FUNCTIONAL GENOMIC STUDY OF

BERMUDAGRASS RESPONSES

TO FUNGAL INFECTION

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FORMAT OF THESIS

This thesis presented in the Molecular of Plant-Microbe Interactions style and format allowing for independent chapters (Chapter II and Chapter III) to be suitable for submission to scientific journals. Two papers have been prepared from research data collected at Oklahoma State University to partially fulfill the requirements for the degree of Doctor of Philosophy. Each paper is complete in itself containing an abstract, introduction, results, discussion, materials and methods, acknowledgments and literature cited sections.

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NOMENCLATURE

cDNA	complementary DNA
DEPC	Diethylene Pyrocarbonate
DMSO	Dimethyl Sulfoxide
ds	double-strand
EST	expression sequence tag
EtBr	Ethidiumbromid
LB	Luria-Bertani
mRNA	messenger RNA
PCR	Polymerase Chain Reaction
R/T	room temperature
rRNA	ribosomal RNA
SDS	spring dead spot
ss	single-strand
SSH	suppression subtractive hybridization

Chapter I

Introduction and Literature Review

Bermudagrass is a major turf-type grass used in the southern United States. Investment in bermudagrass turf is extensive with a yearly replacement value in Oklahoma alone easily exceeds 1.7 billion dollars (Martin 1990). Spring dead spot (SDS), a fungal disease, causes more damage to bermudagrass than any other single disease, particularly in the cooler parts of the warm-season grass regions. It is discouraging to find large areas in beautiful green bermudagrass fairways and lawns dead in the spring because of this disease. Extensive research has been conducted over the past fifty years to develop effective control strategies for spring dead spot (Smith et al. 1989). Unfortunately, there is no successful method to control this disease in bermudagrass.

Identification and characterization of the fungal induced gene transcripts and monitoring of gene expression levels of bermudagrass will allow us to understand which genes are affected by the fungal infection, and how the expression profiles of the genes are related to the symptoms. It will help explain the molecular mechanisms of the plant defense system imposed by pathogen stress, and the importance of the fungal induced genes in the host-pathogen interaction. The research will also increase our understanding about the signal transduction in plant membrane system. The results of our project will provide valuable information for controlling spring dead spot of bermudagrass by plant transformation. The fungal-induced transcript sequence data of bermudagrass will also be informative to other researchers working in the same or related fields such as plant genome sequencing, stress-induced gene cloning, and plant transformation.

BERMUDAGRASS AND THE DISEASE SPRING DEAD SPOT

Introduction of bermudagrass.

Bermudagrass, *Cynodon dactylon*, is a warm-season perennial sod-forming species used widely throughout the southern United States for urban use and recreation. Although it is distributed around the world between the latitudes of 45°N and 45°S, the natural distribution and use is primarily in warmer climatic regions (Harlan et al. 1970). The turf bermudagrass has a high level of variability, salt and drought tolerance, and many desirable aesthetic qualities (Gatschet 1993). In the United States, turf bermudagrass is primarily located in the southeast due to the limited tolerance to the low winter temperature. Within its area of adaptation, bermudagrass is hardy enough to survive with little care and can respond quickly to more intensive management. Bermudagrass cultivars typically produce extensive root systems and are drought tolerant. They also respond well to nitrogen fertilization and produce abundant biomass when soil moisture is adequate.

The two most serious obstacles to growing bermudagrass are its susceptibility to freeze damage and problems with a fungal disease known as spring dead spot (SDS). Many of the highest-quality bermudagrasses are very susceptible to SDS. The symptoms of SDS usually appear in early spring as dormant bermudagrass resumes growth and occur as circular, dead areas ranging from a few centimeters up to several meters in diameter. In severe cases, dead areas coalesce to encompass a much larger area of turf, which may be mistaken for winter killing or winter desiccation. Bermudagrass re-growth into dead areas is often slow and usually follows weed invasion. SDS often occurs in mature bermudagrass (3 or more years old) that was intensively managed at low mowing

heights and high nitrogen fertility (Baird et al. 1998). SDS was first observed in Stillwater, Oklahoma during the spring of 1954 (Wadsworth and Young 1960). Although the disease may have occurred earlier, it did not become a serious problem until the late 50's. This was probably due to the greatly increased use of bermudagrass as a fine turf and high amount of nitrogen fertilization.

Unfortunately, most of high quality bermudagrass cultivars are completely sterile triploid hybrids (2n=3x=27) and sexually isolated, therefore cannot be breed for increased resistance. In different regions, SDS is caused by different fungi; therefore, control of this disease with fungicides has yielded varying results. Tisserat et al. (1991) evaluated the efficiency of selected fungicides for the control of SDS in a naturally infected "Kansas Improved" bermudagrass lawn. Although none of the fungicides tested significantly reduced disease severity, fenarimol and propiconazole tended to reduce the number of infection centers per plot. However, the level of control achieved with these fungicides was too low to be commercially accepted.

Casual organisms of spring dead spot in bermudagrass.

Spring dead spot, a serious patch disease of bermudagrass, was first observed in Oklahoma during 1954 (Smith et al. 1989). The identity of the primary agent(s) has become the subject of a spirited debate that continues today.

In Australia, a disease of similar etiology was first noted on *Cynodon* spp. in 1961 (Smith 1965). This disease also became known as spring dead spot. The first advance in determining the causality of spring dead spot in eastern Australia came from studies conducted by Smith (Smith 1965). Smith proved that the disease in New South Wales

was caused by a soilborne fungus that he initially designated as *Ophiobolus herpotrichus* (Fr.:Fr.) Sacc. & Roum. It was later determined that two distinct fungi were combined within this assignment and that neither of them could be retained within the genus *Ophiobolus*. In 1972, these fungi were described as *Leptosphaeria narmari* J. C. Walker& A. M. Sm. and *L. Korrae* J. C. Walker& A. M. Sm. (Walker and Smith 1972), two new species within the genus *Leptosphaeria* Ces. & De Not., nom. Cons. *L. narmari* is currently the dominant pathogen associated with spring dead spot in Australia.

Helminthosporium spiciferum (Bainier) J. Nicot was one of the first agents associated with spring dead spot in the United States (Wadsworth et al. 1968). Although isolates of this fungus caused leaf spots on bermudagrass seedlings, symptoms of spring dead spot were never reproduced (Lucas 1980). Two decades after Smith made his initial report, scientists in the United States began to develop lines of evidence to support the concept that *Gaeumannomyces*-like extotrophs incite spring dead spot in North America. Agents associated with the disease complex in the United States are now reported to include *L. korrae* (Endo et al. 1985), *Ophiosphaerella herpotricha* (Fr.:Fr.) J. C. Walker (Tisserat et al. 1989), and *G. graminis var. graminis* (McCarty and Lucas 1989).

Taxonomy of pathogen fungi.

Spring dead spot of bermudagrass is caused by a closely related group of ectotrophic root infecting (ERI) fungi. All are classified in Ascomycetes. The ERI fungi possess several morphological features that are useful taxonomic aids for proving the identities. A careful examination of macro-and microscopic symptoms, coupled with isolation of pathogens, is essential before spring dead spot can be diagnosed with certainty. Extensive fungal involvement is readily noticeable on affected bermudagrass

wherever spring dead spot occurs. Dark brown mycelia, typically associated with dead and dying roots, crowns, and stolons, comprise a rich flora of dematiaceous fungi that apparently function as secondary invaders. Such saprophytic fungi are particularly common on turf affected by spring dead spot in the United States (Smith et al. 1989)

Signs of *L. Korrae* and *L. narmari* are evident on infected plants as dark brown mycelia, mycelial aggregates, and flattened, lens-shaped sclerotia (Walker and Smith 1972). Pseudothecia of *L. narmari* develop within leaf sheaths or form superficially on stolons (Smith et al. 1989). They are black, flask-shaped, and occur singly or in clusters. The necks are lined with upwardly projecting periphyses and often have two thickened ridges of cells at the base. Asci are clavate (with a foot-shaped base), bitunicate, and eight-spored. Ascospores are biseriate, pale brown, elliptical to fusiform, and have three to seven septa. No conidial state of *L. narmari* has been found in the field or in culture. Runner hyphae on host tissue are brown, septate, and branched (Smith et al. 1989).

G. graminis var. graminis also produces dark-colored, ectotrophic mycelia. In contrast to the other casual agents of this disease, *G. graminis var. graminis* produces lobed hyphopodia that are formed abundantly on the surfaces of living leaf sheaths and stolons. Perithecia of this fungus are often formed between diseased leaf sheath, like hyphopodia, are visible with a hand lens (Smith et al. 1989).

O. herpotricha belongs to one class of ectotrophic root-infecting fungi associated with patch diseases of turfgrasses. The taxonomy of *O. herpotricha* is listed in Table 1. Pseudothecia of *O. herpotricha* are occasionally found on host tissue in the field (Walker 1981). They are dark brown or black, spherical or flattened at the base, and have a neck that often protrudes through the leaf sheath of the host. Numerous dark brown, thick-

walled hairs surround Pseudothecia. Asci are bitunicate, cylindrical to club-shaped, and are surrounded by hyaline pseudoparaphyses. Ascospores are long, filiform, yellow to pale brown, eight-spored, and contain eight to 20 septa. The ascospores are parallel or loosely twisted within the ascus. Pycidia of *O. herpotricha* are rarely produced in culture. Webster and Hudson (1957) did produce the pycnidial state of *O. herpotricha* on oat agar after an incubation period of 6-10 week. When present, pycnidia resemble the ascocarp and are 200-550 um in diameter. Conidia are cylindrical and contain five to six septa. The spores have a truncate base and taper to a bristle-like apex. Runner hyphae are dark brown, septate, and branched. Hyphae may produce intercalary hyphopodia on host tissue (Tisserat et al. 1989). *O. herpotricha* produces a white, cottony mycelium on potato dextrose agar and malt agar. Cultures turn tan to brown in 3-7 days. Optimum growth ranges from 3.5-4 mm per 24 hour at 20-25 °C (Walker 1981). Tisserat et al. (1989)reported the formation of fertile pseudothecia on stolons and crowns of inoculated bermudagrass plants in the greenhouse. All attempts to initiate pseudothecia of *O. herpotricha* in culture have not been successful (Tisserat et al. 1989).

Table 1. Taxonomy Classification of *O. herpotricha* (Landschoot 1980)

Division: Amastigomycota
Subdivision: Ascomycotina
Class: Ascomycetes
Subclass: Loculoascomycetidae (Loculoascomycetes)
Order: Dothidiales (Pleosporales)
Family: Pleosporaceae
Genus: *Ophiosphaerella*
Species: *O. herpotricha*

Identification of casual organisms of SDS.

Helminthosporium spiciferum is frequently associated with rotted roots in the spring dead spot disease of bermudagrass in 1960's. Wadsworth in Oklahoma State University conducted experiment and showed *H. spiciferum* could be the pathogen that caused spring dead spot of bermudagrass. Smith (1971) conducted the experiments to prove the identity of the casual organism of spring dead spot of bermudagrass in New South Wales. Single ascospore of the *L. narmari* was isolated from bermudagrass and grew on PDA medium at 25 °C for inoculating the seedling and turf. Almost 15 years later, by identifying the signs and symptoms of the spring dead spot on bermudagrass, Endo and Kraussman (1985) concluded that at least one cause of the SDS disease in California is *L. korrae*. McCarty and Lucas (1989) obtained three isolates of a dark-gray, slow-growing fungus isolated from bermudagrass cultivar Tifway with spring dead spot symptoms and tested them for pathogenicity on bermudagrass. One isolate produced typical SDS symptoms on bermudagrass when potted plants were inoculated in the fall and grown outside during the winter. The type of ascus and the length of ascospores produced by the fungus isolated from bermudagrass indicated that the fungus was *Gaeumannomyces graminis (Sacc.) v. Arx.& Oliver var. graminis* by J. Walker (Walker 1981). Even *L. Korrae or L. narmari* are the common causal agent for bermudagrass in many Southern U. S. location, researchers could not recover these two fungi in Kansas from any bermudagrass affected with SDS even numerous attempts have been made (Tisserat et al. 1989). Tisserat et al. (1989) conducted experiment to determine the cause of SDS in Kansas. Diseased bermudagrass roots and stolons were collected from SDS-affected patches, washed in water for 30 min, and finely chopped. The debris was placed

in pot containing vermiculite. Wheat seed was germinated in the pots, and roots were allowed to grow through the debris. After 10-21 days, plants were removed form pots and the roots were gently washed with water to remove the vermiculite. Root pieces were examined microscopically for the presence of ectotrophic hyphae, then surface-sterilized and placed on PDA with lactic acid. Ectotrophic fungi were isolated from diseased roots and maintained on PDA at 25 °C under cool-white fluorescent light. Two ectotrophic fungi, *O. herpotricha* and *G. incrustans* Landschoot & Jackson, were consistently isolated from roots and stolons of bermudagrass affected with SDS in Kansas. In preliminary experiments, only *O. herpotricha* caused extensive root discoloration. *G. incrustans* did not cause extensive root rot or root weight loss of bermudagrass in greenhouse trials. The relationship of *O. herpotricha* and *G. incrustans* to SDS is unclear.

Ophiosphaerella herpotricha is the primary cause of SDS in the Southern Great Plains region including Kansas, Oklahoma and Texas (Tisserat et al. 1989). It has been found that *O. herpotricha* is very active in the fall and in the early spring under cool moist conditions. It may infect bermudagrass whenever soil temperatures are between 10 and 25 °C (Endo et al. 1985). In a growth chamber study (McCarty and Lucas 1988), when compared to uninfected turf, re-growth of infected turf was reduced by 95% following exposure to –5 °C. Furthermore, foliar injury caused by *O. herpotricha* was enhanced as low temperature stress increased. *O. herpotricha* produces dark-colored, ectotrophic mycelia, occasionally with bubble-like structures on roots, stolons, and rhizomes of grasses, partially immersed in dead leaf sheath and stolen tissues have also been observed on bermudagrass affected with SDS (Tisserat et al. 1989).

Although these fungi can be differentiated on the basis of ascospore morphology, ascocarps rarely occur on naturally infected turfgrass and cannot be induced easily in the laboratory. Other physiological and morphological characteristics of these fungi in culture, including colony color and texture, are not unique and cannot be consistently used for differentiation. Recent development of monoclonal antibodies (Nameth et al. 1990) and DNA probes (Tisserat et al. 1991) for *L. Korrae* and DNA amplification techniques for *G. g. var. graminis* (Schesser et al. 1990) has improved identification of these fungi. Tisserat group selected a 1.5-Kb clone from a genomic library as probe to identify *O. herpotricha* because of its specificity and strong hybridization to the total DNA of 29 *O. herpotricha* isolates (Sauer et al. 1993). Later they also discovered using rDNA internal transcribed spacer (ITS) regions to detect *O. herpotricha* and *O. Korrae* (Tisserat et al. 1994). The ITS regions of the rDNA of *O. herpotricha* and *O. Korrae* were amplified with the universal primers ITS4 and ITS5. Amplifications of genomic DNA from *O. herpotricha* isolates always resulted in a single 590-bp fragment. Primers specific for *O. herpotricha* were derived form sequence analyses of the ITS regions and specified amplified DNA of *O. herpotricha* but not from DNA of other fungal or bacterial species. These primers can be used to rapidly diagnose turfgrass patch diseases caused by *O. herpotricha* without culturing the fungi from diseased tissue.

HOST-PATHOGEN INTERACTIONS

Each pathogen has evolved a specific way to invade plants. Some species penetrate surface of plant cell directly by using mechanical pressure or enzymatic attack. Others pass through wound and natural openings, such as stomata or lenticels. Three main attack strategies are developed by the pathogens once they are inside the plant: necrotrophy, in which the plant cells are killed; biotrophy, in which the plant cells remain alive; and hemibiotrophy, in which the pathogen initially keeps cells alive but kills them at later stages of the infection (Buchanan et al. 2000).

Studying the interactions between plants and pathogens should lead us to practical solutions for the control of plant disease in agricultural crops, help elucidate the signaling mechanisms by which plant cells imposed by stress conditions and discover how organisms from different kingdoms communicate with one another.

Plant defense systems.

Unlike animal cells, plants do not have mobility to escape from pathogen attack. However, only a very small proportion of pathogen infections are likely to result in a diseased plant. Besides environmental conditions impact, complex responses from host or non-host frequently save plants from destruction by pathogens.

Generally, the plant cell possesses both preformed and inducible defense strategy towards pathogen infections. Preformed defenses involve large amount of secondary metabolites. Many plants already have different secondary metabolites with antimicrobial properties. These compounds may either be present in their biologically active forms or in most of the case be stored as inactive precursors that are converted to

their active forms by host enzymes in response to pathogen attack or tissue damage. Saponins and glucosinolates are the two well-characterized classes of preformed inhibitors. A biologically active triterpenoid saponin was found in the roots of oat plants, which is highly against *Gaeumannomyces graminis var. tritici*, a serious pathogen for many cereal plant species. In contrast to saponins, glucosinolates, produced by members of Brassicaceae including *Arabidopsis*, become biologically active only in response to tissue damage by the activity of the enzyme myrosinase. Moreover, the concentrations of inhibitors presented in a plant cell are also affected by the pathogen attacking strategies. For example, necrotrophic fungi cause the release of high concentrations of inhibitors from the plants, whereas haustorium-forming biotrophic fungi may never encounter those defenses.

On recognition of the attacking pathogen for some plants, defense systems are activated rapidly. Only minutes are required for each plant cell to switch from normal primary metabolism to a multitude of secondary metabolism defense pathways and to activate novel defense enzymes and genes. Plant defense usually associated with elicitor and pathogen mediated induction of gene expression. Cascades of transcription factors may serve to amplify the input signal or modify the regulation of specific aspects of the complex plant defense response. Therefore, transcriptional activation of specific defense genes is important for the inducible resistance response. Many different trans-acting DNA elements are found involved in the regulation of plant defense genes. These trans-acting DNA-binding proteins are regulated by either a rapid increase in transcript concentrations at steady state or changes in their phosphorylation status. Some incompatible reaction involves host cell death to keep the invasion of the pathogen

localized, which called hypersensitive response (HR). The process ensures only the necessary numbers of plant cells are recruited from primary metabolism into a defensive role and creates unfavorable conditions for pathogen growth and reproduction. At the same time the responding cells detoxify and impair the spread of harmful enzymes and toxins produced by the pathogen. Hypersensitive response is such a fast reaction, which usually occurs within 24 hours of the pathogen attacking and leads either directly or indirectly localized cell death to prevent further spread of the pathogen.

Genetic basis of plant-pathogen interactions.

In the early 1900s, plant breeders recognized that resistance to plant pathogens was often inherited as a single dominant or semidominant trait. Later the inheritances of both plant resistance and pathogen virulence were elucidated. Flor (1947) proposed gene-for-gene model according to his genetic studies on flax and the flax rust pathogen in 1940s. In this model he predicts that plant resistance will occur only when a plant possesses a dominant resistance gene (*R*) and the pathogen expresses the complementary dominant avirulence gene (*Avr*). This model has been proved for most biotrophic plant-pathogen interactions (De Wit 1992; Alfano and Collmer 1996; Jones 2001). R proteins are predicted to have two basic functions: recognizing the corresponding *Avr*-derived signal, and activating downstream signaling pathways to trigger the complex defense response. Studies showed similar mechanisms are activated during R-Avr-mediated defense, non-host-induced resistance and in response to pathogen-derived elicitors. However, the mechanism of interaction between R and Avr protein for activation of plant defense responses is not understood.

Most plant R proteins have structural similarities. By now, many *R* genes were isolated from three monocots (rice, maize and barley) and eight dicots (Arabidopsis, potato, tobacco, tomato, flax, pepper, lettuce, beet) (Hulbert et al. 2001). Nearly all of these were found to belong to one of six main classes including TIR:NB:LRR, CC:NB:LRR, eLRRs, Serine- LRR kinase, Threonine Kinases and SA:CC (Jones 2001). These genes have resistance to a range of pathogens that are taxonomically unrelated. And most of them encode members of the nucleotide-binding (NB)-leucine-rich repeat (LRR) class.

Molecular mechanisms of plant defense responses.

Plants appear to induce a broad-spectrum defense response to most pathogens. With recognizing the pathogen infection, every cellular compartment is recruited into defense and multiple levels of defense reactions in each cell are activated by pathogen attack.

The first level of defense is the immediate responses of invaded cells, which including generation of reactive oxygen species, nitric oxide synthesis, opening of ion channels, protein phosphorylation/ dephosphorylation, cytoskeletal rearrangements, hypersensitive cell death (HR) and other gene inductions.

The HR results in rapid, localized cell death. This rapid response is thought to play a causal role in resistance to some pathogens by blocking biotrophs of access to further nutrients (Heath 2000). Potentially, two mechanisms underlie HR formation. Either the attacked cell initiates a regulated cell death program or the responding cells are rapidly poisoned by the toxic compounds and free radicals they have synthesized and

thus die as a result of necrosis. Recent evidence suggests that both types of cell death may occur during plant defense (Cole et al. 2001).

Reactive oxygen species (ROS) are produced during the early stages of a plant resistance response. ROS is often the first compounds detected, occurring within less than five minutes after pathogen attack (Vanacker et al. 2000). The typical ROS species detected are superoxide($O^{\cdot-}_2$) and hydrogen peroxide (H_2O_2). A plasma membrane-associated NADPH oxidase probably involves in superoxide production. ROS may play several roles in plant defense response. For example, H_2O_2 may be directly toxic to pathogens by producing the extremely reactive hydroxyl radical ($OH^{\cdot}$) with the presence of iron (Chamnongpol et al. 1998). Alternatively, it may contribute to the structural reinforcement of plant cell walls for more resistant to microbial penetration and enzymatic degradation, either by cross-linking various praline compounds to the polysaccharide matrix or by increasing the rate of lignin polymer formation due to the high peroxidase activity (Karkonen et al. 2002). Some ROS also fund to be important in signaling. For example, H_2O_2 induces benzoic acid 2-hydroxylase (BA 2-H) activity, which is required for biosynthesis of salicylic acid (SA) (Pasqualini et al. 2002). Production of ROS may also substantially alter the redox balance in the responding cells, in which the activity of specific plant transcription factors may be regulated by changes of the redox status (Willekens et al. 1997).

Although ROS production is one of the earliest responses to pathogen attack, it is usually insufficient to induce plant cell death. Nitric oxide (NO), a signal molecule used by mammals to regulate various biological processes, has the capacity to induce the plant cell death by ROS (Hancock et al. 2001). NO is known to bind heme and thereby could

inhibit catalase and ascorbate peroxidase,,which detoxify H_2O_2. These finding suggests that NO and ROS play an important synergistic role in the rapid activation of defense responses after pathogen infection. Some study also fund that adding a NO-generating compound to plant cell suspension cultures and leaves leads to the accumulation of mRNAs from several genes involved in defense and cell protection, which indicates the other roles of NO during plant defense (Bolwell 1999).

The second level of defense involves local responses and gene activation. The defense responses in this level are: alterations in secondary metabolic pathways, cessation of cell cycle, synthesis of pathogenesis-related (PR) proteins, accumulation of benzoic and salicylic acid, production of ethylene and jasmonic acid and fortification of cell walls.

Papillae, which are primarily composed of callose and lignin, often form directly beneath the fungi infection site. This structure is thought to act as a physical barrier to block fungal penetration into plant cells. Hydroxyproline-rich glycoproteins (HRGPs) and polygalacturonase-inhibition proteins (PGIPs) are two classes of defense-related extracellular proteins by cell wall reinforcement (Kuc 1990). Preformed HRGPs cross-link rapidly to the wall matrix by reacting with induced H_2O_2. *De novo* HRGP synthesis initiates additional lignin polymerization to further reinforce the cell walls (Davies et al. 1997). PGIPs carry a LRR motif and inhibit a subclass of necrotrophic pathogen cell wall-degrading enzymes, called polygalacturonases (PGs) (Powell et al. 2000).

Benzoic acid (BA) and salicylic acid (SA) are fund in glucoside conjugates and accumulate to high concentrations in the incompatible infection sites (Pasqualini et al. 2002). Both SA and BA are derived from the phenylpropanoid pathway and have many

roles in plant defense responses. A bacterial nahG gene encoding salicylate hydroxylase, the enzyme that converts SA to catechol, was introduced into plant to reduce the producing of SA. The lacks of SA accumulation in these nahG plants abolish resistance phenotype, which indicates the absolute requirement for SA in some incompatible interactions (Donofrio and Delaney 2001).

Jasmonic acid (JA) and ethylene are required for defense against necrotrophic fungi and for induction of some plant defense genes (Jensen et al. 2002). JA is a hormone derived from oxygenated linolenic acid. Increases in JA in response to pathogen/insect attack occur both locally and systemically. Studies showed spraying methyl-JA onto plants increases their resistance to some necrotrophic fungi but not to biotrophic fungi or bacteria. In addition, a subset of the inducible plant defense genes also require a JA-dependent, SA-independent signaling pathway (Leon et al. 2001).

The gaseous hormone ethylene is frequently synthesized during both incompatible and compatible interactions (Rojo et al. 1999). Although investigators have found that ethylene is apparently not required for several *R-Avr* gene-mediated resistance responses, it is required to mediate both resistance against necrotrophic fungal pathogens and non-host resistance against soilborne fungal species, which are unordinary plant pathogens. Another proven role for ethylene in defense is in combination with the signal molecule JA, both of which are required for activation of proteinase inhibitor (PI) genes and certain PR and chitinase genes (Lindsey et al. 2002).

The third level of defense is systemic responses and gene activation, which is also the latest response of the plant for pathogen attack. Large amount of PR proteins such as β-glucanases, chitinases, and peroxidases and other PR proteins are synthesized and

secreted towards the pathogen infection site. In addition, a range of secondary signaling molecules are generated to ensure coordination of the defense response.

PR proteins include chitinases and glucanases, enzymes that degrade structural polysaccharides of fungal cell walls and may reduce fungal growth. Studies showed the transcripts of PR and defense related genes accumulated in incompatible interaction after pathogen attack or elicitor treatment, and same genes are also induced in compatible interactions but much more weakly and slowly (Rauscher et al. 1999). SA mediated signal transduction cascades regulate the transcriptional activations of many PR genes. Ethylene and SA have been shown to act synergistically to further enhance the expression of PR genes (Heil and Bostock 2002).

Lipoxygenase, another PR proteins, may generates secondary signal molecules such as JA and lipid peroxides and produces toxic volatile and nonvolatile secondary metabolites with substantial antimicrobial activity (Bate and Rothstein 1998).

Plant defensins are the third type of defense-related genes with antimicrobial activity. They have similar structure and function with mammal defensin peptides produced after microbial attack (Thevissen et al. 2000). The conserved structural and functional relationship suggests the existence of an ancient conserved strategy involving production of defense compounds in response to microbial attack. The signaling pathway that controls accumulation of plant defensin is mediated by ethylene and JA, but not SA (Park et al. 2002).

Another group of antimicrobial compounds that accumulate rapidly at sites of incompatible pathogen infection are phytoalexins. They are low molecular mass, organic, or inorganic secondary metabolites (Bonhoff et al. 1986). For example,

phenylalanine is diverted into the synthesis of various flavonoid phytoalexins by synthesis of phenylalanine ammonia lyase (PAL), an enzyme that controls a key branch point in the phenylpropanoid biosynthetic pathway (Kamo et al. 2000). Because the synthesis of most phytoalexins requires the activities of numbers of biosynthetic enzymes, the highly coordinated signal transduction events are required. Although primarily discovered in 1940s, the exact role for most phytoalexins in plant defense is unknown.

