mRNA transcripts (Hegde et al. 2000). Patterns of differential gene expression have several applications including identification of tissue-specific, organ-specific, developmental stage-specific, abiotic stress-induced, and biotic stress-induced transcripts (Baldwin et al. 1999). Expression profiling is also an attractive approach to assess and predict gene functions (Cummings and Relman 2000). This is based on the assumption that a gene is transcribed only when and where its function is needed, determining the locations and conditions under which a gene is expressed allows inferences about its function.

Representational difference analysis (RDA) is a form of subtractive hybridization originally developed by Litsitsyn et al. (1993) for use with genomic DNA and modified by Hubank and Schatz (1994) for cDNA capable of isolating

the differences between two complex DNA populations. In this assay, subtractive enrichments and hybridization kinetics act to purify cDNA fragments (the target) present in one population (tester) but not in another (driver). Successive rounds of subtraction and amplification lead to enrichment of the target cDNAs. This technique is popular in biomedical studies, but has been recently adapted to plant genes. Zhu et al. (1997) cloned a developmentally regulated gene PPF-1, the first *Pisum sativum* post floral specific gene, using RDA from cDNA library from short-day grown G2 pea tissue. In the same library, Li et al. (1998) isolated a cDNA named GDA-1 that is expressed in darkness and is very rapidly induced by gibberellic acid. GDA-1 shares similarity with the B2 protein that is expressed during embryogenesis of carrot cells. In the study of Thomas et al. (1997), gene fragments shown to be similar to leaf senescence gene in *Festulolium* have been isolated using a variation of the RDA technique.

Most of the recent studies on expression profiling are based on DNA array hybridization. In the DNA array techniques, gene sequences (probes) are deposited on a solid support, hybridized with labeled copies of nucleic acids from biological samples (targets), and intensities of hybridization signals are used to infer levels of gene expression (Harmer and Kay 2000). This system has high sensitivity and can reliably detect rare transcripts (Bouchez and Hoftei 1998). Different methods for labeling RNA are available and allow a quantitative measurement of transcript abundance. DNA array hybridization analysis stands out from other high throughput techniques because of its simplicity, comprehensiveness and data consistency (Cummings and Relman 2000).

DNA array technology greatly facilitates the elucidation of the molecular mechanisms of plant defense responses. Schenk et al. (2000) studied the expression pattern of 2,375 genes in *Arabidopsis* after inoculation with *Alternaria brassicicola* or treatment with salicylic acid (SA), methyl jasmonate (MJ) and ethylene using microarray. The array was enriched with pathogen-induced genes that were either induced or repressed after inoculation with *Alternaria*. Their results indicated the existence of a substantial network of regulatory interactions and coordination among different defense signaling pathways. The largest number of genes co-induced and co-repressed was found after treatments with SA and MJ, which were previously found to interact in an antagonistic fashion.

Similarly, Maleck et al. (2000) conducted a microarray analysis of 10,000 *Arabidopsis* ESTs representing 7000 genes under 14 different systemic acquired resistance (SAR)-inducing or repressing conditions. They observed that 4.3% of the genes studied were involved in SAR. Cluster analysis of the expression patterns derived groups with common regulation patterns. A common promoter element was identified in genes from the regulon that binds to members of a plant-specific transcription factor family. Their results demonstrated the application of expression profiling in defining the regulatory networks and gene discovery in plants.

SIGNIFICANCE AND OBJECTIVES OF THE STUDY

Wheat (*Triticum aestivum L.*), which is considered to be the world's most important food crop, provides over 20% of the calories and protein in human nutrition (Bushuk 1998). Although world wheat production is among the highest of cereal crops (FAO 1999), yields of cultivated varieties frequently suffer significant losses due to fungal diseases. One of the major fungal diseases of wheat is leaf rust caused by the biotrophic pathogen *Puccinia triticina.* The wheat leaf rust pathogen is adapted to a wide range of climates and disease can be found in diverse wheat growing areas throughout the world (Kolmer 1996). Yield reductions associated with wheat leaf rust were reported to be between 10% (mild epidemic year) to 21.9% (severe epidemic year) from 1976 to 1990 in the United States, 5% to 15% in susceptible cultivars in Canada, 40% in Mexico and 38.6% to 50.5% in Argentina (Moschini and Perez 1998).

The use of genetic resistance is potentially the most effective and environmentally sound approach for reducing yield losses to wheat leaf rust. To date, 46 leaf rust resistance genes (Lr) have been isolated and mapped to specific chromosomes of the wheat genome (Kolmer 1996). Many efforts have been undertaken to introgress Lr genes in wheat breeding materials. However, resistance conferred by a single gene is frequently overcome by the appearance of virulent races in the pathogen population within a short period of time. Among the wheat leaf rust resistance genes, Lr-19 has been very effective against all isolates of *Puccinia triticina* found in the United States. Lr-19 gene is derived from *Agropyron elongatum*, a distant relative of wheat (McIntosh et al. 1993).

Because of the high level of effective resistance, wheat defense mechanisms mediated by the Lr19 resistance gene was selected to analyze the wheat-*P. triticina* interaction. Unraveling the molecular basis of the wheat-*P. triticina* interaction will help to identify targets for genetic engineering of wheat plants and consequently provide a strong foundation for the rational design of disease control strategies.

The main goal of this research was to elucidate wheat defense mechanisms by determining changes in gene expression during leaf rust infection. Specific objectives were (1) to isolate using representational difference analysis genes differentially expressed between leaf rust infected susceptible and resistant wheat near-isogenic lines differing in the Lr-19 resistance gene and; (2) to analyze expression profiles of isolated RDA clones using filter-based macroarrays.

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CHAPTER II

Modulation of Wheat Gene Expression as a Defense Response to Leaf Rust Infection

ABSTRACT

Induction of plant disease resistance involves transcriptional modulation of many genes. Recent advances in the field of genomics provide tools for large-scale expression analysis of interacting genes governing host-defense mechanisms. In this study, the wheat transcriptional defense response to the leaf rust pathogen (*Puccinia triticina*) was analyzed using cDNA representational difference analysis (RDA) coupled with filter-based hybridization macroarray. Subtraction cDNA libraries were produced from infected tissues harvested at 24 and 72 hours after inoculation (hai) using near-isogenic lines differing in the Lr19 resistance gene. Of 163 RDA clones analyzed, 28 unique cDNA sequences were induced (≥2.0-expression ratio) in the resistant line. Sequence analysis of 17 differentially expressed RDA clones revealed similarities to genes with defense-related functions. Eleven cDNA fragments had no significant sequence similarity to any known gene. Increased expression of genes related to oxidative stress, lignification and pathogenesis-related (PR) protein accumulation were the predominant resistance reactions to rust infection. Glutaredoxin-, elongation factor 1α- and a putative zinc finger transcription factor- related genes were found highly expressed at 72 hai and showed expression patterns similar to PR genes. Genes related to non-specific lipid transfer protein, proteinase inhibitor,

polyubiquitin and UDP-glucosyltransferase were found upregulated in the resistant plant along with genes involved in lignification at 24 and 72 hai. Co-induction of RDA fragments with putative defense functions was observed at two time points after infection suggesting the production of many possible immediate defenses to prevent leaf rust pathogen establishment. The potential role of the differentially expressed gene fragments in wheat hypersensitive response is discussed.

Key words: Representational difference analysis (RDA); macroarray; host-pathogen interaction; wheat leaf rust resistance; *Puccinia triticina*; expression profiling; cDNA

INTRODUCTION

Plants react to an invading pathogen by modulating their gene expression to prevent colonization. Hence, a considerable part of the plant genome is devoted to encoding the machineries necessary for pathogen recognition and defense response. A resistant reaction begins with the specific recognition of pathogen encoded elicitor molecules by plant encoded receptor molecules (Keen 1990). This recognition event triggers plant defense responses leading to hypersensitive cell death (Hammond and Jones 1996). Although many studies have been conducted to elucidate the molecular basis of race-specific resistance, the whole pathway from recognition to defense reaction is still unclear. Even the contribution of cell death to microbial inhibition in many pathosystems has not been firmly established (Cole et al. 2001, Yu et al. 1998, Century 1995). In addition, different host plants and their pathogens have developed unique interactions through the course of evolution making it more difficult to harmonize our understanding of the genetic induction of disease resistance. This lack of sustained consistency serves as a strong impetus to characterize specific pathosystems.

Leaf rust is a major fungal disease of wheat that conforms to the gene for gene hypothesis of plant-pathogen interaction. Early studies of the infection process revealed that the leaf rust pathogen (*Puccinia triticina*) infects wheat leaves via stomata and colonizes the intercellular spaces (Romig and Caldwell 1964; Niks 1983a, 1987). With the use of scanning electron microscopy, Hu and Rijkenberg (1998) examined the formation of *P. triticina* infective structures and

observed similarities to other rust fungi within susceptible and resistant hosts. Fungal growth is normally inhibited in plants exhibiting race-specific resistance after penetration but not during germination and appressorial formation (Jacobs 1989). Two types of resistance, termed prehaustorial and posthaustorial, have been characterized based on the presence of aborted infective structures (Niks and Dekens 1991). Prehaustorial resistance, like that derived from *Triticum monococcum* (Jacobs et al 1996), occurs at the early stages of infection prior to haustorial mother cell formation. Posthaustorial resistance, such as that exhibited by near isogenic lines of Thatcher carrying Lr19 and Lr21, occur at later stages of infection after the formation of the haustorial mother cells (Hu and Rijkenberg 1998). The genetic background was perceived to affect the onset and expression of wheat leaf rust resistance in host and non-host plants (Hu and Rijkenberg 1998). Distinct properties associated with structural and biochemical defenses to leaf rust infection in a particular wheat genotype are mediated by interacting changes in gene expression. Characterization of genes whose expression is altered during leaf rust infection will aid in the understanding of molecular mechanisms of the wheat multi-component defense response.

Functional genomics is an attractive approach to study interacting genes in various plant-pathogen interactions. Representational difference analysis (RDA) coupled with cDNA array hybridization has been used in isolating and characterizing differentially expressed genes between two complex cDNA populations (Welford et al. 1998; Chang et al. 1997). The RDA subtractive hybridization technique uses subtractive and kinetic enrichment procedures to

isolate differentially expressed genes in a highly specific manner (Lisitsyn et al. 1993; Hubank and Schatz 1994). A high level of specificity is needed in isolating differentially expressed genes to minimize contamination by false positives. In this study, RDA subtractive hybridization and cDNA filter-based hybridization macroarray were used to identify components of the molecular mechanisms of wheat-leaf rust resistance. A subset of differentially expressed genes was isolated and clones were identified that suggest the possible existence of redox-regulation of wheat defense-related gene expression.

MATERIALS AND METHODS

Plant and Fungal Materials

The leaf rust susceptible wheat cultivar, Thatcher (Th), and its near isogenic derivative Thatcher-19 (Th-19) carrying the Lr19 resistance gene, were used in this analysis. Seeds were sown in 10 X 33 cm plastic pots in a potting soil mix (Metromix 350, Scotts, Marysville, Ohio) and grown for two weeks in an environmental control chamber with a cycle of 16 hr light at 20°C and 8 hr dark at 15°C. Water and 20-20-20 NPK fertilizer with micronutrients (Peters Professional, Spectrum Brands, St. Louis, MO) were applied to sustain normal growth.

A race of *P. triticina,* PBRL (Long and Kolmer 1989), avirulent on Th-19 (resistant) and virulent on Th (susceptible) was obtained from the USDA Cereal Disease Laboratory at the University of Minnesota (courtesy of Dr. David Long). This race was increased on a susceptible wheat cultivar (Danne), and spores were collected using a vacuum cyclone collector, placed in cryotubes and stored in liquid nitrogen until needed. Prior to inoculation, spores were thawed at room temperature for 5 min, heat shocked for 5 min at 45 °C, and then maintained at room temperature (20-25 °C) and high humidity (>90%) for 16 hours.

Plant Infection

Wheat seedlings of resistant (Th-19) and susceptible (Th) near-isogenic lines at the two-leaf growth stage were inoculated with rust spores using the plant-to-plant brushing method (Browder 1971). Susceptible wheat cultivar (Danne) was used as the brush plants and served as the source of inoculum. Brush plants were

inoculated with hydrated rust spores that had been stored in liquid nitrogen. After 11 days, rust pustules were evident on the leaves of the brush plants. Prior to inoculation, seedlings of resistant and susceptible near-isogenic lines were placed in a mist chamber for 1 hr to provide ample moisture to the leaf surfaces. The plants were removed from the mist chamber and inoculated by brushing the infected brush plants lightly over the tops of misted seedlings. After brushing, spores were allowed to settle on the leaves for another 15 min and the inoculated plants were placed back in the mist chamber for another 12-16 hr. Infected seedlings were transferred back to the growth chamber. For macroarray and Northern blot confirmation analyses, leaf samples were harvested at 24 and 72 hours after inoculation (hai) from infected susceptible and infected resistant plants. In the Northern blot time course analysis, leaf samples from infected resistant plants were harvested at 0, 6, 12, 24, and 72 hai. Harvested leaf tissues were ground to a fine powder in liquid nitrogen, placed in 50 ml centrifuge tubes and stored in liquid nitrogen.

Representational Difference Analysis (RDA)

The RDA subtractive hybridization technique was used to generate the 24 and 72 hai subtractive cDNA libraries using infected resistant and susceptible source tissues as described by Hubank and Schatz (1994) (Figure 1). The subtraction process was carried out by multiple rounds of hybridization of tester cDNA (infected resistant) to excess driver cDNA (infected susceptible) in increasing ratios of 1:100, 1:800 and 1:400,000 through three successive rounds.