For each host-pathogen interaction, specific cellular protection mechanisms are activated simultaneously and accompany the defense response to minimize the damage to host cells. Synergies, antagomisms and feedback loops exist both within and between signaling pathways and metabolic pathways to create a complex network that ensures tight coordination of individual defense response. In addition, the plant defense signal transduction network may cross-talk with other plant stress response pathways.

Systemic acquired resistance.

Defense responses are also sometimes elaborated in tissues far from the invasion site and even in neighboring plants. The type of systemic response induced is determined by the identity of the pathogen. Fungi, bacteria, and viruses activate systemically a specific subset of PR-type genes by systemic acquired resistance (SAR) mechanism. For SAR to occur, the initial infection must result in formation of necrotic lesions. Thus, SAR converts genetically compatible plant-pathogen interactions into incompatible ones (Dong 2001).

Various synthetic chemicals induce SAR. Two of the most potent are 2,6-dichloroisonicrotinic acid (INA) and benzo-(1,2,3)-thiodiazole-7-carbothionic acid S-methyl ester (BTH). It has been found that both INA and BTH act independently or downstream of SA in SAR signaling (Molina et al. 1999).

FUNGAL INFECTION OF PLANT CELLS

Fungal pathogenesis.

Pathogenic fungi attack all of the three million flowering plant species. However, a single plant species can be host to only a few fungal species. In turn, most fungi usually have a limited host range. The high degree of specialization of fungal pathogen for individual plant species may be reflected in the different levels of specialization observed in extant plant-fungal interactions (Scheffer 1991). Fungal plant pathogens use a wide range of pathogenesis strategies. The first level may be seen in opportunistic parasites, which enter plants through wounds or require otherwise weakened plants for colonization. A broad host range but a relatively low virulence usually characterizes these fungal species, so that they cause only mild disease symptoms. The next level comprises true pathogens that rely on living plants to grow but that under certain circumstances can survive outside of their hosts. Many of the more serious plant pathogens are found at this level; most are highly virulent on only a limited number of host species. Finally, the highest level of complexity is achieved by obligate pathogens, for which the living host plant is an absolute prerequisite to fulfill their complete life cycle.

Compared with bacteria and viruses using natural openings or wounds for invasion, many true phytopathogenic fungi have evolved mechanisms to actively traverse the plant's outer structural barriers, the cuticle and the epidermal cell wall. Fungi generally secrete a mixture of hydrolytic enzymes, including cutinases, cellulases, pectinases, and proteases to make entrance on plant cell. Because these enzymes are also required for the saprophytic lifestyle, they are unlikely to represent the tools specifically

developed by fungi to implement pathogenesis, and each individual hydrolytic enzyme may not be absolutely necessary for penetration. However, the structure and biosynthetic regulation of these enzymes may be specific needed for a pathogen on a particular host plant.

The cuticle covers the aerial parts of living plants and needs to be pierced before other pathogenetic mechanisms can become effective. Therefore, enzymatic degradation of cutin, the structural polymer of the plant cuticle, has been proved as crucial for fungal pathogenicity, and cutinase is presumed to be a key player in the penetration process (Kolattukudy 1985). In addition, cutinase may also be involved in pre-penetration processes, for example, by altering the adhesive properties of the cuticle and thus facilitating fungal attachment to plant surfaces (Nicholson and Epstein 1991) or by releasing signal molecules required for early fungal development on the plant (Kolattukudy et al.1995).

Combined with hydrolytic enzymes, some fungi have developed a more complex and sophisticated mechanism to penetrate the cuticle of host plants. Phytopathogenic fungi form specialized penetration organs "appressoria" at the tip of their germ tubes, these organs are firmly attached to the plant surface by extracellular adhesives. As it develops, the porosity of the aspersorium wall of mechanically penetrating fungi is markedly reduced by melanin incorporation, allowing high turgor pressure (>8 megapascals; Howard et al. 1991) to build up inside. This pressure is focused effectively on a small area at the base of the appressorium that is kept free of wall material and melanin. From this penetration pore, an infection peg develops and pierces through the cuticle and cell wall, possibly assisted by hydrolytic enzymes (Mendgen and Deising

1993). Studies of the rice blast fungus *Magnaporthe grisea* have illustrated the importance of melanin for infection peg penetration; melanin-deficient mutants are unable to infect intact plants, but some mutants retain pathogenicity on leaves with wounded epidermis (Chumley and Valent 1990; Kubo and Furusawa 1991). Furthermore, melanized appressoria of *M. grisea* were capable of pushing penetration pegs through plastic membranes (Howard et al.1991). These results suggest that melanin is an essential factor for mechanically penetrating fungi.

Other fungal species, including some rusts, have not evolved a direct penetration mechanism and instead bypass the plant cuticle and outer cell wall by entering through the stomata. The mechanisms of these fungi locate stomata openings on the plant surface are not understood (Correa and Hoch 1995). Thus, penetration is likely to be controlled by a combination of different factors, which may include plant surface structures as well as activators or inhibitors of fungal spore germination and germ tube formation.

Plant defense to fungal infection.

Genetic analyses demonstrated that pathogen recognition is often determined by the interaction of plant resistance genes (R gene) with single avirulence genes of the pathogen (Avr gene) (Flor 1971). This gene-for-gene hypothesis may be interpreted in biochemical terms as the interaction of a race-specific pathogen elicitor with either a cultivar-specific plant receptor or alternatively with a cultivar-specific signal transduction compound (Keen 1990). In other words, resistant plant cultivars are capable of utilizing specific features of pathogen races to trigger their defense response.

Several fungal resistance genes including tomato Cf-9 have been cloned and found to encode proteins with putative secretory signal sequences, single trans-membrane domains, and short cytoplasmic tails, indicating their membrane-anchored extracellular localization. In addition, a role of the gene products in recognition is suggested by the occurrence of leucine-rich repeats in the putative extracellular domain. Studies with AVR9 revealed high-affinity binding sites on plasma membranes isolated from Cf-9 plants.

The molecular bases for recognition of potential pathogens by plants outside of gene-for-gene systems are poorly understood. Plants may recognize an aggressor through non-self factors that are present on the fungal surface (e.g., chitin and glucan fragments) or are secreted by the pathogen (e.g., proteins) and/or through self-determinants such as plant cell wall fragments (e.g., oligogalacturonates) that are released by an invading pathogen through the activity of hydrolytic enzymes. After recognition of the pathogen, a multitude of plant resistance-associated reactions is initiated. From a number of fungi, molecules have been isolated that trigger some of these plant defense reactions. These compounds are called elicitors (Knogge 1996). Although some of the more general elicitors such as oligo-N-acetylglucosamines and oligogalacturonates are active in several plants, others appear to be species specific (Ebel and Scheel 1996).

Chitin, a polymer made of ß-1, 4-linked N-acetylglu-cosaminyl residues, is the most important structural component of the cell wall of fungi (Ruiz-Herrera 1992). The polymer contributes to the structural rigidity and osmotic integrity of the fungal cell wall. In addition, the temporal and spatial regulation of cell wall polymer synthesis plays an important role in morphogenesis during fungal growth and development. For instance,

hyphae grow filamentously as a result of the deposition of wall polymers at the hyphal apex, which will later form the fungal infection cushions. Defense responses or related cellular response in many monocots, and some dicots, can be induced by chitin oligomers generated from fungal cell walls by endochitinase (Ishihara et al. 1996; Kaku et al. 1996). Purified chitin fragments can induce phytoalexin biosynthesis and various cellular responses in rice (Yamada et al. 1993; Nojiri et al. 1996; Minami et al. 1996). The rapid and transient nature of some of these cellular responses suggests their involvement in the signal transduction cascade (Stacey and Shibuya 1997). Furthermore, the transcription rates of numerous plant genes have previously shown to be strongly affected by pathogen infection or elicitor treatment (Batz et al. 1998). Oligosaccharide products from fungal cell wall degradation may also act as signal molecules that elicit plant defense mechanisms.

An interesting group of small proteinaceous elicitors, termed elicitins, are secreted by species of *Phytophthora* that cause diseases on various plants (Yu 1995). Because elicitins were also found to be produced by another *Oomycete, Pythium vexam*, they may be ubiquitous in this fungal class (Huet et al. 1995). The purified proteins induce necrosis and other defense reactions at the site of application but also distally after their translocation, thus mimicking the effects of fungal infection. In addition, they trigger SAR in tobacco and other Solanaceous species (Ricci et al. 1989).

In the most common model, elicitor activity is explained by binding to a specific cell surface-localized plant receptor that initiates a defense-related signal transduction cascade. The fungus, in turn, may produce a compound, suppressor, to retain the function of elicitor. This suppressor may interfere directly with elicitor binding, signal

transduction, gene activation, or the activity of defense factors from the plant. However, many research data indicated that the suppressors might not function simply by inhibiting elicitor binding to a receptor in plant cell membranes but rather by affecting the signaling pathway that leads to the activation of the resistance response (Wada et al. 1995).

Chitinases are known extensively for their significant roles in inhibiting fungal ingress by attacking the chitin-containing cell wall of the fungi (Graham and Sticklen 1994). Baird et al. (1998) found that bermudagrass crown tissues from SDS-inoculated field plots had higher total chitinase activity compared to those from un-inoculated areas. Glucanases are involved in pathogen resistance in a manner similar to the chitinases. These two enzyme systems can act synergistically (Jach et al. 1995; Jongedijk et al. 1995). Both enzyme types are expressed as multiple isozymes, each isozyme with a defined function and specificity. Transgenic plants over-expressing other plant chitinases or glucanases show a higher degree of resistance compared to non-transformed plants (Lin et al. 1995; Neuhaus et al. 1992).

Some chitinases and glucanases are also known to express antifreeze activity that is believed to prevent damaging re-crystallization of ice in sensitive plant tissue during freeze-thaw conditions (Hon et al. 1994). It was hypothesized that this cold-induced chitinase might also be involved in plant resistance against fungal attack during cool conditions when the fungus is most active. For many years the close association between cold tolerance and SDS resistance was strongly suspected but never proved. The recent research revealed a strong positive relationship between SDS resistance and cold tolerance in 26 field-grown bermudagrass varieties (Baird and Martin 1996). Varieties that were cold tolerant also appeared to have a high level of SDS resistance. In addition,

Gatschet isolated a 27KD protein (COR protein) from bermudagrass that was highly expressed during prolonged cold temperature acclimation and at higher levels in a cold tolerant variety than in a cold sensitive one (Gatschet et al. 1996). This COR protein was identified as a type b, class II chitinase based on its significant homology to plant chitinases or chitinase precursors (Gatschet et al. 1996). The close association suggests a common molecular mechanism between cold tolerance and SDS resistance. The dual role of chitinases in disease resistance and cold acclimation may help to explain this intriguing relationship.

Other pathogenesis-related (PR) proteins have also been detected based on their defense response. A PR protein has been localized to plasmodesmata between maize parenchyma cells infected with fungal pathogen (Murillo et al. 1997). The distribution of this protein suggests its function in host defense by preventing molecular trafficking between plant cells. A mitogen- or stress-activated protein kinase (M/SAPK) cascades also appear to be involved in responses to wounding and to stimuli such as fungal elicitors (Zhang et al. 1998). M/SAPK-like activity has been demonstrated in tobacco cells treated with a fungal elicitor and in a number of monocot plant species after leaf wounding (Mizoguchi et al. 1997; Stratmann and Ryan 1997). Furthermore, plant inhibitors of fungal enzymes also form part of the plant defense system. Hahn et al. (1989) found that fungal endopolygalacturonase is inhibited in dicotyledons by a polygalacturonase-inhibiting protein.

Another frequently observed response of plants to fungal attack is modification of the plant cell wall adjacent to the invading pathogen (Hardham and Mitchell 1998). There are many examples showed the alternation of the existing wall or the formation of

papilla or wall apposition between the plasma membrane and the existing wall because of the fungal infection (Aist and Bushnell 1991, Smart 1991).

GENE CLONING BY SUBTRACTIVE HYBRIDIZATION

The basis of subtractive hybridization.

Subtractive hybridization is an attractive method for enriching differentially expressed genes. This method was first used by Bautz and Reilly (1966) to purify phage T4 mRNA in the 1960's. In 1984 Lamar and Palmer applied a subtractive hybridization technique to clone probes for the Y chromosome. Since then subtractive cDNA hybridization has been widely utilized to identify and study cDNAs of differentially expressed genes (Agron et al. 2002; Akopyants et al. 1998, Heinrich et al. 1997). This method involves hybridization of cDNA from one sample population (tester) to excess of mRNA (cDNA) from other sample population (driver) and then separation of the unhybridized fraction (target) from hybridized common sequences (Diatchenko et al. 1996). Pure subtractive methodologies are of limited use due to the need for a large quantity of mRNA to drive hybridization to completion as well as the difficulty in cloning the tiny amount of cDNA remaining after hybridization. The method was greatly improved when Duguid and Dinauer (1990) adapted generic linkers to cDNA allowing the selective PCR amplification of tester cDNA between hybridization cycles. Then several cDNA subtraction methods were established. Representational difference analysis (RDA), developed by Lisitsyn et al. (1993) to isolate differences in genomic DNA, has been successfully modified to use cDNA as starting material, thereby adapting to identify genes that are differentially expressed between two populations of cells (Hubank and Schatz 1994). Compared with previously used differential hybridization methods, the RDA technique reduces the complexity of physical separation of single-stranded (ss) and double-stranded (ds) cDNAs, resulting in a higher success rate.

However, RDA requires multiple rounds of subtraction, which is labor intensive and not well suited for the identification of rare messages (Hara et al. 1991). Diatchenko et al. further introduced the technique of Suppression Subtractive Hybridization PCR (SSH PCR) in which differentially expressed genes could be normalized and enriched over 1000-fold in single round of hybridization (Diatchenko et al. 1996). The recent commercialization of an SSH PCR kit "PCR-Select™ DNA Subtraction" by Clontech (CLONTECH Laboratories, Palo Alto, CA, USA) has lead to its increasing popularity in biological research laboratories. Many studies have applied SSH to detect the differentially expressed cDNAs (Kloos et al. 2002; Hinderhofer and Zentgraf 2001; Tkatchenko et al. 2000).

Suppression subtractive hybridization (SSH).

The SSH technique can selectively amplify differentially expressed cDNA fragments and simultaneously suppress non-target DNA amplification. This functionally removes those genes that are represented at equal levels in both states. The result is a pool of cDNAs that represents differentially expressed genes. This method is based on a new technique called suppression PCR described by Siebert et al. (1995). By using this technique, the amplifications of undesirable sequences are selectively suppressed in PCR procedures when long inverted terminal repeats are attached to DNA fragments (Siebert et al. 1995). SSH also combines subtraction and normalization in a single procedure, in which the normalization step equalizes the abundance of cDNAs within the target population and the subtraction step excludes the common sequences between the target and driver population. Therefore, SSH requires only one round of subtractive

hybridization, and can enrich the differentially expressed cDNAs over 1,000-fold (Diatchenoko et al. 1996).

Clontech PCR-Select™ DNA Subtraction is a revolutionary method for finding differentially expressed genes—those genes expressed in one mRNA population but reduced or absent in another. This method is particularly well suited for the identification of target cDNAs that correspond to rare transcripts, which are typically the most difficult to obtain.

Figure 1 details the molecular basis of PCR-Select cDNA subtraction. First, cDNA is synthesized from 0.5–2 ug of poly A^+ RNA from the two types of tissues or cells being compared. The tester and driver cDNAs are digested with *Rsa I*, a four-base-cutting restriction enzyme that yields blunt ends. The tester cDNA is then subdivided into two portions, and each is ligated with a different cDNA adaptor. One end of the adaptor is single-stranded and the other end is double-stranded. The double-stranded terminus is the end that is ligated to the cDNA. Neither terminal is phosphorylated, so only one strand of each adaptor (the 3' end) becomes ligated to the 5' end of each cDNA (via the phosphate group supplied by the end of the cDNA molecule). Once denatured, only one strand of the cDNA is actually attached to adaptor sequence.

Two hybridizations are then performed. In the first, an excess of driver is added to each sample of tester. The samples are then heat denatured and allowed to anneal, generating the type **a**, **b**, **c**, and **d** molecules in each sample (Fig. 1). The concentration of high- and low-abundance sequences is equalized among the type **a** molecules because reannealing is faster for the more abundant molecules due to the second-order kinetics of hybridization. At the same time, the ss type **a** molecules are significantly enriched for

differentially expressed sequences, as cDNAs that are not differentially expressed form type **c** molecules with the driver.

During the second hybridization, the two primary hybridization samples are mixed together without denaturing. Now, only the remaining equalized and subtracted ss tester cDNAs can re-associate and form new type **e** hybrids. These new hybrids are ds tester molecules with different ends, which correspond to the sequences of Adaptors 1 and 2R. Fresh denatured driver cDNA is added (again, without denaturing the subtraction mix) to further enrich fraction **e** for differentially expressed sequences. After filling in the ends by DNA polymerase, the type **e** molecules—the differentially expressed tester sequences—have different annealing sites for the nested primers on their 5' and 3' ends.

The entire population of molecules is then subjected to PCR to amplify the desired differentially expressed sequences. During PCR, type **a** and **d** molecules are missing primer-annealing sites, and thus cannot be amplified. Due to the suppression PCR effect, most type **b** molecules form a pan-like structure that prevents their exponential amplification (see Appendix II for more details). Type **c** molecules have only one primer annealing site and can only be amplified linearly. Only type **e** molecules, which have two different adaptors, can be amplified exponentially. These are the equalized, differentially expressed sequences.

Next, a secondary PCR amplification is performed using nested primers to further reduce any background PCR products and to enrich for differentially expressed sequences. The cDNAs can then be directly inserted into a T/A cloning vector. Alternatively, the Not I (Sma I, Xma I) site on Adaptor I and the Eag I site on Adaptor

2R can be used for site-specific cloning, or the Rsa I site at the adaptor/cDNA junction can be used for blunt-end cloning. Then, differentially expressed RNAs can be identified by sequence and hybridization analysis.

Because the total RNA will produce an excess of cDNA sequences corresponding to ribosomal RNA, it is not suitable for this PCR-based subtraction method. However, this problem can be largely eliminated if the total RNA is pre-amplified to generate mRNA prior to subtraction. In general, the maximum amount of RNA recommended for use is 2.0 μg, performing the procedure with more than 2.0 μg of RNA will not significantly improve the results. However, due to the low purity of plant RNA, Clontech recommend starting with 4 μg poly A^+ RNA for each source of the plant RNA samples. A minimum of 0.5 μg poly A^+ RNA can also be used. However, starting from less than 0.5 μg of RNA will fail to yield a high quality preparation of cDNA. This is a potential disadvantage of the SSH technique, which begins with a few micrograms (2 ug) of mRNA from the two cell populations are needed. In some special cases such quantity of RNAs may be difficult to obtain. Even a pre-amplification step can be incorporated to generate sufficient quantities of both cDNA samples, it may result in the loss of some sequences and produce higher background (Diatchenko et al. 1996).

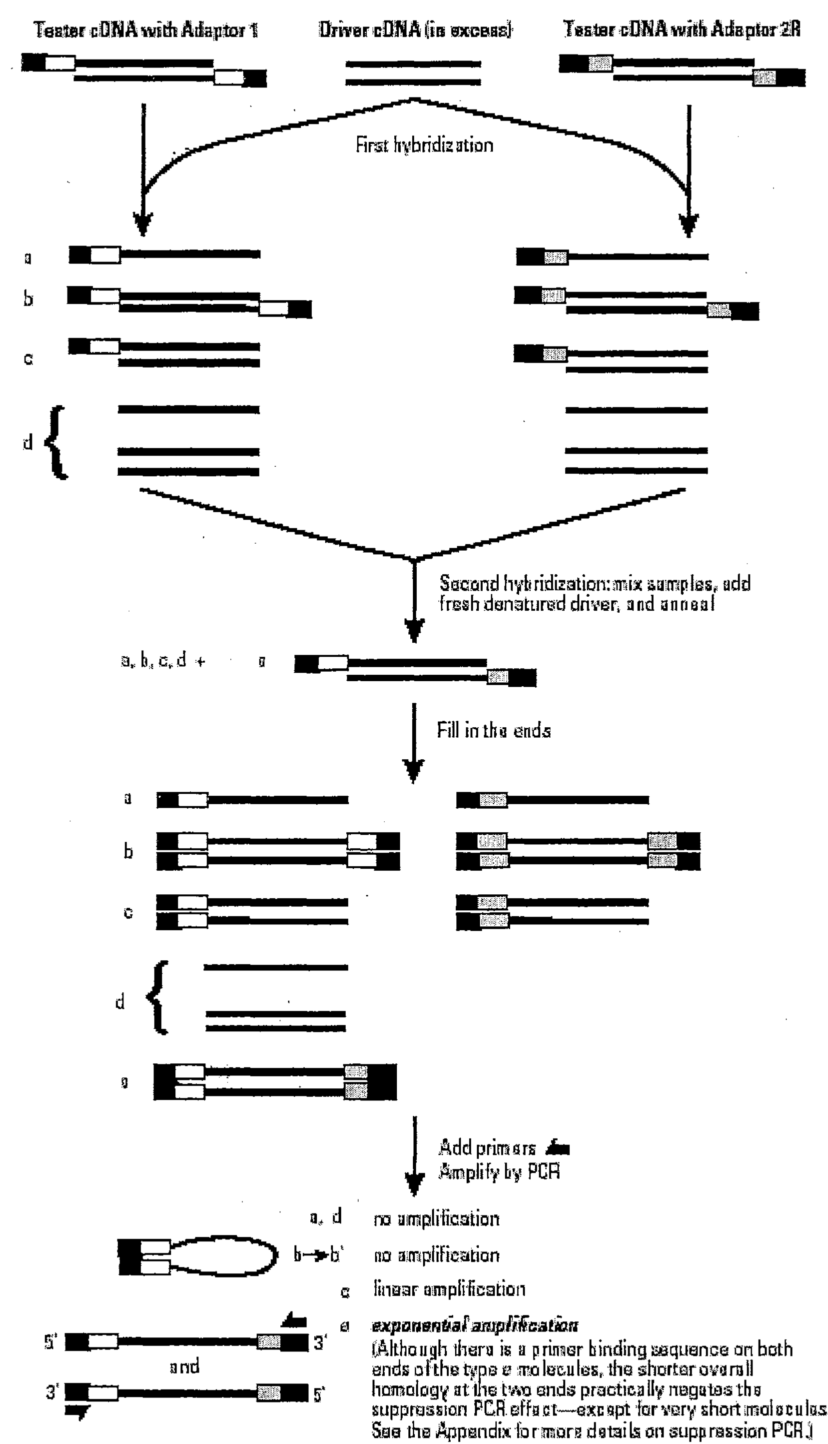

Fig. 1. Overview of the Clontech PCR-Select^{TM} procedure (Clontech User Manual)

Interpretation of SSH results need to be carefully due to the non-differentially expressed genes present in the subtraction library. This may not result from experimental error but maybe caused by the absence of significantly differentially expressed genes between the chosen driver and tester samples. The failure of a SSH PCR library to include a known differentially expressed mRNA may also not be a result of experimental error.

The highly complex samples with different number of cell types and percentage of target cells in the entire cell population may cause problems in performing successful SSH. When a tissue consists many different cell types, RNA purified from this tissue contains an increased number of independent mRNA species. Such a highly complex poly(A)$^+$RNA sample decreases the efficiency of subtraction (Desai et al. 2000). Therefore, SSH cannot be used to subtract genomic DNA, which is usually highly complex. This problem can be solved by representational difference analysis (RDA) by repeated rounds of hybridization and PCR. The repeated hybridization cycles in RDA efficiently enrich the most abundant differentially expressed genes between tester and driver in each round. However, the final subtracted cDNA population consists of only a few sequences. In contrast, SSH generates a more complex population of subtracted cDNA fragments, and the final subtracted pool of cDNAs appears as a smear with or without a few bands on a 2% agarose / EtBr gel (Clontech Manual). This smear reflects the high number of independent clones in the subtracted library.

For efficient subtraction, the SSH method can result in up to a 10,000-fold enrichment of differentially expressed cDNAs theoretically (Gurskaya et al. 1996). Sequences that are most highly enriched during in this PCR-based subtraction represent

genes which are 'turned on" in one state and 'off' in the other (i.e., cDNAs that are present in tester but not in driver). Nevertheless, researchers have used the PCR-Select technique to identify many clones representing genes with 1.5- to 5-fold differences in expression (Gurskaya et al. 1996). As a general rule, PCR-Select is best used to identify genes with at least a five-fold difference in abundance between tester and driver RNA. However, if the tester and driver poly A^+ RNA contain genes with large (e.g., 1,000-fold) and small (e.g., two-fold) differences in abundance, the PCR-Select method will be biased towards the enrichment of genes displaying larger differences in expression.

The incubation time for the first hybridization should be 6–12 hours. If the first hybridization exceeds 12 hours, all of the single-stranded tester molecules will eventually hybridize with one another, and will not be available to form double-stranded hybrids during the second hybridization. If the first hybridization is too short, the ss tester fraction will not be completely equalized, and rare, differentially expressed genes will be lost. The second hybridization should be longer than the first, since the ss tester molecules are present at a very low concentration and require more time to form ds hybrids. The second hybridization may proceed for 12-24 hours. Decreasing the length of both the first and second hybridization steps result in recovery of high-abundance differentially expressed genes with a low background of non-differentially expressed cDNAs. However, this also decreases the probability of obtaining low-abundance differentially expressed genes. Moreover, if the tester does not contain high-abundance differentially expressed genes, decreasing the hybridization time will lead to a higher background. Similarly, increasing the driver: tester ratio will lead to a higher

background, especially if tester and driver lack the mRNAs with significant difference in transcription level.

The stringency of subtraction can be altered by changing the ratio of driver : tester. Increasing the driver: tester ratio allows preferential enrichment of those genes that are highly up-regulated in tester compared to driver and also decreases the background of non-differentially expressed clones in the subtracted library. Nevertheless, cDNAs with smaller differences in expression will be lost during the subtraction as the driver: tester ratio is increased. Likewise, decreasing the ratio allows the identification of those genes that are only slightly up regulated in tester compared to driver; however, a low driver: tester ratio will also increase the occurrence of false positive clones.

Discovering differentially expressed genes by SSH.

Because SSH PCR favors highly differentially expressed genes, the primary application of SSH PCR should be to detect dramatic alteration of gene expression, such as comparison of gene expression after stress condition or gene expression profiling of two different tissues. Although the utilization of SSH is more intensive and advanced in medical studies (Gardmo et al. 2002; Porkka and Visakorpi 2001; Zylka and Reppert 1999), researchers interested in differential gene expression of plants have begun to utilize this technique. Many studies using suppression subtractive hybridization have been published recently.

For example, SSH was used to isolate large numbers of low temperature-induced genes from the cold-treated winter barley (Bahn et al. 2001). One hundred and sixty *blti* (barley low temperature-induced) cDNA clones were obtained. Northern blot analyses

showed that several *blti* clones were differentially expressed by treatment of low temperature, NaCl, dehydration and ABA. Nucleotide sequences showed no sequence homology with the previously reported low temperature-responsive (LTR) barley genes, and the deduced amino acid sequences revealed that one gene *blti2* contains three membrane-spanning regions. These results suggest that *blti2* is a novel transmembrane protein induced by low temperature (Bahn et al. 2001).