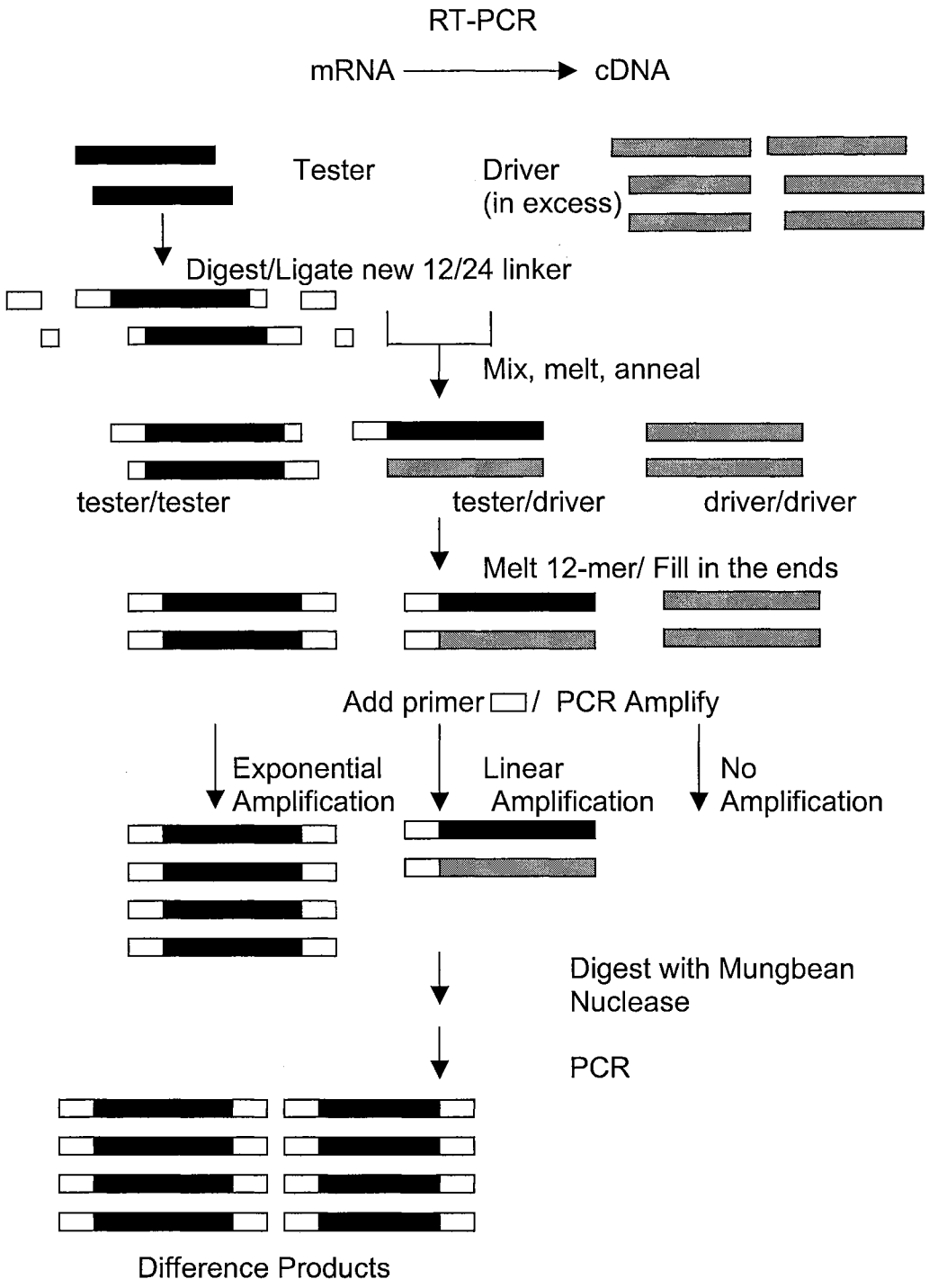


Figure 1. Schematic diagram of cDNA-representational difference analysis (RDA) subtractive hybridization technique based on the procedures described by Hubank and Schatz (1994).

The RDA was initiated by first isolating mRNA from leaf tissues undergoing resistant and susceptible reactions at 24 and 72 hai using Straight A's kit (Novagen, Madison, WI) following the manufacturer's instruction. Two μg of mRNA was converted to cDNA using Superscript RT (Life Technologies, Rockville, MD) and oligo-d (T) primers at 41°C for 1.5 hr. The second strand cDNA synthesis was performed by adding 10 U *E.coli* DNA polymerase (New England Bio-Labs Inc., Beverly, MA), 0.75 U RNase H (Gibco BRL, Grand Island, NY), 2 U *E.coli* DNA ligase (New England Bio-Labs Inc., Beverly, MA) to the first strand synthesis mix and incubating at 15°C for 2 hr and at 22°C for 1 hr. Double stranded cDNA (2 μg) was digested with *Dpn*II restriction endonuclease (New England Bio-Labs Inc., Beverly, MA) at 37°C for 2 hr. The products of digestion were extracted with phenol/chloroform, precipitated with isopropanol/NaOAc and resuspended in 20 μl TE (Tris-EDTA). The resulting digested cDNA (1.2 μg) was then ligated to the R-Bam-12/24 adapters (R-Bam-24 5'-AGCACTCTCCAGCCTCTCACCGAG-3', R-Bam-12 5'-GATCTGCCGTGA-3') in a mixture containing: 130 ng R-Bgl-24 oligo, 6 ng R-Bgl-12 oligo, 10X ligase buffer (New England BioLabs Inc., Beverly, MA) and 31 μl Milli-Q water (Millipore Corp, Bedford, MA). The mixture was incubated at 50°C for 5 min and then cooled to 10°C over a period of 1 hr in a thermocycler (MJ Research, Waltham, MA). Ligation was carried out overnight at 14°C using 1200 U of T4 DNA ligase (New England Bio-Labs Inc., Beverly, MA). cDNA representations were produced by PCR amplification of 200 μl reaction mixture containing 2 μl diluted ligation mixture, 5X PCR buffer (66 mM Tris-HCl, pH 8.8), 4 mM $MgCl_2$, 16 mM

$(NH_4)_2SO_4$, 33 μg/ml BSA), 0.3 mM dNTP, 10 ng R-Bgl-24 primer and 5 U Taq DNA polymerase (Gibco BRL, Grand Island, NY) at 95°C, 1 min and 72°C, 3 min for 20 cycles. The amplified cDNA representations were extracted with phenol/chloroform, precipitated with isopropanol/NaOAc and resuspended in TE at 0.5 μg/μl. R-adapters were removed in the tester and driver representations by endonuclease digestion with *Dpn*II for 4 hr at 37°C. Digested representations were again extracted with phenol/chloroform, precipitated with isopropanol/NaOAc and resuspended in TE at 0.5 μg/μl. Tester representations were gel purified on 1.2% TAE-agarose gel using QiAquick Gel Extraction kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Two μg of gel purified tester (not the driver) was ligated to the J-Bgl-12/24 adapters (J-Bgl-12 5'-GATGTGTTCATG-3', J-Bgl-24 5'-ACCGACGTCGACTATCCATGAACA-3') using T4 DNA ligase overnight at 14°C in the manner described above for adapter ligation. The product of ligation was diluted to 10 ng/μl with TE.

For the first subtractive hybridization, 0.4 μg adapter-ligated tester and 40 μg driver representations (1/100 tester/driver ratio) were combined, heat-denatured at 98°C for 6 min and incubated at 67°C for 20 hr with 1.25M NaCl to form the first subtractive hybridization mixture. The hybridized DNA was diluted with 8 μl TE (Tris-EDTA) containing 5 μg yeast RNA (New England Bio-Labs Inc., Beverly, MA), and thoroughly suspended in 400 μl TE. Subtraction resulted in three classes of double stranded cDNA: tester/tester (those that were exclusive to the tester cDNA population, and differentially expressed), tester/driver (those that were common between tester and driver populations, and not differentially

expressed) driver/driver (those found exclusively in the driver cDNA population, differentially expressed in the driver population). Four 200 μl PCR reactions were set up as described in the generation of representations with 20 μl of diluted hybridization mix and 10 ng J-Bgl-24 primer for 10 cycles of 95°C, 1 min and 70°C, 3 min. This round of PCR resulted in the exponential amplification of the tester/tester hybrids (adapter sequences present on both strands), the linear amplification of the tester/driver hybrids (adaptor sequences on one strand) and no amplification of the driver/driver hybrids (no adaptor sequences). The PCR products were combined, extracted with phenol/chloroform, precipitated with isopropanol/NaOAc and resuspended in 0.2X TE. Twenty microliters of purified products was treated with 20 U mungbean nuclease (New England Bio-Labs Inc., Beverly, MA) for 35 min at 30°C to digest single stranded cDNA. The reaction was stopped by the addition of 50 mM Tris-HCl (pH8.9). A final amplification was set up (as described in generation of representations) using 20 μl mungbean nuclease treated DNA and 10 ng J-Bgl-24 primer for 18 cycles (1 min, 95°C; 3 min, 70°C). Final amplification products were phenol/chloroform extracted, isopropanol/NaOAc precipitated and resuspended at 0.5 μg/μl to form the first difference product (DP1). Another round of enrichment for differentially expressed gene fragments was performed by repeating the procedure starting with the DP1 but using a tester/driver ratio of 1/800 to generate the second difference product (DP2). A third enrichment using a tester/driver ratio of 1/400,000 resulted in the generation of the third difference product (DP3). In order to generate the second (DP2) and third (DP3) difference products,

adapters had to be replaced by *Dpn*II nuclease digestion as indicated above. The J-adapters (J-Bgl-12 5'-GATGTGTTCATG-3', J-Bgl-24 5'-ACCGACGTCGAC TATCCATGAACA-3') from the DP1 were replaced by the N adapters (N-Bgl-12 5'-GATCTTCCCTCG-3' and N-Bgl-24 5'-AGGCAACTGTGCTATCCGAGGAA-3') to form the DP2, and the N adapters were then replaced by the J-adapters to form the DP3. Subtraction and amplification were done as described for DP1, but using a PCR extension temperature of 72°C in subsequent rounds.

Difference Product Library Production

Purified difference products (DP1 through DP3) from 24 and 72 hai tissues were directly cloned into pGem-T easy vector (Promega, Madison, WI) using 2X rapid ligation buffer and 3 U T4 DNA ligase (Gibco BRL, Grand Island, NY) for 16 hours at 4°C. Ligation products were transformed into DH5α competent cells (Gibco BRL, Grand Island, NY) using the manufacturer's protocol. Transformed bacterial cells were spread on LB (Luria-Bertani) ampicillin (100mg/l) (Sigma, St. Louis, MO) agar plates containing X-gal (40μg/ml) for blue-white selection and incubated overnight at 37°C. One hundred white colonies were picked from each library, placed in 6 ml LB medium with ampicillin and incubated overnight at 37°C on a shaker at 225 rpm.

Macroarray Production

Bacterial lysis was performed using glass bead-beating technique as follows. Ten μl of clonal cultures (products of transformation) was added to sterile

phosphate-buffered saline (8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4 and 0.249g KH_2PO_4) and centrifuged for 10 min at 4°C. Seventy μl of sterile Milli-Q water (Millipore Corp, Bedford, MA) and 50 mg sterile 0.1mm glass beads (Biospec Products Inc., Bartlesville, OK) were added in the tube with bacterial pellet. The mixture was agitated in a mini Bead-Beater (Biospec Products, Bartlesville, OK) for 1 min at 5000 rpm. The bacterial lysate was used directly for PCR amplification. Two hundred clones from the second difference product (DP2) of 24 and 72 hai libraries were PCR amplified using 200 μM T7 and SP6 primers, 200 μM dNTPs, 10X PCR buffer (Qiagen, Valencia, CA), 5X Q solution (Qiagen, Valencia, CA), and 2.5 U Taq DNA polymerase (Qiagen, Valencia, CA) for 40 cycles of 94°C, 1 min, 54°C, 1 min and 72°C, 1 min, and then ethanol precipitated. Inserts that showed consistent amplification were selected for hybridization macroarray analysis. A total of 163 amplified inserts, 91 from 24 hai library and 72 from 72 hai library, were blotted onto the Hybond^{TM} N^+ nylon membrane (Amersham Life Science, Arlington Height, IL) using 384-pin replicator (Nalge Nunc International, Rochester, NY). Wheat actin gene fragments (650 bp) representing 3 spots and pBluescript (Stratagene, Austin, TX) plasmid DNA representing 2 spots were used as positive and negative controls in the macroarray, respectively. The membranes were UV crosslinked, sterilized with 95% ethanol for 10 min and washed sequentially with 2X SSC, 0.1% SDS and 2X SSC for 10 min.

Macroarray Hybridization

Fluorescein-labeled probes were produced using tester (resistant) and driver (susceptible) cDNA representations (see RDA- generation of representations) as substrates. Two hundred ng of cDNA representations was random-prime labeled using the Gene Images Kit (Amersham Pharmacia Biotech, Piscataway, NJ) at 37°C for 2 hr. Fluorescein-labeled susceptible (driver) and resistant (tester) cDNA representations were used to probe the macroarray blots. Hybridization buffer was prepared from 20X SSC, 0.5X liquid block (Gene Images kit), 10% SDS and 0.5X dextran sulfate. Membranes were prehybridized using 35 ml hybridization buffer in acrylic trays for 1 hour at 60°C. Probes were added to prehybridization buffer and the mixture incubated overnight. Following hybridization, two sequential washes with 1x SSC, 0.1% SDS for 15 min and 0.5X SSC, 0.1% SDS for another 15 min were performed at 60°C with gentle agitation. After stringency washes, membranes were incubated with gentle agitation for 1 hr at room temperature in approximately 1 ml per cm^2 of a 1 to 10 dilution of liquid block in buffer A (100mM Tris-HCl and 300 mM NaCl pH 9.5). Membranes were then transferred to 25 ml of freshly prepared 0.5% (w/v) bovine serum albumin in buffer A containing the anti-fluorescein conjugate (Gene Images kit). Unbound conjugate was removed by washing three times for 10 min in 0.3% (v/v) Tween 20 in buffer A at room temperature with agitation. Excess wash buffer was removed from the membranes and the membranes were placed on top of a sheet of saran wrap. Three milliliters of CDP-Star (Gene Images Kit) was applied on to the membranes and allowed to react for 5 min.

Excess detection reagents were removed from the membranes and the membranes were transferred to plastic bags for autoradiographic film exposure. Membranes were exposed to autoradiographic film BioMaX MS-2 (Kodak, Rochester, NY) at room temperature. A preliminary experiment was performed to determine the response of the hybridization signals to increasing concentrations of the spotted clones (200 ng, 500 ng, 1 ug, 1.5 ug, and 2.0 ug) using mean optical density and adjusted volume (volume X mean optical density). A linear response was shown with increasing spot concentrations when using adjusted volume based on size and mean intensity of the spots. Adjusted volume was used in macroarray expression analysis. Hybridization signals were visualized at different exposure times (5 sec, 30 sec, 5 min, 10 min, 15 min, 2 hr, 12 hr and 24 hr) and selected exposure time that exhibited detectable differential induction and within the linear range of the film.

Expression Analysis

Hybridization signals from the autoradiograph were analyzed using GS-700 Imaging Densitometer (Bio-rad, Richmond, CA). Macroarray spots were predefined by manually circling each spot based on the sizes of the spots. Adjusted volume of each spot was quantified using densitometry and the Molecular Analyst (Bio-rad, Richmond, CA) software. Expression data between blots were normalized using hybridization signals of the negative and positive controls. The negative control adjusted volume data were used to subtract the background level from signal intensities of all the spots. After subtracting the

background, expression data between blots were adjusted based on the signal intensities of the actin positive control. Normalization was performed not only between treatments (resistant and susceptible; 24 and 72 hai) but also between replications of a particular treatment. The average adjusted volume of the two-replicate macroarray blots was used to calculate expression ratio (resistant/susceptible) between resistant and susceptible. A minimum cut-off ratio of 2.0 was used to identify differentially expressed RDA fragments. Expression ratios, correlation and scatter plot analysis were calculated using Microsoft Excel (Microsoft Inc., Redmond, WA). Correlation and scatter plot analyses were performed by comparing and plotting the normalized expression data of the two replications of macroarray experiments.