Hinderhofer and Zentgraf (2001) used SSH studying the differential expression of genes during leaf senescence in *Arabidopsis thaliana (L.) Heynh.* After high-throughput screening, several differentially expressed cDNA clones were isolated from SSH library, including a transcription factor of the WRKY family, WRKY53. This transcription factor contained the WRKY domain, a 60-amino-acid domain with the conserved WRKYGQK motif at the N-terminal end, together with a novel zinc-finger motif. The mRNA level of WRKY53 increased substantially within the rosette leaves of a 6-week-old plant, was constant in all leaves of a 7-week-old plant and decreased again in 8-week-old plants. This indicates that WRKY53 is expressed at a very early time point of leaf senescence and might therefore play a regulatory role in the early events of leaf senescence (Hinderhofer and Zentgraf 2001).

The suppression subtractive hybridization (SSH) method was also used to isolate developmentally regulated genes during carnation flower maturation (Kim et al. 1999). Carnation flower maturation-related clones obtained by the SSH were serially assigned as CFMI (carnation flower maturation-induced) clones. Northern blot analysis showed that several CFMI clones were differentially expressed during flower development. One of the clones, CFMI-3, showed similarity to various animal secretary phospholipases A2

(PLA2). Full sequence analysis reveals that the CFMI-3 contains a Ca^{2+} binding domain, a PLA2 active site, and 12 conserved Cys residues, which is a distinct characteristic of PLA2. Amino acid sequence alignment of CFMI-3 to various putative plant PLA2s confirmed that the CFMI-3 cDNA is the full-length putative PLA2 cDNA identified in plant species (Kim et al. 1999).

Capsaicinoids responsible for pungency of chili pepper are synthesized exclusively in the placenta tissue of the fruit. Kim et al. (2001) tried to understand elementary step in the capsaicinoid biosynthesis by studying differentially expressed genes in capsaicinoid biosynthesis pathway. A cDNA library was constructed from the placenta of a highly pungent pepper, *Capsicum chinense cv. Habanero* using the suppression subtractive hybridization (SSH). Thirty-nine cDNA clones from about 400 subtracted clones were selected through dot blot analysis and according to their nucleotides sequence. Sequence information of the chosen clones was evaluated by comparing it with DNA and protein databases. Results showed that the cDNA clones could be divided into 4 groups; cDNAs with similarities in genes encoding metabolic enzymes including acyl transferase and fatty acid alcohol oxidase (Group I), putative cell wall proteins (Group II), biotic and abiotic stress-inducible proteins (Group III), and lastly, cDNAs with no similarity (Group IV). Northern blot revealed that all cDNA clones were differentially expressed in pungent pepper. In addition, the cDNA clones of Groups I and IV were proved to be differentially or preferentially expressed in the placenta of pungent pepper (Kim et al. 2001).

SSH was also used to study plant pathogens gene expression. For example, Ectomycorrhiza development alters gene expression in the fungal and plant symbionts.

Voiblet and colleagues (2001) identified large number of genes expressed exclusively or predominantly in the symbiosis to study the development of the ectomycorrhizal symbiosis. They constructed a cDNA library of 4-day-old Eucalyptus *globulus-Pisolithus tinctorius ectomycorrhiza* and sequenced 850 cDNAs cloned randomly obtained through suppression subtractive hybridization (SSH). Based on the absence of a database match, 43% of the ectomycorrhiza ESTs are coding for novel genes. At the developmental stage analyzed (fungal sheath formation), the majority of the identified sequences represented 'housekeeping' proteins, i.e. proteins involved in gene/protein expression, cell-wall proteins, metabolic enzymes, and components of signaling systems. The cDNAs was then arrayed and screened to identify symbiosis-regulated genes by using differential hybridization. Comparisons of signals from free-living partners and symbiotic tissues revealed significant differences in expression levels (differential expression ratio >2.5) for 17% of the genes analyzed. No ectomycorrhiza-specific gene was detected. The results successfully provide the first global picture of the cellular functions operating in ectomycorrhiza, and demonstrate the use of the cDNA array combines SSH systems as general approach for dissecting symbiosis development (Voiblet et al. 2001).

THE STUDY OF PLANT GENE EXPRESSION WITH MICROARRAYS

Introduction of DNA microarray technology.

Controlling gene expression is one of the key regulatory mechanisms used by living cells. RNA-based gene expression study has provided clues to gene function for several decades. Measurement of mRNA levels has proven to be an important method although the final activity of a gene is determined by the encoded protein. Large amount of sequence data including complete genome sequences and ESTs (expressed sequence tags) from different organisms are available now days. However, sequence information alone is not sufficient for a full understanding of gene function. Direct monitoring of large numbers of mRNAs in parallel will provide valuable information about the expression and regulation of these genes. Therefore, the development of large-scale and genome-wide analyses of gene expression patterns is required to assign the function of the sequences. Several novel methods are currently available: 1). Sequence-based such as SAGE (Velculescu et al. 1995), MPSS (Brenner et al. 2000), 2). Fragment-based such as AFLP (Bachem et al. 1996) and 3). Hybridization-based such as macro- and microarrays (Schena et al. 1995, Lockhart et al. 1996, Desprez et al. 1998).

Microarray technology is a hybridization-based method combining miniaturization and the application of fluorescent dyes for labeling (Southern, 1996; O'Donnell-Maloney et al. 1996; Ginot, 1997). The utilization of fluorescent dyes helps combine two differently labeled samples in a single competitive hybridization experiment to reduce experimental error. Therefore, relative expression levels of large numbers of genes can be determined simultaneously with a high degree of sensitivity. Two major strategies for microarray have been developed to scale up the analysis of DNA samples.

In one format, oligonucleotides are synthesized *in situ* or by conventional means followed by immobilization of the chip. Originally, Stephen Fodor at Affymax, Inc. develops this method for the precise spacially directed synthesis of combinatorial peptide libraries (Fodor et al. 1991). As oligonucleotide arrays allow highly sensitive detection of DNA mismatches, they are well suited for DNA variation analysis as well. However prior sequence knowledge as well as complicated design and production methodologies are required for producing such arrays (Lipshutz et al. 1999). An alternative method, cDNA microarrays, allows a more flexible design for the fabrication of microarrays, in which pre-synthesized nucleic acids such as PCR-amplified cDNA clones are mechanically deposited onto a solid surface (Duggan et al. 1999). This methodology is originally developed in the laboratories of Patrick Brown and Ron Davis at Stanford and later commercialized by Dari Shalon at Synteni, Inc. (Schena et al. 1995; Shalon et al. 1996). The principle of the DNA microarray is the same as arrayed DNA samples on porous membranes (ie. dot blots), which are used routinely in molecular biology (Ross et al. 1992). With this method arrays can be produced containing up to a few hundred thousand distinct elements (Fodor et al. 1991).

The principal of assay used with both types of array is similar and based on the specific hybridization of a labeled sample to the immobilized nucleic acids (probe) on the array. As a result, the complex mixture of nucleic acids isolated from the biological sample under study is spatially separated into the specific mRNAs. The physical separation on the array then enables the individual quantification of many specific mRNAs in a single hybridization experiment. Furthermore, the independent detection of fluorescent signals at specific wavelengths allows simultaneous analysis of multiple dyes

and thus mixed samples. Once data are collected and normalized, expression ratios are obtained for each individual gene, representing relative expression levels for the samples investigated. Finally, biological meaning is obtained from the comparison between samples and genes across one or multiple experiments and the combination with related biological knowledge.

Expression profiling using microarrays is currently being performed for numerous organisms, including several plant species, using an assortment of biological samples. The scale of these experiments ranges from a few hundred genes to genome-wide coverage (e.g. *Saccharomyces cerevisiae*, Lashkari et al. 1997, *Drosophila melanogaster*, Zou et al. 2000, and *Caenorhabditis elegans,* Jiang et al. 2001).

Principle of cDNA microarray analysis.

Like other hybridization-based analysis methods in molecular biology, the specificity of microarray technology relies on the selective and differential hybridization of nucleic acids. In array-based methods, complex mixtures of labeled polynucleotides, such as cDNA derived from RNA, are hybridized with large numbers of individual elements (e.g. unique PCR products in cDNA microarrays) attached to a solid surface. Efficient expression analysis using microarrays requires the development and successful implementation of a variety of laboratory protocols and strategies for fluorescence intensity normalization. The process of expression analysis can be broadly divided into three stages: *(i)* probe preparation and array fabrication; *(ii)* sample preparation and hybridization; and *(iii)* data collection, normalization and analysis.

Applications of microarrays in plant gene expression profiling.

Microarrays are currently used for two main applications: gene expression studies and DNA variation analysis. Plant microarrays have been proved an important tool for research in plant science field. The systematic, nonbiased, accurate and large-scale acquisition of data using microarray technology enables new experimental approaches for plant molecular biologists. Microarray technology already provides a global overview of biological mechanisms that were investigated in a 'gene by gene' manner.

The most common use of these techniques is to determine patterns of differential gene expression or to compare differences in mRNA expression levels between identical cells subjected to different stimuli or between different cellular phenotypes or developmental stages. Recently, cDNA microarrays have been developed and used to quantify differential gene expression by hybridizing a complex mRNA-derived probe onto an array of PCR products (Schena et al. 1995; Lockhart et al. 1996; Welford et al. 1998; Schenk et al. 2000).

Microarray assays may be directly integrated into functional genomic approaches aimed both at assigning function to identified genes, and to studying the organization and control of genetic pathways acting together to make up the functional organism. The rationale behind this approach is that genes showing similarity in expression pattern may be functionally related and under the same genetic control mechanism. Therefore, a common strategy undertaken in early microarray studies was to analyze data by clustering genes into groups based on their expression profiles as scored in multiple experiments (Brown and Botstein 1999). In most cases, gene clusters comprise both known and unknown genes allowing researchers to associate putative functions to the

unknown genes by employing the concept of 'guilt by association'. At present, both cDNA microarrays and oligonucleotide microarrays are used for gene expression monitoring. The first demonstration of the use of cDNA microarrays for quantitative monitoring of gene expression described the differential expression between *Arabidopsis* leaf and root tissues using a small, 45-element array (Schena et al. 1995). Essentially, microarrays may be used to analyze any kind of variability in gene expression between given samples. These differences can be either naturally occurring or induced. Natural variation may occur between different plant cultivars, tissues, developmental stages, environmental conditions or during circadian rhythm. Microarray experiments, in which the response to drought and cold stresses (Seki et al. 2001), mechanical wounding and insect feeding (Reymond et al. 2000), herbivory (Arimura et al. 2000) and nitrate treatments (Wang et al. 2000) were analyzed further, have already demonstrated the capability of microarray-assisted expression studies to identify novel response genes including those encoding regulatory factors. A review on genomic studies of plant response to stress (especially biotic stress) is presented in the next section.

Genome-wide expression profiling at the transcript level is one of the most exciting tools to study the cell and its integrative processes. Firstly, it is possible to measure transcript levels of every gene. This is something that is not yet feasible for proteins or metabolites. Secondly, expression patterns of genes can provide strong clues to elucidate their function. This assumption is based on numerous examples in which gene function was tightly connected to precise expression patterns under certain conditions. Consequently, global observation of gene expression patterns allows evaluation of the association between conditions of gene expression and function as well

as the generality and strength of this link. Clearly, genome-wide expression data are linked to the study of promoters and regulatory elements that determine the levels of specific gene transcription. Understanding the information conveyed by the promoters will influence our ability to comprehend similarities in expression profiles. A broad picture of genes coordinately expressed in a cell might provide a dynamic molecular view and help to understand the operative biochemical and regulatory networks. The ability to monitor simultaneously the expression of a large set of genes is one of the main spin-offs of genome sequencing efforts. Current reports on genome-wide expression analysis in plants also describe the use of microarrays (either oligonucleotide or cDNA) and already cover approximately one-third of the *Arabidopsis* genome (Wisman and Ohlrogge 2000; Zhu and Wang 2000). *Arabidopsis* and possibly rice microarrays representing entire genomes will soon also be commercially available or provided as a service to the scientific community (Wisman and Ohlrogge 2000; Zhu and Wang 2000).

The flexible nature of the fabrication and hybridization methods of cDNA microarrays allows the application of the technology to non-model organisms. An early example of the application of cDNA microarrays to a non-model plant described the use of strawberry microarrays containing 1701 cDNAs for analyzing gene expression during fruit development (Aharoni et al. 2000). A significant product of these experiments was the identification and characterization of a novel gene involved in fruit flavor production. This clearly demonstrated the capability of microarray expression profiling to link gene to function, particularly when an exceptionally complicated and poorly described biological process is of interest. Results from microarrays representing genes derived from a range of sources, from lower plants to trees will no doubt be reported in the near

future. As a consequence, microarray technology will effectively narrow the gap in molecular biology between model species and less exploited plant species.

Microarrays composed specifically of all yeast intergenic regions were used thus limiting the use of the method to organisms with sequenced genomes. In addition, such an approach would be difficult to apply in situations such as in higher plants where the genome is larger, the intergenic regions are more extensive and the promoter regions are difficult to define.

The future of plant microarrays.

Microarray expression studies are producing massive quantities of gene expression and other functional genomics data, which provide key insights into gene function and interactions within and across metabolic pathways (Young 2000). However, most applications have only allowed the identification of genes differentially expressed at significant levels (at least 2-fold). The true challenge will be to use it to identify genes that are consistently up- or down-regulated by 10% or 20%, yet play significant roles in the differentially expression due to the physiological and biochemical changes of cells.

However, unlike genome sequence data, which have standard formats for presentation and widely used tools and database, much of the microarray data generated so far remain inaccessible to the broader research community (Brazma et al. 2001). Several factors contribute to the barrier to widespread access to microarray data. The field is young and has only recently approached the maturity needed to identify important aspects of the data. In addition, gene expression data are more complex than sequence data where they are meaningful only in the context of a detailed description of the

conditions under which they were generated. In contrast to an organism's genome, there are as many transcriptomes as there cell types multiplied by environmental conditions (Aach et al. 2000). Moreover, because microarrays do not measure gene expression levels in any objective units, comparing gene expression data is more difficult. In fact, most measurements report only relative changes in gene expression, using a reference, which is rarely standardized. Finally, different microarray platforms and experimental designs produce data in various formats and units and are normalized in different ways. All of which make comparison and integration of these data an error-prone exercise (Quackenbush 2001). Therefore, it is necessary to define the minimum information that must be reported, in order to ensure the interpretability of the experimental results generated using microarrays as well as their potential independent verification. MIAME, the Minimum Information About a Microarray Experiment, was a standard established by Brazma and colleagues (Brazma et al. 2001) to facilitate recoding and reporting microarray-based gene expression data. According to MIAME standard, six sections need to be concerned for a published microarray-based gene expression experiment: experimental design, array design, samples, hybridizations, measurements, and normalization controls (Brazma et al. 2001).

Even the basic methods are well established, however, the microarray technology is still being refined. The high cost and poor repeatability of the entire array are main disadvantages for the common use of microarray. The full potential of this technology also depends on the establishment of public databases to house microarray expression data so that a maximum number of researchers can bring their expertise and intuition to bear on the interpretation of the expression data (Richmond and Somerville, 2000).

Although applications of gene expression arrays are extensive, it has been realized that it is not simply mRNA levels but also the amount and modification of expressed proteins within the particular cellular context that determine true gene activity. Therefore, it is most important to couple transcriptome data to other functional knowledge derived from DNA, protein and metabolite analyses. Protein expression data obtained either by 2D gel analysis coupled to mass spectrometry or other more sensitive methods may provide clues to the mode of regulation when coupled to gene expression data (Dutt and Lee, 2000). High-throughput protein interaction assays such as those performed for all yeast open reading frames (Uetz et al. 2000) will link protein partners to microarray gene expression clusters.

STUDY OF PLANT DISEASE RESISTANCE WITH DNA MICROARRAYS

Plants are constantly challenged with biotic and abiotic stresses and have evolved a diversity of constitutive and inducible responses in order to adapt and survive in the environment (Kombrink and Somssich 1995). Understanding the specific changes in gene expression of signaling pathways and the genes involved in towards certain stresses has become general approaches to find defence strategies for plants. Induced defence has received a lot of attention and a large number of genes encoding defence-related proteins have been identified over the years. Many of these genes are induced when the plant is attacked by microbial pathogens, viruses or insects (Dong 1998; Eisen and Brown 1999). However, the integration of all different responses into a global knowledge of plant disease resistance, the precise control of pathway cross-talk (Genoud and Metraux 1999) or a complete catalogue of defence-related genes is still lacking. DNA microarray is already a standard tool for genome-wide monitoring of gene expression in animal studies and is starting to contribute to the field of plant biology. Plant biologists have rapidly recognized the importance of DNA microarray technology, as illustrated by ambitious genomic programs (Richmond and Somerville 2000). The recent advent of tools enabling the global analysis of gene expression coupled to the genome sequencing of model species like *Arabidopsis* or rice provide answers to more general questions. Completion of both *Arabidopsis* and rice genomes are opening the way to genome-wide microarrays, which will become essential tools for discovering the function of genes. There is large potential of DNA microarray technology could impact research in plant defense.

Initial experiments demonstrated that microarrays are suited for plant genes. These reports included the comparison of gene expression between roots and leaves with 48 (Schena et al. 1995) and 1 400 *Arabidopsis* ESTs (Ruan et al. 1998), the difference between two *Arabidopsis* accessions using 673 cDNA clones (Kehoe et al. 1999), and the comparison of light-grown and dark-grown *Arabidopsis* seedlings with 432 ESTs spotted on high-density filter arrays (Desprez et al. 1998). Another study with 1701 randomly chosen cDNA clones from strawberry and 480 from petunia allowed the discovery of a key enzyme involved in flavor biogenesis (Aharoni et al. 2000) and showed that microarrays are not restricted to *Arabidopsis* genes. The first large-scale analysis was performed to identify nitrate responsive genes using 5524 unique cDNA clones representing approximately a quarter of the *Arabidopsis* genome (Wang et al. 2000). Novel nitrate-induced genes were found and multiple responses to nitrate were observed at the transcript level, illustrating the power of such global investigation for gene discovery and for the analysis of regulatory networks. Since then many large-scale gene expression studies of plant defense response to either abiotic or biotic stress have been done by using DNA microarrays. There is no doubt that in the near future, a flow of microarray data covering all aspects of plant biology will be produced. The first results from experimental analyses of plant defence with microarrays are already appearing.

For examples, DNA microarrays were used to monitor transcript abundance and expression patterns in rice exposed to high salinity. On the basis of root cDNA libraries and expressed sequence tags (ESTs) from the moderately salt-tolerant rice line, 1728 microarray elements derived from the roots of stressed plants were assembled to monitor changes in transcripts compared with unstressed plants after salt shock. The results

indicate a progression of regulated functions such that different categories of transcripts show regulation at different time points (Kawasaki et al. 2001).

Similar study was done to monitor responses to drought and salinity in barley by microarray hybridization of 1463 DNA elements derived from cDNA libraries of drought-stressed plants (Ozturk et al. 2002). Nearly 15% of all transcripts were either up- or down-regulated under drought stress. Transcripts that showed significant up-regulation under drought stress are by jasmonate-responsive, metallothionein-like, late-embryogenesis-abundant (LEA) and ABA-responsive proteins. Most drastic down-regulation in a category was observed for photosynthesis-related functions. However, a number of functionally unknown transcripts from cDNA libraries of drought-stressed plants showed up-regulation by drought but down-regulation by salt stress, which illustrated the power of microarray on transcript profiling of large amount of genes in different growth conditions and environments.

Full-length cDNAs are essential for functional analysis of plant genes. A cDNA microarray using 1300 full-length Arabidopsis cDNAs from plants in different conditions, such as drought-treated, cold-treated, or unstressed plants, and at various developmental stages from germination to mature seed was made to identify drought- and cold-inducible genes and target genes of DREB1A/CBF3, a transcription factor that controls stress-inducible gene expression. In total, 44 and 19 cDNAs for drought- and cold-inducible genes, respectively, were isolated, 30 and 10 of which were novel stress-inducible genes that have not been reported as drought- or cold-inducible genes previously. Twelve stress-inducible genes were identified as target stress-inducible genes of DREB1A, and six of them were novel. These results show that full-length cDNA

microarray is a useful material with which to analyze the expression pattern of Arabidopsis genes under drought and cold stresses, to identify target genes of stress-related transcription factors by combining the expression data with the genomic sequence data (Seki et al. 2001).

Complementary results obtained with other high throughput techniques, such as serial analysis of gene expression (SAGE) and sequencing, already provide valuable resources on when, where and at what level transcripts are found in a given plant or tissue (Ewing et al. 1999; Matsumura et al. 1999). Plants have to deal with a vast range of pathogens and it is not known what proportion of the genome is allocated to defense. Recent data from the sequencing of the *Arabidopsis* genome help in addressing this question. In a detailed analysis of 1.9 Mb contiguous sequences from chromosome 4, 14 % of genes with predicted or known functions were classified as involved in disease or defence (Bevan et al. 1998). This figure is potentially biased by the presence of a cluster of several putative resistance genes and by the relatively small size of the sequence that was analyzed but it already indicates that a sizeable fraction of the genome might contain defence genes. When sequencing of chromosomes 2 and 4 was completed, a functional analysis revealed that respectively 2 and 12 % of the known genes were included in the disease and defence category (Lin et al. 1999; Mayer et al. 1999). The difference between chromosomes may reveal a non-homogeneous representation of defence-related genes along chromosomes. When the complete *Arabidopsis* genome is annotated accurately, a more precise estimation of the plant defense genes will be established. By comparison, analysis of 110 Mb *Arabidopsis* genomic sequence predicts that more than 5 % of the genes are transcription factors (Riechmann and Ratcliffe 2000).

However, gene function predicted by sequence annotation must be confirmed experimentally. A thorough microarray study using ca. 10 000 *Arabidopsis* ESTs, representing 7 000 genes (25–30 % of all *Arabidopsis* genes), has recently been undertaken and described transcript profiles during systemic acquired resistance (SAR), a defense reaction known to develop in systemic leaves after an initial pathogen attack of local leaves (Maleck et al. 2000). The authors compared several experiments in which SAR was induced and scored ESTs differentially expressed in at least two conditions (induction equal or greater than 2.5-fold). They observed that 4.3 % of the genes (300 out of 7 000) were involved in the SAR response. When whole genome-microarrays are available, experiments with plants treated by various pests or pathogens will help in characterizing the full complement of genes that a plant uses for its defense.

Plant defense responses are diverse and complex and their study will undoubtedly benefit from DNA microarray technology. So far, only a few examples of the use of microarrays in the study of plant pathogenesis are available but they already illustrate the versatility of this technology. An initial study was using an Affymetrix chip containing oligonucleotide probes for 1 500 maize genes identified 117 genes that showed a consistent change after various treatments with the fungal pathogen *Cochliobolus carbonum* (Baldwin et al. 1999). The interaction between the incompatible fungal pathogen *Alternaria brassicicola* and *Arabidopsis* was recently examined with microarrays containing 2 375 selected ESTs (Schenk et al. 2000). The array was enriched with defence associated and regulatory genes but the results showed that a substantial fraction of these genes were either induced (168) or repressed (39) after inoculation with the pathogen, many of them having no previous described function.

When the authors compared fungal infection with treatments with the defense-related molecules salicylic acid (SA), methyl jasmonate (MJ), or ethylene, they observed a surprisingly large number of genes that were regulated by multiple treatments, unraveling a high degree of co-ordination among different signaling pathways. About 150 defence-related *Arabidopsis* genes were analyzed on the timing, dynamics, and regulation of the expression in response to mechanical damage or insect attack (Reymond et al. 2000). To characterize the signaling pathway associated with the wound response, the defined mutant *coi1-1*, which is insensitive to jasmonic acid (JA) was used. The result showed that JA signaling was necessary for the induction of half of the genes normally induced by wounding.

Availability of known *Arabidopsis* mutants will certainly be a major asset when combined with microarray analyses. Moreover, a thorough characterization of these mutants will be possible at the gene expression level. For example, Reymond (2001) found jasmonate does not induce a set of genes that are normally induced in wild-type plants in *coi1-1* mutant plants that are insensitive to jasmonate, which indicates the COI1 gene product controls the induction of all JA-inducible genes. Another example on the use of defined mutant in plant-pathogen interactions combined with microarray analysis comes from the work of Maleck et al. (2000) on SAR. Comparing wild-type plants with mutants unable to mount a SAR response could identify sets of SAR specific genes.

Numerous studies have shown that transcription factors are important in regulating plant responses to environmental stress. However, specific functions for most of the genes encoding transcription factors are unclear. The DNA microarray technology is also used in the functional analysis of transcription factors. The comparative analysis

of promoter sequences of genes that share similar expression profiles will provide researchers with candidate regulatory sequences. This approach has been validated with microarray data from yeast expression profiles (Roth et al. 1998) and has uncovered novel putative motifs (Livesey et al. 2000). In their microarray study of SAR, Maleck et al. (2000) identified a common promoter element in a group of genes that were coordinately regulated. Among those genes was PR1, a well known marker of SAR. All promoters from this PR1 regulator were enriched in W boxes, motifs that are binding sites for WRKY transcription factors (Eulgem et al. 2000). In a preliminary analysis of microarray experiments on wounded *Arabidopsis* leaves (Reymond et al. 2000), conserved regulatory sequences were found in promoters of clustered genes. In addition, microarrays will help in the identification of genes whose expression is controlled by known transcription factors. For instance, a transcription factor could be overexpressed or silenced in transgenic plants and the effect on gene expression measured by microarray analysis. This was achieved successfully in yeast for defining target genes modulated by transcription factors involved in oxidative stress or sporulation (Chu et al. 1998). Recently, mRNA profiles generated from microarray experiments were used to deduce the functions of genes encoding 402 known and putative Arabidopsis transcription factors at different developmental stages and under various stress conditions (Chen et al. 2002). Transcription factors potentially controlling downstream gene expression in stress signal transduction pathways were identified by observed activation and repression of the genes after certain stress treatments. The mRNA levels of a number of previously characterized transcription factor genes were changed significantly in connection with other regulatory pathways, suggesting their multifunctional nature. The expression of 74 transcription

factor genes responsive to bacterial pathogen infection was reduced or abolished in mutants that have defects in salicylic acid, jasmonic acid, or ethylene signaling. This observation indicates that the regulation of these genes is mediated at least partly by these plant hormones and suggests that the transcription factor genes are involved in the regulation of additional downstream responses mediated by these hormones. Among the 43 transcription factor genes that are induced during senescence, 28 of them also are induced by stress treatment, suggesting extensive overlap responses to these stresses. Statistical analysis of the promoter regions of the genes responsive to cold stress indicated unambiguous enrichment of known conserved transcription factor binding sites for the responses. A highly conserved novel promoter motif was identified in genes responding to a broad set of pathogen infection treatments. This observation strongly suggests that the corresponding transcription factors play general and crucial roles in the coordinated regulation of these specific regulators (Chen et al. 2002).

Another particularly promising way of using microarrays for understanding the mechanisms of pathogenesis will be to compare the responses induced by various pests or microorganisms. For instance, an analysis of transcript profiles after challenging plants with different pathogens or stimuli might answer the question concerning host discrimination between pathogens and might help identifying transcript profiles among host responses. The studies were conducted by looking at defense gene expression in plants that were treated with known signal molecules (methyl jasmonate, methyl salicylate), wounded, or challenged with insects and microbial pathogens (Reymond 2001). A microarray analysis with 150 defence-related *Arabidopsis* ESTs was performed and a two-way clustering of gene expression ratios and experimental conditions was

carried-out in order to group genes with similar behavior as well as to group experiments with similar transcript profiles. This type of investigation allows the identification of diagnostic genes that are only induced in specific conditions while others may respond in a less discriminating manner. For example, it was found that the expression profile of methyl salicylate-treated plants resembles that of plants infected with *P. syringae* avrRPM1 while methyl jasmonate-treated plants show transcript profiles more related to wounding or insect challenge. This is in accordance with the current idea that jasmonate- and salicylate- dependent signaling pathways regulate the responses to different classes of pathogens (Glazebrook 1999). It will be interesting to compare different pathogens for their effects on inducible gene expression. A similar analysis has proven to be very useful in comparing different treatments inducing SAR in *Arabidopsis* plants (Maleck et al. 2000). The authors observed that related experimental conditions yielded similar expression profiles. This approach is already being taken for the analysis of animal-pathogen interactions (Manger and Relman 2000) and is providing important information for classification of human tumors based on variations in gene expression (Ross et al. 2000). A larger effort will be necessary before pathogen specific-transcript profiles can be defined but this opens the perspective of being able to precisely diagnose plant diseases at the molecular level, which will be playing a central role for agriculture. Recently, the first complete genome sequence of a plant pathogen was achieved (Simpson 2000). As other genomes are sequenced, it will soon be possible to have the complete sets of genes of both the host and the pathogen on the same microarray, producing a unique molecular view of the coordinated interaction between the plant and its aggressor.