Cluster Analysis of Expression Data

Cluster analysis was performed using the NT-SYS software (Exeter Software, NY) based on the expression data from macroarrays probed with labeled representations from resistant and susceptible at 24 and 72 hai. Data standardization using the NT-SYS software prior to clustering was performed by subtracting the mean expression across all treatments from each data point and dividing the result by the standard deviation. Standardization is a common procedure to eliminate scale differences in the data to measure similarity based on the shape of expression patterns rather than the magnitude of expression changes. Euclidean distance coefficients were calculated using the NT-SYS software algorithm based on standardized expression data of the RDA clones in

the infected resistant and susceptible plants at both time points after infection. A dendrogram was constructed based on the Euclidean distance matrix using the unweighted pair group mean arithmetic (UPGMA) clustering algorithm of NT-SYS software.

Sequencing Analysis of RDA clones

Bacterial cultures of the identified differentially expressed clones at 24 and 72 hai libraries were grown overnight in 5 ml LB medium. Plasmids with RDA inserts were isolated from DH-5α bacterial cells using Qiagen Plasmid Isolation kit (Qiagen, Valencia, CA). Sequencing of RDA fragments was performed using a 373 Applied Biosystems DNA Sequencer (Applied Biosystems, Foster, CA) at the Recombinant DNA/Protein Core Facility of the Oklahoma State University. Database nucleic acid and amino acid sequence comparisons were conducted using BLASTX and BLASTN search algorithms (Altschul et al. 1997) from the National Center for Biotechnology Information (NCBI), Bethesda, MD.

Northern Blot Analysis

Total RNA was isolated from 1 g of infected leaf tissues following the protocol of the TotallyRNA kit (Ambion, Austin, TX). For Northern blot confirmation analysis, leaf samples were harvested from resistant and susceptible infected near-isogenic lines at 24 and 72 hai. Northern blot time course analysis was also conducted using leaf samples harvested from resistant infected plants at 0, 6, 12, 24 and 72 hai. Northern analysis of extracted RNA

was performed according to the protocol of the NorthernMax-Gly kit (Ambion, Austin, TX). Twenty μg of total RNA in glyoxal loading buffer was heated at 50°C for 30 min and run on a 1% (w/v) glyoxal-based agarose gel according to the kit directions (Ambion, Austin, TX) at 65 V for 3 hours. Equalized loading and RNA quality were verified under UV light. The electrophoresed RNA was transferred to a Hybond^{TM} N^+ nylon membrane (Amersham Life Science, Arlington Height, IL) by downward blotting for 2 hr using a turbo blotter (Schleicher and Schuell, Keene, NH). The blotted membranes were UV crosslinked at 1200 μJoules.

Probe preparation was performed using Strip-EZ DNA labeling kit (Ambion, Austin, TX). RDA clones related to blue copper binding protein, glutathione-S-transferase, caffeic-O-methyltransferase, polyubiquitin, chitinase II precursor, thaumatin-like protein, glutaredoxin and β-D-glucan exohydrolase were selected as probes in Northern blot confirmation analysis. RDA clone related to blue copper binding protein was used as a probe in Northern blot time course analysis. Twenty-five ng of RDA clones was denatured at 95°C for 5 min and labeled using 10X decamer solution, 5X buffer-dATP/-dCTP, 10X dCTP, 2 mCi/ml α-^{32}P dATP, 5 U Exonuclease-free Klenow and 25 μl nuclease-free water (Strip-EZ DNA) at 37°C for 30 min. Labeled probes were purified using QiAquick PCR Purification kit (Qiagen, Valencia, CA).

Hybridization of probes to Northern blots was performed according to the protocol of the NorthernMax-Gly kit (Ambion). Prehybridization was performed by adding 10 ml of ULTRAhyb hybridization buffer (Ambion, Austin, TX) per 100 cm^2 of membrane at 42°C. Labeled probes (10^6 cpm per ml) were denatured at 90°C

for 10 min and added to the prehybridization blots. Hybridization was performed overnight at 42°C with gentle agitation. After hybridization, membranes were washed with low stringency wash solution #1 (NorthernMax-Gly) for 10 min and high stringency wash solution #2 (NorthernMax-Gly) for 15 min at 42°C. Excess wash solution was removed and membranes were transferred to plastic bags for autoradiography. Hybridized membranes were autoradiographed using Biomax MS-2 films (Kodak, Rochester, NY) for 12-16 hr at -80 °C. Optimum exposure time was selected by visualizing the hybridization signals using different exposure times (1h, 3h, 6h, 1 day, 2 days, and 1 week).

After autoradiography, hybridized probes were removed from the membranes following the instructions of Strip-EZ DNA kit (Ambion). At room temperature, 1X probe degradation buffer (200X probe degradation buffer, 100X degradation dilution buffer and 10 ml nuclease-free water, Strip-EZ DNA) was added to the container with the membrane and incubated for 2 min. Membranes with probe degradation buffer were transferred to the oven-shaker at 68°C for 10 min. Probe degradation buffer was replaced with 1X blot reconstitution buffer (100X blot reconstitution buffer, 20% SDS and 10ml nuclease-free water, Strip-EZ DNA), added to the container with membranes and incubated in the oven-shaker at 68°C for 10 min. Final wash was performed using 0.1% SDS at 68°C for 10 min with gentle agitation.

For Northern blot analysis, autoradiographic film was scanned with the GS-700 Imaging Densitometer (Bio-rad, Richmond, CA). Densitometer data was imported into the Molecular Analyst software to determine signal strength within

the band. Adjusted volume (OD x mm x mm) was used as a measure of signal strength. Fold-induction (R/S - 1) was calculated based on the resistant and susceptible hybridization signals.

RESULTS

RDA Subtraction Products Analysis

To analyze wheat resistance-related responses to infection by *P. triticina*, gene fragments were isolated by cDNA-RDA subtractive hybridization between infected resistant and susceptible wheat near-isogenic lines. Selective amplification of differentially expressed cDNA fragments at 24 and 72 hai was shown by agarose gel electrophoresis of the subtraction products (Figure 2). Gene fragments common to the resistant and susceptible cDNA populations were subtracted out in multiple cycles of RDA subtraction and amplification. Decreasing complexity based on the presence of smeared products and the number of distinct bands of the RDA amplicons was found with increasing rounds of subtraction. Smearing of DNA fragments was observed in DP1 that may indicate the presence of non-specific amplicons. Selective amplification of resistance-related RDA fragments was revealed by the formation of distinct bands in DP2 and DP3. Very few bands were amplified in the DP3 due to the utilization of a very high stringency of subtraction (1/400,000 tester to driver ratio). DP2 subtraction products were chosen for further analysis because of the level of specificity and the number of bands amplified. Previous studies have shown that RDA products contained not only differentially expressed genes but also non-differentially expressed transcripts that evaded subtraction (Gress et al. 1997; Chang et al. 1998). The presence of non-differentially expressed fragments in RDA subtraction products suggests the need for further expression analysis to minimize inclusion of false positives.

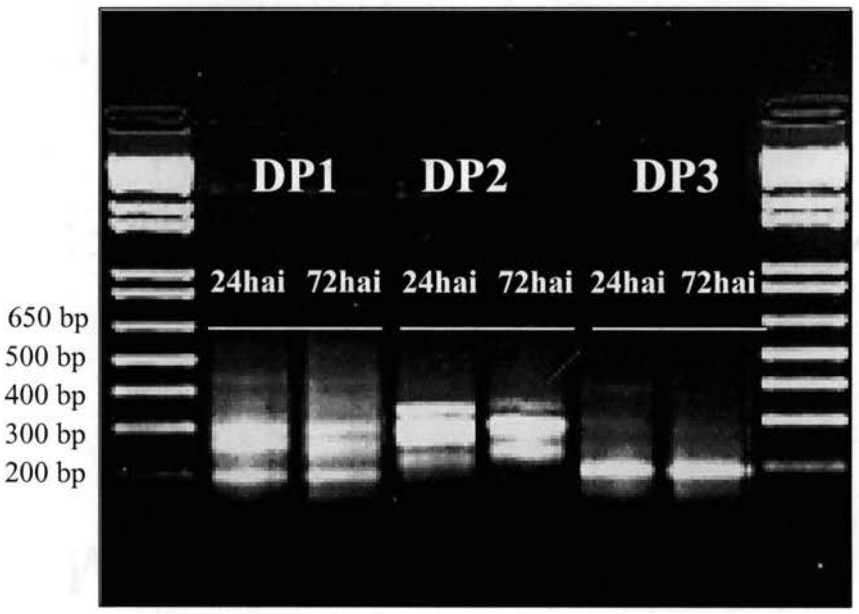


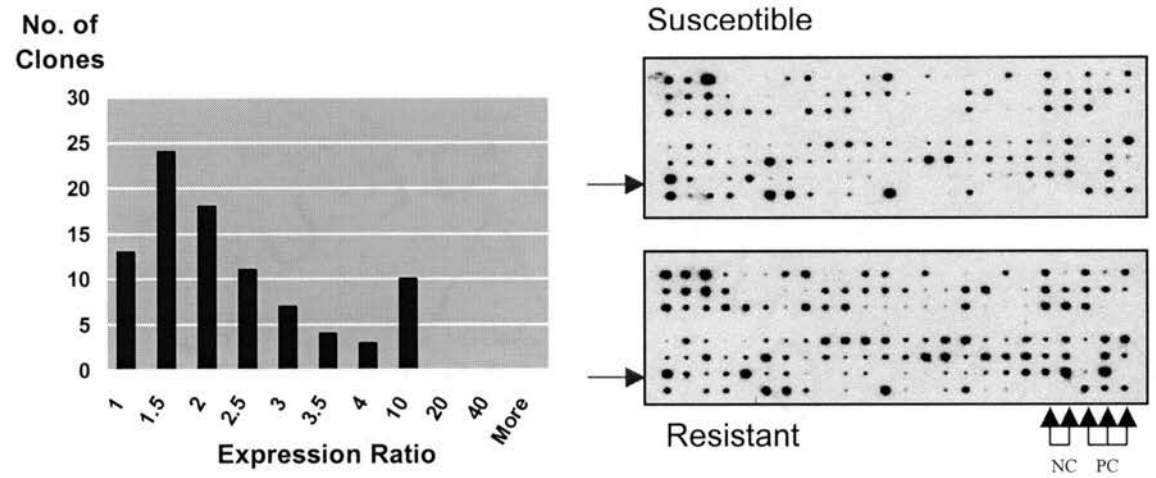
Figure 2. Agarose gel electrophoresis of representational difference analysis (RDA) subtraction products. Subtractive hybridization was done using infected resistant and susceptible leaf tissues at 24 and 72 hours after inoculation (hai). DP1, DP2 and DP3 were the difference products of first, second and third subtractions, respectively. The subtracted amplicons range from 0.1kb to 0.5kb. Equal amounts of difference products were loaded in 1.0% agarose gel.

Macroarray Expression and Sequence Analyses

To verify differential expression of RDA clones, macroarray hybridization analysis was performed with cDNA derived from two time points after inoculation (Figure 3). Two hundred clones from DP2 libraries from 24 and 72 hai samples were initially isolated and PCR amplified. Of the total clones amplified, 91 and 71 clones that showed good amplification were selected from 24 and 72 hai libraries, respectively. Selected clones were blotted onto the nylon membrane for macroarray hybridization analysis.

Fluorescein-labeled cDNA representation, a PCR amplified restriction endonuclease digested cDNA, was used to probe the macroarray blots. Similar expression intensities of positive control actin genes were seen between macroarrays probed with labeled cDNA representations from susceptible and resistant plants at 24 and 72 hai, suggesting an equal amplification of tester and driver cDNA representations. Non-specific hybridization was not evident in macroarray blots as plasmid DNA negative controls showed no detectable signal. Expression ratios of individual RDA clones ranged from 0.6 to 10.1 in the 24 hai library and 2.0 to 47.5 in the 72 hai library. High correlation was found in the expression data between the two replications of macroarray experiments at 24 and 72 hai with coefficients equal to $r=0.91$ and $r=0.90$, respectively (Figure 4). The overall gene expression of all isolated cDNA fragments (combined resistant and susceptible) at 72 hai was higher ($P<0.001$) compared to 24 hai. Based on the normalized signal intensities of all isolated RDA clones, the average gene expression for resistant infected tissue on the macroarray at 72 hai was

A.) 24 hai



B.) 72 hai

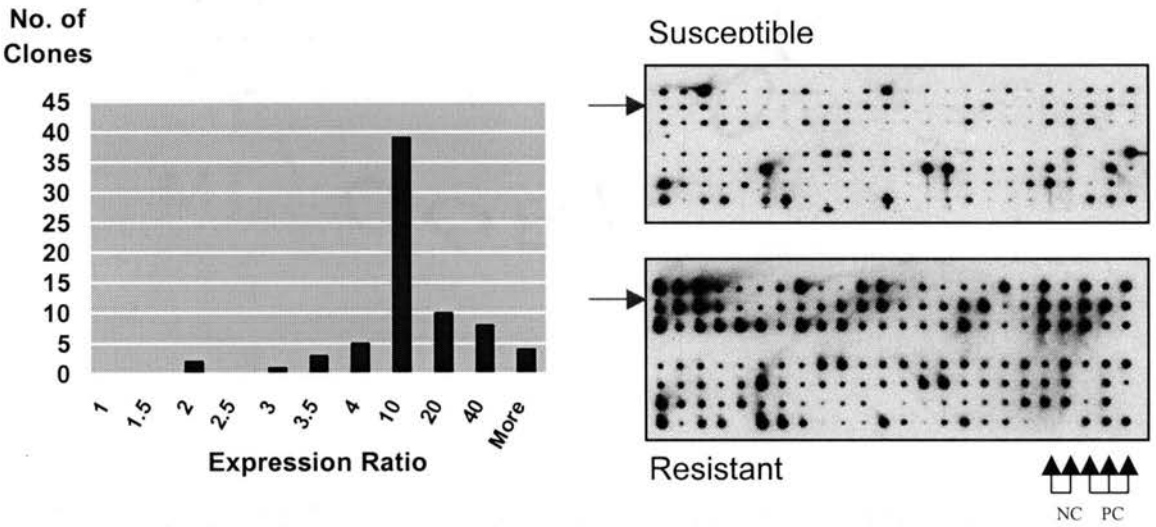
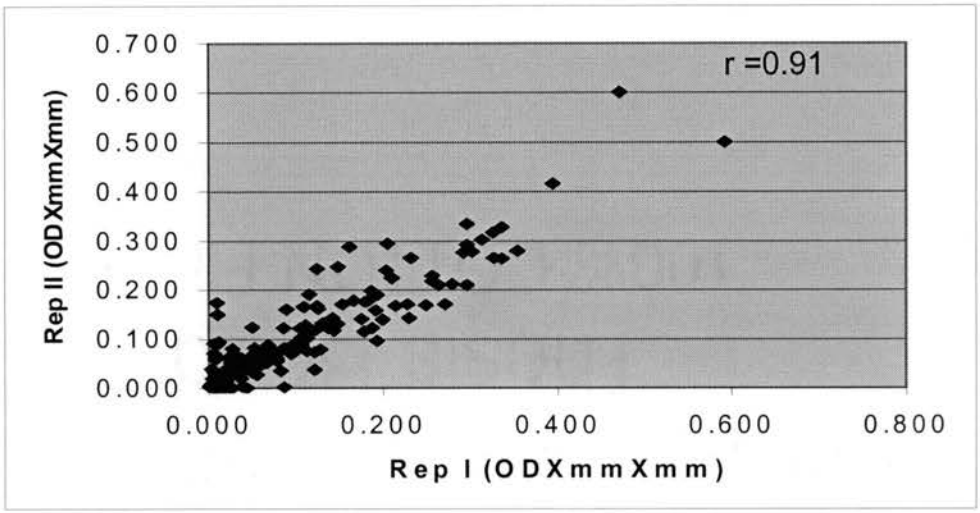


Figure 3. Histograms and macroarray blots of differential induction of RDA clones at (A) 24 and (B) 72 hours after inoculation (hai) in susceptible and resistant wheat near-isogenic lines. NC and PC represent the negative control plasmid DNA and positive control actin gene, respectively. RDA clones isolated from the library of that particular time point of infection are indicated by the arrows. Autoradiography of resistant and susceptible hybridized membranes was performed using the same film. Mean expression data (adjusted volume) were measured based on the spot intensities and normalized based on the controls. Expression ratio was estimated by dividing resistant over susceptible spot intensities.