One of the challenges facing the research community will be to deal with the flow of data generated by whole genome expression studies (Ermolaeva et al. 1998). Efforts to normalize expression data as well as to deposit the information in public databases are clearly in need. One such example is the database developed at Stanford University (http://genome-www4.stanford.edu/MicroArray/SMD/index.html). This database hosts data from multiple microarray analyses in different organisms and offers several useful query tools. It will be important in the future to be able to compare expression data across many different experimental, geographical or technical platforms.

It is evident that the measurement of transcript level only reflects one aspect of a biological process and that change in protein and metabolite levels need also to be investigated. In order to fully understand complex defence responses, input from proteomic and metabolomic studies will be essential. Although technologically more demanding, such approaches are currently being developed for plants (Fiehn et al. 2000; Roessner et al. 2000). This metabolic profiling technique has also recently permitted the discovery and quantification of fatty acid-derived molecules that accumulate during wounding and pathogenesis (Vollenweider et al. 2000). The role of these molecules as biological regulators implicated in defence will be tested by microarray analysis, highlighting a potential application of transcript profiling methods for discovering the function of new metabolites. When the repertoires of transcripts and metabolites measured in a single experiment increase to genome-scale levels, the challenge will be to integrate these complex databases and to extract meaningful biological information.

In the next few years, DNA microarrays will certainly become a standard tool in each laboratory. The technology could be used in two to answer different types of

questions. First, microarrays containing a representation of the whole plant genome will be served to identify the expression pattern of genes of unknown function, to define specific sets of genes responding to various stresses or stimuli, to provide a global view on metabolic processes, and to assist in comparing wild type and mutant plants. Because the production and routine use of whole genome-microarrays might be financially too demanding for most research groups, genomic studies providing access to large microarrays might develop further and allow the screening for genes of specific interest. Second, after a few screening experiments with large-scale microarrays, each research group could narrow down on a subset of favorite genes which they could study using conventional techniques or which they could print on small-scale specific microarrays. This second approach will be quite useful for a deep and thorough analysis of the expression patterns of hundreds of genes and would be affordable for most research groups. Such studies may include more detailed characterization of expression patterns, including replication of multiple experiments and time-course analyses. The strategy of fabricating custom arrays tailored to a specific biological question has the advantage of being easier to control at the production side (less genes to prepare and manipulate) while reducing the amount of data to process and integrate. Another source of candidate genes involved in specific plant responses and which might also constitute specific microarrays will come from differential screening methods, such as differential display and RNA fingerprinting (cDNA-AFLP), or might simply be constituted on the basis of literature search or in silico analyses of SAGE and ESTs databases. For species for which no genome sequencing program is expected in the near future, these tools will help in designing custom microarrays. Dedicated microarrays containing a well-defined set of

defence-related genes have already demonstrated their utility for the study of wound- and insect inducible gene expression and the involvement of signal molecules in the wound and pathogen responses (Reymond et al. 2000). As more knowledge accumulates on the genes involved in plant defence, custom microarrays tailored for specific responses (e.g. SAR, wound, induced systemic resistance, insect-specific, pathogen specific, etc.) might be used for fundamental or applied research.

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Chapter II

Differential Gene Expression in Bermudagrass Associated with Resistance to the Spring Dead Spot Fungus *Ophiosphaerella herpotricha*

Bermudagrass [*Cynodon dactylon* (L.) Pers.] is extensively used for turf, forage, and soil stabilization in the southern United States. Spring dead spot (SDS), caused by *Ophiosphaerella herpotricha,* is a serious fungal disease of bermudagrass turf. This research was conducted to identify bermudagrass genes conferring resistance to SDS. Suppression subtractive hybridization (SSH), systematic sequencing of cDNA clones, and cDNA microarrays were used to study the relative abundance of transcripts corresponding to sets of clones derived from cultivars which are resistant (Yukon) and susceptible (Jackpot) to this pathogen. A total of 154 contigs were generated from sequence comparison of 250 bermudagrass ESTs. These contigs were grouped into seven different categories according to their putative functions. Global gene expression associated with fungal resistance was studied by a cDNA microarray with 834 bermudagrass SSH clones. Expression profiles of resistant and susceptible plants from tissues collected during the fall and spring were compared by probing this array. During the fall and spring seasons, approximately 13% and 19%, respectively, of the genes from the SSH library displayed more than a 2-fold differential expression between the two cultivars. Eighty-nine responsive genes from both cultivars were grouped into 6 clusters according to their fall and spring expression patterns.

Additional keywords: fungal resistance, bermudagrass, SSH, expression profiling.

Bermudagrass, *Cynodon dactylon*, is a warm-season perennial sod-forming species used widely for turf, forage, and soil stabilization in the southern United States. Within this region, bermudagrass is hardy enough to survive with little care and can respond quickly to intensive management. Bermudagrass cultivars typically produce extensive root systems and are drought tolerant. They also respond well to nitrogen fertilization and produce abundant biomass when soil moisture is adequate (Gatschet, 1993).

Spring dead spot (SDS) is a serious patch disease of bermudagrass that was first observed in Oklahoma during 1954 (Wadsworth and Young 1960). The symptoms of SDS usually appear in early spring as dormant bermudagrass resumes growth and symptoms occur as circular, dead areas ranging from a few centimeters up to several meters in diameter (Smith et al. 1989). In the Southern Great Plains region (including Kansas, Oklahoma and Texas) the primary causal agent of SDS is an ectotrophic root-infecting fungus, *Ophiosphaerella herpotricha* (Tisserat et al. 1989). *O. herpotricha* produces dark-colored, ectotrophic mycelia, occasionally with bubble-like structures on roots, stolons, and rhizomes of grasses, partially immersed in dead leaf sheath and stolen tissues have also been observed on bermudagrass affected with SDS (Tisserat et al. 1989). *O. herpotricha* is very active in the fall and in the early spring under cool moist conditions. It may infect bermudagrass whenever soil temperatures are between 10 and 25 °C (Endo et al. 1985). Bermudagrass cultivars differ substantially in response to infection by *O. herpotricha*, ranging from highly tolerant to highly susceptible, but none has been immune (Martin et al. 2001).

Disease resistance in plants depends on the ability of the host to recognize pathogens and initiate defense mechanisms that limit infection. Therefore, resistance is associated with a plant defense response that involves an integrated set of genes (Schenk et al. 2000). However, the analyses of plant resistance to pathogen infection have traditionally focused on one or a few genes at any one time (Jones 2001), whereas the integration of all responses into a global analysis of plant disease resistance, the precise control of pathway cross-talks (Genoud and Metraux 1999) or a complete catalogue of defense-related genes is still lacking. DNA microarrays are already standard tools for genome-wide monitoring of gene expression in animal studies and they are starting to contribute to the field of plant biology. Initial experiments have demonstrated that microarrays are suited for plant genes (Schena et al. 1995, Ruan et al. 1998, Kehoe et al. 1999). The first results from experimental analyses of plant defense to either biotic or abiotic stress with microarrays are beginning to appear (Schenk et al. 2000, Seki et al. 2001, Kawasaki et al. 2001, Ozturk et al. 2002).

In this study, putative differentially expressed gene transcripts were identified by suppression subtractive hybridization (SSH) from two bermudagrass cultivars with different levels of resistance. We have used cDNA microarrays to examine the abundance and expression changes of 834 bermudagrass transcripts in both resistant and susceptible cultivars during the fall and spring to dissect the molecular bases of resistance to this pathogen. Our results demonstrated the presence of potential networks of regulatory interactions between genes in response to infection by this pathogen. This information provides insights into targets for manipulation for generating genetically improved bermudagrass with enhanced resistance to SDS.

RESULTS

Suppression subtractive hybridization cDNA library.

We isolated 310 cDNA clones from Yukon and Jackpot that were infected with the fungus *Ophiosphaerella herpotricha* by using suppression subtractive hybridization (SSH) to identify cultivar specific genes that are differentially expressed for fungal pathogen resistance or susceptibility. A set of 221 clones was generated by SSH in which mRNA from infected crown tissue of Yukon was used as 'tester' and the mRNA from infected crown tissue of Jackpot was used as 'driver'. This set of cDNA clones was enriched for genes over-expressed in Yukon when compared to Jackpot. To obtain clones that were over expressed in the susceptible cultivar, a reverse subtraction was performed. In this case, mRNA from infected crown tissue of Jackpot was used as 'tester' and mRNA from infected crown tissue of Yukon was used as 'driver'. This set of cDNA clones was enriched for genes over-expressed in susceptible cultivar. Eighty-nine clones were selected from this reverse subtraction.

Ninety-eight of 310 clones were further characterized by colony PCR to check the cloning efficiency. Approximately 10% clones did not have cDNA inserts. Those with inserts had sizes ranging from 55 to 800 bp with an average size of 400 bp.

Sequencing and data mining.

Upon assembling the readable sequences obtained from the 5' or 3' ends of 310 cDNAs, 250 bermudagrass EST sequences were generated from the two subtractions (forward and reverse). The inserts of clones with sequences longer than 100 bp were submitted to GenBank dbEST (Boguski et al 1993) at the National Center for

Biotechnology Information. A total of 231 clones (176 from Yukon and 55 from Jackpot) were deposited in dbEST (http://www.ncbi.nlm.nih.gov). Sequence analysis revealed that these 250 clones belonged to 154 different contigs. Sixty-two (40.3%) of them matched previously described genes and 37 contigs (24%) aligned with genes of known function in GenBank. Plant defense genes previously cloned from other plant species were isolated from bermudagrass using SSH. An example is phenylalanine ammonia lyase (PAL), which had previously been characterized as a differentially expressed gene for resistance (Cheng et al. 2001). The sequences of the other 92 contigs (59.7%) did not match entries in the GenBank and are likely to be novel. Genes with related functions were grouped in six separate categories: 1) genes involved in oxidative burst /stress or apoptosis; 2) antimicrobial genes; 3) genes of low molecular weight defense signals; 4) genes involved in cell signaling and communications, 6) genes for cell maintenance and development; or 6) other (unknown) functions (Table 1).

Microarray analysis.

Intensities in hybridizations with probes from two samples labeled with Cy5 or Cy3 were compared using a scatter plot (Fig. 1). Hybridization of different microarrays with the same mRNA samples labeled with swapped dyes identified the same differentially expressed genes (Fig. 2). In addition, similar values were obtained for all clones printed in duplicate (Fig. 2).

Differential gene expression in response to fall infection.

O. herpotricha infects bermudagrass crown tissue in late fall when soil temperature drop to 10 °C to 25 °C (Endo et al. 1985). However, the disease symptoms are not apparent until the following spring. To understand the difference in fungal resistance between these two cultivars, gene expression profiles were studied in crown tissues sampled during the fall 2000 and spring 2001. To investigate differences in gene expressions between resistant (Yukon) and susceptible (Jackpot) cultivars during fall infection, probes were prepared from the two cultivars (Yukon infected and Jackpot infected) sampled in late fall (November) of 2000. An example of one such hybridization is shown in Figure 2, in which the Yukon cDNA probe is labeled with Cy-3 fluorochrome (red) and Jackpot is labeled with Cy-5 fluorochrome (green). Clones with a green color were induced in Yukon, and clones represented by a red color were induced in Jackpot. A dye swap experiment was conducted side by side to the original hybridization to reduce uneven coupling effect of two fluorescence dyes. As shown in Fig 2 B, the same genes were identified. Yellow fluorescence indicates equal expression for the two cultivars. A scatter plot of expression data (median signal intensity) form Yukon (x axis) *vs*. Jackpot (y axis) indicates similar number of differentially expressed genes in the two cultivars during this season (Fig. 1 A).

A marked change was seen in the global pattern of gene expression (Fig. 1). Confidence intervals for gene expression ratios were considered significant for a two-fold change or when the log base two ratio > |1|. By inspecting the log base two ratios more than "+1.0" or less than "-1.0" in signal intensity between Yukon/Jackpot hybridizations revealed significant differences in transcript concentrations between the two cultivars.

Therefore, genes were regarded as "Yukon-induced" only if the $\log_2$ ratio of Yukon/Jackpot was >1.0, and when this ratio was < -1.0 genes were considered to be "Jackpot-induced". Based on this criterion, 106 genes were differentially expressed between the two cultivars during fall infection by this pathogen. The mRNA levels for 57 genes were induced in Yukon, whereas mRNA levels for 49 genes were induced in Jackpot (Appendix VII). This may be a conservative estimate of percentage of differentially expressed genes between two cultivars, because of the stringent criteria used for their selection. Several plant stress-inducible protein genes were found among these highly induced genes for resistant cultivar (Appendix VII).

Differential gene expression in response to spring infection and re-growth.

Probes were prepared from resistant and susceptible cultivars (Yukon infect and Jackpot infect) sampled in early spring (April) of 2001 to study differences in gene expression between these two cultivars. A dye swap experiment was also conducted to reduce bias associated with uneven incorporation of the two fluorescence dyes. The corresponding genes on these arrays showed similar expression ratios with reversed colors in the dye-swap experiments (data not show). Scatter plots indicate that more transcripts on the array were over-expressed in Yukon than in Jackpot (Fig. 1 B). A total of 154 genes were differentially expressed during spring infection and re-growth ($\log_2$ ratio larger than 1 or smaller than -1). One hundred and four genes were induced in Yukon, whereas 50 genes were induced in Jackpot (Appendix VIII).

As expected, most of the highly induced known genes for Yukon belonged to potential housekeeping or structural genes, that is, genes encoding proteins involved in

cell-wall formation (phenylalanine ammonia-lyase), carbon metabolism (ATP synthase) and maintenance (ribosomal functions; anion channeling; translation). Several plant genes, designated as stress-inducible protein genes, such as ethylene receptor (Klee and Tieman 2002), catalase (Polidoros et al. 2001), and ascorbate peroxidase (Jimenez et al. 1997), were found among these highly expressed genes.

Cluster analysis of fall and spring gene expression data.

In order to understand the responses of different cultivars to fungal infection during the fall and spring, expression data from genes differentially expressed during fall 2000 and spring 2001 were clustered together based only on the observed expression pattern without regard to any known biology. A list of genes clustered for the two seasons is presented in Table 2. To obtain greater insight into the function of known or novel genes, a nonhierarchical algorithm (k-mean) (Eisen et al. 1998) was used for these 89 genes to group these clones according to similar expression patterns across seasons (Fig. 3). Six different patterns of transcript regulation were identified (Fig. 4).

Cluster A contains 37 genes constitutively over-expressed in Yukon in both the fall and spring. Several genes involved in defense responses to stress were included in this cluster. For example, catalase and ascorbate peroxidase are in this cluster. Transcripts that encode proteins involved in cell signaling and communication, such as those that have homology to ADP-ribosylation factor and wheat HMG1/2-like high mobility group protein, were also present in this cluster.

Genes induced in Yukon during the fall and induced in Jackpot during the next spring were sorted in cluster B. Only eight genes belong to this cluster. A transcript in

this cluster has homology to fused-ccdB, an active cytotoxic killer gene that was cloned from *E. coli.* (Bernard et al. 1994).

There were 12 genes grouped in cluster C, in which expression level was significantly different for the two cultivars between the seasons. These genes were induced in Jackpot in fall but had greater expression in Yukon during spring. An example in this cluster include a gene that encodes the ATP synthase A chain protein, which is involved in cell maintenance and developmental processes. ATP synthase catalyses the reaction between adenosine diphosphate (ADP) and inorganic phosphate to form ATP with the use of energy from a transmembrane proton-motive force generated by respiration or photosynthesis (Wehrle et al. 2002).

Clusters D1, D2, and D3 contained 32 transcripts, which were over-expressed only in the susceptible cultivar. Because the expression levels of these genes differed from fall and spring, three sub-clusters (D1, D2 and D3) were established based on these subtle differences in expression patterns (Fig. 4). Fourteen transcripts were present in D1; the expression levels of these genes were not very high. Except for genes encoded for two ribosomal proteins, two metabolism related genes, branched chain alpha-keto acid dehydrogenase and ubiquitin-fusion degradation protein were also clustered in this category. Sub-cluster D2 contained 11 transcripts. Their expression Log_2 ratios were between 2 to 3 and did not significantly differ between seasons. Genes that code for homologs of formate dehydrogenase and C3HC4-type zinc fingers protein were in this category. Seven genes were included in D3 that were highly induced in Jackpot for both seasons, and with decreased expression level from fall to spring. A gene that codes for a protein similar to rice cysteine proteinase was over-expressed in Jackpot during both the

fall and spring. A transcript, which showed the highest expression in Jackpot with a $\log_2$ ratio greater than 6x for both seasons, showed similarity to an unknown. Although the expression profiles for these 32 genes were not identical in the susceptible cultivar according to the k-means analysis, these subtle differences in expression patterns may not be biologically significant.

DISCUSSION

SSH Library.

In our SSH library, the largest category (12.4%) of identified sequences corresponded to genes coding for cell maintenance and development which includes transcripts such as those coding for ribosomal proteins, ATPases, and the ubiquitin / proteasome pathway components. For example, 8 ESTs coded for various plant ribosomal proteins. Although the expression of these genes varied between two cultivars, they are likely involved in the highly active metabolism in crown tissue of bermudagrass. A significant proportion of transcripts (7.8%) involved in cell signaling and cell communication (e.g. ADP-ribosylation factors; voltage-dependent anion channel protein 1a; signal peptidase; HMG1/2-like high mobility group protein). Six contigs which belong to the oxidative burst /stress, apoptosis, pathogen/stress defense, antimicrobial, and low molecular weight defense signals categories were identified in this SSH library. These contigs include genes that encode proteins homologous with cysteine proteinase, ascorbate peroxidase, catalase, phenylalanine ammonia-lyase, ethylene receptor, and auxin binding protein. All of these genes have been previously implicated in abiotic or biotic defense responses.

The cDNA libraries generated by SSH are usually a very rich source of new sequences that either share partial homology to known genes or lack any homology to known genes (Hinderhofer and Zentgraf 2001). In our SSH subtraction libraries, 59.7% did not match any entries in GenBank, which is nearly two fold greater than the average (30%) number of new sequences discovered in SSH libraries (Desai et al. 2000). Moreover, a significant number of differentially expressed mRNAs have homology with

ESTs or genomic sequences (Voiblet et al. 2001). We have analyzed more than 300 clones from the subtracted library to be sure that genes representing low-abundance transcripts are not lost (Yang et al. 1999).

Although the cDNA library constructed by SSH was normalized, highly expressed genes may still be selected more than once, suggesting that there is a small degree of redundancy (Kim et al. 2001, Desai et al. 2000). The most abundant transcript corresponds to a novel gene, which codes for a homologue of unknown function protein from *Arabidopsis thaliana*. This transcript has 14 replicates and is highly represented in the resistant cultivar (Yukon). In addition, the SSH library also contained large amounts of rare transcripts such as signal peptidase and auxin binding protein. These findings suggested that our suppression-subtraction libraries contain both high and low-abundance differentially expressed cDNAs. The microarray data demonstrate that out of 154 contigs selected by SSH from both forward and reverse libraries, 102 contigs (75 from Yukon and 27 from Jackpot) had been induced in either cultivar for both spring and fall season experiments. Two-thirds of the SSH clones were differentially expressed as confirmed by microarray analysis. These results indicate that suppression subtractive hybridization was able to significantly enrich the cDNA libraries with differentially expressed genes.

Bermudagrass fungal resistance.

The most well understood and documented mechanism for plant resistant responses to pathogen attack is the "gene-for-gene" model (Flor 1971), in which plant resistance to a pathogen can be characterized genetically in terms of a dominant resistance gene (R gene) in the plant and a corresponding avirulence gene (avr gene)

present in the pathogen in order for a resistance response to occur (review by Hulbert et al. 2001). There is clearly not enough evidence to support a gene-for-gene relationship in the *O. herpotricha* - bermudagrass interaction. As highlighted above, no bermudagrass cultivar has been found which is immune to this disease; resistance is a measure of the degree of susceptibility. Therefore, the source of the resistance could be polygenic. Genes that have altered expression in compatible and incompatible plant-pathogen interactions have been characterized by microarray analysis (Baldwin et al. 1999). Therefore, comparing the gene expression profiles of two cultivars with different resistance to a fungus could help us to understand the molecular bases of bermudagrass resistance to *O. herpotricha* infection. Comparing the genes induced in Jackpot (susceptible cultivar) in both fall and spring, a similar number (49 for fall/50 for spring) were obtained for both seasons. Whereas the number of genes induced in Yukon (resistant) for spring (104 genes) was almost double to that found in the fall (57 genes). The known function of genes differentially expressed in Yukon only during spring include transcripts encode for ATP synthase, methionine synthase, methyltransferase CmuC, transcription factor SF3, LLS1 protein, voltage-dependent anion channel protein, ubiquinol--cytochrome-c reductase, phenylalanine ammonia-lyase. Among those genes, methionine synthase, ubiquinol--cytochrome-c reductase, and phenylalanine ammonia-lyase are well known for their functions in pathogen/stress defense. This might explain the resistance of Yukon, in which there are more defense related genes induced on during symptom development and recovery in spring. These genes may help the plant overcome fall fungal infection to suppress or minimize symptom development in the spring resulting in smaller infection patches associated with the resistant cultivar Yukon. Even

though the interaction between the resistant cultivar and fungus is genetically compatible, the resistant plant slows or retards symptom development. As a consequence, tissue damage is kept to a minimum even though the plants are heavily infected. Our results tend to support this mechanism of resistance to *O. herpotricha.*

The cluster analysis of gene expression for both seasons revealed groups of genes with similar behavior (Figure 4). One implication of a common temporal pattern of expression is that genes might share similar or related roles in cellular processes, or the same signal molecules might regulate them. Cluster A contains 37 genes over-expressed in Yukon during both fall 2000 and spring 2001. Among the genes clustered in this group, 11 genes have shown certain level of homology with genes of known function in GenBank. Interestingly, approximately two-thirds (7 out of 11) genes encode proteins involved in cell signaling and communication (HMG1/2-like high mobility group protein, ADP-ribosylation factor, and transcription factor SF3), or defense (ethylene receptor) as well as oxidative burst related genes (ascorbate peroxidase, catalase). Therefore, the greater resistance of Yukon might be the result of enhanced signal transduction that leads to improved defense responses which reduce infection or symptom development. Obviously, special emphasis should be given to this cluster of genes for future functional analysis to understand the resistance response of bermudagrass to this pathogen.

MATERIALS AND METHODS

Plant materials and pathogen treatment.

The SDS resistant cultivar Yukon and susceptible cultivar Jackpot were used in this study. Plots were seeded on July 5, 1995 using 1 pound of pure live seed per 1,000 square feet. The soil was a silty clay loam with a pH range of 6.9 to 7.0. Grasses were planted in a randomized complete block design with three replications. Mean phosphorus and potassium soil test levels were kept in the optimum range for turf growth, with average readings of 41 and 167 parts per million, respectively. All plots were inoculated on September 26, 1997 using *O. herpotricha* isolate KS 188 (from Dr. Tisserat, Kansas State University). Disease was evaluated by the diameter of each patch appeared at all inoculation sites for these two cultivars during the three-year field study. No symptoms were present at the control sites on the inoculation process (Martin et al. 2001). In order to isolate transcripts from plant material, infected tissues were sampled on the edges of the infection patch of three duplicated inoculation site in each three infection patches for each cultivar. Each replicated sample was then pooled together for general representation of the tissue. Clay attached to root and crown was rinsed away by running water. Crowns tissues were harvested, further cleaned with water, and then submerged in RNAlater (Ambion, Houston, TX, USA) for 10 minutes before stored in -80°C.

mRNA isolation and suppression subtractive hybridization.

The mRNA was isolated from bermudagrass crown tissues of Yukon infected and Jackpot infected plants by using Straight A's mRNA Isolation System (Novagen, Madison, WI, USA) following the manufacturer's protocol. Differentially expressed

genes were isolated by using PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) starting with 2 ug of poly A^+ RNA from bermudagrass tissues (Yukon Infected *vs*. Jackpot Infected). In the last step of secondary PCR, a seven-minute 72 °C extension was applied to ensure that all PCR products were full length and 3' adenylated. Three sets of subtractions were performed, including both experimental forward and reverse as well as control subtractions following the manufacturer's instructions.

Cloning and sequencing.

The subtracted cDNA population was cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA). PCR products were inserted into pCR2.1-TOPO vector in salt solution and transformed with One Shot TOP10F´ Chemically Competent *E. coli*. Bacterial clones were picked and inoculated in to LB broth with 50 ug/ml ampicillin. Cells were cultured at 37 °C with 250 rpm shaking overnight. Then 1 ml of cell culture for each clone was stored at -80°C in 15% glycerol. Plasmid DNA from SSH clones was purified by using QIAprep Spin Miniprep kit (Qiagen, Valencia, CA, USA) and sequenced. Sequencing was performed with the BigDye Terminator Cycle sequencing kit (PE Biosystems, Foster City, CA, USA). Of the 310 sequencing reactions carried out, all sequences were primed using the M13 reverse primer present in pCR2.1-TOPO plasmid and thus read from the m3G cap into the 5' end of each cDNA. The sequencing reaction products were analyzed using ABI 3700 DNA Analyzer or ABI 373A automated DNA sequencer. Raw sequence data were edited using the SEQUENCHER (version 3.1.1) (GeneCodes Corporation, Ann Arbor, MI, USA). All sequence outputs obtained from the automated sequencers were checked visually to

confirm overall quality of peak shape and correspondence with base calls. Leading and trailing vector and polylinker sequences from SSH, and sequences with more than 3% ambiguous base calls were removed. Sequences shorter than 100 bp were not further analyzed. Groups of sequences were assembled into clusters using the contig routine of SEQUENCHER.

Homology comparisons and database construction.

The sequences in either Standard Chromatogram Format (SCF) or FASTA format were loaded into PipeOnline, a web-based resource designed by the Oklahoma State University Bioinformatics Group to assist investigators in the determination of metabolic and biological function from large-scale DNA sequence data. Within the PipeOnline program a base-caller program PHRED (Ewing and Green 1998; Ewing et al. 1998) converts SCF file to FASTA format. PipeOnline produced a new set of contig-assembled files using PHRAP. Assembled sequences were compared against a local NCBI non-redundant protein database using BLASTn. The resulting output files were automatically collected, parsed, formatted, assembled, indexed, and uploaded to a local server by the PipeOnline database assembly module. Functional sorting of the input DNA sequences was achieved through a proprietary sorting method that utilizes functional information gathered from public databases. Function was estimated using the Metabolic Pathways database (MPW) functional dictionary (Selkov et al. 1998) obtained from WIT (Overbeek et al. 2000). Although the BLAST scores and P values were considered, the assessment of whether a given homology was significant was determined by investigator judgment, not by absolute numerical cut-offs. All bermudagrass EST sequences generated from this

experiment have been deposited in dbEST at the National Center for Biotechnology Information (NCBI accession numbers BQ825893- BQ826455, BG322272- BG322368, and BG354696). The entire collection of submitted ESTs and homology comparisons have been submitted to dbEST (http://www.ncbi.nlm.nih.gov).