A.) 24 hai



B.) 72 hai

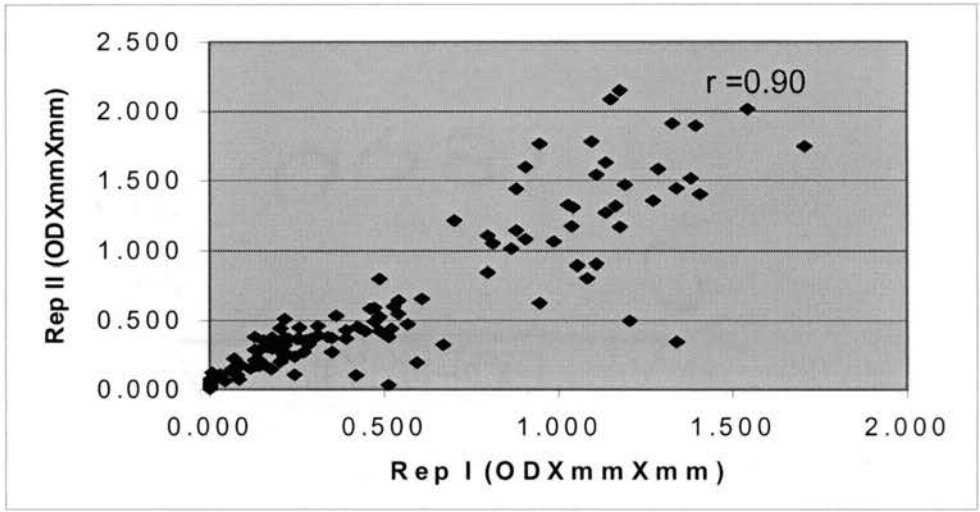


Figure 4. Scatter plots of adjusted volume data (ODXmmXmm) of the two independent macroarray experiments at (A) 24 and (B) 72 hours after inoculation (hai). Data were normalized based on the expression of positive control actin gene. Normalized expression data of replication I were plotted against replication II data at 24 and 72 hai. High correlation was found between the two replications of 24 and 72 hai macroarray experiments with correlation coefficients of r=0.91 and r= 0.90, respectively. Correlation was calculated by comparing the expression data of all RDA clones in two replications of macroarray experiments using Microsoft Excel.

significantly higher compared to susceptible infected tissues at 24 and 72 hai using a t-test ($P< 0.001$) (Figure 5).

Of the 163 RDA clones analyzed, 118 were found induced in the resistant line with differential expression ratios greater than or equal to 2.0 at 24 and 72 hai. Sequence analyses of the induced clones revealed many redundant clones with only 28 unique cDNA fragments (Table 1). The 28 nonredundant sequences represent 24% of all the differentially expressed clones sequenced. Apparently, the high degree of redundancy found in the RDA subtraction products may have resulted from the combination of high tester/driver subtraction ratio and PCR amplification during the enrichment process. Induced clones isolated from the 24 hai subtraction library showed sequence similarities to oxidative stress, anti-microbial, lignification and photosynthetic- related genes. Blue copper binding protein (BCBP), polyubiquitin and caffeic-O-methyltransferase (COM) cDNA fragments were found highly expressed in the resistant line at the early stage of pathogen infection with average expression ratios of 6.2, 4.0 and 2.4, respectively (Figure 6). In contrast, induced RDA fragments isolated from 72 hai subtraction library showed similarities to transcripts associated with regulation of gene expression and pathogenesis-related proteins. Chitinase II precursor, thaumatin-like protein (TLP) and putative Zn-finger transcription factor (ZnTF) related RDA clones showed average expression ratios of 5.1, 4.4 and 24.0, respectively (Figure 7). As shown by the error bars, variability was found in the hybridization signals of the redundant clones of highly expressed RDA fragments. The variability could be attributed to the effect of location in the array

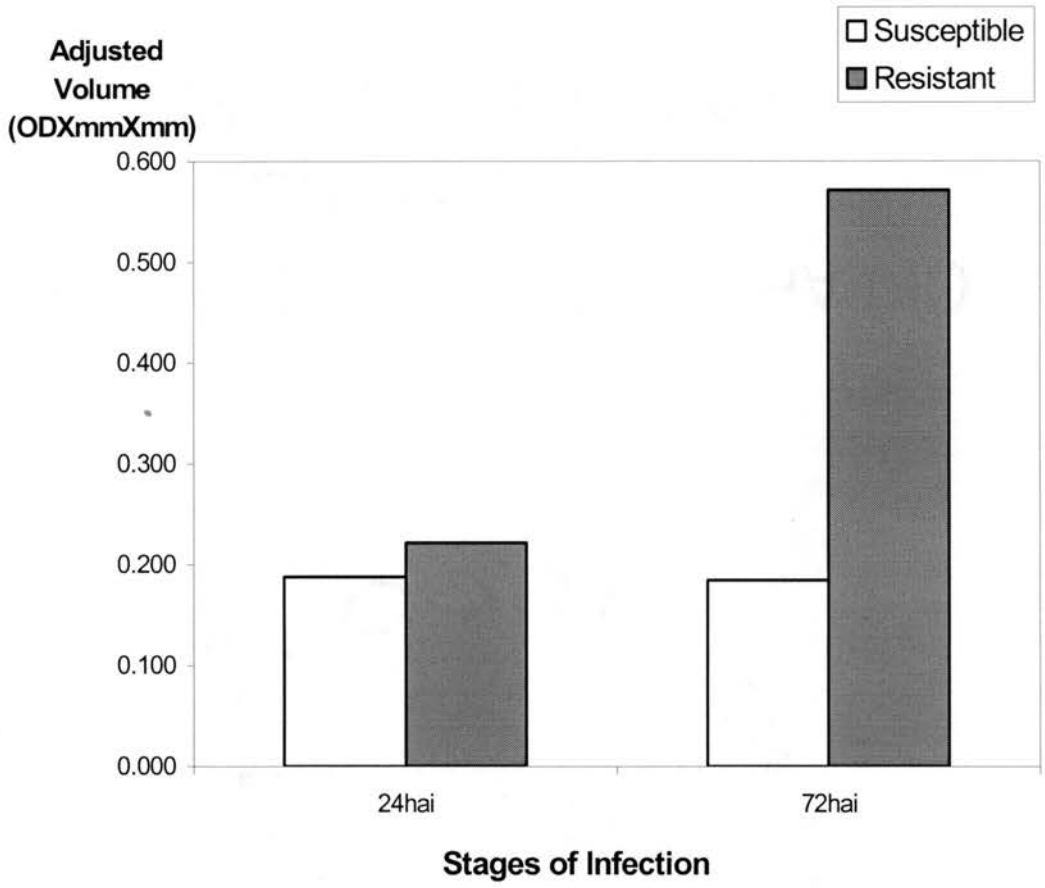


Figure 5. Overall expression of isolated RDA clones at 24 and 72 hours after inoculation (hai) in resistant and susceptible wheat near isogenic lines. Macroarray data were normalized based on the positive control. Mean expression data was calculated based on the normalized signal intensities (adjusted volume) of all clones. T-test was used in comparing mean expression data of resistant and susceptible at 24 and 72 hai. Significant t-test ($P<0.001$) was found in the comparison of the mean expression data (combined resistant and susceptible) at 24 hai and 72 hai. The average gene expression for resistant plant at 72 hai is significantly higher ($P< 0.001$) compared to the average expression data for susceptible plant at 24 and 72 hai.

Table 1. Unique expressed sequence tags (ESTs) isolated from representational difference analysis (RDA) subtraction experiments.

RDA Clone	Clone Length (bp)	Matching Sequences from Database	Organism	E-value*
DP2-24hai				
DP2-24-43	278	Blue copper-binding protein	Wheat	1×10^{-39}
DP2-24-33	174	Peroxidase	Soybean	5×10^{-06}
DP2-24-37	213	Gluthathione-S-transferase	Rice	1×10^{-10}
DP2-24-56	245	Bowman-Birk protease inhibitor	Wheat	4×10^{-44}
DP2-24-64	201	Uclacyanin	Arabidopsis	0.028
DP2-24-10	178	Caffeic-O-methyltransferase	Barley	5×10^{-27}
DP2-24-26	204	Putative UDP-Glucosyltransferase	Rice	2×10^{-09}
DP2-24-44	148	Rubisco	Wheat	1×10^{-19}
DP2-24-45	175	Non-specific Lipid Transfer Protein	Barley	1×10^{-11}
DP2-24-29	201	Polyubiquitin	*Pinus sylvestris*	8×10^{-21}
DP2-24-6	290	Unknown		
DP2-24-5	124	Unknown		
DP2-24-16	167	Unknown		
DP2-24-65	124	Unknown		
DP2-24-12	230	Unknown		
DP2-24-63	161	Unknown		
DP2-72hai				
DP2-72-1	239	Chitinase II precursor	Barley	1×10^{-30}
DP2-72-5	178	β-D glucan Exonuclease β-glucosidase	Barley	5×10^{-27}
DP2-72-7	178	Peroxidase	Soybean	2×10^{-07}
DP2-72-2	178	Thaumatin-like Protein	Rice	9×10^{-34}
DP2-72-6	140	Glutaredoxin	*Arabidopsis*	2×10^{-05}
DP2-72-32	198	Putative Zn-finger TF	Rice	7×10^{-32}
DP2-72-25	210	Elongation Factor 1α	Cassava	1×10^{-04}
DP2-72-30	171	Unknown		
DP2-72-43	324	Unknown		
DP2-72-23	230	Unknown		
DP2-72-29	290	Unknown		
DP2-72-15	183	Unknown		

* Expect value- The top most hit of the BLAST search was selected.

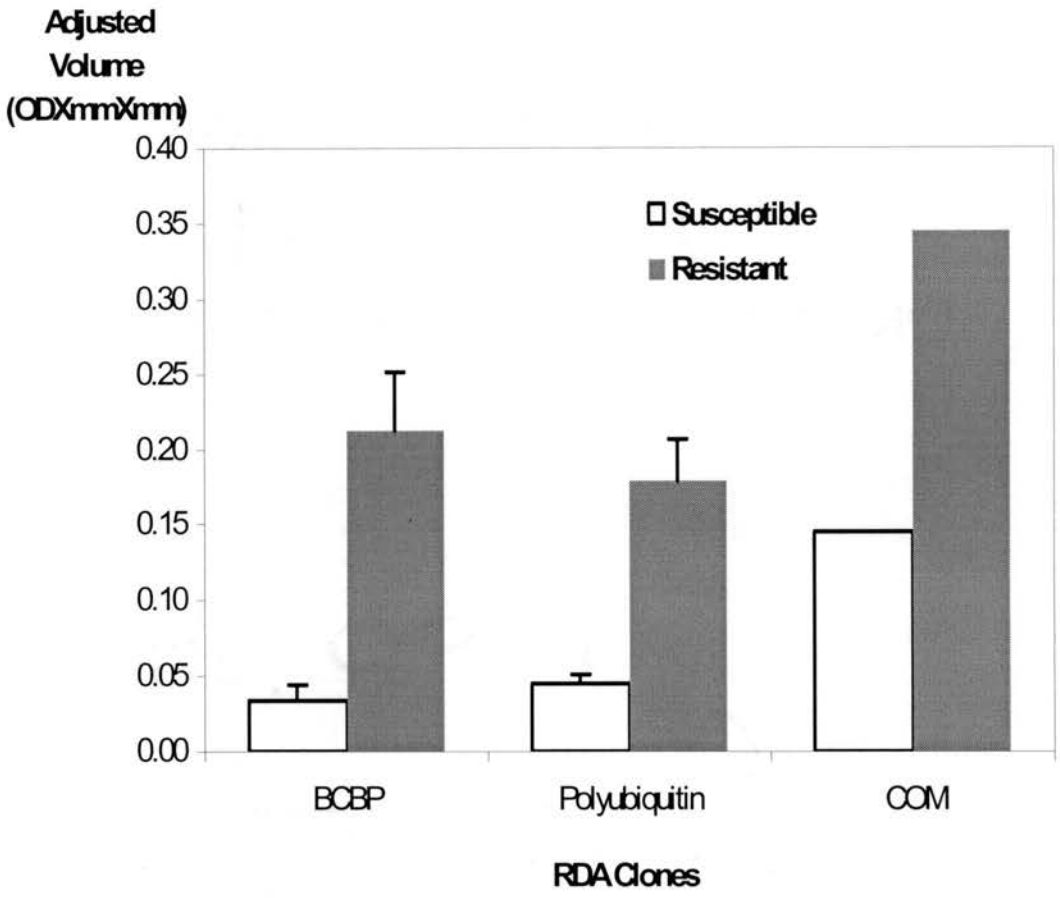


Figure 6. Differential expression of highly expressed RDA clones isolated from 24 hours after inoculation (hai) library. Expression data of BCBP and polyubiquitin were taken from 6 and 3 redundant clones within the array, respectively, while expression data of COM were based on single clone. Abbreviated highly expressed clones were as follows: BCBP- Blue copper binding protein and COM- Caffeic-O -methyltransferase.