DNA microarray construction.

Plasmid DNA was isolated from all 834 SSH clones that selected from two independent subtraction libraries (Yukon infected *vs.* Jackpot infected, Jackpot infected *vs.* Jackpot non-infected) by using Biomek 2000 Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA, USA). The plasmid DNA was then PCR amplified by following two step PCR program: 92 °C for 2 min; then 10 cycles of 95 °C for 20 sec, 52 °C for 20 sec and 72 °C for 1 min and 45 sec; following with 25 cycles of 95 °C for 20 sec, 49 °C for 20 sec and 72 °C for 5 min and 20 sec, which amplified more copies of the specific fragments with lower annealing temperature; another 72 °C extension step was added for 7 min and followed by 4 °C. The ArrayIt PCR Purification Kit (TeleChem, Int. Sunnyvale, CA, USA) was used to clean PCR products in 96-well micro-plate centrifugation format. Approximately 0.2 to 1 ug/ul cleaned PCR products in 384 well titer plates were re-suspended in 5 ul water and shook for one and half hours. An equal volume of 100% DMSO (5 ul) was added into the PCR products 2 hours before spotting. The final concentration of DMSO was 50%. The PCR products (0.1-0.5 ug/ul) were spotted on Telechem SuperAmine slides (TeleChem, Int. Sunnyvale, CA, USA) using the MicroGrid apparatus (BioRobotics, Hudson, NH, USA). Sixteen ArrayIt Stealth Micro Spotting Pins (TeleChem, Int. Sunnyvale, CA, USA) were used for the array printing.

The whole array harbors 12X4 sub-array and each sub-array contains 8X9 spots. Each clone was printed in duplicate adjacent to each other at 0.4 mm spacing to increase the reliability of experiment. The slides were dried on arrayer for 10-15 minutes after the spotting was complete. UV crosslinking (300 milliJoules) was used to fix the DNA on the surface of the slides. Printed microarrays were washed to remove unbound material and double-stranded DNA by submerging in boiling water for 2 minutes and cooling in ice-cold ethanol for 3 minutes, and then all slides were spun dried. Slides were stored under vacuum at room temperature.

Expression profiling.

Total RNA was isolated from Yukon infected and Jackpot infected bermudagrass crown tissue collected in fall 2000 and spring 2001 by ToTALLY RNA isolation kit (Ambion, Houston, TX, USA). Because of the high polysaccharides contamination, bermudagrass total RNA was further purified with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) spin column following manufacturer's instructions. Due to the limited sample amount and low RNA yield, the purified total RNA was amplified by using MessageAmp aRNA kit (Ambion, Houston, TX, USA) to generate a sufficient amount of RNA for the expression studies. The two-step indirect labeling method, Amino Allyl cDNA Labeling Kit (Ambion, Austin, TX, USA), was used to generate fluorescent dye labeled nucleotides for array hybridizations. The cDNA was made from Yukon infected and Jackpot infected samples aRNAs by reverse transcription. In the aRNA procedure one of the nucleotides (dTTP) was partially substituted with an analog containing a reactive primary amino group (i.e. amino allyl dUTP). After the reverse

transcription reaction, the amino modified cDNA was coupled to the fluorescent dye by incubation of the cDNA with the succinimidyl ester-derivitized reactive free dye, e.g. Cy3 or Cy5 mono-reactive NHS-ester (Amersham, Piscataway, NJ, USA). The coupling reaction was terminated by addition of hydrozylamine, and the reaction was passed through a NucAway Spin Column to remove the free dye. Paired Cy3 / Cy 5 labeled samples were mixed before the spin column purification step. The labeled cDNA was then concentrated by ethanol precipitation. For pre-hybridization, each slide was incubated in 50 ml of pre-hybridization solution (5 X SSC, 0.1% SDS, 1% BSA) for 1 to 2 hours at 42 °C. Then slide was washed in ultra pure water for three times (1 minute each) and span dried. For hybridization, 45 ul of Glassy hybridization solution (Clontech, Palo Alto, CA, USA) was added to the labeled probe pellet and incubated in 65 °C with occasional tabbing till the pellet dissolved. The probe mixture was then boiled for 3 minutes for denaturizing and applied to one end of the slide. A cover slip was carefully laid on the top of the array to distribute probe solution evenly on the slide. To maintain the humidity of the hybridization chamber, 30 ul of 3 X SSC was added to both end of the chamber. The hybridization was conducted in a water bath for 16 to 20 hours at 65 °C with hybridization chamber wrapping in aluminum foil. Two dye swap experiments were conducted side by side to reduce uneven coupling effect of two fluorescence dyes. Following hybridization, the microarray was placed immediately into wash solution I (1 X SSC, 0.1% SDS), and washed at R/T for 5 minutes with the cover slip slide off the array gently. The slide was then transferred into wash solution II (0.1 X SSC, 0.1% SDS) and washed at R/T for 5 minutes. The final wash was conducted in wash solution III (0.05 X SSC) at R/T for 5 minutes. Array slides were scanned by

ScanArray 3000 laser scanner (GSI Lumonics, Watertown, MA, USA) at pixel size resolution of 10x10 microns. Data from each fluorescence channel was collected and stored as a separate 16- bit TIFF image.

Data collection and processing.

Signal extraction and spot quantification were conducted by GenePix Pro 4.0 (Axon Instruments, Union City, CA, USA). The intensity for each spot was calculated by the median of local background-subtracted signal. The log base two transformation of the expression ratio was used to adjust the ratio for more reliable detection. Confidence intervals for gene expression ratios are generally accepted as significant for a two-fold change or when the log base two ratio is above "+1.0" or below "–1.0" (post-normalization). Normalization was conducted to account for unbalanced Cy3-Cy5 labeling of cDNA or unbalanced scanning. Normalized "Ratio of Medians" was generated based on global normalization by GenePix Pro 4.0. A normalization factor was calculated based on the total measured fluorescence signal. This factor was then applied to the entire column of data (Ratio of Medians) to make the median ratio equal to a value of 1.0 (ie. red = green), and then the data were filtered for poor quality features such as small size or background interference. Normalized data were saved as Microsoft Excel files and further processed to identify differentially expressed genes according to the following criteria: both background subtracted median (F633 Median - B633 or F543 Median - B543) intensities greater than 300 or at least one background subtracted median (F543 Median - B543 or F633 Median - B633) intensity greater than 1000. Among all these post-normalization data set, log based two ratio above "+1.0" (two fold increase on

Ratio of Medians) or below "–1.0" (two fold decrease on Ratio of Medians) was used to select "up" or "down" regulated genes. The expression intensity of each differentially regulated gene was calculated by the average value of the two duplicated spots, two dye swap experiments as well as identical contigs. Expression data from fall 2000 and spring 2001 were clustered together and compared by Genesis from the Graz University of Technology in Graz, Austria for non-hierarchical cluster (k-means) analysis. The k-means clustering generated 6 clusters based on their expression patterns. Centroids were calculated for each group corresponding to the average of the expression profiles. Individual genes were reassigned to the group in which the centroid was the most similar to the gene. Group centroids are then recalculated, and the process for each experiment was iterated (repeated) 2000 times until the group compositions converge. The text output files for cluster analysis were generated which contained tables of the corresponding clustered gene ID's and expression ratios for each experiment.

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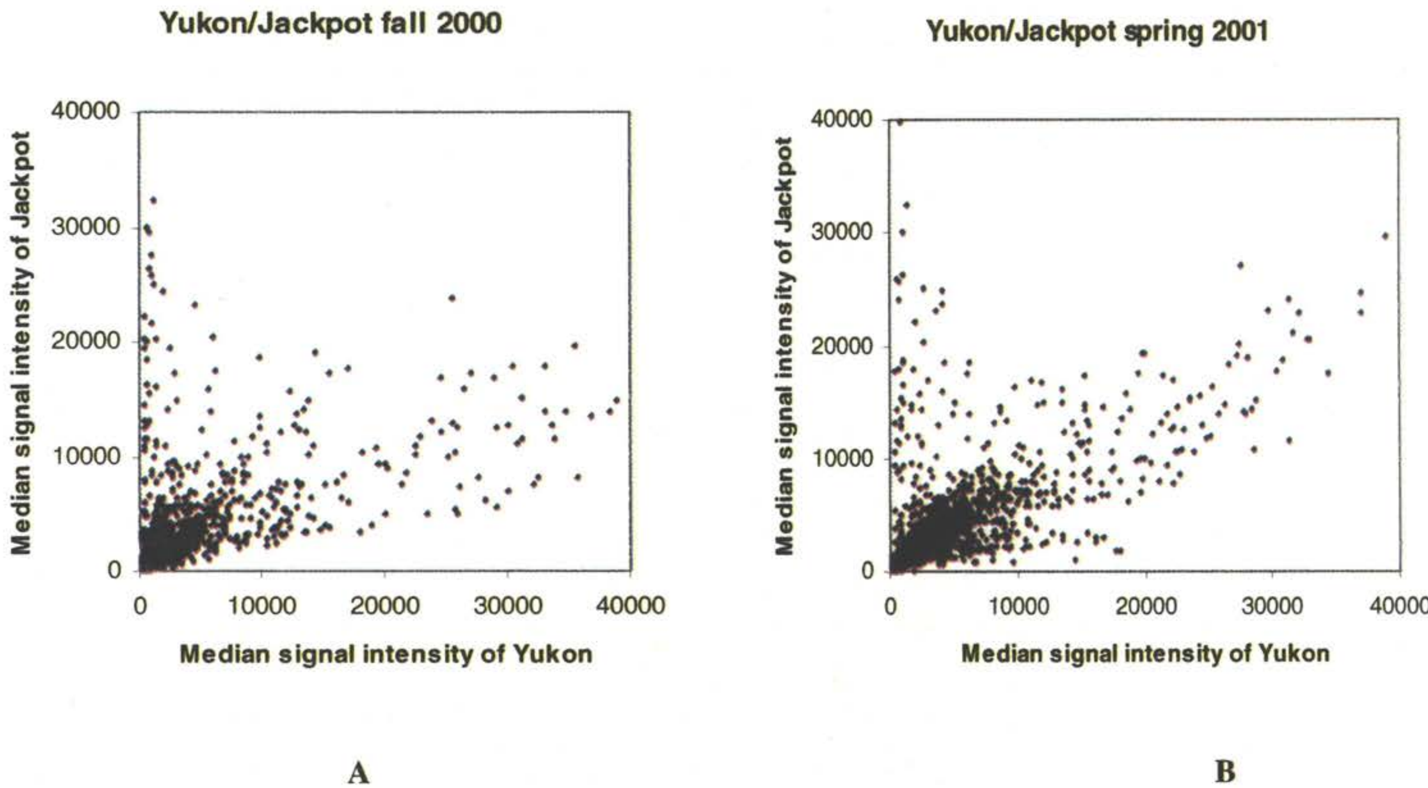

Fig. 1. Scatter plot comparing the spot intensities in hybridizations with probes from Yukon (x axis) and Jackpot (y axis). Data from images of both Cy dye were plotted as the median signal minas background signal intensity after normalization of clones spotted in duplicate.

(A) Scatter plot of signal intensities of two cultivars sampled in fall 2000.

(B) Scatter plot of signal intensities of two cultivars sampled in spring 2001.

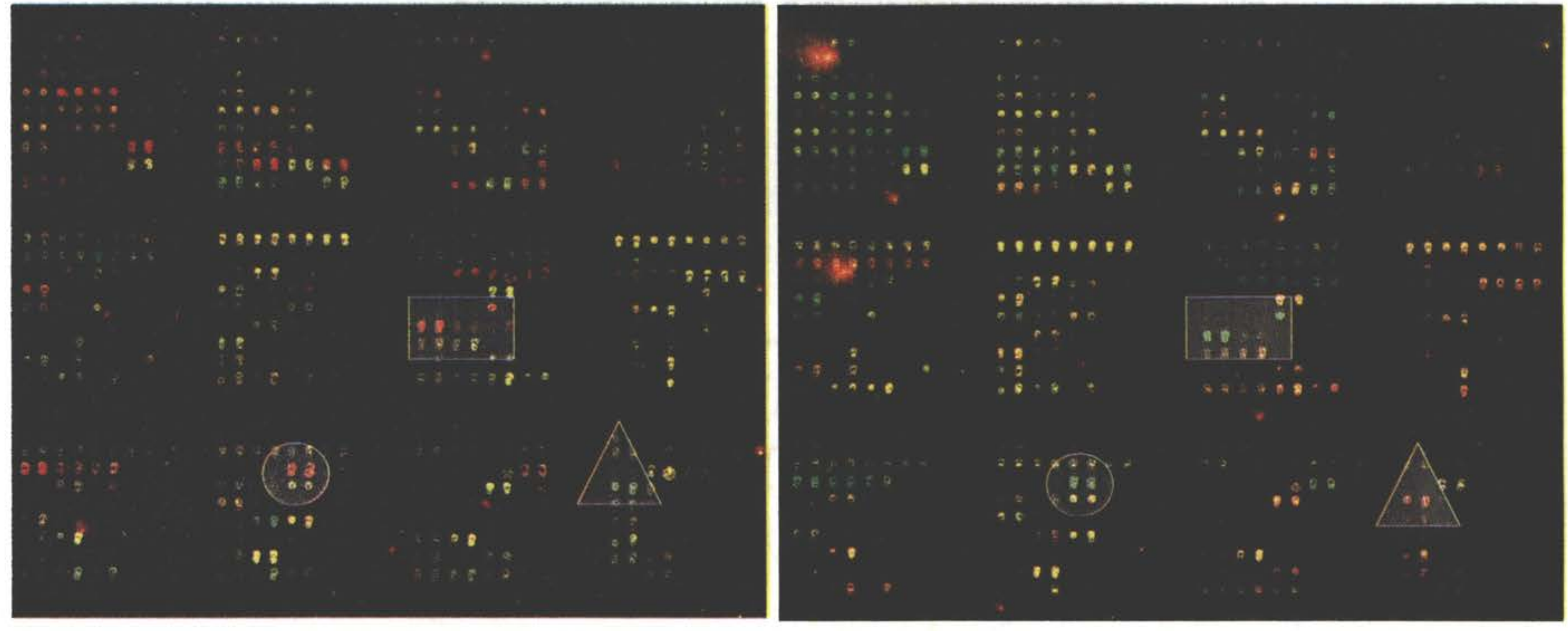

Fig. 2. Comparison of dye swap experiments of Yukon/Jackpot fall 2000 RNA expression. A: RNA from Yukon labeled with Cy3 and RNA from Jackpot labeled with Cy5. Clones showed green color were induced in Yukon, and clones showed red color were induced in Jackpot. B: dye-swap experiment of A. RNA from Yukon labeled with Cy5 and RNA from Jackpot labeled with Cy 3. Clones showed red color were induced in Yukon, and clones showed green color induced in Jackpot. Same genes were corresponded in between two experiments with reversed color indicated by different shape of marks.

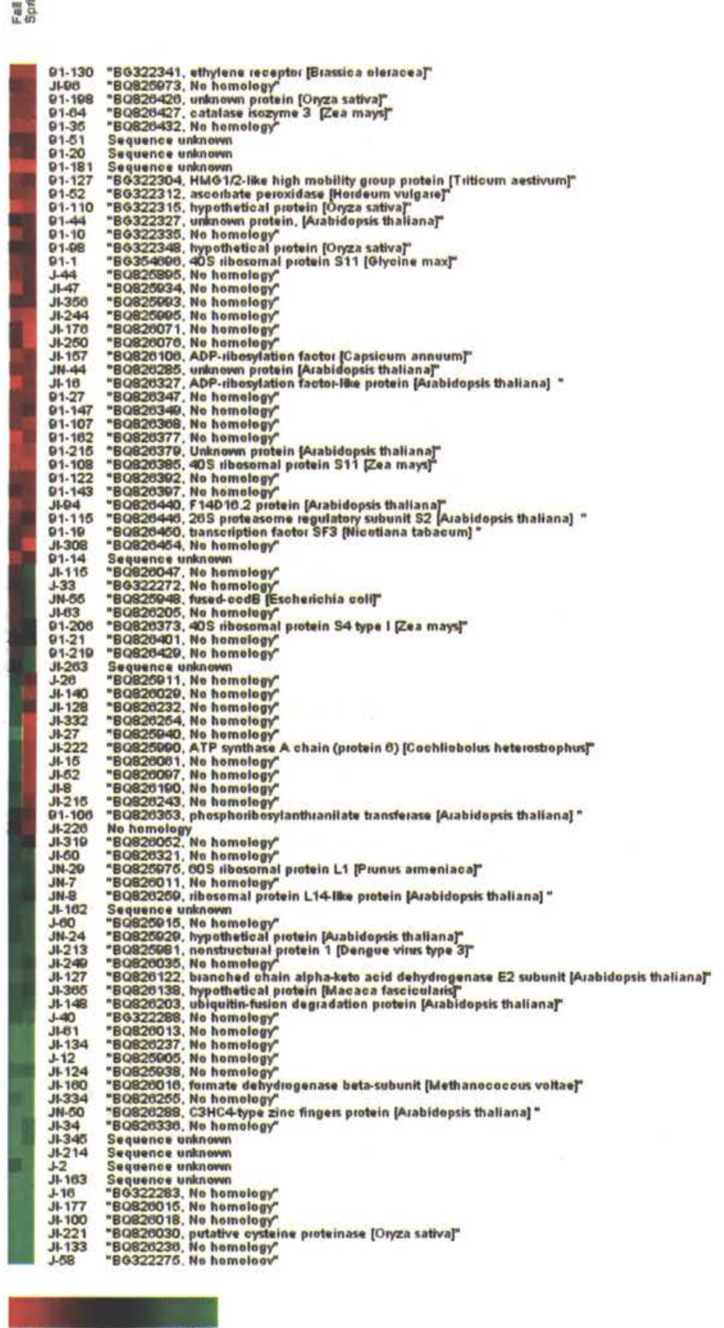

Fig 3. Tree view of cluster analysis by k-mean on 89 transcripts in different response to fungal infection for resistant and susceptible cultivars in fall 2000 and spring 2001. Clustering was performed by Genesis. The color saturation reflects the magnitude of the $\log_2$ expression ratio (Yukon/Jackpot) for each transcript with clone number (transcript number), GenBank EST accession number, and annotation. Transcripts are grouped into patterns **(A)** to **(D)** according to their expression profiles of Yukon induced (red) and Jackpot induced (green) at different time points. Each gene is represented by a single row of colored boxes, and each time point is represented by a single column.

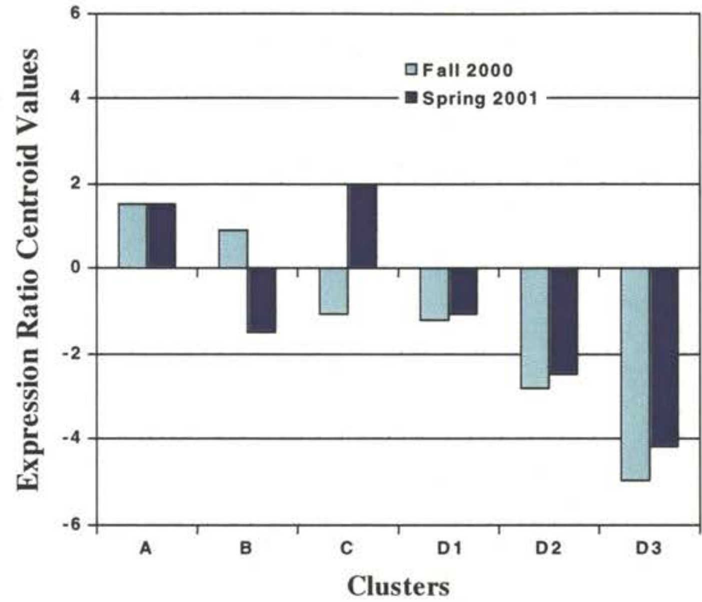

Fig. 4. Centroid views of cluster analysis by k-mean for Yukon and Jackpot in two seasons. The centroid value (bars) represents the average of expression of all genes present in each cluster. The expression ratios (y axis) of transcripts (log-2) for differentially expressed genes for Yukon or Jackpot are presented by bars for each season. Log_2 Ratio = 0 expression no change, Log_2 Ratio > 0 induced in Yukon, Log_2 Ratio < 0 induced in Jackpot.

(A) Genes constitutively over-expressed in Yukon for both season. (B) Genes were induced in Yukon in fall and response more to Jackpot in next spring. (C) Genes were induced in Jackpot in fall and response more to Yukon during the following spring. (D) Including D1, D2 and D3. Genes induced in Jackpot, expressions had slightly change between two seasons.

Table 1. Functional Categories of Transcripts of Bermudagrass Represented in Yukon/Jackpot SSH Libraries

Major Functional Categories	Number of Unique Contig	Percentage %
Oxidative burst /stress or apoptosis	3	1.9
Anti-microbial genes	1	0.6
Low molecular weight defense signals	2	1.3
Cell signaling and communications	12	7.8
Cell maintenance and development	19	12.4
Others - Unclassified	25	16.3
No homology	92	59.7
Total	154	100

Table 2. Cluster Analysis of Yukon and Jackpot Gene Expression Profiles from Fall 2000 and Spring 2001

Clone ID	Log_2 Ratio		Cluster	Function Classification	Accession No., Annotation
	Fall 2000	Spring 2001			
91-130	1.63	1.82	A	defense	BG322341, ethylene receptor [Brassica oleracea]
JI-96	1.21	1.27	A		BQ825973, No homology
91-198	1.45	1.42	A		BQ826426, unknown protein [Oryza sativa]
91-64	1.43	1.34	A	oxidative burst	BQ826427, catalase isozyme 3 [Zea mays]
91-35	1.96	1.81	A		BQ826432, No homology
91-51	1.08	1.16	A		Sequence unknown
91-20	1.29	1.12	A		Sequence unknown
91-181	2.9	3.14	A		Sequence unknown
91-127	1.34	1.12	A	signaling	BG322304, HMG1/2-like high mobility group protein [Triticum aestivum]
91-52	1.28	1.5	A	oxidative burst	BG322312, ascorbate peroxidase [Hordeum vulgare]
91-110	2.47	1.73	A		BG322315, hypothetical protein [Oryza sativa]
91-44	0.78	1.23	A		BG322327, unknown protein, [Arabidopsis thaliana]
91-10	1.94	1.11	A		BG322335, No homology
91-98	0.82	0.63	A		BG322348, hypothetical protein [Oryza sativa]
91-1	1.27	1.22	A	maintenance	BG354696, 40S ribosomal protein S11 [Glycine max]
J-44	1.56	1.17	A		BQ825895, No homology
JI-47	2.12	1.01	A		BQ825934, No homology
JI-356	1.08	1.11	A		BQ825993, No homology
JI-244	1.59	2.03	A		BQ825995, No homology
JI-176	1.02	3.76	A		BQ826071, No homology
JI-250	1.02	2.42	A		BQ826076, No homology
JI-157	1.81	1.47	A	signaling	BQ826106, ADP-ribosylation factor [Capsicum annuum]
JN-44	1.55	1.5	A		BQ826285, unknown protein [Arabidopsis thaliana]
JI-16	2.28	1.48	A	signaling	BQ826327, ADP-ribosylation factor-like protein [Arabidopsis thaliana]
91-27	1.54	1.28	A		BQ826347, No homology
91-147	1.5	1.11	A		BQ826349, No homology
91-107	1.55	1.59	A		BQ826368, No homology

91-162	2.33	1.54	**A**		BQ826377, No homology
91-215	2.21	1.96	**A**		BQ826379, unknown protein [Arabidopsis thaliana]
91-108	2.12	2.06	**A**	maintenance	BQ826385, 40S ribosomal protein S11 [Zea mays]
91-122	1.35	1.38	**A**		BQ826392, No homology
91-143	1.03	1.39	**A**		BQ826397, No homology
JI-94	1.61	1.46	**A**	others	BQ826440, F14D16.2 protein [Arabidopsis thaliana]
91-115	1.45	0.6	**A**	maintenance	BQ826446, 26S proteasome regulatory subunit S2 [Arabidopsis thaliana]
91-19	1.09	1.37	**A**	signaling	BQ826450, transcription factor SF3 [Nicotiana tabacum]
JI-308	1.12	2.31	**A**		BQ826454, No homology
91-14	1.61	1.01	**A**		Sequence unknown
JI-115	1.15	-1.46	**B**		BQ826047, No homology
J-33	0.64	-1.19	**B**		BG322272, No homology
JN-55	1.06	-1.16	**B**	others	BQ825948, fused-ccdB [Escherichia coli]
JI-63	1.04	-1.24	**B**		BQ826205, No homology
91-206	1.19	-0.03	**B**	maintenance	BQ826373, 40S ribosomal protein S4 type I [Zea mays]
91-21	-0.01	0	**B**		BQ826401, No homology
91-219	1.21	-1.49	**B**		BQ826429, No homology
JI-263	0	-1.44	**B**		Sequence unknown
J-26	-1.08	1.01	**C**		BQ825911, No homology
JI-140	-1.31	1.7	**C**		BQ826029, No homology
JI-128	-1.71	1.2	**C**		BQ826232, No homology
JI-332	-1.28	2.37	**C**		BQ826254, No homology
JI-27	-2.42	2.18	**C**		BQ825940, No homology
JI-222	-1.61	1.94	**C**	maintenance	BQ825990, ATP synthase A chain (protein 6) [Cochliobolus heterostrophus]
JI-15	-1.37	1.92	**C**		BQ826061, No homology
JI-52	-1.92	1.66	**C**		BQ826097, No homology
JI-8	-1.16	1.81	**C**		BQ826190, No homology
JI-215	-1.42	1.27	**C**		BQ826243, No homology
91-106	-0.62	1.13	**C**	maintenance	BQ826353, phosphoribosylanthranilate transferase [Arabidopsis thaliana]
JI-226	-0.01	1.55	**C**		No homology
JI-319	-1.24	-0.37	**D1**		BQ826052, No homology

JI-50	-1.18	-1.29	**D1**		BQ826321, No homology
JN-29	-1.06	-1.04	**D1**	maintenance	BQ825975, 60S ribosomal protein L1 [Prunus armeniaca]
JN-7	-1.36	-1.04	**D1**		BQ826011, No homology
JN-8	-1.03	-0.56	**D1**	maintenance	BQ826259, ribosomal protein L14-like protein [Arabidopsis thaliana]
JI-162	-1.22	-1.56	**D1**		Sequence unknown
J-60	-1.02	-1.79	**D1**		BQ825915, No homology
JN-24	-1.21	-1.36	**D1**		BQ825929, hypothetical protein [Arabidopsis thaliana]
JI-213	-1.35	-1.61	**D1**		BQ825981, nonstructural protein 1 [Dengue virus type 3]
JI-249	-1.45	-1.12	**D1**		BQ826035, No homology
JI-127	-1.99	-1.28	**D1**	maintenance	BQ826122, branched chain alpha-keto acid dehydrogenase E2 subunit [Arabidopsis thaliana]
JI-365	-1.41	-1.2	**D1**		BQ826138, hypothetical protein [Macaca fascicularis]
JI-148	-1.66	-1.49	**D1**	maintenance	BQ826203, ubiquitin-fusion degradation protein [Arabidopsis thaliana]
J-40	-1.73	-2.16	**D1**		BG322288, No homology
JI-61	-3.97	-3.29	**D2**		BQ826013, No homology
JI-134	-3.92	-2.43	**D2**		BQ826237, No homology
J-12	-2.96	-2.86	**D2**		BQ825905, No homology
JI-124	-2.21	-2.14	**D2**		BQ825938, No homology
JI-160	-2.77	-2.49	**D2**	maintenance	BQ826016, formate dehydrogenase beta-subunit [Methanococcus voltae]
JI-334	-2.24	-2.39	**D2**		BQ826255, No homology
JN-50	-2.47	-3.98	**D2**	signaling	BQ826288, C3HC4-type zinc fingers protein [Arabidopsis thaliana]
JI-34	-2.49	-2.25	**D2**		BQ826336, No homology
JI-345	-2.78	-2.09	**D2**		Sequence unknown
JI-214	-2.55	-2.89	**D2**		Sequence unknown
J-2	-1.94	-2.38	**D2**		Sequence unknown
JI-163	-6.08	-6.38	**D3**		Sequence unknown
J-16	-5.28	-4.03	**D3**		BG322283, No homology
JI-177	-4.96	-4.04	**D3**		BQ826015, No homology
JI-100	-4.42	-4.37	**D3**		BQ826018, No homology
JI-221	-4.66	-3.15	**D3**	oxidative burst	BQ826030, putative cysteine proteinase [Oryza sativa]
JI-133	-5.1	-5.93	**D3**		BQ826236, No homology
J-58	-4.34	-3.26	**D3**		BG322275, No homology

Chapter III

Differential Gene Expression in Bermudagrass in Response to the Spring Dead Spot Fungal Pathogen *Ophiosphaerella herpotricha*

Bermudagrass [*Cynodon dactylon* (L.) Pers.] is extensively used for turf, forage, and soil stabilization in the southern United States. Spring dead spot (SDS) caused by *Ophiosphaerella herpotricha* is a serious fungal disease of bermudagrass turf. Suppression subtractive hybridization (SSH), systematic sequencing of cDNA clones, and cDNA microarrays were used to study the relative abundance of transcripts corresponding to sets of clones from fungal infected and control crown tissue of a susceptible bermudagrass cultivar. A total of 206 contigs were generated from sequence comparison of 512 bermudagrass ESTs from both forward and reverse SSH libraries. These contigs were grouped into six categories according to their putative functions. Insights into the global gene expression during the progression of this disease were obtained by constructing a cDNA microarray with 834 bermudagrass clones. Expression profiles from both fall and spring seasons were studied and compared to each other. About 8% of the genes selected from SSH library represented by the array displayed greater than 2-fold differential expression. These thirty-one genes were grouped into 6 clusters according to their expression patterns during two seasons.