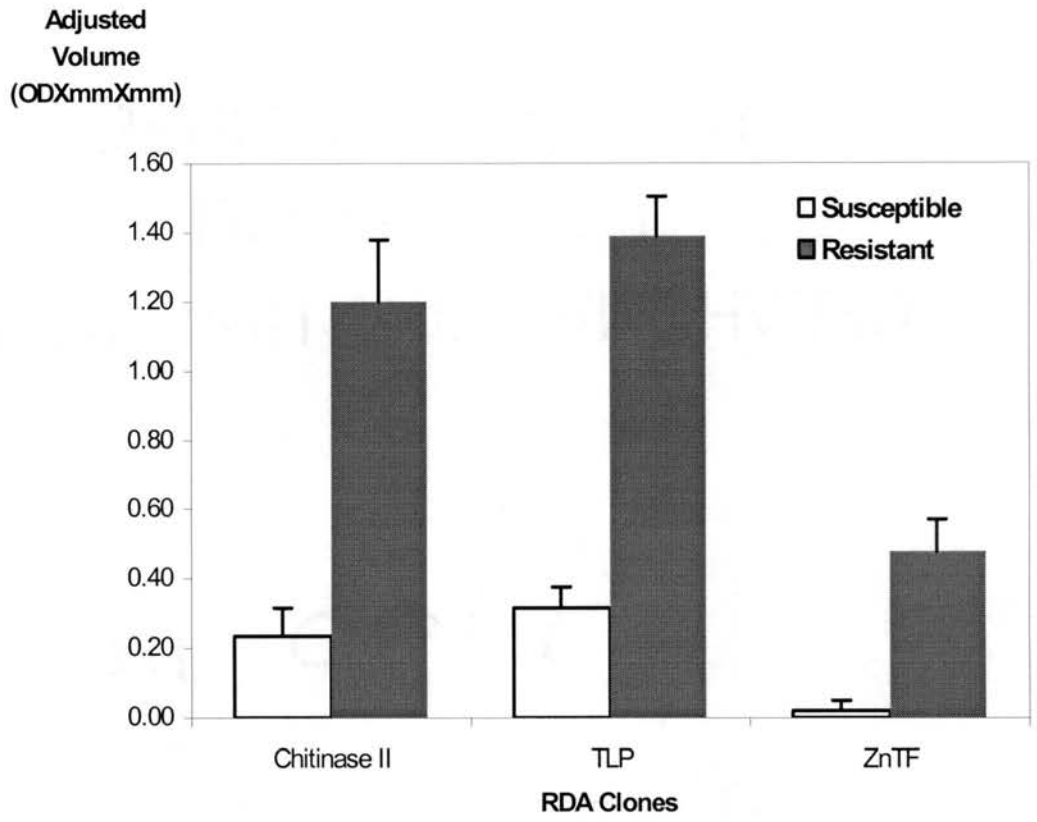


Figure 7. Expression of highly induced clones at 72 hours after inoculation (hai) in the resistant and susceptible isogenic lines. Mean adjusted volumes of Chitinase II, TLP and ZnTF were calculated based on the expression data of 8, 11 and 12 redundant clones within the array, respectively. RDA clones were as follows: Chitinase II- Chitinase II precursor, TLP- Thaumatin-like protein and ZnTF- a putative zinc finger transcription factor.

and the differential length of RDA redundant clones. Eleven clones showed no significant similarities to sequences in the database, which constituted 39% of the total induced unique cDNA-RDA fragments.

Venn Diagram of Gene Induction

A Venn diagram of putative genes shows overlapping and non-overlapping induction based on the expression ratios at the two time points after pathogen inoculation (Figure 8). Putative genes with non-overlapping induction showed differential expression only at one time point of pathogen infection. Uclacyanin, glutathione-S-transferase (GST), Rubisco and 6 unknowns were found differentially expressed only at 24 hai. Elongation factor-1α, Zn-finger transcription factor, β-D-glucan exohydrolase, chitinase II precursor, thaumatin-like protein, glutaredoxin and 5 unknowns were highly induced only at 72 hai. Overlapping induction was exhibited by putative genes found differentially expressed at two time points after pathogen inoculation. Clones representing non-specific lipid transfer protein, caffeic-O-methyltransferase, UDP-glucosyltransferase, polyubiquitin, BCBP and proteinase inhibitor were isolated from 24 hai library but were also induced at 72 hai. Most RDA gene fragments isolated from the 72 hai library were not differentially induced at 24 hai, the exception being the peroxidase gene fragment which was isolated at both 24 and 72 hai.

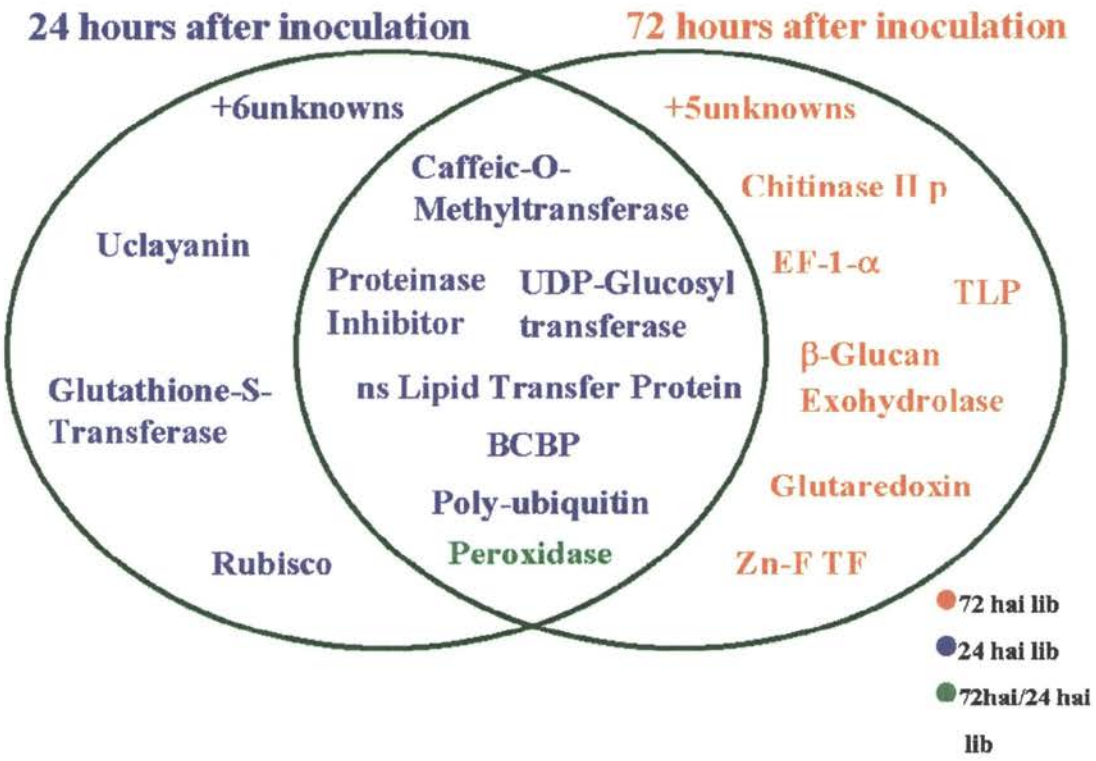


Figure 8. Venn diagram of overlapping and non-overlapping induction of isolated cDNA-RDA clones. Induction was calculated based on normalized macroarray data at 24 and 72 hours after inoculation (hai). Differential induction of clones isolated from 24 hai library was also measured at 72 hai and vice versa. Induction was based on cut-off expression ratio of 2.0. Abbreviated RDA clones that showed overlapping induction was BCBP- blue copper binding protein and abbreviated RDA clones that showed non-overlapping induction were TLP- thaumatin-like protein, EF-1α- elongation factor 1α, chitinase II precursor and Zn-TF- putative zinc finger transcription factor.

Cluster Analysis of Susceptible and Resistant Macroarray Data

Figure 9 represents a cluster analysis of 28 clones based on standardized macroarray expression data at 24 and 72 hai in susceptible and resistant wheat near-isogenic lines. A representative RDA clone from each redundant gene set was selected for cluster analysis. Cluster analysis groups the RDA gene fragments based on similarities of expression patterns. The groupings may indicate coregulation of genes based on the assumption that shared expression implies shared pattern of regulation (Cummings and Relman 2000, Bassett et al. 1999, Eisen et al. 1998). Euclidean distance estimates were used in the UPGMA cluster algorithm to present expression pattern similarities of the RDA gene fragments as dendrogram. The dendrogram revealed four main clusters at distance coefficients of 1.5 to 1.7. Cluster A contains gene fragments with putative defense functions and 5 with unknown functions. Glutaredoxin, elongation factor 1-alpha and Zn-finger transcription factor-related RDA clones clustered together with cDNA fragments related to PR genes. Non-specific lipid transfer protein, proteinase inhibitor, β-D-glucan exohydrolase and caffeic-O-methyltransferase formed a separate subcluster within cluster A. An RDA clone related to peroxidase clustered with defense-related gene fragments and separated from oxidative stress-related gene fragments. Two peroxidase-related RDA clones isolated at different time points after infection showed similar expression pattern. Cluster B contains a cDNA fragment related to Rubisco, indicating a unique expression pattern different from gene fragments with putative defense functions. RDA clones with putative roles in oxidative stress

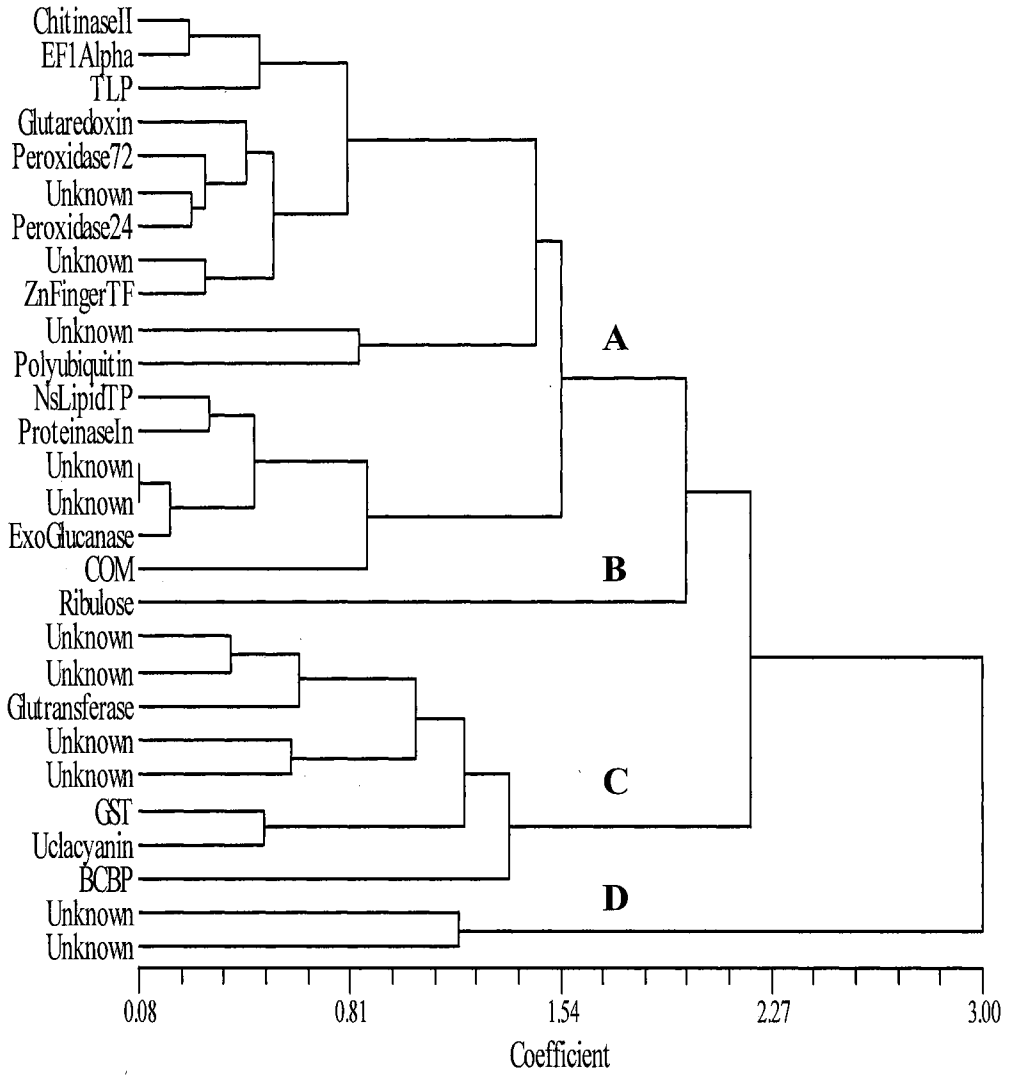


Figure 9. Cluster analysis of macroarray expression data of the induced RDA clones in susceptible and resistant cultivars at 24 and 72 hai. Euclidean distance was estimated based on standardized expression data and used in unweighted pair group mean arithmetic (UPGMA) cluster algorithm to produce dendrogram. Dendrogram was divided into four major clusters; (A) resistance-related cluster, (B) photosynthetic-related cluster, (C) oxidative stress-related cluster and (D) unknown function cluster. Abbreviated RDA clones were as follows: Chitinase II- chitinase II precursor, EF1Alpha- elongation factor 1α, TLP- thaumatin-like protein, ZnFingerTF- zinc-finger transcription factor, NsLipidTP- non-specific lipid transfer protein, ProteinaseIN- proteinase inhibitor, Exoglucanase- β-D-glucan exohydrolase, COM-caffeic-O-methyltransferase, Ribulose- Ribulose bisphosphate carboxylase/oxygenase, Glutransferase-UDP-glucosyltransferase, GST-glutathione-S-transferase and BCBP- blue copper binding protein.

grouped together in cluster C along with 4 genes of unknown function. BCBP showed a different expression pattern among gene fragments within cluster C. Two RDA clones with unknown functions formed cluster D.

Northern Analysis of RDA Clones

To confirm the macroarray data, eight cDNA-RDA gene fragments were used as probes in the Northern blot analysis. Figure 10 shows the autoradiograms of several Northern blots probed with the indicated ^{32}P labeled RDA clonal inserts. The total ethidium bromide stained RNA indicates near equal RNA loading of the gel. The northern blot analysis confirmed the results of macroarray analysis in the selected RDA clones. BCBP, UDP-glucosyltransferase and caffeic-O-methyltransferase showed 2.0-fold induction in the resistant compared to the susceptible at 24 hai. Polyubiquitin showed the highest induction at 24 hai of about 3-fold increase in expression in the resistant relative to the susceptible lines. Chitinase II precursor and thaumatin-like protein were upregulated in the resistant infected plants compared to the susceptible infected plants at 72 hai with 3.0-fold induction. β-D-glucan exohydrolase and glutaredoxin cDNA-RDA fragments were also found differentially expressed at 72 hai with 2-fold induction in the resistant compared to susceptible infected plants.

To check the temporal pattern of defense-related gene expression, blue copper binding protein RDA fragment was selected as a representative probe in a time course northern blot analysis. Leaf tissues of the infected resistant plants undergoing hypersensitive resistance were sampled at 0, 6, 12, 24 and 72 hai.

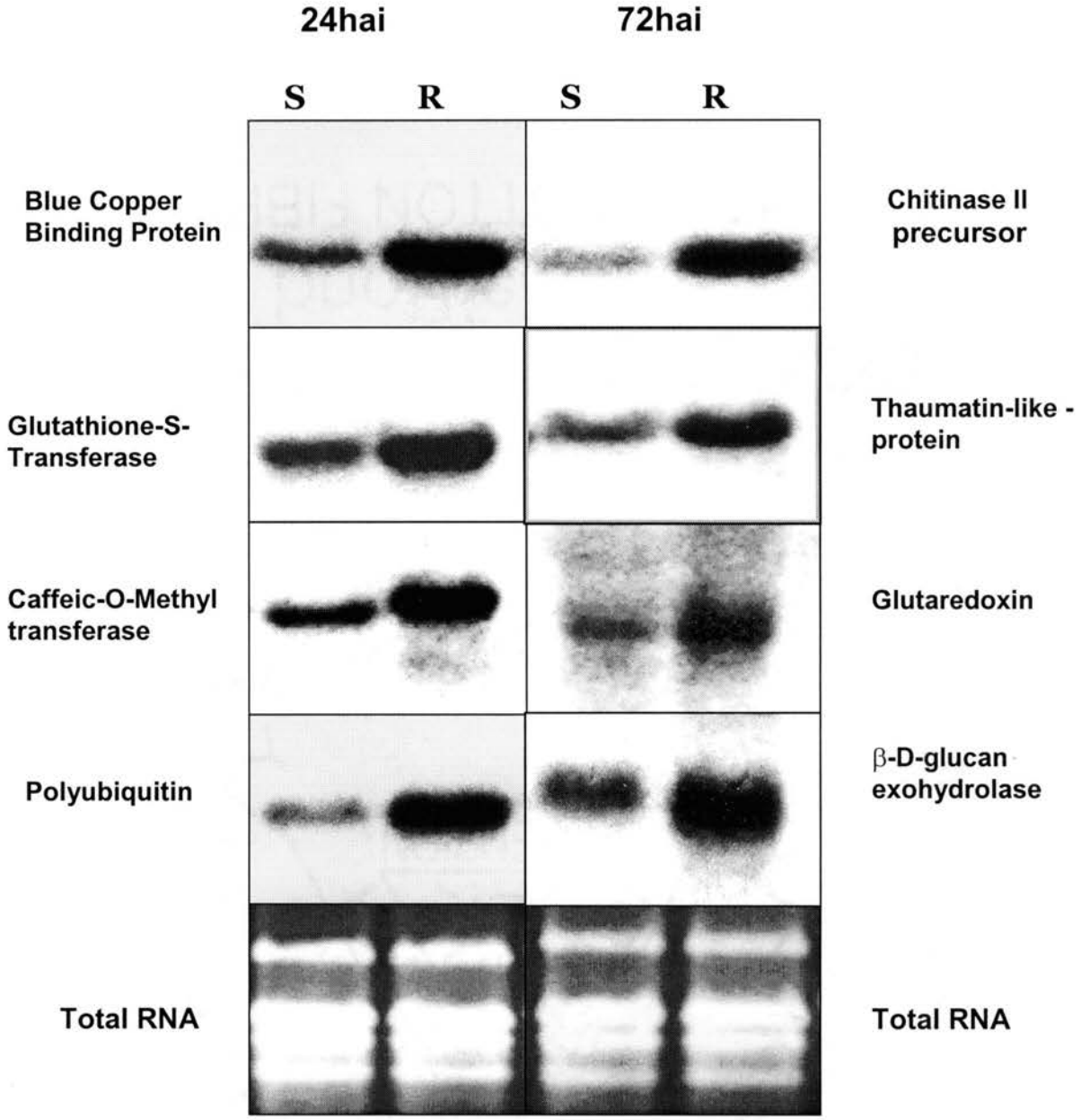


Figure 10. Northern blot analysis of differentially expressed RDA clones in the susceptible and resistant wheat lines. Equal amounts of total RNA from leaf samples at 24 and 72 hours after inoculation (hai) were electrophoresed on glyoxal-based agarose gel and transferred onto nylon membrane. Four clones found induced in macroarray analysis were used in each infection stage as probes. Differential induction was analyzed using Bio-rad Imaging Densitometer.

Total RNA was blotted onto the membrane and probed with a ^{32}P-labeled BCBP cDNA fragment. BCBP was found initially induced at 6 hai and highly induced at 24 and 72 hai (Figure 11).

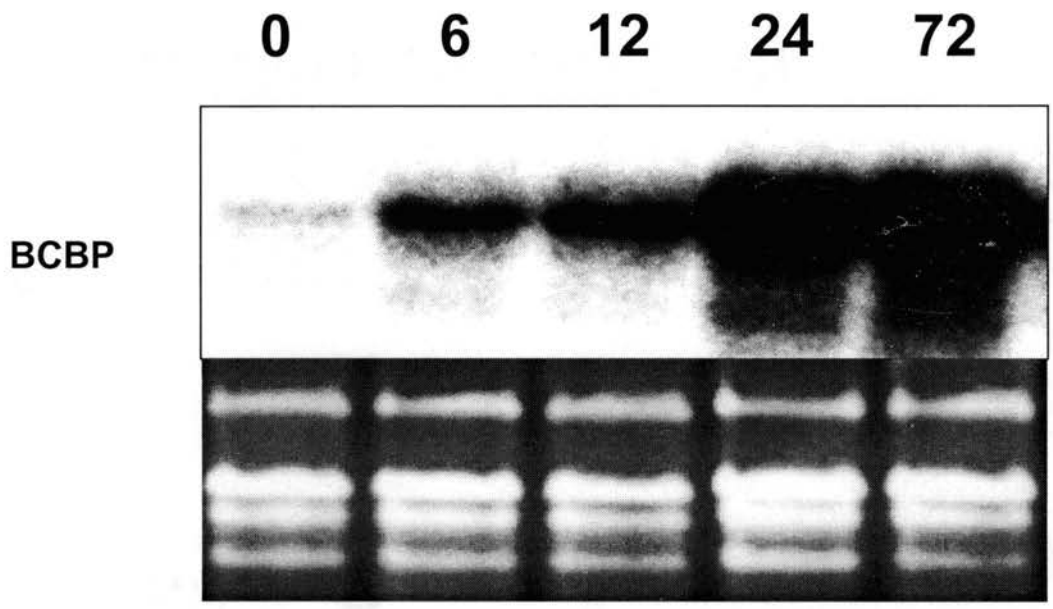


Figure 11. Genetic induction of blue copper binding protein (BCBP)-related RDA clone in wheat resistant line undergoing hypersensitive resistance. Total RNA of leaf rust infected Th-19 was isolated from tissues harvested at 0, 6, 12, 24, and 72 hai. Equal amounts of total RNA were electrophoresed in a denaturing agarose gel and transferred onto the nylon membrane. RDA clone was α^{32} dATP-labeled and hybridized onto the RNA blot.

DISCUSSION

This study describes the utility of combining cDNA-RDA subtraction and filter-based macroarray in dissecting the wheat-*P. triticina* interaction. RDA subtraction and amplification allowed enrichment of some pathogen-induced genes that are differentially expressed in the infected wheat near-isogenic lines. Thatcher (Th) and its near isogenic derivative (Th-19) differ in the Lr-19 gene, and this resistance gene should mediate changes in gene expression during pathogen infection. As shown by macroarray analysis, the overall gene expression in the infected resistant plant increased rapidly from 24 to 72 hai. Rapid induction of defense-related genes has been suggested to contribute in the effectiveness of resistant plants in controlling disease (Maleck 2000).

A threshold ratio of 2.0 was used as a criterion for differential expression to avoid exclusion of biologically relevant genes in low abundance. In the analysis of the expression ratios, a subset of differentially expressed genes with putative defense related functions was identified. Gene fragments found highly expressed in the resistant infected plant at 24 hai have sequence similarities to genes involved in the oxidative stress, which is one of the early responses to pathogen infection as mediated through the oxidative burst (Lamb and Dixon 1997). BCBP, uclacyanin, GST and peroxidase RDA gene fragments were found induced as early as 24 hai. The specific functions of BCBP and uclacyanin are still unclear but both have copper-binding domains and are possibly involved in redox control and lignin formation (Nersissian et al. 1998). Furthermore, the induction of BCBP has also been shown to be upregulated in response to

drought stress (Cho, 1997), ozone (Langebartels et al. 2000) and aluminum toxicity (Ezaki et al. 2000). GST and peroxidase are major enzymes involved in cellular regulation of redox potential and have been found upregulated during plant defense (Maleck et al. 2000; Jabs et al. 1996). Aside from its anti-oxidant function, other isoforms of peroxidase were shown to be involved in H_2O_2 production (Bolwell and Wojtaszek 1997) and lignin formation (Ezaki et al. 2001). In addition to oxidative stress induced genes, genes related to caffeic-O-methyltransferase, proteinase inhibitor, non-specific lipid transfer protein, UDP-glucosyltransferase and polyubiquitin were also found induced at 24 hai. Caffeic-O-methyltransferase is one of the major enzymes involved in the biosynthesis of lignin precursors, and subsequent lignification increases the strength of cell walls and may prevent fungal penetration (Guo 2001). Non-specific lipid transfer protein was transcriptionally activated in response to pathogen attack and shown to inhibit pathogen growth *in vitro* (Jung and Hwang 2000, Molina et al 1993). Glucosyltransferases are involved in the production of cyanogenic glucosides (Jones et al. 1999), lignin (Lim et al 2001) and salicylic glucosylation (Lee and Raskin 1999). Proteinase inhibitor and polyubiquitin are both involved in regulation of protein activities associated with physiological and biochemical changes induced by pathogen infection (Stevens et al. 1996). RDA fragment related to ribulose bisphosphate carboxylase/oxygenase (Rubisco) was also found differentially expressed at 24 hai. Induction of Rubisco in the resistant plant may occur at the early stage of pathogen infection to produce the necessary assimilates needed for the energy-requiring induction of defense responses.

Induced genes at 72 hai were mostly related to regulation of gene expression and pathogenesis-related proteins. Chitinase II precursor, β-D-glucan exohydrolase and thaumatin-like protein genes were highly induced at the later stages of pathogen infection. β-D-glucan exohydrolase has been reported to hydrolyze (1-3)-β-D-glucans and (1-3), (1-6)-β-D-glucans present in fungal cell walls (Hrmova and Fincher, 1998). Another possible function of exoglucanase in the wheat-leaf rust interaction is to catalyze self-hydrolysis of the native cell wall due to cessation of growth at the site of pathogen infection. β-glucans found in cell walls of grass species accumulate during enlargement but are extensively hydrolyzed when cell growth ceases (Carpita and Gibeaut 1993). Chitinase II and thaumatin-like protein have been found to degrade chitin and permeabilize the cell wall of fungal pathogens, respectively (Sclumbaum et al. 1986; Abad et al. 1996). RDA clones related to these proteins showed similar temporal patterns and magnitudes of expression and are two of the most abundant clones in the library. The combined effect of these two PR proteins may significantly contribute to the abortion of leaf rust infective structures at later stages of pathogen infection. Interestingly, elongation factor 1α RDA clone clustered together with chitinase II precursor and thaumatin-like-protein related fragments. Elongation factor 1 α (EF1α) delivers aminoacyl tRNA to the ribosomes during translation. Since high induction of defense-related proteins has been observed in response to pathogen attack (Van Loon 1976), the demand for Ef-1α should also be high and expression should be computationally similar to the highly expressed defense-related genes as shown in the cluster analysis. In addition to its function

in protein synthesis, Ef-1α has been suggested also to be involved in actin filament bundling (Yang et al. 1990), microtuble severing (Shiina et al. 1994) and oxidative stress-induced apoptosis (Chen et al. 2000).

The isolation of glutaredoxin RDA clone at 72 hai and its expression pattern may suggest involvement in redox-regulation of plant defense-related gene expression (Figure 12). Glutaredoxin is a small disulfide-reducing enzyme that has been found to modulate activities of many mammalian transcription factors like NF-κB, AP-1, NFI (Hirota et al. 2000, Bandyopandhyay et al. 1998) during the hyperoxic state, a condition of an increased oxygen concentration in the cell. These glutaredoxin-modulated mammalian transcription factors regulate the expression of various genes related to the immune response and apoptosis (Wang et al. 1996, Van-Antwerp et al. 1996). Oxidation of sulfhydryl groups can activate or deactivate DNA binding function of many transcription factors thus effecting changes in the expression profile of their target genes (Grant and Loake 2000). The cluster analysis showed the grouping of glutaredoxin, a putative Zn finger transcription factor and a peroxidase for possible coregulation of expression. Redox-regulation of the putative Zn-finger transcription factor may be involved in the expression of wheat PR genes or inhibition of hypersensitive cell death. Cysteine residues in the DNA-intercalating domains of Zn-finger transcription factors are the primary targets for oxidative inactivation (Wu et al. 1996). Novel Zn finger proteins encoded by *Arabidopsis lsd1* gene (Dietrich et al. 1997) and WRKY transcription factors (Eulgem et al. 2000) have been proposed to negatively regulate hypersensitive cell death and positively activate PR genes,

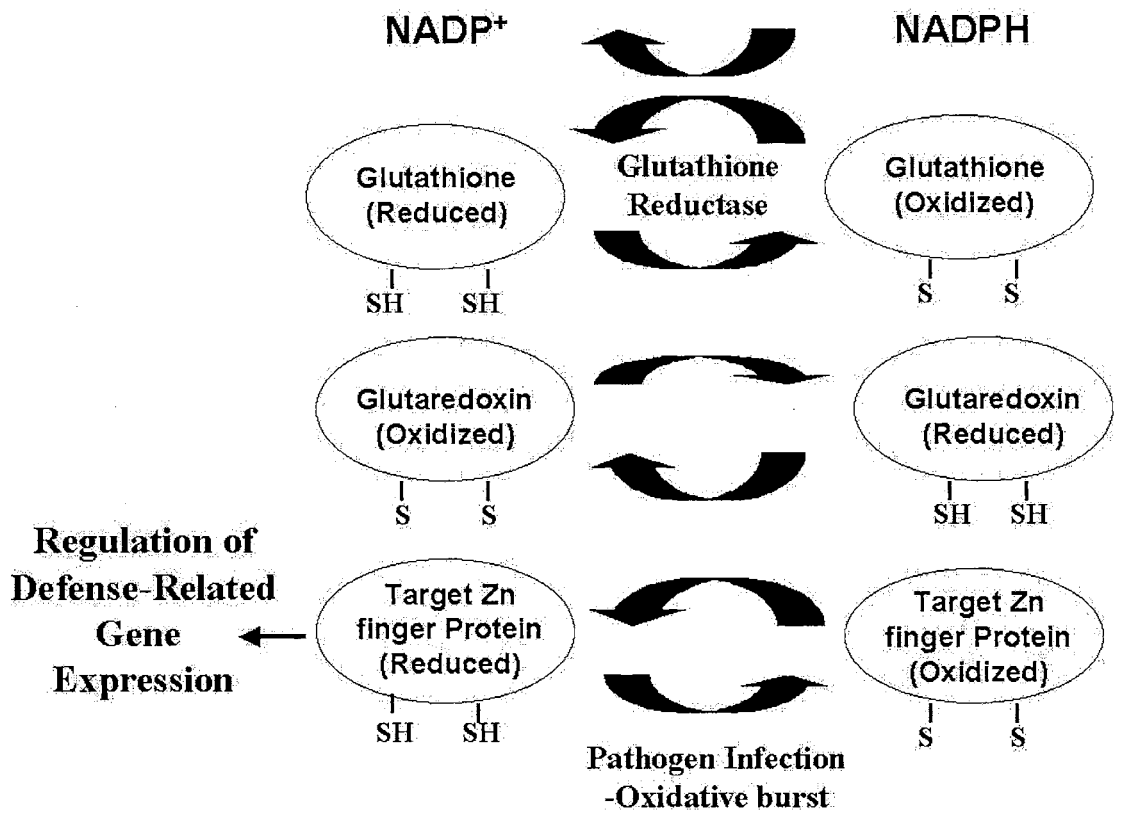


Figure 12. Model for the possible mechanism of redox-regulation of defense-related gene expression. Extracellular followed by intracellular production of reactive oxygen species (ROS) have been shown to occur during pathogen infection. Intracellular ROS cause oxidative inactivation of many proteins. Zinc finger proteins are the primary targets of oxidative inactivation due to the presence of cysteine residues affecting DNA binding activity. Reduced glutathione, with the action of glutathione reductase and NADPH, donates electron to glutaredoxin, which in turn reduces zinc finger proteins. Reduction of zinc finger proteins enhances DNA binding activity, thus regulating gene expression.

respectively. Database search for sequence similarity of the DP2-72-32 RDA clone showed two significant hits: a putative Zn-finger transcription factor in rice (E value = 7 X 10^{-32}) and a putative CCCH-type zinc finger protein in Arabidopsis (E value = 2 X 10^{-4}). Sequence analysis of these two zinc finger proteins showed a consensus sequence related to mammalian CCCH-type zinc-finger domain (see Appendix A). Tristetrapolin, a mammalian CCCH type zinc-finger protein, was thought to function as a transcription factor but was recently shown to destabilize mRNA related to tumor necrosis factor α, a programmed cell death receptor (Lai and Blackshear 2001). Due to the similarity of DP2-72-32 clone to genes with CCCH-type zinc finger consensus sequence, DP2-72-32 RDA clone may not be a zinc finger transcription factor but may be a gene with mRNA binding activity that degrades cell death-related mRNAs to prevent the spread of the hypersensitive response.