Additional keywords: defense response, bermudagrass, SSH, expression profiling.

Bermudagrass, *Cynodon dactylon*, is a warm-season perennial sod-forming species used widely throughout the southern United States for turf, forage, and soil stabilization. Within its area of adaptation, bermudagrass is hardy enough to survive with little care and can respond quickly to more intensive management. Bermudagrass cultivars typically produce extensive root systems and are drought tolerant. They also respond well to nitrogen fertilization and produce abundant biomass when soil moisture is adequate (Smith et al. 1989).

Spring dead spot (SDS), is a serious patch disease of bermudagrass which was first observed in Oklahoma during 1954 (Wadsworth and Young 1960). The symptoms of SDS usually appear in early spring as dormant bermudagrass resumes growth and occur as circular, dead areas ranging from a few centimeters up to several meters in diameter (Smith et al. 1989). In the Southern Great Plains region (including Kansas, Oklahoma and Texas) the primary causal agent of spring dead spot is an ectotrophic root-infecting fungus, *Ophiosphaerella herpotricha* (Tisserat et al. 1989). *O. herpotricha* produces dark-colored, ectotrophic mycelia, occasionally with bubble-like structures on roots, stolons, and rhizomes of grasses, partially immersed in dead leaf sheath and stolen tissues have also been observed on bermudagrass affected with SDS (Tisserat et al. 1989). It has been found that *O. herpotricha* is very active in the fall and in the early spring under cool moist conditions. It may infect bermudagrass whenever soil temperatures are between 10 and 25 °C (Endo et al. 1985). Although much effort has been placed on resistance breeding, no *O. herpotricha* immune cultivars have been developed (Martin et al. 2001).

Induced defense has received much attention and a large number of genes encoding defense-related proteins have been identified. Many of these genes are induced when the plant is attacked by microbial pathogens, viruses or insects (Dong 1998; Eisen et al. 1998). Therefore, plant defense response is associated with an integrated set of genes (Schenk et al. 2000). However, the analyses of plant defense response to pathogen infection have traditionally focused on one or a few genes at any one time (Jones 2001), whereas the integration of all different responses into a global knowledge of plant disease resistance, the precise control of pathway cross-talk (Genoud and Metraux 1999) or a complete catalogue of defense-related genes are still lacking. DNA microarray analysis is a standard tool for genome-wide monitoring of gene expression in animal studies and is starting to contribute to the field of plant biology. Initial experiments have demonstrated that microarrays are well suited for studying plant genes (Schena et al. 1995, Ruan et al. 1998, Kehoe et al. 1999). The first results from experimental analyses of plant defense to either biotic or abiotic stress with microarrays are beginning to appear (Schenk et al. 2000, Seki et al. 2001, Kawasaki et al. 2001, Ozturk et al. 2002).

In this study, putatively induced gene transcripts were identified by suppression subtractive hybridization (Diatchenko et al. 1996) of mRNA isolated from fungal infected and non-infected crown tissue of the bermudagrass susceptible cultivar "Jackpot." The gene expression profiles of fungal infected *vs.* non-infected plants in both the fall and spring were studied to characterize differential gene expression in the host during the progression of this disease.

RESULTS

Suppression subtractive hybridization cDNA library.

A total of 524 cDNA clones were selected from the susceptible bermudagrass cultivar Jackpot by using the suppression subtractive hybridization (SSH) technique (Diatchenko et al. 1996). Subtractions were conducted between mRNAs frominfected by the fungus *Ophiosphaerella herpotricha* and non-infected (control) crown tissues. A set of 379 clones was generated by SSH in which mRNA from infected tissue was used as 'tester' and the mRNA from control tissue was used as 'driver'. This set of cDNA clones should be enriched for genes induced in response to infection. A reverse subtraction was also conducted by exchanging the "tester" and "driver" (e.g. control tissue = tester, infected tissue = driver) to obtain genes repressed in response to infection. The reverse library contained 145 clones. The average size of the inserts was about 400 bp.

Sequencing and data mining.

Upon assembling the readable sequences obtained from the 5' or 3' ends of 524 cDNAs, 512 bermudagrass EST sequences were generated from two subtractions (forward and reverse). Clones longer than 100 bp were submitted to GenBank dbEST database (Boguski et al. 1993). A total of 432 clones (325 from infected plants and 107 from control plants) were deposited in dbEST at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Nucleotide similarity searches and data mining were performed by batch processing using PipeOnline (See Material and Methods). The 512 clones were assembled into 206 contigs according to their sequence similarity. Sequence analysis

revealed 97 of 206 contigs (47.1%) did not match any entries in the GenBank database and are likely to be novel. The remaining 109 contigs (52.9%) had homologies that matched previously described genes in GenBank. There were 15 contigs with unknown or hypothetical proteins. The PipeOnline program categorizes sequences based on a functional dictionary derived from microbial metabolism. Genes of potentially related function were grouped into five categories: 1) genes involved in oxidative burst /stress or apoptosis; 2) antimicrobial genes; 3) genes for cell signaling and communications, 4) genes involved in cell maintenance and development; or 5) other (unknown) functions. All functional categories are listed in Table 1 with the number of unique contigs as well as the percentage included in each group.

Differential gene expression during fall infection.

Probes were prepared from infected and control plants sampled in late fall (November) of 2000 to study changes of gene expression in response to fall infection and regrowth. A dye swap experiment was conducted to reduce variation due to uneven coupling of the two fluorescence dyes. The same genes were identified in the two experiments as indicated by reversed color (Figure 1).

Genes were designated as up-regulated only if the $\log_2$ ratio of Infected/Control was >1.0. Genes were designated down-regulated when this ratio was < -1.0. Based on this criterion, there were 65 genes differentially expressed in response to fall infection (Appendix IX). mRNA levels for 36 genes were up-regulated, whereas mRNA levels for 29 genes were down-regulated as indicated in the scatter plot (Fig 2A). Because of the

stringent criteria used for these designations, the percentage of differentially expressed genes in response to fungal infection could be higher.

Differential gene expression in response to spring infection and re-growth.

Probes were prepared from infected and control plants sampled in early spring (April) of 2001 to study changes of gene expression in response to spring infection and regrowth. A dye swap experiment was also conducted to reduce variation due to uneven coupling of the two fluorescence dyes. The same genes were identified in the two experiments as indicated by reversed color (data not shown). A total of 65 genes showed differential expression patterns in the spring ($\log_2$ larger than 1 or smaller than -1). (Appendix X). Among them, 63 genes were up-regulated, whereas only 2 genes were down-regulated in this season, as illustrated in the Figure 2B scatter plot.

Cluster Analysis of Two Seasons

In order to understand seasonal bermudagrass responses to fungal infection, expression data from differentially expressed genes during fall 2000 and spring 2001 were analyzed using nonhierarchical algorithm (k-mean) to identify similar expression patterns across seasons (Fig. 3) (Eisen et al. 1998). A list of differentially expressed genes for the two seasons is presented in Table 2. The expression profiles were represented by four patterns by the cluster analysis. These clusters represent transcripts that are differentially expressed between the two seasons as indicated by their centroid views (Fig. 4).

A total of 15 genes were grouped in Cluster A. Although all 15 genes were up-regulated by fungal infection, the expression patterns were not identical. Therefore, three sub-clusters (A1, A2 and A3) were established. Nine genes that were up-regulated for both seasons are in sub-cluster A1. At least two of these genes are involved in cell signaling and communications (C3HC4-type zinc fingers protein and ADP-ribosylation factor). Another two known genes code for proteins that have homology with ATP synthase and branched chain alpha-keto acid dehydrogenase, both of these are involved in cell maintenance and development. Two transcripts are present in A2 with expressions slightly decreasing from fall to spring. The one gene with a known function codes for a protein related to voltage-dependent anion channel. A3 contained 4 transcripts up-regulated for both seasons including a DnaJ-related protein ZMDJ1 from maize. Expression of these genes slightly increased from fall to spring.

Genes that were not differentially expressed in the fall and were up-regulated in spring were sorted into cluster B. Only 6 genes belong to this cluster including ubiquitin-fusion degradation protein and ribosomal protein which are related to cell maintenance and development.

Cluster C had 9 genes that were down-regulated in fall and up-regulated in following spring. A cysteine proteinase present in this cluster has putative function related to oxidative burst / stress and apoptosis. Two other known function genes had homology with genes for formate dehydrogenase beta-subunit and ATPases from *Arabidopsis thaliana.* Both of these genes are involved in cell maintenance and development.

There is only one gene, that was down-regulated for both fall and spring seasons in response to fungal infection (Cluster D). However, the sequence of this gene does not match with any gene in GenBank and is therefore novel with an unknown function.

The cluster analysis of gene expression with time course revealed six groups of genes with similar behavior (Fig. 3). One implication of a common temporal pattern of expression is that genes might share similar or related roles in cellular processes, or the same signal molecules might regulate them.

DISCUSSION

SSH Library.

Several pathogen defense genes, such as cysteine proteinase, ascorbate peroxidase, and a class III chitinase were isolated from the infected/control SSH library. These genes function in oxidative burst/stress, apoptosis or antimicrobial genes, respectively. About 29% of contigs belong to cell maintenance and development. The ATPase synthase gene was represented with 17 different contigs. Fifteen contigs (7.3%) were identified with functions related to cell signaling and communication proteins. Most signaling genes are expected to be rare transcripts. However, a transcript homologous to the C3HC4-type zinc fingers protein cloned from *Arabidopsis thaliana* was highly represented in the SSH library and shown to be induced after infection. Similar cases were also observed for several other genes (ADP-ribosylation factor, ATP synthase, branched chain alpha-keto acid dehydrogenas, and formate dehydrogenase). Kim et al. (2001) has demonstrated that SSH would be highly ineffective in profiling gene expression changes in diseased *vs.* normal tissues or over an experimental time course where small changes in gene expression are more likely to be physiologically relevant. In addition, for effective enrichment by SSH PCR the target mRNA must be at least 0.01% of the total mRNA (Ji et al. 2002), thus low abundance genes such cytokines, and receptors, which are key regulators of many pathological processes might not be detected by this method.

Despite the 60% redundancy of this subtraction library, microarray hybridization identified 70 unique contigs (62 from infected plants and 8 from control plants) that showed differential expression (either up or down-regulated) due to fungal infection in

both seasons. Therefore, out of 206 unique contigs assembled from SSH clones, 34% were differentially expressed. This demonstrates that subtractions between infected and control plants were successful and differentially expressed genes were enriched in this library.

Bermudagrass responses to fungal infections.

In order to understand molecular mechanisms of bermudagrass-fungal interactions, expression profiles of *O. herpotricha* infected bermudagrass that were sampled in two seasons (fall 2000 and spring 2001) were studied with a cDNA array that contained 834 bermudagrass ESTs. More than one hundred genes represented by this array were differentially expressed in response to fungal infected bermudagrass for both seasons. Although the biology of the defense response is uncertain, these results prompt us to hypothesize that altered gene expressions are the result of defense related gene activation. It is likely that these genes play very important roles in plant-pathogen interactions and defense responses to fungal infection.

Among genes with altered expression patterns, eighteen in the fall and twenty-one in the following spring had known functions. Eight responsive genes have a role in cell signaling and communications. Besides genes that encode transcription and translation factors, genes involved in signal recognition and transduction were also included in this group (e.g. ADP-ribosylation factor, high mobility group protein, and voltage-dependent anion channel). Another 17 genes were involved in cell maintenance or development, including genes that encode variable ribosomal proteins, several transport related genes (ATPase, DnaJ, Ferredoxin) and genes involved in protein (amino acid transport protein,

ubiquitin-fusion degradation like protein), carbon (branched-chain alpha-keto acid dehydrogenase, formate dehydrogenase), biosynthesis and secondary metabolite (phosphoribosylanthranilate transferase) pathways. We have identified four genes which have previously been implicated in plant defense and belong to two function categories; either oxidative burst/ stress related genes (catalase, ascorbate peroxidase, and cysteine proteinase) or low molecular weight defense signals (ethylene receptor). Interestingly, most of these genes were down-regulated in susceptible cultivar when infected by *O. herpotricha*. Our results implicate this functional group of genes in the defense response. This suggests that the susceptible cultivar cannot mount a successful defense response due to the repression of genes for key enzymes or proteins envolved in the oxidative burst response or pathways controlled by ethylene signaling.

Plant defense is often associated with elicitor and pathogen mediated induction of gene expression. Cascades of transcription factors and signaling molecules serve to amplify the input signal or modify the regulation of specific aspects of the complex plant defense response (Zhu 2002). Therefore, transcriptional activation of specific defense genes is important for the inducible resistance response. Chen et al. (2002) found that there are more transcriptional factor genes expressed in roots under stress condition compared when compared to leaf and flowers. In this study, several genes belonging to different families of transcription factors were found to be differentially expressed in response to fungal infection (root specific *SCARECROW* gene, C3HC4-type zinc fingers, and transcription factor SF3). In addition to the transcription factor genes, several root-specific genes, such as root ferredoxin (Matsumura et al. 1997), root specific amino acid transport protein AAP2 and AAP3 (Fischer et al. 1995), as well as non-green tissue

formate dehydrogenase (Suzuki et al. 1998) were identified in our subtraction library and show induction in response to fungal infection

Interaction of biotic and abiotic stresses on gene expression.

In any study of plant disease it is important to consider the contribution of each of the multiple interacting events that occurs upon pathogen infection. Many studies unveil the intimate link between pathogen responses and abiotic stress detection. For instance, it has been found that a large fraction of wound-inducible genes in *Arabidopsis* were also regulated by water stress (Reymond et al. 2000). The number of examples of similar defense gene induction common to both biotic and abiotic stresses are increasing (Durrant et al. 2000; Timmusk and Wagner 1999; van De Ven et al. 2000). Research has shown that symptoms of bermudagrass spring dead spot increase with cold winter temperatures. However, there is no direct molecular evidence to link pathogen infection with cold acclimation or freezing tolerance. Chitinases are well known for their roles as pathogenesis-related (PR) proteins. Gatschet et al. (1996) demonstrated increased chitinase levels in crown tissue of freeze-tolerant bermudagrass. Three bermudagrass chitinase genes were cloned and were found to be induced by cold acclimation and dehydration stresses (drought and ABA) (de los Reyes et al. 2001). These bermudagrass chitinase genes (genomic DNA clones) were printed on our bermudagrass array to study their expression in response to fungal infection. Among the three chitinases, one of them, *cht1* (AF105426), was induced in response to fungal infection. The relationship between cold acclimation and fungal infection still needs to be verified experimentally.

MATERIALS AND METHODS

Plant materials and pathogen treatment.

The spring dead spot (SDS) susceptible cultivar Jackpot was used in this study. Plots were seeded on July 5, 1995 using 1 pound of pure live seed per 1,000 square feet. The soil was a silty clay loam with a pH range of 6.9 to 7.0. Grasses were planted in a randomized complete block design with three replications. Mean phosphorus and potassium soil test levels were kept in the optimum range for turf growth, with average readings of 41 and 167 parts per million, respectively. All plots were inoculated on September 26, 1997 using *O. herpotricha* isolate KS 188 (by Dr. Tisserat, Kansas State University). Disease was evaluated by the diameter of each patch. Spring dead spot appeared at all inoculation sites during the three-year field study. No symptoms were present at the mock inoculation sites (Martin et al. 2001). In order to isolate transcripts from plant material, infected tissues were sampled on the edges of the infection patch of three inoculation sites in each of three replications. All samples were pooled for mRNA extraction. Crowns tissues were harvested, cleaned, and treated with RNALater (Ambion, Houston, TX, USA) for 10 minutes at room temperature. All samples were subsequently stored at -80°C.

mRNA isolation and suppression subtractive hybridization

The mRNA was isolated from bermudagrass crown tissues with Straight A's mRNA Isolation System (Novagen, Madison, WI, USA) following the manufacturer's protocol. Differentially expressed genes were isolated by using PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) starting with 2 ug of poly A^{+} RNA from

tissues being compared (Jackpot infected *vs*. Jackpot control). In the last step of secondary PCR, a seven-minute 72 °C extension was applied to ensure that all PCR products were full length and 3' adenylated. Three sets of subtractions were performed, including both experimental forward and reverse as well as control subtractions following the manufacturer's instructions.

Cloning and sequencing.

The subtracted cDNA population was cloned by TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA). PCR products were inserted into pCR2.1-TOPO vector in salt solution and transformed with One Shot TOP10F′ *E. coli*. Bacterial clones were picked and inoculated in to LB broth with 50 ug/ml ampicillin. Cells were cultured at 37 °C with 250 rpm shaking overnight. Then 1 ml of cell culture for each clone was stored at -80°C in 15% glycerol. Plasmid DNA from SSH clones was purified by using QIAprep Spin Miniprep kit (Qiagen, Valencia, CA, USA) and sequenced. Sequencing was performed with the BigDye Terminator Cycle sequencing kit (PE Biosystems, Foster City, CA, USA). The sequencing reactions were primed using the M13 reverse primer present in pCR2.1-TOPO vector and thus read from the m3G cap into the 5' end of each cDNA. The sequencing reaction products were analyzed using a ABI 3700 DNA Analyzer or ABI 373A automated DNA sequencer. Raw sequence data were edited using SEQUENCHER (version 3.1.1) (GeneCodes Corporation, Ann Arbor, MI, USA). All sequence outputs were checked visually to confirm overall quality of peak shape and correspondence with base calls. Leading and trailing vector and polylinker sequences from SSH, and sequences with more than 3% ambiguous base calls were removed.

Sequences shorter than 100 bp were not further analyzed. Groups of sequences were assembled into clusters using the contig routine of SEQUENCHER.

Homology comparisons and database construction.

The sequences in either Standard Chromatogram Format (SCF) or FASTA format were loaded into PipeOnline, an experimental Web-based resource designed by the Oklahoma State University Bioinformatics Group to assist investigators in the determination of metabolic and biological function from large-scale DNA sequence data. A base-caller PHRED (Ewing and Green 1998; Ewing et al. 1998) was applied to convert SCF file to FASTA format. Then a new set of contig-assembled files were assembled using PHRAP within PipeOnline. Assembled sequences were compared against a local NCBI non-redundant protein database using BLASTn. The resulting output files were automatically collected, parsed, formatted, assembled, indexed, and uploaded to a local server by the PipeOnline database assembly module. Functional sorting of the input DNA sequences was achieved through a proprietary sorting method that utilizes functional information gathered from public databases. Function has been estimated using the Metabolic Pathways database (MPW) functional dictionary (Selkov et al. 1998) obtained from WIT (Overbeek et al. 2000). Although the BLAST scores and P values were considered, the assessment of whether a given homology was significant was determined by investigator judgment, not by absolute numerical cut-offs. All bermudagrass EST sequences generated from the subtraction library have been deposited in dbEST at the National Center for Biotechnology Information (NCBI accession numbers BQ825893-BQ826455, BG322272-BG322368, and BG354696).

DNA microarray assembling.

Plasmid DNA was isolated from 834 SSH clones recovered from two independent subtraction libraries (Jackpot infected *vs.* Jackpot control, Yukon infected *vs.* Jackpot infected,) by using Biomek 2000 Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA, USA). The plasmid DNA was then PCR amplified by following two step PCR program: 92 °C for 2 min; then 10 cycles of 95 °C for 20 sec, 52 °C for 20 sec and 72 °C for 1 min and 45 sec; following with 25 cycles of 95 °C for 20 sec, 49 °C for 20 sec and 72 °C for 5 min and 20 sec, which amplified more copies of the specific fragments with lower annealing temperature; another 72 °C extension step was added for 7 min and followed by 4 °C. The ArrayIt PCR Purification Kit (TeleChem, Int. Sunnyvale, CA, USA) was used to clean PCR products in 96-well micro-plate centrifugation format. Approximately 0.2 to 1 ug/ul cleaned PCR products in 384 well titer plates were re-suspended in 5 ul water and shook for one and half hours. An equal volume of 100% DMSO (5 ul) was added into the PCR products 2 hours before spotting. The final concentration of DMSO was 50%. PCR products (0.1-0.5 ug/ul) were spotted on Telechem SuperAmine slides (TeleChem, Int. Sunnyvale, CA, USA) using the MicroGrid apparatus (BioRobotics, Hudson, NH, USA). Sixteen ArrayIt Stealth Micro Spotting Pins (TeleChem, Int. Sunnyvale, CA, USA) were used for the array printing. The whole array harbors 12x4 sub-array and each sub-array contains 8x9 spots. Each clone was printed in duplicate adjacent to each other at 0.4 mm spacing to increase the reliability of experiment. Slides were dried on arrayer for 10-15 minutes after the spotting was complete. UV crosslinking (300 milliJoules) was used to fix the DNA on the surface of the slides. Printed microarrays were washed to remove unbound material

and double-stranded DNA by submerging in boiling water for 2 minutes and cooling in ice-cold ethanol for 3 minutes, and then all slides were spun dried. Slides were stored under vacuum at room temperature.

Expression profiling.

Total RNA was isolated from bermudagrass Jackpot fungal infected and control plant crown tissue collected in fall 2000 and spring 2001 by ToTALLY RNA total RNA isolation kit (Ambion, Houston, TX, USA). Because of the high polysaccharides contamination, bermudagrass total RNA was further purified with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) spin column following manufacturer's instructions. Due to the limited sample amount and low RNA yield, the purified total RNA was amplified by using MessageAmp aRNA kit (Ambion, Houston, TX, USA) to generate a sufficient amount of aRNA for the expression studies. The two-step indirect labeling method, Amino Allyl cDNA Labeling Kit (Ambion, Austin, TX, USA), was used to generate fluorescent dye labeled nucleotides for array hybridizations. The cDNA was made from plant samples (Jackpot infected and Jackpot control) aRNAs by reverse transcription, one of the nucleotides (dTTP) was partially substituted with an analog containing a reactive primary amino group (i.e. amino allyl dUTP). After the reverse transcription reaction, the amino modified cDNA was coupled to the fluorescent dye by incubation of the cDNA with the succinimidyl ester-derivitized reactive free dye, e.g. Cy3 or Cy5 mono-reactive NHS-ester (Amersham, Piscataway, NJ, USA). The coupling reaction was terminated by addition of hydrozylamine, and the reaction was passed through a NucAway Spin Column to remove the free dye. Paired Cy3 / Cy 5 labeled

samples were mixed before the spin column purification step. The labeled cDNA was then concentrated by ethanol precipitation. For pre-hybridization, each slide was incubated in 50 ml of pre-hybridization solution (5 X SSC, 0.1% SDS, 1% BSA) for 1 to 2 hours at 42 °C. The slide was washed in ultra pure water for three times (1 minute each) and span dried. For hybridization, 45 ul of GlassHyb hybridization solution (Clontech, Palo Alto, CA, USA) was added to the labeled probe pellet and incubated in 65 °C with occasional tabbing till the pellet dissolved. The probe mixture was then boiled for 3 minutes for denaturizing and applied to one end of the slide. A cover slip was carefully laid on the top of the array to distribute probe solution evenly on the slide. To maintain the humidity of the hybridization chamber, 30 ul of 3 X SSC was added to both ends of the chamber. The hybridization was conducted in a water bath for 16 to 20 hours at 65 °C with the hybridization chamber wrapped in aluminum foil. Two dye swap experiments were conducted to account for uneven coupling of the two fluorescence dyes. Following hybridization, the microarray was placed immediately into wash solution I (1 X SSC, 0.1% SDS), and washed at room temperature. The slide was then transferred to wash solution II (0.1 X SSC, 0.1% SDS) and washed at room temperature for 5 minutes. The final wash was conducted in wash solution III (0.05 X SSC) at room temperature for 5 minutes.

Data collection and processing.

Array slides were scanned with a ScanArray 3000 laser scanner (GSI Lumonics, Watertown, MA, USA) at pixel size resolution of 10x10 microns. Data from each fluorescence channel was collected and stored as a separate 16-bit TIFF images. Signal extraction and spot quantification were conducted by GenePix Pro 4.0 (Axon

Instruments, Union City, CA, USA). The intensity for each spot was calculated as the median of the background-subtracted signal. A log base two transformation was used to detect the magnitude of change between the two conditions tested. Confidence intervals for gene expression ratios were accepted as significant for a two-fold change or when the log base two ratio is above "+1.0" or below "–1.0" (post-normalization). Normalization was conducted to account for unbalanced Cy3-Cy5 labeling of cDNA or unbalanced scanning. Normalized "Ratio of Medians" was generated based on global normalization by GenePix Pro 4.0. A normalization factor was calculated based on the total measured fluorescence signal. This factor was then applied to the entire column of data (Ratio of Medians) to make the median ratio equal to a value of 1.0 (ie. red = green), then the data were filtered for poor quality features such as small size or background interference. Normalized data were saved as Microsoft Excel files and further processed to identify differentially expressed genes according to the following criteria: both background subtracted median (F633 Median - B633 or F543 Median - B543) intensities greater than 300 or at least one background subtracted median (F543 Median - B543 or F633 Median - B633) intensity greater than 1000. Among all these post-normalization data set, log based two ratio above "+1.0" (two fold increase on Ratio of Medians) or below "–1.0" (two fold decrease on Ratio of Medians) was used to select "up" or "down" regulated genes. The expression intensity of each differentially regulated gene was calculated by the average value of the two duplicated spots, two dye swap experiments as well as identical contigs. Expression data from fall 2000 and spring 2001 were clustered together and compared by Genesis from the Graz University of Technology in Graz, Austria for non-hierarchical cluster (k-means) analysis. The k-means clustering was generated by 6

clusters based on their expression patterns. Centroids were calculated for each group corresponding to the average of the expression profiles. Individual genes were reassigned to the group in which the centroid was the most similar to the gene. Group centroids are then recalculated, and the process for each experiment was iterated (repeated) 2000 times until the group compositions converge. The text output files for cluster analysis were generated which contained tables of the corresponding clustered gene ID's and expression ratios for each experiment.