Coordinated and overlapping expression of defense-related genes is possibly a plant strategy to produce synergistic effects to control pathogen growth. Synergistic interaction has been shown by chitinase and β-1,3-glucanase in transgenic tomato plants (Jongedijk et al. 1995) and by non-specific lipid transfer protein and thionins in barley (Molina et al. 1993). The combined effect of the two genes enhanced protection against disease. Co-expression of wheat defense-related genes was found at 24 and 72 hai. Overlapping expression of genes suggests that resistant wheat plants produce many possible immediate defenses to prevent leaf rust pathogen establishment.

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CHAPTER III

CONCLUSIONS: SUMMARY AND FUTURE DIRECTIONS

Transcriptional profiling is an attractive tool to study the complexity of plant-pathogen interactions. Analysis of gene expression provides a molecular description of the events that occur during infection. The goal of this study was to understand the molecular mechanisms of wheat defense responses to leaf rust pathogen attack through gene expression profiling.

A combination of cDNA-RDA subtractive hybridization and macroarray analysis was used to isolate differentially expressed gene fragments between near-isogenic lines of wheat differing in resistance to leaf rust infection. Sequence analysis showed that induced genes have putative roles in oxidative stress, lignification, gene regulation, photosynthesis, and PR protein accumulation. Based on the expression pattern of isolated RDA clones, transcriptional reprogramming in wheat during leaf rust pathogen ingress showed a considerable degree of complexity similar to other cereal-fungal pathosystems.

It is clear in this study that we can draw meaningful inferences from gene expression data using computational analysis. Genes with putative functions in oxidative stress, lignification and pathogenesis-related protein accumulation were the predominant reactions during leaf rust infection. Glutaredoxin and a putative Zn-finger transcription factor related genes shared the same expression pattern and are predicted to have roles in regulating defense-related gene expression or cell death. Accumulation of reactive oxygen species is an important response to

pathogen infection in some pathosystems but there has been no report yet on the mechanisms of redox-regulation of transcription factors that modulate plant defense-related gene expression. In mammalian systems, redox-regulation of many transcription factors related to defense has been reported in many studies (Hirota et al. 2000, Bandyopandhay et al. 1998).

Although only a subset of defense-related genes was analyzed at two time points after infection, this study provides a significant contribution in describing some aspects of the molecular basis of wheat leaf rust resistance. More in-depth inferences can be obtained if RDA fragments are analyzed under several different conditions and additional time points after pathogen infection. Such data sets may be analyzed using various methods with increasing depth of inference such as principal component analysis and self-organizing map (see Appendix B).

Because there are still many pathogen-induced RDA clones with unknown functions, future experiments might discover other pathways important in the genetic control of the HR in wheat. Knowledge of the exact function and the potential targets of the isolated RDA clones will provide important insights toward understanding the mechanisms of the wheat defense response. Isolation of full-length cDNA sequences will be necessary in future experiments on validation of functions. The use of knockouts and antisense strategies in wheat will be the critical areas of research for the ultimate assignment of gene function. More complete understanding of wheat fungal defense responses will require a genome-wide expression analysis of all genes but is viewed with considerable challenges. By knowing the molecular details of the wheat-wheat leaf rust

interaction, scientists can identify host defense strategies and characterize the cues to which they respond and mechanisms by which they are regulated. Specifically, the information on molecular mechanisms of the wheat defense response will help breeders and biotechnologists to identify targets for genetic manipulation and consequently provide a strong foundation for the rational design of environment-friendly and consumer-accepted biotechnological strategies for developing wheat germplasm with improved leaf rust resistance.

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APPENDIX A

A Pathogen-Induced Putative Zn-finger Transcription Factor: Its Isolation in Leaf Rust Infected Resistant Wheat Plant

ABSTRACT

The RACE (rapid amplification of cDNA ends) method was used to isolate the 3' cDNA end of the DP2-72-32 RDA gene fragment induced in the wheat leaf rust infected resistant plants in the gene expression profiling experiment. Nested PCR using gene specific primer 2 (GSP2) produced amplicons corresponded to the 3'-end of the gene. Database search for sequence similarity of the 3' cDNA-end of the gene showed only two significant hits: a putative Zn-finger transcription factor in rice (E value = 1 X 10^{-14}) and a putative CCCH-type Zn-finger protein in *Arabidopsis* (E value = 2 X 10^{-04}). Sequence alignment of the Zn-finger transcription factor and CCCH-type Zn-finger protein revealed a highly conserved sequence of Cys-His in the form of YSGTA-$CX_7CX_5CX_3H$. This zinc finger domain is related to the YKTEL-$CX_8CX_5CX_3H$ conserved region of a small family of mammalian CCCH-type Zn-finger proteins found to destabilize mRNA encoding tumor necrosis factor α (TNFα). Due to its relatedness to CCCH-type Zn-finger protein that regulates mammalian apoptosis, this DP2-72-32 RDA clone may bind to and destabilize pro-death mRNAs to regulate the spread of the hypersensitive response.

INTRODUCTION

Cell death is one of the obvious manifestations of hypersensitive resistance. Although it is common in many plant-pathogen interactions, the pathway leading to the expression of programmed cell death remains to be elucidated. Many studies have shown the uncoupling of resistance and cell death (Cole et al. 2001, Yu et al. 1998), suggesting that these two components of hypersensitive response are conditioned by different set of genes.

Zn-finger proteins have been implicated in the regulation of programmed cell death in both plants and animals. In the plant system, Dietrich et al. (1997) isolated a novel zinc finger protein called LSD1 that appears to negatively regulate hypersensitive cell death at the level of transcription. The mechanism of regulation could involve either repression of prodeath (see Appendix B) pathway or activation of antideath (see Appendix B) pathway. In the mammalian system, a small class of zinc-finger proteins has been shown to regulate apoptosis (see Appendix B). Tristetrapolin (TTP), a prototype of CCCH zinc-finger proteins, has been found to destabilize genes involved in tumor necrosis factor, a programmed cell death receptor (Lai et al. 1999). TTP initiates the degradation after binding of the tandem zinc-finger domain to the AU-rich region (ARE) in the 3' UTRs of the target mRNAs (Lai et al. 2002).

In the study of the wheat-*Puccinia triticina* interaction, an RDA clone was isolated in the subtractive hybridization experiment (refer to Chapter II) that showed sequence similarity to zinc-finger proteins. This gene fragment was highly induced in the infected resistant wheat line at 72 hai. To study the role of

this zinc-finger related RDA clone in hypersensitive resistance, isolation of full-length cDNA sequence was necessary. This study describes the initial effort in isolating the full-length sequence of the putative Zn-finger transcription factor RDA clone using 3'RACE.

MATERIALS AND METHODS

Plant Infection

The wheat resistant line, Th-19, containing the Lr-19 resistance gene, was used in the analysis. Seeds were sown in 10X10 cm pots with Metro-mix (Scotts, Marysville, OH) potting medium and grown for two weeks in a growth chamber with 16/8 hr light/dark cycle at 20° and 15°C, respectively. Seedlings at the two-leaf stage were infected with *P. triticina* using a plant-to-plant brushing method as described by Browler (1971). The infected susceptible wheat cultivar, Danne, was used as the brush plants that served as a source of inoculum. Brush plants were inoculated with rust spores that had been stored in liquid nitrogen. Rust pustules developed on the leaves of the brush plants 11 days after inoculation. Th-19 seedlings were placed in a mist chamber 1 hr prior to inoculation. The plants were removed from the mist chamber and inoculated by brushing the infected brush plants lightly over the tops of misted resistant seedlings. Spores were allowed to settle on the leaves for 15 min and the inoculated plants were incubated in the mist chamber for another 12-16 hr. Infected seedlings were transferred back to the growth chamber. Leaf tissues were harvested at 72 hai from the infected Th-19 seedlings and ground in liquid nitrogen. Ground samples were stored in liquid nitrogen.

RACE Cloning

Sequence information on the DP2-72-32 RDA clone was used to design primers for 3' rapid amplification of the cDNA end (RACE). Two gene specific primers were designed using MacVector 6.0 software (Oxford Molecular Group). Total RNA was isolated from ground leaf tissues using the TotallyRNA kit (Ambion, Austin, TX) following the manufacturer's protocol. 3' RACE kit (Life Technologies, Rockville, MD) was used to obtain the 3' cDNA end of the gene. First strand cDNA synthesis was performed in 5 μg total RNA with 10 μM adapter primer using SuperScript II RT (Life technologies, Rockville, MD) at 42°C for 50 min. After the first strand cDNA synthesis, the original RNA template was degraded with RNase H (Life Technologies, Rockville, MD) for 20 min at 37°C. The transcribed cDNA was then used as the template for PCR. The 50 μl reaction contained 20 mM Tris-HCL, 50 mM KCL, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 200 nM GSP1 (gene specific primer 1), 200 nM UAP (Universal Amplification Primer) and 2.5 U *Taq* DNA polymerase (Life Technologies, Rockville, MD). PCR amplification was performed using the following conditions: 94°C, 3 min; 35 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min; and a final 10 min extension at 72°C. PCR products were diluted in 1:10000 using sterilized Milli-Q water (Millipore, Bedford, MA). A 2 μl aliquot of the diluted reaction was used as template for nested amplification. Similar PCR conditions as the first amplification was employed for nested PCR using gene specific primer 2 (GSP2). The 3' RACE-nested PCR products were gel-purified and ligated into pGEM-T easy vector (Promega, Madison, WI). The ligated plasmid vector was transformed into

the DH5α competent cells (Life Technologies, Rockville, MD) following the manufacturer's protocol. Three microliters of ligation reaction were added directly to competent cells. Competent cells were heat-shocked for 20 seconds at 37°C and incubated on ice for 2 min. Luria-Bertani (LB) medium was added and incubated in the shaker at 225 rpm for 1 hr at 37°C. Bacterial cultures were spread on LB containing ampicillin (100mg/l) agar plates with X-gal (40μg/ml) and incubated overnight at 37°C in a shaker at 225 rpm. White colonies were picked, placed in 6 ml LB medium with ampicillin and incubated overnight at 37°C in a shaker at 225 rpm. Plasmid DNA was isolated using Qiagen Plasmid Isolation kit (Qiagen, Valencia, CA) and inserts were sequenced at the Recombinant DNA/Protein Core Facility, Oklahoma State University, using a 373 Applied Biosystems DNA Sequencer (Applied Biosystems, Foster, CA). Database nucleotide and amino acid sequence comparisons were conducted using BLASTN and BLASTX (Altschul et al. 1997), respectively. Conserved sequence domains were searched using RPS-BLAST (Altschul et al. 1997) and Pfam databases.

RESULTS AND DISCUSSION

Two PCR primers were designed based on the nucleotide sequence of DP2-72-32 RDA clone for RACE. Sequences and locations of the gene specific primers are shown in Figure 1. The second gene specific primer (GSP2) was used in nested amplification. RACE products were cloned and 10 individual plasmids were sequenced. Two sequences were identified with different end points (Figure 2). One is longer than the other by 17 nucleotides from termination codon to polyadenylation site. In almost all plant genes, the position of cleavage can be quite heterogeneous within a single transcription unit (Rothnie 1996). Extensive deletion and linker analysis showed that the processing site is controlled by an upstream sequence called the near upstream element (NUE) (Mogen et al. 1992). In the two 3'-end sequences isolated in this experiment, the NUE is most probably TGTAAAT at 14-15 nt upstream of poly(A) site. Duplication of TGTAAAT was found in the longer RACE product.

The two sequences, although different in length, have the same coding region. The 3'-end RACE products coded for a predicted 82 amino acid sequence (Figure 3). The predicted amino acid sequence showed significant similarity to two sequences in the database: a putative zinc finger transcription factor in rice and a putative CCCH-type zinc finger protein in *Arabidopsis* (Table 1). Sequence comparison of these two proteins revealed three tandem zinc finger conserved sequences; $CX_{12}CX_{10}CX_3H$, YSGTA-$CX_7CX_5CX_3H$ and GVFECWLHPARYRTQP-$CX_5CX_4CX_3H$ (Figure 4). The spatial arrangements of the Cys and His residues in the zinc finger domain are highly conserved in the

5'

DP2-72-92 GATCC TCACT TGGTC ACGGT GACCA CCGCC TCTGG

CGCCA CCATA ACCAT GGAGC CCATG GACCT CGGG**C**

GSP1
TCATA GCAGA GGAGC AGCCT GTGGA GAGGG TAGAG

TCCGG GAGAG CCCTC CGCGC AAAGG TATTC GAGAG

GSP2
GCTCA GCAAA GAAGC CACCG TCTGC AACGA CACCA

3'

TCGCT GCCGC AGCCG TGGAA GTT

Figure 1. Sequences and locations of the gene specific primers (GSP1 and GSP2) for 3' rapid amplification of cDNA ends (RACE). MacVector 6.0 was used in designing primers following the recommendations in the 3'RACE kit. Gene specific primer 1 (GSP1) was consisted of 21 bases with 57% GC and melting temperature of 58°C. Gene specific primer 2 (GSP2) was consisted of 18 bases with 55% GC and melting temperature of 55°C. GSP1 and GSP2 were used in the first and nested PCR reactions, respectively.

A.) RACE product 1

GCTCAGCAAAGAAGCCACCGTCTGCAACGACACCATCGCTGCCGCAGCCG
TGGAAGTTCCGACCTCAGCCGCTCCTGACGTCGGCTGGGTCTCCGATCTCA
CCAAC**TAA**AGCAATCAAGGTGGTCAGGTGTTGCTAGATCGGTTTGCAATTC
CTATTCCTTGTAAGATACTCCTATTCCT**TGTAAAT**ATGTTCTTCCTTCCTATG
TAAAAAAAAAAAAAAAAAA

B.) RACE product 2

GCTCAGCAAAGAAGCCACCGTCTGCAACGACACCATCGCTGCCGCAGCCG
TGGAAGTTCCGACCTCAGCCGCTCCTGACGTCGGCTGGGTCTCCGATCTCA
CCAAC**TAA**AGCAATCAAGGTGGTCAGGTGTTGCTAGATCGGTTTGCAATTC
CTATTCCTTGTAAGATACTCCTATTCCT**TGTAAAT**ATGTTCTTCCTTCCTA**TG**
TAAATTACCAAGATGTTGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAA

Figure 2. Two products of 3' rapid amplification of cDNA ends (RACE) with different polyadenylation sites. The first sequence (A) has 97 nucleotides from termination codon to the poly(A), while the second sequence (B) has 115 nucleotides from the termination codon to the poly(A). TGTAAAT sequence is possibly the near upstream element that controls the polyadenylation site.

D P H L V T V T T A S G A T I T M
GATCCTCACTTGGTCACGGTGACCACCGCCTCTGGCGCCACCATAACCATG

E P M D L G L I A E E Q P V E R V
GAGCCCATGGACCTCGGGCTCATAGCAGAGGAGCAGCCTGTGGAGAGGGT

E S G R A L R A K V F E R L S K E
AGAGTCCGGGAGAGCCCTCCGCGCAAAGGTATTCGAGAGGCTCAGCAAAG

A T V C N D T I A A A A V E V P
AAGCCACCGTCTGCAACGACACCATCGCTGCCGCAGCCGTGGAAGTTCCG

T S A A P D V G W V S D L T N *
ACCTCAGCCGCTCCTGACGTCGGCTGGGTCTCCGATCTCACCAAC**TAA**AGC
AATCAAGGTGGTCAGGTGTTGCTAGATCGGTTTGCAATTCCTATTCCTTGTA
AGATACTCCTATTCCT**TGTAAAT**ATGTTCTTCCTTCCTATGTAAAAAAAAAAA
AAAAAAA

Figure 3. Nucleotide and deduced amino acid sequences of the 3' cDNA end of the DP2-72-32 RDA clone. The 3' cDNA end was isolated using rapid amplification of cDNA ends (RACE) method. The termination codon and predicted near upstream element (NUE) of the gene are in bold letters.

Table 1. Similarity of the 3' cDNA end of the DP2-72-32 RDA clone with sequences in the database.

Sequences	% Identities	% Positives	E-value
(AP002746) Putative zinc finger transcription factor (*Oryza sativa*)	53	63	1×10^{-14}
(AC005169) Putative CCCH-type zinc finger protein (*Arabidopsis thaliana*)	50	58	2×10^{-04}
(NC_003075) Putative protein (*Arabidopsis thaliana*)	50	57	0.037

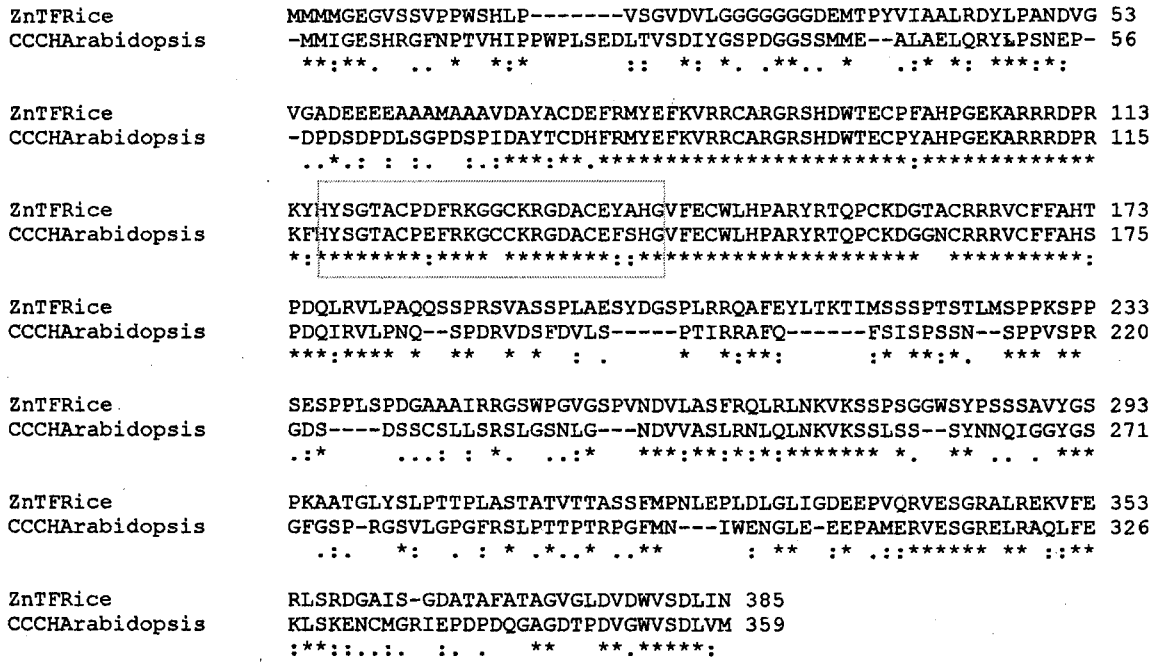

Figure 4. Alignment of DP2-72-32 RDA clone similar sequences in the database. Database search found significant sequence similarities of DP2-72-32 cDNA fragment to putative zinc finger transcription factor in rice (E value =1 X 10^{-14}) and putative CCCH-type zinc finger protein in *Arabidopsis* (E value = 2 X 10^{-04}). Sequence alignment of related genes was done using Clustal W and showed a conserved region related to YKTELCX_8CX_5CX_3H, a mammalian CCCH-type zinc finger domain. Consensus sequences are shown in asterisk and CCCH-type related zinc finger domain is enclosed in a box.

two proteins. The YSGTA-$CX_7CX_5CX_3H$ zinc finger domain, where X refers to variable amino acids, is related to YKETL-$CX_8CX_5CX_3H$ CCCH-type zinc finger consensus sequences in mammalian cells. CCCH-type zinc finger proteins in mammalian cells have been found to destabilize mRNA activity. Tristetrapolin, a prototype of the small family of CCCH-type zinc finger proteins in mammalian cells, was originally thought to be transcription factor but was recently found to initiate degradation of mRNAs related to tumor necrosis factor (Lai et al. 1999).

Because of its sequence similarity to the CCCH-type protein that regulates mammalian apoptosis, the DP2-72-32 RDA clone is possibly a gene that negatively regulates hypersensitive cell death. Our hypothesis is that the CCCH-type zinc finger protein and pro-death genes may be induced in the plant cells within and outside of the pathogen infection site (Figure 5). In *lsd1 Arabidopsis* mutant, the presence of superoxide is a necessary and sufficient signal for cell death (Dietrich et al. 1997, Morel and Dangl 1997, Jabs et al. 1996). Cell death occurs only at the site of pathogen infection probably because of the very high level of H_2O_2 entering the cells. H_2O_2 can be converted to a more reactive oxygen species, the hydroxyl radical, in the presence of iron or copper through a Fenton type reaction. Reactive oxygen species in the cell may deactivate the mRNA binding activity of the CCCH-type zinc finger protein allowing the expression of cell death genes. In areas surrounding the infection site, the level of reactive oxygen species entering the cells may not be very high. Induction of glutaredoxin may be enough to neutralize the level of oxidation in the CCCH-type zinc finger protein, thus enhancing their mRNA binding activity and triggers

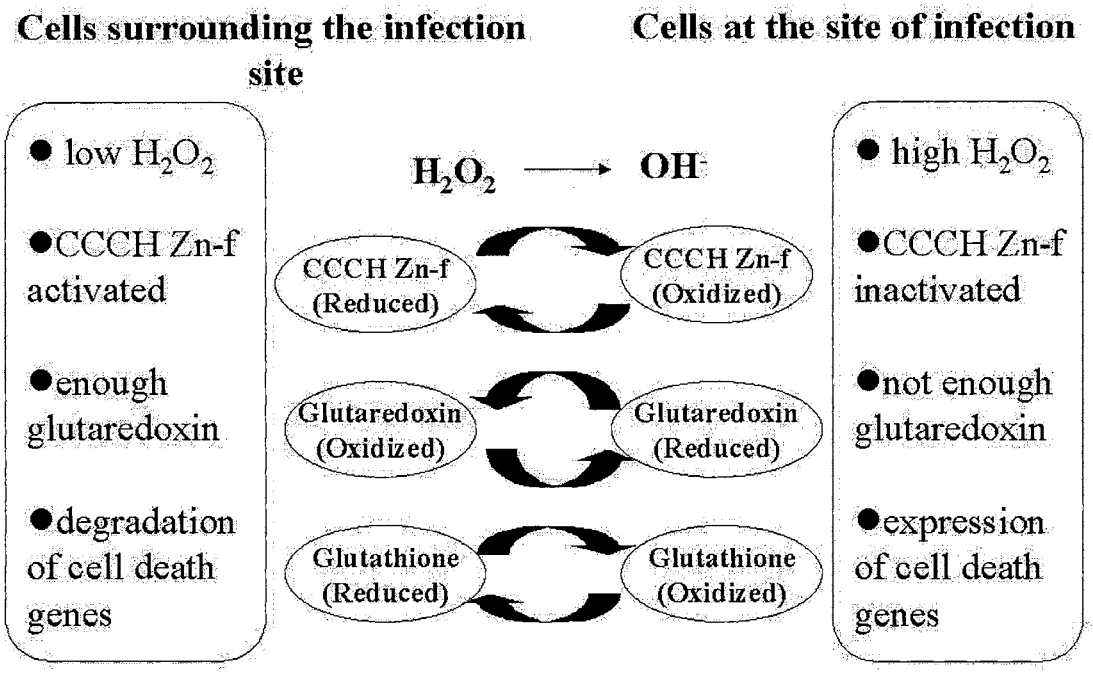

Figure 5. Model for the possible mechanism of redox-regulation of the hypersensitive cell death. In this hypothesis, intracellular levels of reactive oxygen intermediates and glutaredoxin regulate the mRNA binding activity of CCCH-type zinc finger protein that destabilizes mRNA related to cell death.

destabilization of mRNA related to cell death. Destabilization of cell death mRNA may prevent the spread of hypersensitive response surrounding the infection site. Isolation and functional analysis of the full-length cDNA sequence of the DP2-72-32 RDA clone with sequence similarity to a putative zinc finger protein are needed to investigate this hypothesis.

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APPENDIX B

Glossary of Terms

Algorithm	A computational strategy for accomplishing an objective.
Ankyrin	A globular protein that links spectrin and an integral membrane protein in the erythrocyte plasma membrane.
Ankyrin repeat	Tandemly repeated module of about 33 amino acids known to function as protein-protein interaction domains.
Anti-death genes	Genes that protect cells from undergoing cell death.
Apoptosis	A programmed cell death in mammalian cells that can be induced by a variety of stimuli including growth factor, UV, chemotherapeutic agents or by a family of transmembrane proteins called death receptors. It is characterized by shrinkage of the cells, membrane inversion and exposure of phosphatidylserine, blebbing, fragmentation of nucleus, chromatin condensation and DNA degradation. In termination phase, membrane -enclosed vesicles, the small remainders of the cell ("apoptotic bodies") are engulfed by phagocytes which prevents an inflammatory reaction.
Average-linkage clustering	The distance between clusters is calculated using average values. The most common technique is unweighted pair-group method average (UPGMA). The average distance is calculated from the distance between each point in a cluster and all other points in another cluster. The two clusters with the lowest average distance are joined together to form a new cluster.

BLAST	The Basic Local Alignment Search Tool is a fast technique for detecting ungapped subsequences that match a given query sequence.
Blotting	A technique used for transferring DNA or RNA to a suitable matrix, such as nylon membrane.
Brush Plants	Infected susceptible (Danne) plants that serve as sources of inoculum in plant-to-plant brushing method.
cDNA	Complementary DNA; synthesized from an mRNA template by the enzyme reverse transcriptase. Generation of representation in RDA is derived from double stranded cDNA.
cDNA arrays	cDNA clone inserts are printed onto a nylon membrane using 384 replicator and subsequently hybridized to labeled probes. The probes are pools of cDNAs derived from mRNA.
cDNA Representations	cDNA-RDA relies on the generation, by restriction enzyme digestion and PCR amplification, of simplified versions of the cDNA populations under investigation.
Clones	A group of cells or RDA fragments derived from a single ancestor.
Cluster Analysis	Computational algorithm for identification of patterns in gene-expression data.
Driver	Susceptible cDNA representations that drive the subtraction in RDA.
Distance	A measure of dissimilarity between two RDA clones based on macroarray gene expression data.
ESTs	Expressed Sequence Tags; a sampling of sequence from a cDNA.

E-value	Expect value: the expected number of hits with a score equal to or greater than the score for a given hit.
Gene family	Consists of a set of genes whose exons are related; the members were derived by duplication and variation from some ancestral gene.
Hyperoxic State	An increased concentration of oxygen in the cell; is characteristic of cells undergoing oxidative stress induced apoptosis.
Leucine-rich repeat	Short sequence motifs present in a number of proteins which contains leucines or other hydrophobic amino acids at regular interval; LRR motifs usually involved in protein-protein interactions.
Normalization	The process of removing the sources of variation in macroarray experiments that affect the measured gene expression levels. The normalization factor is used to adjust the data to compensate for experimental variability and to balance the hybridization signals from the two samples being compared.
Nucleotide binding site	Characterized by several sequence motifs found in animal ATP and GTP-binding proteins.
Pfam database	Pfam database of protein domains and HMM developed by Washington State University in St. Louis. It is a large collection of multiple sequence alignments and hidden Markov models covering many common protein domains.
Principal Component Analysis	It is a mathematical technique that exploits factors to pick out patterns in the data, while reducing the effective dimensionality of gene expression space without significant loss of information.
Pro-death genes	Genes that induce cells from undergoing cell death.

Protein domain	A domain is independent region of the protein. It may represent a functional unit that is identified with a particular activity of the protein, for example, its ability to perform a certain catalytic activity, to bind certain ligand or interact with other types of domains.
RPS-BLAST	Reverse position-specific BLAST algorithm; The query sequence is compared to a position -specific score matrix prepared from the underlying conserved domain alignment.
Self-organizing maps	It is a neutral-network-based divisive clustering approach. A SOM assigns genes to a series of partitions on the basis of the similarity of their expression vectors to reference vectors that are defined for each partition.
Similarity	A measure of how similar one sequence or expression is to another; depends on a scoring matrix.
Tester	Resistant cDNA representations containing the target genes.
Toll-Interluekin Receptor	Intracellular signaling domain found in MyD88, interleukin 1 receptor and Toll receptor.
Zinc finger protein	Protein that has a repeated motif of amino acids with characteristic spacing of cysteines that may be involved in binding zinc; is characteristic of some proteins that bind DNA and/or RNA.

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