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We thank Dr. Ned A. Tisserat of Department of Plant Pathology at Kansas State University for inoculating the bermudagrass plots used in this experiment. Special thanks also go to Dr. Patricia Ayoubi of the Microarray Core Facility in the Department of Biochemistry and Molecular Biology at Oklahoma State University for her assistance with the array data analysis. This research was supported by The United States Golf Association (USGA) grant No. 1998-11-119 and the Oklahoma Agricultural Experiment Station.

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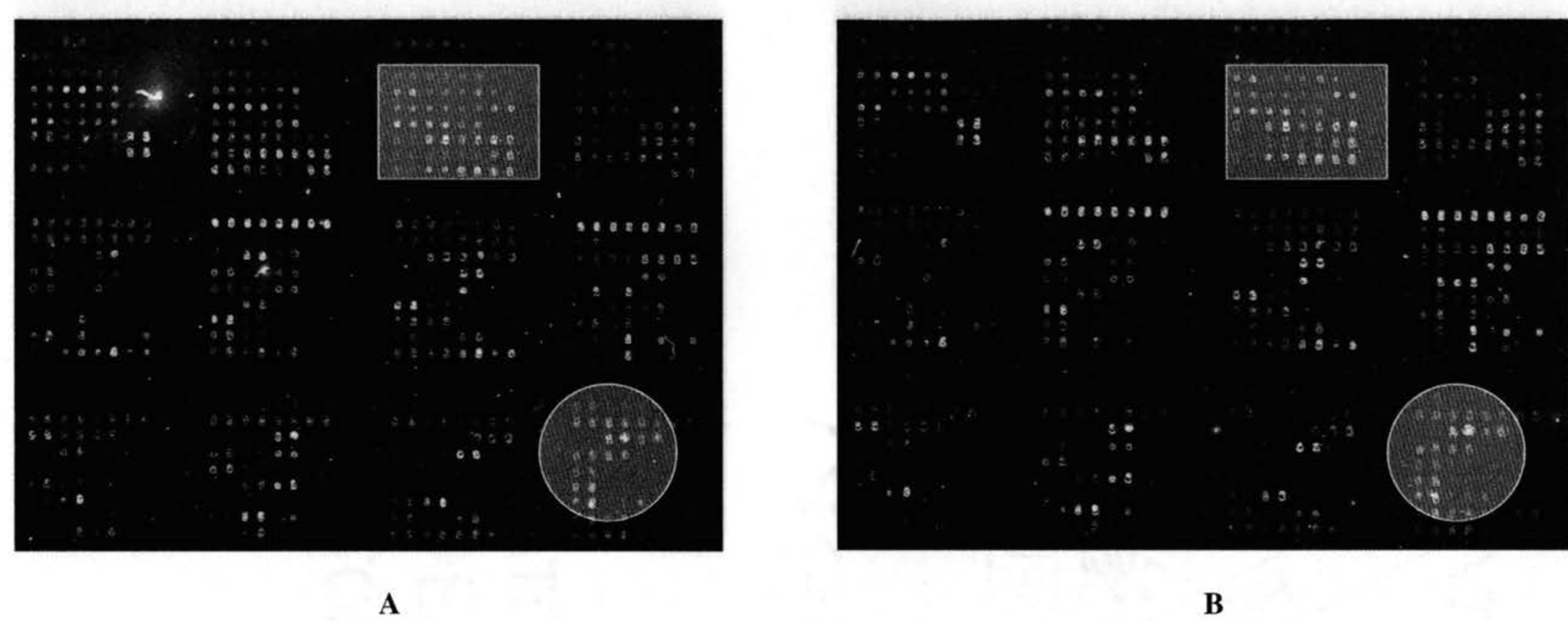


Fig. 1. Comparison of dye swap experiments of fall 2000 mRNA expression. **(A)** RNA from infected plant labeled with Cy5 and mRNA from control plant labeled with Cy3. Clones showed red color were up-regulated, and clones showed green color were down-regulated. **(B)** dye-swap experiment of (A). RNA from control plant labeled with Cy5 and RNA from infected plant labeled with Cy 3. Clones shown in green were up-regulated, and clones shown in red were down-regulated. The same genes between the two experiments with reversed color are highlighted with different shapes. .

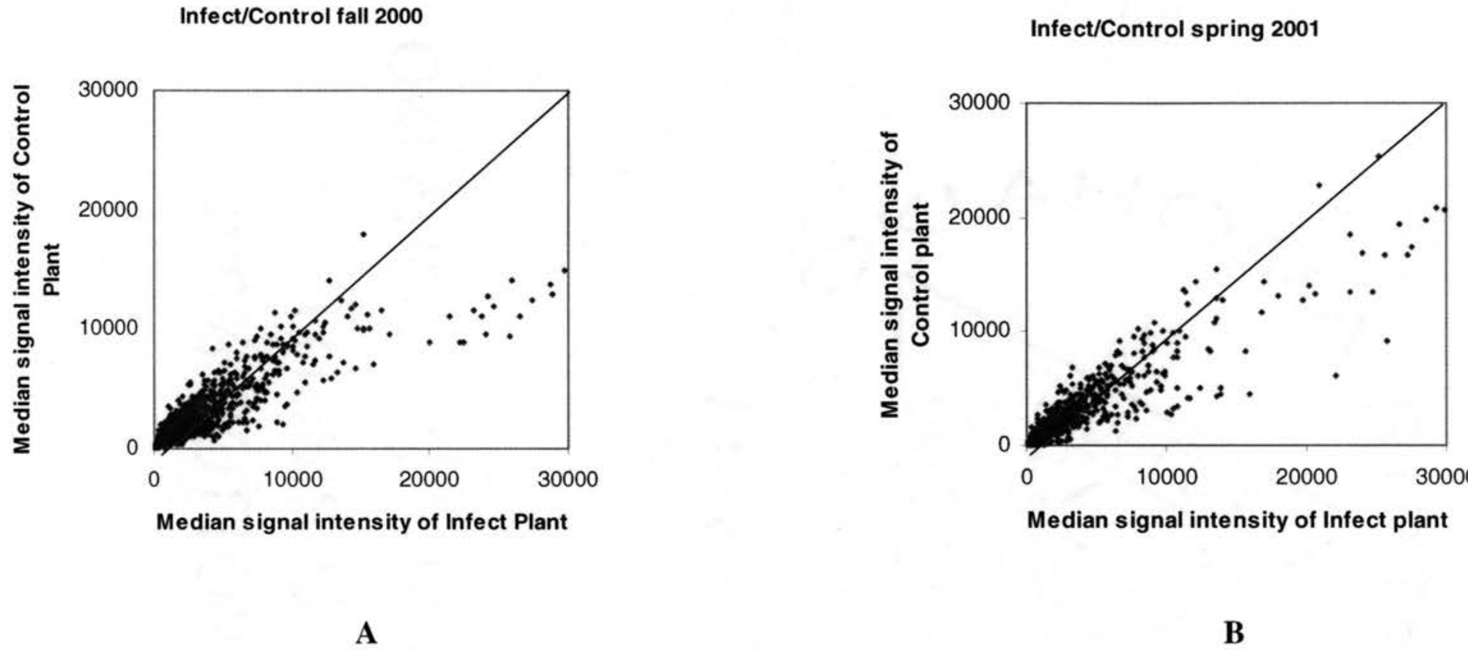


Fig. 2.. Scatter plots comparing the spot intensities in hybridizations with probes from infected (x axis) and control (y axis) tissues. Data from images of both Cy dyes were plotted as the median signal minus background signal intensity after normalization of clones spotted in duplicate.

(A) Scatter plot of signal intensities of two tissues sampled in fall 2000.

(B) Scatter plot of signal intensities of two tissues sampled in spring 2001

Fig. 3. Tree view of cluster analysis by k-means on 31 transcripts in response to fungal infection for infected and non-infected tissues in fall 2000 and spring 2001. The color saturation reflects the magnitude of the log $_2$ expression ratio (Infected/Control) for each transcript with clone number (transcript number), GenBank EST accession number, and annotation. Transcripts are grouped into 6 patterns **(A)** to **(D)** according to their expression profiles of up-regulated (red) and down-regulated (green) at different time points. Each gene is represented by a single row of colored boxes, and each time point is represented by a single column.

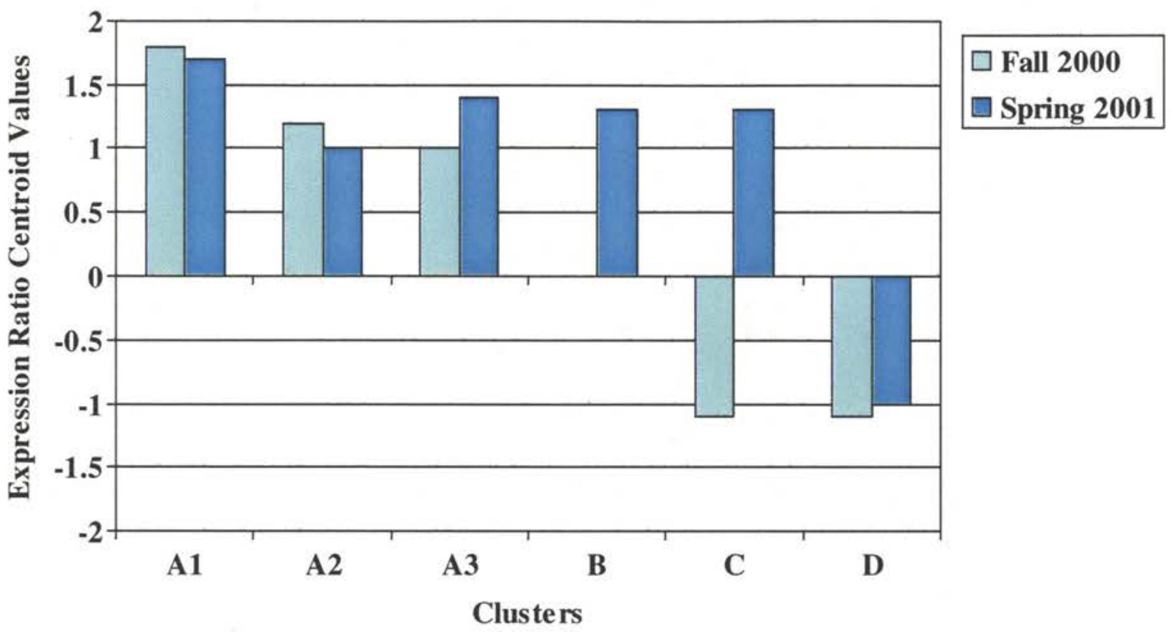


Fig. 4. Centroid views of cluster analysis by k-mean for infected and control plants in the two seasons. The centroid value (bars) represents the average of expression of all genes present in each cluster. The expression ratios (y axis) of transcripts (log-2) for differentially expressed genes for Infect/Control are represented in each cluster (x axis) for fall 2000 and spring 2001. Log_2 Ratio = 0 expression no change, Log_2 Ratio > 0 up-regulated, Log_2 Ratio < 0 down-regulated.
(**A**) Including A1, A2 and A3. Genes were up-regulated for both fall and spring. (**B**) Genes had no response in fall and up-regulated in spring. (**C**) Genes were down-regulated in fall and up-regulated in following spring. (**D**) Gene was down-regulated for both seasons.

Table 1. Functional Categories of Transcripts of Bermudagrass Represented in Infected / Control SSH Libraries

Major Functional Categories	Number of Unique Contig	Percentage %
Oxidative burst /stress or apoptosis	2	1.0
Antimicrobial genes	1	0.5
Cell signaling and communications	15	7.3
Cell maintenance and development	60	29.1
Others - Unclassified	31	15.0
No homology	97	47.1
Total	206	100

Table 2. Cluster analysis of Infected / Control (Jackpot) expression in Fall 2000 and Spring 2001

Clone ID	Log_2 Ratio		Cluster	Function Classification	Accession No.and Annotation
	Fall 2000	Spring 2001			
JI-105	1.48	2.09	**A1**	maintenance	BQ826177, ATP synthase A chain (protein 6) [Cochliobolus heterostrophus]
JI-34	1.67	1.63	**A1**		BQ826336, No homology
J-12	1.85	1.8	**A1**		BQ825905, No homology
JI-27	2.58	1.15	**A1**		BQ825940, No homology
JI-198	1.81	1.39	**A1**	signaling	BQ826026, C3HC4-type zinc fingers protein [Arabidopsis thaliana]
JI-295	1.97	1.49	**A1**		BQ826066, No homology
JI-127	1.51	1.94	**A1**	maintenance	BQ826122, branched chain alpha-keto acid dehydrogenase E2 subunit [Arabidopsis thaliana]
JI-332	1.73	1.97	**A1**		BQ826254, No homology
JI-197	1.37	1.92	**A1**	signaling	BQ826240, ADP-ribosylation factor [Capsicum annuum]
91-4	1.27	1.1	**A2**	signaling	BG322322, voltage-dependent anion channel protein 1a [Zea mays]
JI-292	1.54	1.18	**A2**		Sequence Unknown
JI-347	1.05	1.4	**A3**	maintenance	BQ826267, 60S ribosomal protein L37 [Lycopersicon esculentum]
JI-238	1.05	1.41	**A3**		No homology
JI-215	1.04	1.3	**A3**		BQ826243, No homology
91-161	1.04	1.19	**A3**	maintenance	BQ826412, DnaJ-related protein ZMDJ1 [Zea mays]
JI-319	0.05	1.26	**B**		BQ826052, No homology
JI-100	0	0.97	**B**		BQ826018, No homology
JI-172	-0.01	1.64	**B**	maintenance	BQ826201, ubiquitin-fusion degradation protein [Arabidopsis thaliana]
91-208	0.01	1.27	**B**		BQ826361, (AC011765) unknown protein; [Arabidopsis thaliana]
JI-345	0.01	1.2	**B**		Sequence Unknown
91-129	0	1.12	**B**	maintenance	BG322340, (AF118149) ribosomal protein S7 [Secale cereale]
J-3	-1.17	1.4	**C**		BQ825893, No homology
J-68	-1.03	1.46	**C**		BQ825920, No homology
JI-163	-1.26	2.19	**C**		Sequence Unknown
JI-346	-1.11	1.84	**C**	oxidative burst	BQ826031, putative cysteine proteinase [Oryza sativa]
JI-187	-1.08	1.19	**C**	maintenance	BQ826123, formate dehydrogenase beta-subunit [Methanococcus voltae]
91-87	-1.08	1.13	**C**	maintenance	BG322297, ATPases associated with cellular activites [Arabidopsis thaliana]
JN-4	-1.08	1.17	**C**		BQ825946, hypothetical protein [Arabidopsis thaliana]
JI-251	-1.01	1.07	**C**		BQ826014, No homology

91-123	-1.01	1.03	**C**	Sequence Unknown
91-10	-1.06	-1.07	**D**	BG322335, No homology

APPENDIX

APPENDIX I Bermudagrass Cultivar and Treatment Layout.

Resistant cultivar Yukon (yellow marked) and susceptible cultivar Jackpot (red marked) were randomly planted in bermudagrass cultivar evaluation trail. Each cultivar was replicated three times. There are four treatment zones inside each plot, including three different fungal inoculations (LN = *Leptosphaeria narmari*, OH = *Ophiosphaerella herpotricha*, OK= *Ophiosphaerella Korrae*) and a control zone. The fungal infected bermudagrass crown tissues were collected from Ophiosphaerella herpotricha inoculation zone with three replicates of 10 cm diameter core of sample.

			Jackpot OH OK Control LN
Yukon OH LN OK Control			
		Jackpot OK Control LN OK	
	Yukon Control LN OH OK		
Yukon LN OH Control OK			
			Jackpot Control OK OH LN

Appendix II **Primer Sequences.**

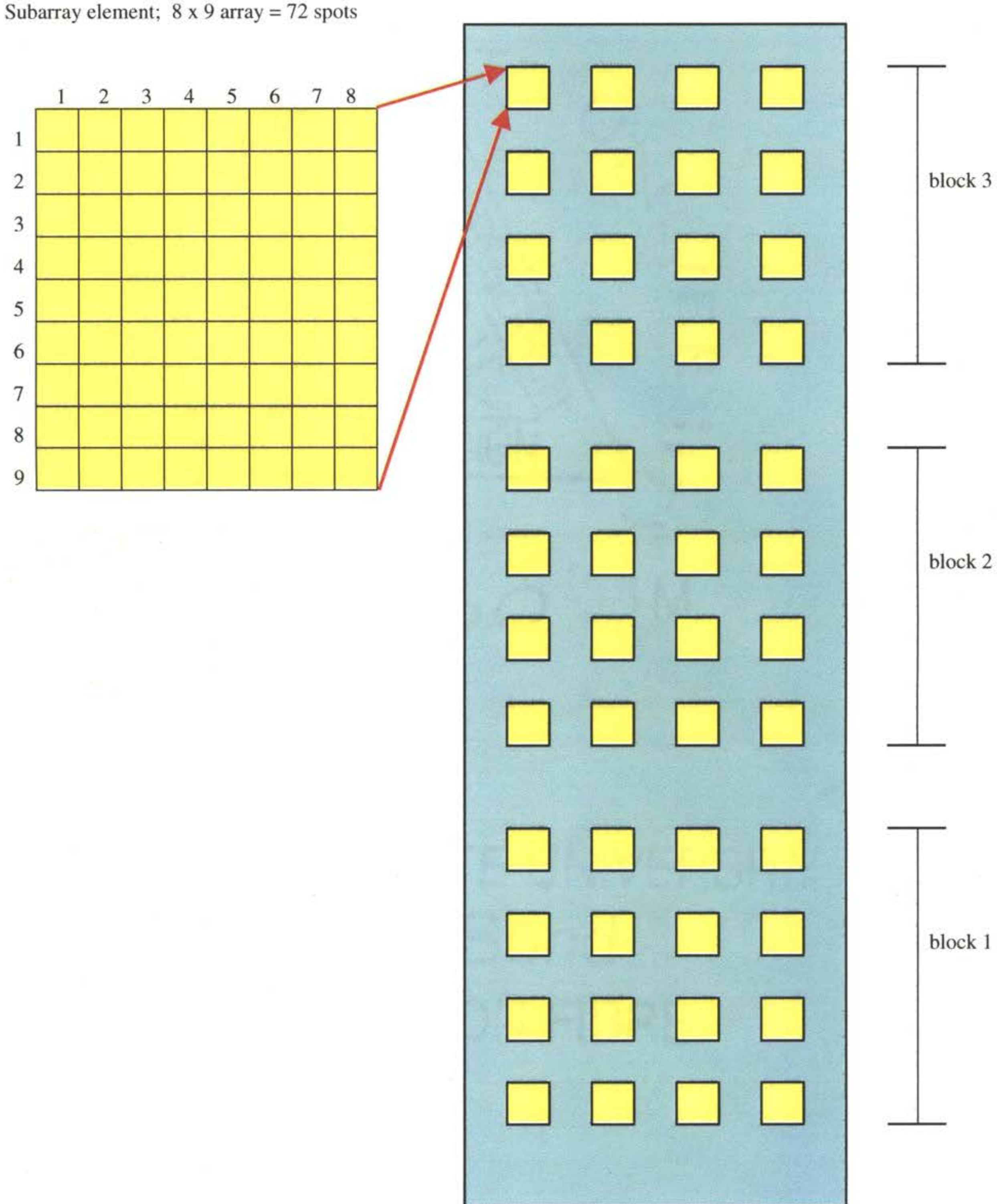
M13 Forward	5'	-GTT TTC CCA GTC ACG AC -3'
-21M13	5'	-TG TAA AAC GAC GGC CAG T-3'
M13 Reverse	5'	-CA GGA AAC AGC TAT GAC C -3'

Control Primers:	**G3PDH 5' Primer**	5'-ACCACAGTCCATGCCATCAC-3'
	G3PDH 3' Primer	5'-TCCACCACCCTGTTGCTGTA-3'

Appendix III Unix Code for Creating PipeOnline Database 112001_FASTA.

```
bash-2.03$ pwd
/export/htdocs/html/users/yzhang
bash-2.03$ chmod -R 777 112001_FASTA
bash-2.03$ pwd
/export/htdocs/html/users/yzhang
bash-2.03$ cd 112001_FASTA
bash-2.03$ pwd
/export/htdocs/html/users/yzhang/112001_FASTA
bash-2.03$ ls -l
total 204
-rwxrwxrwx  1 yzhang  edupol   30972 Nov 20 15:25 91-fasta-1
-rwxrwxrwx  1 yzhang  edupol   19974 Nov 20 15:25 91-fasta-2
-rwxrwxrwx  1 yzhang  edupol   31478 Nov 20 15:25 91-fasta-3
-rwxrwxrwx  1 yzhang  edupol   20176 Nov 20 15:25 J-fasta
bash-2.03$ cat * > new.seq
bash-2.03$ ls -l
total 428
-rwxrwxrwx  1 yzhang  edupol   30972 Nov 20 15:25 91-fasta-1
-rwxrwxrwx  1 yzhang  edupol   19974 Nov 20 15:25 91-fasta-2
-rwxrwxrwx  1 yzhang  edupol   31478 Nov 20 15:25 91-fasta-3
-rwxrwxrwx  1 yzhang  edupol   20176 Nov 20 15:25 J-fasta
-rw-r--r--  1 yzhang  edupol  102600 Nov 20 15:27 new.seq
bash-2.03$ chmod -R 777 new.seq
bash-2.03$ ls -l
total 428
-rwxrwxrwx  1 yzhang  edupol   30972 Nov 20 15:25 91-fasta-1
-rwxrwxrwx  1 yzhang  edupol   19974 Nov 20 15:25 91-fasta-2
-rwxrwxrwx  1 yzhang  edupol   31478 Nov 20 15:25 91-fasta-3
-rwxrwxrwx  1 yzhang  edupol   20176 Nov 20 15:25 J-fasta
-rwxrwxrwx  1 yzhang  edupol  102600 Nov 20 15:27 new.seq
bash-2.03$ pwd
/export/htdocs/html/users/yzhang/112001_FASTA
```

Appendix IV Microarray Layout.



Appendix V Microarray Printing Program.

Source:

5 Plates	1728 Samples	Last Plate	12 / 24
Plate Type	Nunc 384 TeleChem		

Target:

Spot Pattern

Current Spot Pattern: 8 X 9 at 0.4 mm spacing, custom format

Target Area

55 Copies TeleChem Slides

Wash:

Wash Tool 3 Cycles

5 Seconds in left bath

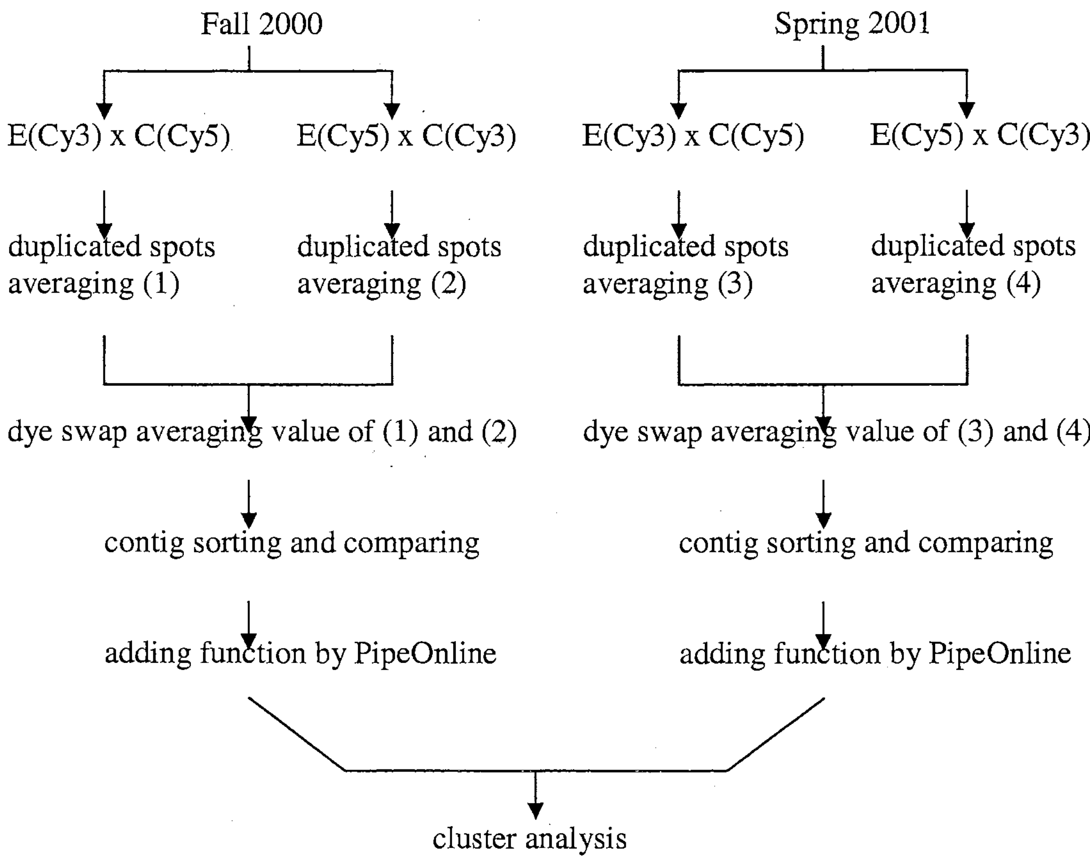
5 Seconds in right bath

10 Seconds in dryer

Tool:

Microspotting 16 pin tool

Appendix VI Array Data Processing and Cluster Analysis.



In experiment Yukon Infect x Jackpot Infect, E =Yukon infect, C = Jackpot Infect. In experiment Jackpot Infect x Jackpot Non-infect, E = Jackpot Infect, C = Jackpot Non-infect. The expression intensity of each differentially regulated gene was calculated by the average value of the two duplicated spots and then averaged again by two dye swap experiments. Genes belong to the same contig were compared, the expression of one clone was used to represent the default value of each contig. Expression data from Fall 2000 and Spring 2001 were clustered together based only on the observed expression pattern.

Appendix VII Differentially Expressed Genes from Yukon and Jackpot in Responses to Fall Infection.

Clone ID	Accession No.	Log_2 Ratio Yukon/Jackpot	HSP	Annotation
91-181		2.90		Sequence unknown
91-162	BQ826377	2.33		No homology
JI-16	BQ826327	2.28	143	ADP-ribosylation factor-like protein [Arabidopsis thaliana]
91-215	BQ826379	2.21	496	Unknown protein [Arabidopsis thaliana]
91-108	BQ826385	2.12	684	40S ribosomal protein S11[Zea mays]
JI-47	BQ825934	2.12		No homology
JN-57	BQ826292	2.00	424	hypothetical protein [Oryza sativa]
91-35	BQ826432	1.96		No homology
91-10	BG322335	1.94		No homology
91-62	BQ826419	1.92		No homology
JI-116	BQ826099	1.81	150	ADP-ribosylation factor [Capsicum annuum]
91-161	BQ826412	1.79	697	DnaJ protein homolog ZMDJ1 [Zea mays]
JI-40		1.71		No homology
JI-93	BQ825942	1.65	286	ribosomal protein L11 [Homo sapiens]
91-130	BG322341	1.63	94	ethylene receptor [Brassica oleracea]
JI-94	BQ826440	1.61	340	(AC068602) F14D16.2 [Arabidopsis thaliana]
91-14		1.61		Sequence unknown
91-13	BQ826394	1.60		No homology
JI-244	BQ825995	1.59		No homology
91-67	BG322360	1.56		No homology
91-214	BQ826367	1.55		No homology
JN-34	BQ825969	1.55	359	unknown protein [Arabidopsis thaliana]
91-119	BQ826390	1.54		No homology
91-126	BG322337	1.54		No homology
91-147	BQ826349	1.50		No homology
91-198	BQ826426	1.45	342	unknown protein [Oryza sativa]
91-115	BQ826446	1.45	243	26S proteasome regulatory subunit S2 [Arabidopsis thaliana]

91-64	BQ826427	1.43	264	catalase isozyme 3 [Zea mays]
91-122	BQ826392	1.35		No homology
91-127	BG322304	1.34	81	HMG1/2-like high mobility group protein [Triticum aestivum]
91-20		1.29		Sequence unknown
91-52	BG322312	1.28	565	ascorbate peroxidase [Hordeum vulgare]
JN-89	BQ825932	1.28		No homology
91-1	BG354696	1.27	204	40S ribosomal protein S11 [Glycine max]
91-46	BQ826405	1.23		No homology
91-116	BQ826388	1.22		No homology
JI-96	BQ825973	1.21		No homology
91-219	BQ826429	1.21		No homology
91-206	BQ826373	1.19	257	40S ribosomal protein S4 type I [Zea mays]
91-34	BQ826436	1.18		No homology
JI-115	BQ826047	1.15		No homology
91-218	BQ826410	1.14		No homology
J-30	BQ825914	1.14		No homology
JI-308	BQ826454	1.12		No homology
91-8		1.09	463	transcription factor SF3/ LIM domain protein WLIM2 [Nicotiana tabacum]
JI-356	BQ825993	1.08		No homology
91-51		1.08		Sequence unknown
JN-110	BQ825949	1.06	142	fused-ccdB [Escherichia coli]
91-144	BQ826398	1.06		No homology
91-123		1.06		Sequence unknown
JI-63	BQ826205	1.04		No homology
91-143	BQ826397	1.03		No homology
91-120	BQ826449	1.03	284	rev interacting protein mis3 - like [Arabidopsis thaliana]
JI-261	BQ826455	1.02		No homology
JI-176	BQ826071	1.02		No homology
91-186	BG322338	1.01		No homology
91-135		1.01		Sequence unknown
91-48	BQ826344	0.00	176	putative ubiquitin protein [Oryza sativa]

J-60	BQ825915	-1.02		No homology
JN-8	BQ826259	-1.03	293	ribosomal protein L14-like protein [Arabidopsis thaliana]
JN-29	BQ825975	-1.06	150	60S ribosomal protein L1 [Prunus armeniaca]
J-26	BQ825911	-1.08		No homology
JI-347	BQ826267	-1.13	117	60S ribosomal protein L37 [Lycopersicon esculentum]
91-201		-1.13		Sequence unknown
JI-8	BQ826190	-1.16	123	No homology
JI-342	BQ826130	-1.18		No homology
J-52	BG322292	-1.20	87	unknown protein [Arabidopsis thaliana]
JN-24	BQ825929	-1.21	143	hypothetical protein [Arabidopsis thaliana]
JI-293	BQ825980	-1.22	100	nonstructural protein 1 [Dengue virus type 3]
JI-162		-1.22		Sequence unknown
JI-319	BQ826052	-1.24		No homology
JI-332	BQ826254	-1.28		No homology
JI-191	BQ826239	-1.30		No homology
JI-352	BQ826027	-1.31		No homology
JN-3	BQ826009	-1.36		No homology
JI-15	BQ826061	-1.37	133	No homology
JI-365	BQ826138	-1.41	84	hypothetical protein [Macaca fascicularis]
J-8	BG322293	-1.41		No homology
JI-215	BQ826243	-1.42		No homology
JI-249	BQ826035	-1.45		No homology
JI-148	BQ826203	-1.66	269	ubiquitin-fusion degradation protein-like [Arabidopsis thaliana]
JI-71	BQ826171	-1.67	204	ATP synthase A chain (protein 6) [Cochliobolus heterostrophus]
JI-128	BQ826232	-1.71		No homology
J-40	BG322288	-1.73		No homology
JI-131	BQ826234	-1.75		No homology
JI-52	BQ826097	-1.92		No homology
J-2		-1.94		Sequence unknown
JI-127	BQ826122	-1.99	481	branched chain alpha-keto acid dehydrogenase E2 subunit [Arabidopsis thaliana]
JI-322	BQ826252	-2.06	122	formate dehydrogenase beta-subunit [Methanococcus voltae]

JI-124	BQ825938	-2.21		No homology
JI-334	BQ826255	-2.24		No homology
JI-27	BQ825940	-2.42		No homology
JI-102	BQ826040	-2.48		No homology
JI-318	BQ825953	-2.49		No homology
JI-112	BQ826150	-2.52	171	hypothetical zinc fingers protein [Arabidopsis thaliana]
JI-214		-2.55		Sequence unknown
JI-345		-2.78		Sequence unknown
J-12	BQ825905	-2.96		No homology
JI-134	BQ826237	-3.92		No homology
JI-61	BQ826013	-3.97		No homology
J-1	BG322276	-4.34		No homology
JI-100	BQ826018	-4.42		No homology
JI-221	BQ826030	-4.66	147	putative cysteine proteinase [Oryza sativa]
JI-177	BQ826015	-4.96		No homology
JI-133	BQ826236	-5.10		No homology
J-16	BG322283	-5.28		No homology
JI-163		-6.08		Sequence unknown
spike1		1.49		Ambion spike gene 1
spike2		-1.10		Ambion spike gene 2
spike3		1.11		Ambion spike gene 3
spike4		0.06		Ambion spike gene 4
spike5		1.66		Ambion spike gene 5
spike6		1.20		Ambion spike gene 6
spike7		1.36		Ambion spike gene 7
spike8		0.12		Ambion spike gene 8
Cht1		-1.36		Bermudagrass Chtinase genomic DNA clone 1
Cht2		1.18		Bermudagrass Chtinase genomic DNA clone2

Appendix VIII Differentially Expressed Genes in Yukon and Jackpot in Response to Spring Infection and Regrowth.

Clone ID	Accession No.	Log_2 Ratio Yukon/Jackpot	HSP	Annotation
JI-176	BQ826071	3.76		No homology
91-181		3.14		Sequence unknown
JI-295	BQ826066	2.92		No homology
JI-250	BQ826076	2.42		No homology
JI-332	BQ826254	2.37		No homology
JI-308	BQ826454	2.31		No homology
JI-331	BQ826075	2.23		No homology
JI-27	BQ825940	2.18		No homology
91-108	BQ826385	2.06	684	40S ribosomal protein S11 [Zea mays]
JI-244	BQ825995	2.03		No homology
JI-204		2.01		No homology
91-215	BQ826379	1.96	496	Unknown protein [Arabidopsis thaliana]
JI-71	BQ826171	1.95	204	ATP synthase A chain (protein 6) [Cochliobolus heterostrophus]
JI-15	BQ826061	1.92	133	No homology
91-130	BG322341	1.82	94	ethylene receptor [Brassica oleracea]
JI-8	BQ826190	1.81	123	No homology
91-35	BQ826432	1.81		No homology
J-5		1.74		Sequence unknown
91-202	BQ826340	1.73	97	hypothetical protein [Oryza sativa]
JI-140	BQ826029	1.70		No homology
JI-292		1.69		Sequence unknown
JI-52	BQ826097	1.66		No homology
91-76	BQ826364	1.62	785	methionine synthase protein [Sorghum bicolor]
JI-273		1.60		Sequence unknown
91-107	BQ826368	1.59		No homology
JI-287	BQ825996	1.55		No homology
JI-226		1.55		No homology

91-162	BQ826377	1.54		No homology
JI-42		1.53	92	hypothetical protein Y39G8C.b [Caenorhabditis elegans]
91-52	BG322312	1.50	565	ascorbate peroxidase [Hordeum vulgare]
JN-2	BQ826037	1.50	82	methyltransferase CmuC [Aminobacter sp. IMB-1]
JN-34	BQ825969	1.50	359	unknown protein [Arabidopsis thaliana]
JI-238		1.49		No homology
JI-16	BQ826327	1.48	143	ADP-ribosylation factor-like protein [Arabidopsis thaliana]
JI-68	BQ826098	1.47	150	ADP-ribosylation factor [Capsicum annuum]
JI-94	BQ826440	1.46	340	(AC068602) F14D16.2 [Arabidopsis thaliana]
JN-52	BQ826290	1.44		No homology
91-198	BQ826426	1.42	342	unknown protein [Oryza sativa]
91-43		1.41		Sequence unknown
91-143	BQ826397	1.39		No homology
91-122	BQ826392	1.38		No homology
91-19	BQ826450	1.37	463	transcription factor SF3 protein [Nicotiana tabacum]
91-64	BQ826427	1.34	264	catalase isozyme 3 [Zea mays]
91-160	BQ826411	1.34		No homology
91-83	BG322310	1.33	108	LLS1 protein [Oryza sativa]
91-99	BG322344	1.31		No homology
JI-137	BQ826161	1.30		No homology
JI-149	BQ825983	1.28		No homology
91-27	BQ826347	1.28		No homology
JI-28	BQ826208	1.27		No homology
JI-215	BQ826243	1.27		No homology
JI-96	BQ825973	1.27		No homology
91-15	BG322319	1.26	507	voltage-dependent anion channel protein 1a [Zea mays]
JI-278	BQ826128	1.26		No homology
JI-25	BQ826120	1.25		No homology
91-159	BQ826409	1.24		No homology
J-25	BG322284	1.24		No homology
91-44	BG322327	1.23	167	unknown protein [Arabidopsis thaliana]

JI-108	BQ826223	1.23	98	larval glue protein Lgp3 precursor [Drosophila virilis]
91-151	BQ826403	1.23		No homology
91-50		1.23		Sequence unknown
91-1	BG354696	1.22	204	40S ribosomal protein S11 [Glycine max]
J-10		1.22		Sequence unknown
91-2		1.21		Sequence unknown
JI-128	BQ826232	1.20		No homology
91-158	BQ826408	1.20		No homology
91-100	BG322336	1.19	622	SCARECROW gene regulator/ phytochrome A signal transduction 1 protein [Arabidopsis thaliana]
J-48		1.19		No homology
JI-240	BQ826245	1.18		No homology
91-142	BQ826396	1.17	257	unknown protein [Oryza sativa]
JI-87	BQ826082	1.17		No homology
J-44	BQ825895	1.17		No homology
J-32	BQ825916	1.16		No homology
91-51		1.16		Sequence unknown
J-39		1.16	83	Hypothetical protein [Oryza sativa]
J-55		1.15		Sequence unknown
JI-378	BQ826275	1.14	133	CG2839 gene product [Drosophila melanogaster]
J-57	BQ825922	1.14		No homology
JN-85		1.14		Sequence unknown
91-106	BQ826353	1.13	122	putative phosphoribosylanthranilate transferase [Arabidopsis thaliana]
91-127	BG322304	1.12	81	HMG1/2-like high mobility group protein [Triticum aestivum]
91-20		1.12		Sequence unknown
JI-156	BQ826153	1.12	486	ubiquinol--cytochrome-c reductase iron-sulfur subunit [Zea mays]
JI-356	BQ825993	1.11		No homology
91-210	BQ826376	1.11		No homology
91-10	BG322335	1.11		No homology
91-147	BQ826349	1.11		No homology
J-28		1.10		Sequence unknown

91-187	BQ826387	1.09		No homology
JI-59		1.08		Sequence unknown
J-51		1.07		Sequence unknown
91-189	BQ826350	1.06	138	putative protein [Arabidopsis thaliana]
91-220	BQ826413	1.05	84	protein translation initiation factor SUI1 homolog[Oryza sativa]
JN-58	BQ826293	1.04	488	60S ribosomal protein L9 [Oryza sativa]
JI-31	BQ826230	1.04		No homology
J-47	BG322282	1.04	248	phenylalanine ammonia-lyase [Oryza sativa]
J-7		1.04		Sequence unknown
91-3	BG322356	1.03		No homology
J-24		1.03		Sequence unknown
JI-47	BQ825934	1.01		No homology
J-26	BQ825911	1.01		No homology
91-14		1.01		Sequence unknown
91-7		1.01		Sequence unknown
91-6	BG322362	1.00	332	eukaryotic translation initiation factor 5 [Zea mays]
91-115	BQ826446	0.60	243	26S proteasome regulatory subunit S2 [Arabidopsis thaliana]
JN-38	BQ825972	0.01	690	11332.4 [Oryza sativa]
91-206	BQ826373	-0.03	257	40S ribosomal protein S4 type I [Zea mays]
91-87	BG322297	-1.00	330	ATPases associated with cellular activites [Arabidopsis thaliana]
JN-29	BQ825975	-1.04	150	60S ribosomal protein L1 [Prunus armeniaca]
JN-7	BQ826011	-1.04		No homology
JI-303	BQ826220	-1.05		No homology
JN-61	BQ826295	-1.10	284	unknown protein [Oryza sativa]
JN-23	BQ825967	-1.12	132	ferredoxin [2Fe-2S] root [Oryza sativa]
JI-249	BQ826035	-1.12		No homology
JI-132	BQ826235	-1.14		No homology
JN-55	BQ825948	-1.16	142	fused-ccdB [Escherichia coli]
91-207	BQ826374	-1.17	107	putative protein [Arabidopsis thaliana]
JI-109	BQ826224	-1.18	88	regulatory protein E2 [Human papillomavirus type 20]
JI-22	BQ826212	-1.19		No homology

J-33	BG322272	-1.19		No homology
JI-365	BQ826138	-1.20	84	hypothetical protein [Macaca fascicularis]
JN-53	BQ825952	-1.21	87	probable antigen 4 - Mycobacterium leprae (fragment)
J-42	BG322289	-1.22	122	putative ribosomal protein L18a cytosolic [Oryza sativa]
JN-111	BQ826304	-1.24	103	60S ribosomal protein L5 [Solanum melongena]
JI-63	BQ826205	-1.24		No homology
JN-100	BQ825926	-1.27		No homology
JI-127	BQ826122	-1.28	481	branched chain alpha-keto acid dehydrogenase E2 subunit [Arabidopsis thaliana]
91-68	BG322363	-1.28		No homology
JI-50	BQ826321	-1.29		No homology
JN-24	BQ825929	-1.36	143	hypothetical protein [Arabidopsis thaliana]
JN-5	BQ826257	-1.38	654	rac GTPase activating protein 2 [Lotus japonicus]
JI-263		-1.44		Sequence unknown
JI-115	BQ826047	-1.46		No homology
91-219	BQ826429	-1.49		No homology
JI-148	BQ826203	-1.49	269	ubiquitin-fusion degradation protein-like [Arabidopsis thaliana]
JI-162		-1.56		Sequence unknown
JI-213	BQ825981	-1.61	100	nonstructural protein 1 [Dengue virus type 3]
J-60	BQ825915	-1.79		No homology
JI-345		-2.09		Sequence unknown
JI-322	BQ826252	-2.14	122	formate dehydrogenase beta-subunit [Methanococcus voltae]
JI-124	BQ825938	-2.14		No homology
J-40	BG322288	-2.16		No homology
JI-34	BQ826336	-2.25		No homology
J-2		-2.38		Sequence unknown
JI-334	BQ826255	-2.39		No homology
JI-134	BQ826237	-2.43		No homology
J-12	BQ825905	-2.86		No homology
JI-214		-2.89		Sequence unknown
JI-221	BQ826030	-3.15	147	putative cysteine proteinase [Oryza sativa]
J-58	BG322275	-3.26		No homology

JI-61	BQ826013	-3.29		No homology
JN-50	BQ826288	-3.98	171	C3HC4-type zinc fingers protein protein [Arabidopsis thaliana]
J-16	BG322283	-4.03		No homology
JI-177	BQ826015	-4.04		No homology
JI-100	BQ826018	-4.37		No homology
JI-133	BQ826236	-5.93		No homology
JI-163		-6.38		Sequence unknown
spike1		1.51		Ambion spike gene 1
spike2		0.56		Ambion spike gene 2
spike3		1.29		Ambion spike gene 3
spike4		1.26		Ambion spike gene 4
spike5		1.89		Ambion spike gene 5
spike6		1.27		Ambion spike gene 6
spike7		1.52		Ambion spike gene 7
spike8		-0.03		Ambion spike gene 8
Cht1		-0.08		Bermudagrass Chitnase genomic DNA clone 1
Cht2		-1.29		Bermudagrass Chitnase genomic DNA clone 2
Cht3		1.67		Bermudagrass Chitnase genomic DNA clone 3

Appendix IX Expression Profile of Infected / Non-infected (Jackpot) in Fall 2000.

Clone ID	Accession No.	Log_2 Ratio Infected/Non-infected	HSP	Annotation
JI-27	BQ825940	2.58		No homology
JI-52	BQ826097	2.01		No homology
JI-295	BQ826066	1.97		No homology
J-12	BQ825905	1.85		No homology
JI-376	BQ826274	1.81	171	C3HC4-type zinc fingers protein [Arabidopsis thaliana]
JI-332	BQ826254	1.73		No homology
JI-34	BQ826336	1.67		No homology
JI-176	BQ826071	1.66		No homology
JI-308	BQ826454	1.65		No homology
JI-273		1.59		Sequence Unknown
JI-331	BQ826075	1.57		No homology
JI-292		1.54		Sequence Unknown
JI-42		1.54	92	hypothetical protein Y39G8C.b [Caenorhabditis elegans]
JI-250	BQ826076	1.52		No homology
JI-127	BQ826122	1.51	481	branched chain alpha-keto acid dehydrogenase E2 subunit [Arabidopsis thaliana]
JI-71	BQ826171	1.48	204	ATP synthase A chain (protein 6) [Cochliobolus heterostrophus]
91-100	BG322336	1.43	622	SCARECROW gene regulator/ phytochrome A signal transduction 1 protein [Arabidopsis thaliana]
JI-8	BQ826190	1.41	123	No homology
JI-132	BQ826235	1.40		No homology
JI-187	BQ826123	1.35	117	formate dehydrogenase beta-subunit [Methanococcus voltae]
JI-226		1.32		No homology
JI-356	BQ825993	1.30		No homology
91-4	BG322322	1.27	507	voltage-dependent anion channel protein 1a [Zea mays]
91-127	BG322304	1.26	81	HMG1/2-like high mobility group protein [Triticum aestivum]
JI-140	BQ826029	1.24		No homology
JI-197	BQ826240	1.23	133	ADP-ribosylation factor [Capsicum annuum]
JN-52	BQ826290	1.11		No homology

JI-131	BQ826234	1.10		No homology
JI-137	BQ826161	1.10		No homology
91-137	BG322343	1.07	136	amino acid transport protein AAP2 [Arabidopsis thaliana]
JI-347	BQ826267	1.05	117	60S ribosomal protein L37 [Lycopersicon esculentum]
JI-238		1.05		No homology
91-161	BQ826412	1.04	697	DnaJ protein homolog ZMDJ1[Zea mays]
JI-215	BQ826243	1.04		No homology
JI-364	BQ826270	1.03		No homology
J-57	BQ825922	1.03		No homology
J-47	BG322282	0.01	248	phenylalanine ammonia-lyase [Oryza sativa]
JI-156	BQ826153	0.01	486	ubiquinol--cytochrome-c reductase iron-sulfur subunit [Zea mays]
91-129	BG322340	0.00	764	ribosomal protein S7 [Secale cereale]
JI-172	BQ826201	-0.01	269	ubiquitin-fusion degradation protein-like [Arabidopsis thaliana]
JI-251	BQ826014	-1.01		No homology
91-128	BG322368	-1.01		No homology
91-123		-1.01		Sequence Unknown
91-42		-1.02		Sequence Unknown
J-68	BQ825920	-1.03		No homology
JN-64	BQ826277	-1.05	152	hypothetical protein [Sorghum bicolor]
91-10	BG322335	-1.06		No homology
91-211	BQ826346	-1.06		No homology
JN-4	BQ825946	-1.08	147	hypothetical protein [Arabidopsis thaliana]
91-87	BG322297	-1.08	330	ATPases associated with cellular activites [Arabidopsis thaliana]
JI-187	BQ826123	-1.08	115	formate dehydrogenase beta-subunit [Methanococcus voltae]
J-30	BQ825914	-1.10		No homology
JI-346	BQ826031	-1.11	147	putative cysteine proteinase [Oryza sativa]
91-143	BQ826397	-1.15		No homology
91-110	BG322315	-1.17	190	hypothetical protein [Oryza sativa]
J-3	BQ825893	-1.17		No homology
91-187	BQ826387	-1.19		No homology
91-130	BG322341	-1.22	94	ethylene receptor [Brassica oleracea]

J-29	BQ825912	-1.24	150	ADP-ribosylation factor-like protein [Arabidopsis thaliana]
91-203	BQ826371	-1.25		No homology
91-116	BQ826388	-1.26		No homology
JI-163		-1.26		Sequence Unknown
91-24	BQ826417	-1.27		No homology
JI-244	BQ825995	-1.29		No homology
JN-124	BQ825968	-1.30	132	ferredoxin [2Fe-2S] root [Oryza sativa]
91-162	BQ826377	-1.41		No homology
J-40	BG322288	-1.43		No homology
91-207	BQ826374	-1.46	107	putative protein [Arabidopsis thaliana]
91-64	BQ826427	-1.73	264	catalase isozyme 3 [Zea mays]
spike1		1.70		Ambion spike gene 1
spike2		1.39		Ambion spike gene 2
spike3		1.45		Ambion spike gene 3
spike4		1.60		Ambion spike gene 4
spike5		2.50		Ambion spike gene 5
spike6		1.48		Ambion spike gene 6
spike7		1.83		Ambion spike gene 7
spike8		1.31		Ambion spike gene 8
Cht1		1.26		Bermudagrass Chitnase genomic clone 1

Appendix X Expression Profile of Infected / Non-infected (Jackpot) in Spring 2001.

Clone ID	Accession No.	Log_2 Ratio Infect/Non-infect	HSP	Putative Function
JI-163		2.19		Sequence unknown
JI-133	BQ826236	2.10		No homology
JI-71	BQ826171	2.03	204	ATP synthase A chain (protein 6) [Cochliobolus heterostrophus]
JI-332	BQ826254	1.97		No homology
JI-127	BQ826122	1.94	481	branched chain alpha-keto acid dehydrogenase E2 subunit [Arabidopsis thaliana]
JI-197	BQ826240	1.92	133	ADP-ribosylation factor [Capsicum annuum]
JI-221	BQ826030	1.84	147	putative cysteine proteinase [Oryza sativa]
J-12	BQ825905	1.80		No homology
JI-214		1.72		Sequence unknown
JI-34	BQ826336	1.63		No homology
JI-104	BQ826140	1.58		No homology
JI-295	BQ826066	1.49		No homology
91-140	BQ826395	1.46		No homology
JI-97	BQ826148	1.44		No homology
JI-172	BQ826201	1.44	269	ubiquitin-fusion degradation protein-like [Arabidopsis thaliana]
JI-50	BQ826321	1.41		No homology
JI-238		1.41		No homology
JI-347	BQ826267	1.40	117	60S ribosomal protein L37 [Lycopersicon esculentum]
J-33	BG322272	1.40		No homology
91-186	BG322338	1.40		No homology
91-21	BQ826401	1.39		No homology
JI-112	BQ826150	1.38	171	C3HC4-type zinc fingers protein [Arabidopsis thaliana]
JI-134	BQ826237	1.35		No homology
JI-249	BQ826035	1.34		No homology
JI-278	BQ826128	1.33		No homology
JI-365	BQ826138	1.30	84	hypothetical protein [Macaca fascicularis]
JI-215	BQ826243	1.30		No homology

91-167	BG322325	1.27	167	unknown protein [Arabidopsis thaliana]
JI-119	BQ826139	1.26		No homology
JI-319	BQ826052	1.26		No homology
91-189	BQ826350	1.26	138	putative protein [Arabidopsis thaliana]
91-63		1.26		Sequence unknown
91-19	BQ826450	1.22	463	transcription factor SF3/ LIM domain protein WLIM2 [Nicotiana tabacum]
91-219	BQ826429	1.20		No homology
91-106	BQ826353	1.20	122	putative phosphoribosylanthranilate transferase [Arabidopsis thaliana]
JI-345		1.20		Sequence unknown
91-161	BQ826412	1.19	697	DnaJ protein homolog ZMDJ1 [Zea mays]
JN-8	BQ826259	1.19	293	ribosomal protein L14-like protein [Arabidopsis thaliana]
JI-292		1.18		Sequence unknown
JI-200	BQ826316	1.18	116	formate dehydrogenase beta-subunit [Methanococcus voltae]
JN-4	BQ825946	1.17	147	hypothetical protein [Arabidopsis thaliana]
JI-108	BQ826223	1.17	98	larval glue protein Lgp3 precursor [Drosophila virilis]
JI-259	BQ825974	1.16		No homology
JI-240	BQ826245	1.16		No homology
91-34	BQ826436	1.16		No homology
JI-27	BQ825940	1.15		No homology
JI-334	BQ826255	1.14		No homology
91-87	BG322297	1.13	330	ATPases associated with cellular activites [Arabidopsis thaliana]
91-212	BQ826366	1.12	210	actin [Oryza sativa]
91-129	BG322340	1.12	764	ribosomal protein S7 [Secale cereale]
91-5	BG322320	1.10	507	voltage-dependent anion channel protein 1a [Zea mays]
91-6	BG322362	1.09	332	eukaryotic translation initiation factor 5 [Zea mays]
91-68	BG322363	1.08		No homology
JN-120	BQ826297	1.07	567	ascorbate peroxidase [Hordeum vulgare]
JI-177	BQ826015	1.07		No homology
JI-315	BQ825939	1.07		No homology
JI-63	BQ826205	1.06		No homology
91-124	BQ826393	1.06		No homology

91-123		1.03		Sequence unknown
JI-162		1.03		Sequence unknown
J-2		1.02		Sequence unknown
91-206	BQ826373	1.01	257	40S ribosomal protein S4 type I [Zea mays]
JN-58	BQ826293	1.01	488	60S ribosomal protein L9 [Oryza sativa]
JI-100	BQ826018	0.97		No homology
91-146	BQ826400	0.01	227	RUB1 conjugating enzyme [Lycopersicon esculentum]
91-26		0.01	534	cleft lip and palate associated transmembrane protein-like [Arabidopsis thaliana]
91-119	BQ826390	0.00		No homology
91-13	BQ826394	0.00		No homology
91-10	BG322335	-1.07		No homology
JI-158	BQ826039	-1.16		No homology
spike1		1.57		Ambion spike gene 1
spike2		1.32		Ambion spike gene 2
spike3		1.39		Ambion spike gene 3
spike4		1.25		Ambion spike gene 4
spike5		1.98		Ambion spike gene 5
spike6		1.48		Ambion spike gene 6
spike7		1.67		Ambion spike gene 7
spike8		1.16		Ambion spike gene 8
Cht1		1.51		Bermudagrass Chitnase genomic clone 1

VITA

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